Vol. 13, No. 1, 2022
ISSN 2233-4203/ e-ISSN 2093-8950
ARTICLE www.msletters.org | Mass Spectrometry Letters

Metabolic Signatures of Adrenal Steroids in Preeclamptic Serum and Placenta Using Weighting Factor-Dependent Acquisitions

Chaelin Lee[1,2†], Min-Jeong Oh[3†], Geum Joon Cho[3], Dong Jun Byun[1,2], Hong Seog Seo[4]*, and Man Ho Choi[1]*

[1]*Molecular Recognition Research Center, Korea Institute of Science and Technology, Seoul 02792, Korea*
[2]*Department of Chemistry, Korea University, Seoul 02841, Korea*
[3]*Department of Obstetrics and Gynecology, Korea University Guro Hospital, Seoul 08308, Korea*
[4]*Cardiovascular Center, Korea University Guro Hospital, Seoul 08308, Korea*

Received January 07, 2022, Revised February 11, 2022, Accepted February 24, 2022
First published on the web March 31, 2021; DOI: 10.5478/MSL.2022.13.1.11

Abstract : Although translational research is referred to clinical chemistry measures, correct weighting factors for linear and quadratic calibration curves with least-squares regression algorithm have not been carefully considered in bioanalytical assays yet. The objective of this study was to identify steroidogenic roles in preeclampsia and verify accuracy of quantitative results by comparing two different linear regression models with weighting factor of 1 and $1/x^2$. A liquid chromatography-mass spectrometry (LC-MS)-based adrenal steroid assay was conducted to reveal metabolic signatures of preeclampsia in both serum and placenta samples obtained 15 preeclamptic patients and 17 age-matched control pregnant women (33.9 ± 4.2 vs. 32.8 ± 5.6 yr, respectively) at 34~36 gestational weeks. Percent biases in the unweighted model ($w_i = 1$) were inversely proportional to concentrations (-739.4 ~ 852.9%) while those of weighted regression ($w_i = 1/x^2$) were < 18% for all variables. The optimized LC-MS combined with the weighted linear regression resulted in significantly increased maternal serum levels of pregnenolone, 21-deoxycortisol, and tetrahydrocortisone ($P < 0.05$ for all) in preeclampsia. Serum metabolic ratio of (tetrahydrocortisol + allo-tetrahydrocortisol) / tetrahydrocortisone indicating 11β-hydroxysteroid dehydrogenase type 2 was decreased ($P < 0.005$) in patients. In placenta, local concentrations of androstenedione were changed while its metabolic ratio to 17α-hydroxyprogesterone responsible for 17,20-lyase activity was significantly decreased in patients ($P = 0.002$). The current bioanalytical LC-MS assay with corrected weighting factor of $1/x^2$ may provide reliable and accurate quantitative outcomes, suggesting altered steroidogenesis in preeclampsia patients at late gestational weeks in the third trimester.

Keywords : adrenal steroid, cortisol metabolism, 17,20-lyase, weighting factor, preeclampsia

Introduction

Reliable quantitative results in clinical studies are highly affected by selecting the optimal weighting factor for linear and quadratic calibration curves. Although an unweighted linear regression model has been commonly used, appropriate weighting factors such as $1/x$, $1/x^2$, $1/y$, and $1/y^2$ could reduce a percent relative error (%RE) versus concentration, thus improving the precision in quantification.[1] Dynamic ranges in bioanalytical assays are usually more than one order of magnitude with data points at each concentration having unequal variances (σ^2). The accuracy of instrumental responses decreases in the low range while it increases proportionally to sample concentration.[1-3] These heteroscedastic errors could lead to incorrect quantitative information in biomedical applications.

Preeclampsia is characterized by the new onset of pregnancy-induced hypertension and proteinuria after 20 weeks of gestation with complications in 3~4% of pregnancies that may lead to about 50,000 maternal deaths worldwide a year.[4,5] Physiological changes of maternal hypothalamic-pituitary-adrenal (HPA) axis in pregnancy are associated with maternal and fetal outcomes.[6] Dysregulation of maternal HPA-axis also can affect metabolic signatures of adrenal steroids.[7,8] Preeclampsia patients show decreased plasma levels of mineralocorticoids.[8,9] High cortisol levels induced by distress conditions could be associated with preeclampsia.[10] In addition, maternal cortisol-to-cortisone ratio is decreased in serum but increased in placenta of patients with preeclampsia.[11-13] However, metabolic changes

*Reprint requests to Man Ho Choi
https://orcid.org/0000-0003-1017-1156
E-mail: mh_choi@kist.re.kr

This article is part of the special issue in honor of Dr. Bong Chul Chung.

of adrenal steroids of preeclampsia have not been extensively investigated to date.

Biologically active steroid hormones are mostly presented at trace levels in various biological specimens including blood and tissue samples. Due to similar chemical structures, their analytical selectivity can be hampered by matrix complexity derived from endogenous interference and isobaric components.[14] Therefore, levels of steroids should be calculated by accurate calibration curves with the correct weighting factor in addition to proper sample pretreatment protocols. In particular, a weighting factor of $1/x^2$ has been recently recommended for mass spectrometry-based bioanalytical methods[2,3] as it produces lower b value for lower background signal and better stability against unweighted linear calibration equation ($y = ax + b$).

Here, a comprehensive profiling of adrenal steroids using liquid chromatography-mass spectrometry (LC-MS) was applied to both serum and placenta samples obtained from 15 preeclampsia patients and 17 age-matched pregnant women. In conjunction with verification of quantitative results in accuracy by comparing two different linear regression models with weighting factor of 1 and $1/x^2$, altered metabolic signatures of preeclampsia were identified and their physiological roles were discussed.

Experimental

Reagents

Reference standards of adrenal steroids (Table 1) were purchased from Sigma (St. Louis, MO, USA) and Steraloids (Newport, RI, USA). Internal standards (9,11, 12,12,-d_4-F for eleven corticoids; 2,2,4,6,6,17α,21, 21,21,-d_9-Prog and 2,2,4,6,6,21,21,21,-d_8-17α-OHProg for eight progestogens; 16,16,17-d_3-T for three androgens; 2,2,3,4, 4,6-d_6-DHEA for DHEA; 16,16,17-d_3-TS for DHEA-S and

Table 1. Comparison of percent bias* at four concentrations with different weighting factors.

Compounds (abbreviation)	Unweighted ($w_i = 1$)				Weighted ($w_i = 1/x^2$)			
	LOQ	Low	Medium	High	LOQ	Low	Medium	High
Mineralocorticoids								
Pregnenolone (Preg)	-68.27	-20.12	-1.25	-1.84	4.21	5.13	-3.77	-6.56
Pregnenolone sulfate (Preg-S)	-56.47	-20.94	9.61	5.92	-2.63	-2.85	5.73	0.56
Progesterone (Prog)	-400.87	-70.32	6.97	5.42	-12.45	7.49	7.89	5.75
11-Deoxycorticosterone (DOC)	-91.74	-2.42	-0.19	-7.81	-2.95	11.26	-4.08	-11.65
Corticosterone (B)	-271.01	-52.67	9.94	3.61	-17.09	-2.20	10.53	3.47
18-Hydroxycorticosterone (18-OHB)	22.65	-3.04	-0.37	-1.52	17.99	-7.07	-4.67	-5.78
Tetrahydroaldosterone (THAldo)	-83.81	-14.01	4.66	-2.03	2.53	6.48	4.13	-3.12
Glucocorticoids								
17α-Hydroxypregnenolone (17α-OHPreg)	179.34	-42.44	11.60	9.78	-15.68	-8.97	2.42	-0.39
17α-Hydroxyprogesterone (17α-OHProg)	-91.73	-20.68	2.14	0.94	9.80	5.21	3.83	1.86
11-Deoxycortisol (11-deoxyF)	712.63	131.41	-5.22	-1.01	-6.98	-5.98	2.18	7.99
21-Deoxycortisol (21-deoxyF)	119.34	29.25	2.10	2.70	0.56	7.87	4.70	5.51
Cortisol (F)	213.80	-33.37	-2.29	0.24	14.98	4.85	-9.65	-7.99
Cortisone (E)	8.18	5.02	-11.44	-6.05	6.69	-0.57	-17.03	-12.00
Tetrahydrocortisol (THF)	248.07	66.34	-2.09	0.98	12.24	13.13	2.39	7.63
Allotetrahydrocortisol (allo-THF)	135.49	29.94	-2.68	0.36	11.75	12.13	2.60	8.05
Tetrahydrocortisone (THE)	297.59	69.37	-1.67	1.95	6.49	16.01	2.41	7.16
20α-Dihydrocortisol (20α-DHF)	852.91	173.66	-6.70	-7.07	16.58	9.37	-5.10	-4.18
18-Hydroxycortisol (18-OHF)	421.03	80.67	-2.76	-4.97	3.98	2.87	3.47	1.78
6β-Hydroxycortisol (6β-OHF)	119.55	35.28	-7.64	-12.72	-16.48	13.09	-2.87	-8.00
Androgens								
Dehydroepiandrosterone (DHEA)	100.81	-15.06	12.57	5.43	14.09	1.51	6.39	-1.93
Dehydroepiandrosterone sulfate (DHEA-S)	22.74	3.72	0.80	0.25	-4.43	0.25	2.70	2.59
Androstenedione (Adione)	516.30	-113.82	-3.12	-9.82	-14.34	-15.56	-5.12	-12.35
Testosterone (T)	739.38	-137.66	8.35	-2.55	9.27	1.40	-3.11	-13.73

*Values are expressed as mean of four experiments.

Preg-S; 17α,21,21,21,-d_4-Preg for Preg; and 21,21,21-d_3-17α-OHPreg for 17α-OHPreg) were obtained from C/D/N isotopes (Pointe-Claire, QC, Canada).

Stock solutions of all reference standards were prepared at a concentration of 1 mg/mL in a mixture of high-performance liquid chromatography (HPLC)-grade methanol and chloroform (9:1, *v/v*; Burdick & Jackson, Muskegon, MI, USA). Working solutions were prepared at concentrations ranging from 0.02 to 1 μg/mL. All standard solutions were stored at -20°C until used.

Study subjects and sample collection

Samples were obtained at 34-36 weeks of gestation. This study was approved (KUGH1644-002) by the medical ethics committee of Korea University Guro Hospital, Seoul, South Korea). Preeclampsia was diagnosed based on a blood pressure of at least 140/90 mmHg on two or more separate occasions and the development of proteinuria of at least 300 mg in a 24 h collection or the presence of greater than 2+ of protein on a catheterized urine specimen.

Biospecimens and data used in this study were provided by the Biobank of Korea University Guro Hospital, a member of the Korea. Blood samples were allowed to clot before centrifugation at 2,000 g for 10 min. All aliquots of serum were stored at -80°C. Biopsies were taken from the central region (1 cm^3) of placenta tissue on the maternal area. After washing maternal blood from the sample with saline, biopsy samples were immediately frozen in liquid nitrogen and stored at -80°C until use.

Analytical procedure

Quantitative profiling of adrenal steroids was performed based on a previous method.[15] In brief, serum sample (200 μL) was spiked with 20 μL of an internal standard mixture (d_4-F, d_8-17α-OHProg, and d_4-Preg, 0.2 μg/mL; d_9-Prog and d_3-17α-OHPreg, 0.1 μg/mL; d_3-TS, 1 μg/mL; d_3-T, 0.02 μg/mL; d_6-DHEA, 0.5 μg/mL). After dilution with 1.8 mL phosphate buffer (0.2 M, pH 7.2), the sample was incubated with 50 μL of β-glucuronidase at 55°C for 1 h. To reduce matrix interference including water soluble peptides and proteins, the hydrolyzed sample was loaded onto an Oasis HLB cartridge preconditioned with 4 mL of methanol and water, respectively. After washing the cartridge twice with 0.7 mL of 10% methanol, the sample was eluted twice with 1 mL of absolute methanol. Combined eluates were evaporated under a stream of nitrogen at 40°C. The dried extract was reconstituted with 50 μL of methanol and centrifuged at 14,000 rpm for 5 min using an Ultrafree-MC centrifugal filter. Then 50 μL of 10% dimethyl sulfoxide (DMSO) was added to the Ultrafree-MC filter and centrifuged at 14,000 rpm for 5 min. Finally, an aliquot (5 μL) was injected into the LC-MS system in both MRM and SIM modes.

Placental steroids were extracted according to a previous protocol.[16] Then 2 mg of placenta sample was spiked with 20 μL of IS mixtures (d_4-F, d_8-17α-OHProg, and d_4-Preg, 0.2 μg/mL; d_9-Prog and d_3-17α-OHPreg, 0.1 μg/mL; d_3-TS, 1 μg/mL; d_3-T, 0.02 μg/mL; d_6-DHEA, 0.5 μg/mL). After adding 0.6 mL of methanol, the mixture was sonicated for 20 min. The sample was then homogenized using a TissueLyser (Qiagen, Hilden, Germany) at 25 Hz for 5 min each with 3 zirconia beads twice and centrifuged at 12,000 rpm for 10 min. After transferring the supernatant to a glass tube, methanol was removed in a nitrogen stream at 40 °C and enzymatic hydrolysis step was conducted followed by the same procedures used for the serum assay.

Calibration and validation sets

Calibration sets of serum and placenta samples were prepared at nine and seven different concentrations, respectively, using negative control samples spiked with 23 steroids. Steroid-free serum and placenta samples were prepared as described previously[16,17] with minor modifications. Placental tissue samples (50 mg) were added to 1 mL of chloroform: methanol (1:1, *v/v*) and pulverized using a TissueLyser at 25 Hz for 5 min with 4 zirconia beads. Then, the sample was centrifuged at 12,000 rpm for 10 min and the solution was discarded twice. The sample was then mixed with 1 mL of chloroform: 0.6 M methanolic HCl (1:1, *v/v*), centrifuged at 12,000 rpm for 10 min, and the organic solvent was discarded twice. To remove the remaining HCl, the sample was washed with 1 mL of 20% ethanol five times and the sample was frozen at -80°C until needed.

Statistical analysis

All data analyses were performed using SPSS version 22.0 (SPSS Inc., Chicago, IL, USA) and Prism (v. 8; GraphPad Software Inc., San Diego, CA, USA). Parameters (slope and y-intercept) for both unweighted (w_i = 1) and weighted ($w_i = 1/x^2$) least squares ($y = ax + b$) were obtained with the following formulas on Excel 2016 spreadsheets (Microsoft Corp., Seattle, WA, USA):

$$\text{slope } (a_w) = \frac{\Sigma w_i \cdot \Sigma(w_i \cdot x_i \cdot y_i) - \Sigma(w_i \cdot x_i) \cdot \Sigma(w_i \cdot y_i)}{\Sigma w_i \cdot \Sigma(w_i \cdot x_i^2) - (\Sigma(w_i \cdot x_i))^2},$$

$$\text{y-intercept } (b_w) = \frac{\Sigma(w_i \cdot x_i^2) \cdot \Sigma(w_i \cdot y_i) - \Sigma(w_i \cdot x_i) \cdot \Sigma(w_i \cdot x_i \cdot y_i)}{\Sigma w_i \cdot \Sigma(w_i \cdot x_i^2) - (\Sigma(w_i \cdot x_i))^2}$$

Percent bias was calculated by dividing the bias by the theoretical value and multiplying by 100. Quantitative results are expressed as mean ± standard deviation (SD). Group differences were compared using the Mann-Whitney U test. *P* values of less than 0.05 were considered statistically significant.

Results

To validate the applicability of different linear regression models between unweighted (w_i = 1) and weighted (w_i = 1/

x^2) factors, quantitative results from LC-MS based profiling of adrenal steroids were compared with four test sample sets (Table 1). Percent biases in the unweighted model were inversely proportional to concentrations (-739.4–852.9% for LOQ; -137.7–173.7% for low; -11.4–12.6% for medium; -12.7–9.8% for high), while those in the weighted model were < 18% for all concentrations.

When both linear regression models were applied to clinical samples, the unweighted linear regression showed significantly increased serum levels for six adrenal steroids of Preg, 21-deoxyF, THE, 18-OHF, 6β-OHF, and DHEA ($P < 0.01$ for all, except THE and DHEA, $P < 0.04$) in

Table 2. Quantitative results* of serum adrenal steroids with different weighting factors.

Compounds	Unweighted ($w_i = 1$)			Weighted ($w_i = 1/x^2$)		
	Control ($n = 17$)	Preeclampsia ($n = 15$)	P value	Control ($n = 17$)	Preeclampsia ($n = 15$)	P value
Mineralocorticoids						
Preg	1.70 ± 1.26	2.98 ± 1.04	0.007	1.50 ± 1.23	2.18 ± 0.99	0.044
Preg-S	54.64 ± 39.15	52.61 ± 23.99	0.911	59.33 ± 44.19	55.21 ± 25.87	1.000
Prog	107.87 ± 65.61	127.76 ± 39.19	0.350	114.88 ± 70.07	128.26 ± 39.19	0.602
DOC	2.65 ± 0.13	0.76 ± 0.52	<0.0001	0.29 ± 0.14	0.23 ± 0.10	0.331
B	5.97 ± 4.15	7.40 ± 12.52	0.176	6.33 ± 4.20	7.70 ± 11.47	0.390
18-OHB	1.25 ± 0.94	1.07 ± 1.07	0.576	1.35 ± 0.94	0.96 ± 0.98	0.216
THAldo	1.97 ± 1.19	1.89 ± 1.48	0.655	2.01 ± 1.19	1.59 ± 1.42	0.153
Glucocorticoids						
17α-OHPreg	4.16 ± 2.53	4.06 ± 3.01	0.576	4.34 ± 2.45	3.95 ± 2.86	0.278
17α-OHProg	5.55 ± 3.74	6.26 ± 3.48	0.655	5.74 ± 3.83	6.36 ± 3.33	0.710
11-deoxyF	3.20 ± 1.31	3.03 ± 1.05	0.852	1.79 ± 1.38	1.35 ± 1.18	0.313
21-deoxyF	6.96 ± 2.02	10.57 ± 4.21	0.008	4.86 ± 2.39	8.61 ± 4.94	0.014
F	197.22 ± 70.88	194.57 ± 171.64	0.189	187.68 ± 67.53	200.38 ± 178.42	0.370
E	41.43 ± 14.14	42.29 ± 16.18	0.737	42.57 ± 14.59	45.02 ± 17.63	0.628
THF	10.13 ± 4.45	13.70 ± 6.41	0.097	8.94 ± 4.86	12.45 ± 7.20	0.202
allo-THF	10.18 ± 5.86	10.66 ± 4.89	0.941	9.49 ± 6.05	9.74 ± 5.09	0.882
THE	10.17 ± 5.08	16.97 ± 9.25	0.013	5.60 ± 2.91	10.07 ± 5.59	0.008
20α-DHF	9.14 ± 2.87	9.28 ± 4.99	0.502	8.43 ± 2.90	7.72 ± 5.38	0.142
18-OHF	0.93 ± 0.24	2.17 ± 0.46	<0.0001	0.51 ± 0.24	0.57 ± 0.33	0.602
6β-OHF	2.00 ± 1.04	2.89 ± 0.73	0.001	1.72 ± 0.98	1.38 ± 0.64	0.331
Androgens						
DHEA	6.36 ± 9.91	6.82 ± 4.41	0.037	6.98 ± 8.91	6.01 ± 4.14	0.370
DHEA-S	468.21 ± 276.24	562.29 ± 331.40	0.478	534.53 ± 317.12	598.48 ± 351.60	0.576
Adione	3.86 ± 2.02	6.89 ± 6.33	0.076	3.51 ± 1.89	6.29 ± 5.74	0.076
T	1.18 ± 0.63	1.95 ± 1.86	0.478	0.99 ± 0.58	1.78 ± 1.70	0.278
Metabolic ratios						
Preg-S/Preg	42.66 ± 32.63	18.69 ± 6.35	0.003	65.48 ± 69.83	28.44 ± 10.85	0.027
Prog/Preg	85.48 ± 92.33	47.28 ± 20.83	0.313	128.75 ± 189.35	68.66 ± 31.26	0.602
DOC/Prog	78.18 ± 126.92	6.63 ± 5.43	<0.0001	4.75 ± 5.39	1.91 ± 0.81	0.018
6β-OHF/F	1.04 ± 0.33	2.14 ± 1.23	<0.001	0.93 ± 0.31	0.85 ± 0.35	0.710
18-OHF/F	0.51 ± 0.14	1.67 ± 1.12	<0.0001	0.27 ± 0.09	0.33 ± 0.17	0.331
F/11-deoxyF	69.29 ± 36.61	59.29 ± 31.17	0.455	254.87 ± 352.46	187.21 ± 149.66	0.370
F/21-deoxyF	30.67 ± 14.99	17.52 ± 9.47	0.006	50.29 ± 36.53	24.64 ± 14.44	0.011
F/E	4.93 ± 1.75	4.26 ± 2.04	0.064	4.57 ± 1.62	4.12 ± 1.98	0.114
(THF+allo-THF)/THE	2.41 ± 1.50	1.61 ± 0.58	0.030	4.01 ± 2.83	2.41 ± 0.92	0.027

* Results are expressed as ng/mL in concentration.

Table 3. Quantitative results* of placental adrenal steroids with different weighting factors.

Compounds	Unweighted ($w_i = 1$)			Weighted ($w_i = 1/x^2$)		
	Control ($n = 17$)	Preeclampsia ($n = 15$)	P value	Control ($n = 17$)	Preeclampsia ($n = 15$)	P value
Mineralocorticoids						
Preg	395.77 ± 368.70	262.97 ± 236.52	0.455	395.77 ± 368.70	255.69 ± 236.55	0.411
Prog	9760.66 ± 6163.19	13499.90 ± 7458.29	0.216	4473.59 ± 2755.01	4899.97 ± 2638.40	0.628
DOC	41.23 ± 32.80	72.20 ± 42.44	0.064	33.28 ± 29.27	35.84 ± 33.98	0.941
Glucocorticoids						
17α-OHProg	104.19 ± 49.51	119.90 ± 65.31	0.551	69.37 ± 55.93	100.42 ± 68.71	0.142
11-deoxyF	43.35 ± 32.97	30.25 ± 22.21	0.132	28.94 ± 29.37	25.38 ± 20.83	1.000
E	390.59 ± 197.58	433.12 ± 230.92	0.710	366.91 ± 206.19	421.24 ± 241.55	0.602
Androgens						
Adione	35.74 ± 34.90	25.49 ± 13.25	0.911	42.92 ± 34.91	19.67 ± 14.90	0.030
Metabolic ratios						
DOC/Prog	5.06 ± 4.43	5.01 ± 2.30	0.628	8.84 ± 8.07	6.33 ± 3.21	0.737
17α-OHProg/Prog	18.42 ± 19.63	12.04 ± 9.73	0.433	23.76 ± 23.04	23.24 ± 14.57	0.628
Adione/17α-OHProg	3.47 ± 3.58	2.74 ± 2.28	0.576	7.66 ± 6.04	3.59 ± 5.50	0.002

* Results are expressed as ng/g in concentration.

preeclampsia patients, whereas DOC was remarkably decreased ($P < 0.0001$). In contrast, the $1/x^2$ regression model resulted in altered levels of only two steroids (Preg, 21-deoxyF and THE; $P < 0.05$ for all) among seven in the unweighted linear regression (Table 2).

Metabolic ratios were also calculated based on quantities of individual steroids corresponding to related enzyme activities. Altered metabolic ratios of both 6β-OHF/F and 18-OHF/F were found ($P < 0.001$ for both) in only the unweighted linear regression (Table 2). Other metabolic ratios of Preg-S/Preg ($P = 0.003$ for $w_i = 1$ and $P = 0.027$ for $w_i = 1/x^2$), DOC/Prog ($P < 0.0001$ vs. $P = 0.018$), F/21-deoxyF ($P = 0.006$ vs. $P = 0.011$), and (THF+allo-THF)/THE ($P = 0.011$ vs. $P < 0.005$) were significantly changed in both regression models.

Seven adrenal steroids were detected in placenta. They showed no statistically significant differences in either regression model except that levels of Adione were decreased ($P = 0.030$) in preeclamptic patients with the weighted linear regression model (Table 3). Metabolic ratio of Adione/17α-OHProg was also decreased in preeclampsia ($P = 0.002$), indicating 17,20-lyase activity.

Discussion

Functional steroidogenesis is essential in pregnancy and its preeclamptic changes have been extensively studied.[7,8,18,19] Some discrepancies in quantitative findings could be linked to sampling and methodological issues. The devised weighted linear regression with weighting factor of $1/x^2$ in LC-MS based quantitative profiling suggests that metabolic signatures of adrenal steroids in serum and placenta might provide reliable physiological indicators for preeclampsia at late pregnancy weeks of 34~36 in the third trimester.

The unweighted linear regression showed unacceptable percent biases for most steroids analyzed at low concentrations (Table 1). In particular, both 6β-OHF and 18-OHF are relatively high polar compound with fast elution order and these molecules could be easily interfered chromatographically by matrix effects during elution, which cause increase background signal (higher b value) and produce inaccurate quantitative outcomes in unweighted linear calibration equation ($y = ax + b$) at low concentrations. In general the variance of each data point may be quite different when the range in x-values is large, but the simple regression model considers that all the y-values have equal variances. Larger deviations at larger levels tend to affect the regression line more than smaller deviations correlated with smaller concentration (heteroscedasticity) resulting in the inaccuracy in the lower end of the calibration range.[1] In method validation in this study (Table 4), the calibration linearities with the unweighted model were better than those calculated based on the weighted. The linear regression equation usually produces high calibration linearity ($r > 0.097$) and lower accuracy than 5%, but the straight-line model was systematically rejected at the 95% confidence level on the basis of the Lack-of-fit and Mandel's fitting test. The quantitative results obtained from the quadratic regression model did not differ significantly from the theoretical values, whereas those derived from the linear regression model were systematically biased.[20]

Serum results obtained between two different weighting

Table 4. Method validation for 27 adrenal steroids in placental tissue.

Compounds	LOQ[a] (ng/mL)	Calibration range (ng/g)	Linearity (r^2)		Precision[b] (%CV)	Accuracy[b] (%bias)	Recovery[C] (%)
			Unweighted (w_i = 1)	Weighted (w_i = $1/x^2$)			
Mineralocorticoids							
Preg	2.0	2.0-500	0.999	0.996	4.8	99.8	57.7
Preg-S	2.0	2.0-500	1.000	0.997	5.2	102.7	65.0
Prog	5.0	5.0-5000	1.000	0.958	5.9	102.8	53.6
DOC	0.2	0.2-200	1.000	0.973	8.4	101.0	83.0
B	0.2	0.2-200	0.999	0.974	11.3	98.6	113.4
18-OHB	0.5	0.5-200	1.000	0.937	8.7	96.3	118.8
Aldo	2.0	2.0-200	0.999	0.940	6.9	97.1	86.9
THAldo	0.5	0.5-200	0.999	0.980	8.7	99.2	88.8
Glucocorticoids							
17α-OHPreg	0.5	0.5-200	1.000	0.970	14.3	100.7	113.2
17α-OHProg	0.5	0.5-200	0.999	0.985	6.2	102.9	100.2
11β-OHP	0.5	0.5-200	0.999	0.991	8.0	99.8	98.9
11-deoxyF	0.1	0.1-200	1.000	0.990	8.6	101.2	81.8
21-deoxyF	0.1	0.1-200	1.000	0.989	7.5	101.5	99.1
THS	5.0	5.0-200	1.000	0.992	13.1	103.8	88.5
F	0.2	0.2-500	0.999	0.978	7.8	102.6	97.9
E	0.2	0.2-500	1.000	0.970	8.0	98.9	81.0
THF	0.5	0.5-200	0.999	0.940	10.0	113.1	100.2
allo-THF	1.0	1.0-200	1.000	0.980	12.3	102.7	102.5
THE	0.2	0.2-200	1.000	0.984	4.1	106.9	81.2
20α-DHF	0.1	0.1-200	0.999	0.986	6.3	102.0	110.4
18-OHF	0.1	0.1-200	1.000	0.997	6.3	100.3	110.1
6β-OHF	0.1	0.1-200	1.000	0.981	9.5	101.9	100.2
Androgens							
DHEA	1.0	1.0-200	0.999	0.994	5.5	100.5	75.8
DHEA-S	1.0	1.0-200	1.000	0.997	3.7	100.0	81.4
Adione	0.1	0.1-200	1.000	0.978	11.1	96.9	81.7
T	0.1	0.1-200	1.000	0.986	12.6	96.9	80.3
DHT	0.2	0.2-200	1.000	0.991	4.8	105.3	82.6

[a] The limit of quantification was measured according to an S/N ratio > 10.

[b] Precision and accuracy are expressed as the mean values of data obtained from four different concentrations based on the weighted calibration linearities.

[c] Recovery is expressed as the mean values of data obtained from three different concentrations.

factors were comparable in quantitative outcomes (Table 2). DOC is one intermediate in aldosterone biosynthesis. Its excessive production may lead to mineralocorticoid-derived hypertension that can occur in patients with 11β-hydroxylase or 17α-hydroxylase deficiencies, congenital adrenal hyperplasia, DOC-producing adrenal tumors, or in patients taking 11β-hydroxylase inhibitors.[21–23] However, plasma levels of DOC were not associated with pregnancy-induced hypertension.[8,24] Urinary DOC levels are not significantly different between preeclampsia and control.[25] Here, DOC serum level was remarkably decreased in preeclampsia patients, leading to a decreased DOC/Prog ratio known to be responsible for 21-hydroxylase activity. However, those were not significant in the weighted model (Table 2).

Serum levels of both 6β-OHF and 18-OHF in preeclampsia were significantly increased in the unweighted model, but not changed in the weighted model (Table 2). In our previous

study, serum 6β-OHF in the third trimester pregnant women with preeclampsia was found to be increased than that in the control.[18] Such discrepancy in results might also be caused by different linear regression models used. As one of hybrid adrenal steroids, 18-OHF showed increased serum level in patients with primary aldosteronism, a leading cause of secondary hypertension. It can also be used to differentiate subtyping of adrenal diseases.[26] However, no experimental findings with maternal 18-OHF have been reported in preeclampsia to date. According to metabolic enzymes of both hydroxylated cortisols to cortisol, 6β- and 18-hydroxylases may not also remarkable with preeclampsia at 34~36 gestational weeks in the weighted model.

Preeclampsia patients presented significantly higher serum levels of Preg, 21-deoxyF, and THE than those of controls in both linear regression models (Table 2). Blood levels of Preg in preeclampsia patients did not differ from those in controls,[8,27] consistent with our data. However, previous studies used blood samples collected at the second trimester of pregnancy 24~29 weeks,[8] while we collected samples at the third trimester. They also measured levels of serum Preg using a competitive enzyme immunoassay known to cause overestimation by cross-reactivity with endogenous steroids.[27] Consistent with a previous finding showing increased Preg in maternal blood,[7] increased serum levels of Preg in preeclampsia ($P < 0.05$ for both unweighted and weighted models) in the present study affected the metabolic ratio of Preg-S/Preg which was remarkably decreased regardless of weighting factors ($P < 0.03$ for both linear regression models).

Although serum cortisol levels were not significantly different between patients with preeclampsia and controls in the present study, consistent with results in maternal plasma,[8] its precursor 21-deoxyF and one of 5β-reduced metabolites, THE, were remarkably increased in patients with preeclampsia ($P < 0.02$ for both weighting factors; Table 2). These findings may reveal metabolic signatures of cortisol in preeclampsia. Levels of cortisol 21-hydroxylase as a metabolic enzyme represented by F/21-deoxyF ratio were decreased in preeclampsia patients, while levels of another cortisol biosynthetic pathway from 11-deoxyF were not significantly different between patients with preeclampsia and controls ($P = 0.455$ in unweighted model and $P = 0.370$ in weighted model). In general, maternal regulation of 11β-hydroxysteroid dehydrogenase (11β-HSD) can protect the fetus from excessive glucocorticoids and its type 2 (11β-HSD2) can convert active cortisol to inactive cortisone.[28] The activity of 11β-HSD2 can be also expressed by the metabolic ratio of (THF+allo-THF)/THE.[29] Normotensive pregnant women maintain inactivation of placental cortisol. The metabolic ratio of (THF+allo-THF)/THE in maternal sera of preeclampsia patients was decreased in the present study. Such result is expected for the placenta.[28,29]

In the placenta, there were no compatible results between unweighted and weighted models (Table 3). In contrast to an increased tendency of placental DOC level in patients with preeclampsia in the unweighted model ($P = 0.064$), it was not statistically significant in the weighted regression ($P = 0.941$) which might be due to matrix interference. DOC concentrations between serum and placenta were also not correlated in both patients and controls ($r < 0.18$, $P > 0.6$). Due to its curative effect on preeclampsia model,[30] increased levels of placental 17α-OHProg in preeclampsia patients need to be confirmed in further experiment. Adione in rat placenta could be a predominant androgen for ovarian production of estrogens during pregnancy.[31] It is mainly produced via Δ^4-steroidogenic pathway.[32] However, its metabolic activity in human placenta has remained unclear to date, while CYP17 mRNA and protein expression have recently been found in human trophoblasts.[33] The CYP17A1 gene regulates two distinct steroidogenic enzymes of 17α-hydroxylase and 17,20-lyase, although the association between its polymorphism and the risk of preeclampsia has been controversial.[34,35] In addition to decreased placental levels of Adione in preeclampsia in the weighted model ($P = 0.030$), the placental metabolic ratio of Adione/17α-OHProg indicating 17,20-lyase

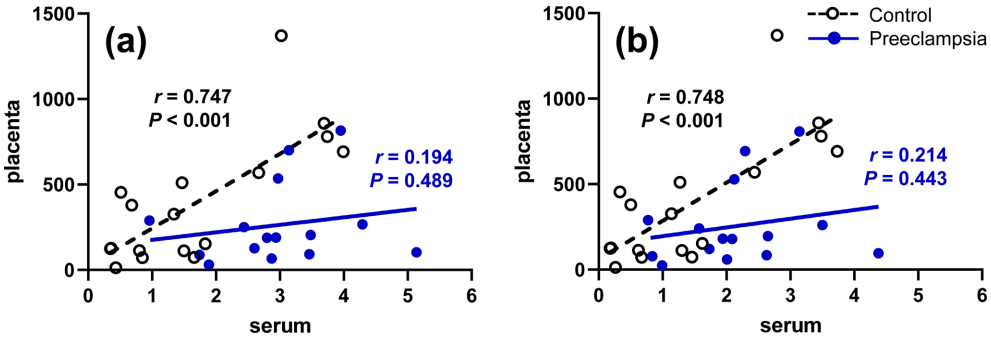

Figure 1. Scatter plots with linear fitted curves and Pearson correlations between serum and placental pregnenolone levels. Both (a) unweighted and (b) weighted regression models showed positive correlations between serum and placenta pregnenolone concentrations ($P < 0.001$ for both), whereas those of preeclampsia patients showed no significant correlations (blue colored).

was significantly decreased in preeclampsia patients (P = 0.002), while 17α-hydroxylase activity expressed by 17α-OHProg/Prog ratio was not different between patients and controls (Table 3).

Correlations of adrenal steroids between serum and placenta were not observed in wither the unweighted or the weighted model (data not shown). In contrast, positive correlations of pregnenolone between serum and placenta levels were clearly found in healthy pregnant women (r = 0.747 and 0.748, $P < 0.001$ in both linear regression experiments; Fig. 1). This might imply metabolic disturbance in pregnenolone production at late gestational weeks of preeclampsia. Dysfunction of 11β-HSD2 is a hallmark of preeclampsia physiology.[28] Maternal serum metabolic ratio of (THF+allo-THF)/THE was significantly decreased in preeclampsia patients. It may indicate that the fetus is not protected from maternal glucocorticoid excess. In the placenta, decreased 17,20-lyase activity may explain the lack of androgen production in patients with preeclampsia.

This study has some limitations. First, the number of subjects was relatively small. Therefore, covariables such as age and BMI were not included in our statistical analyses. However, quantitative results were compared between age-match groups. In addition, gestational diabetes as one of serious complications of pregnancy known to cause increased blood pressure was excluded from the preeclampsia group. Second, only seven adrenal steroids were detected in placenta against 23 serum steroids monitored. In addition, associations between serum and placenta steroid signatures were not fully investigated. Third, amniotic concentrations of adrenal steroids were not compared for both maternal serum and placenta to fully understand the maternal-placental-fetal axis in preeclampsia physiology.

Despite these limitations, the present work successfully provided reliable quantification of adrenal steroids from both serum and placenta samples with minimized matrix interference. It was achieved by introducing a correct weighting factor of $1/x^2$ for linear calibration curve combined with comprehensive LC separation and selective MS detection assay. Comparative metabolic signatures of adrenal steroids in both serum and placenta clearly revealed impaired steroidogenesis in preeclampsia at late gestational weeks of 34~36 in the third trimester.

Conclusions

An optimized LC-MS coupled to corrected linear regression approach revealed metabolic signatures of adrenal steroids in preeclampsia based on exact quantitative outcomes. Based on individual quantity of adrenal steroids, altered steroidogenic activity of 11β-HSD2 was found in maternal serum. In addition, 17,20-lyase deficiency was observed in placenta of preeclampsia patients. However, large-scale randomized trials are needed to confirm these findings.

Acknowledgement

This study was supported by a grant from the Korea Institute of Science and Technology Institutional Program (Project No. 2E31093) and the Bio & Medical Technology Development Programs (NRF-2016M3A9B6902060) through the Ministry of Science and ICT.

References

1. Almeida, A. M.; Castel-Branco, M. M.; Falcão, A. C. *J. Chromatogr. B* **2002**, 774, 215, DOI: 10.1016/s1570-0232(02)00244-1.
2. Gu, H.; Liu, G.; Wang, J.; Aubry, A. F.; Arnold, M. E. *Anal. Chem.* **2014**, 86, 8959, DOI: 10.1021/ac5018265.
3. Kim, J. Y. *Yakhak Hoeji* **2016**, 60, 304, DOI: 10.17480/psk.2016.60.6.304.
4. Ghulmiyyah, L.; Sibai, B. *Semin. Perinatol.* **2012**, 36, 56, DOI: 10.1053/j.semperi.2011.09.011.
5. Mol, B. W. J.; Roberts, C. T.; Thangaratinam, S.; Magee, L. A.; de Groot, C. J. M.; Hofmeyr, G. J. *Lancet* **2016**, 387, 999, DOI: 10.1016/S0140-6736(15)00070-7.
6. Duthie, L.; Reynolds, R. M. *Neuroendocrinology* **2013**, 98, 106, DOI: 10.1159/000354702.
7. Hertig, A.; Liere, P.; Chabbert-Buffet, N.; Fort, J.; Pianos, A.; Eychenne, B.; Cambourg, A.; Schumacher, M.; Berkane, N.; Lefevre, G. *Am. J. Obstet. Gynecol.* **2010**, 203, 477, DOI: 10.1016/j.ajog.2010.06.011.
8. Berkane, N.; Liere, P.; Lefevre, G.; Alfaidy, N.; Nahed, R. A.; Vincent, J.; Oudinet, J. P.; Pianos, A.; Cambourg, A.; Rozenberg, P.; Galichon, P.; Rousseau, A.; Simon, T.; Schumacher, M.; Chabbert-Buffet, N.; Hertig, A. *Placenta* **2018**, 69, 40, DOI: 10.1016/j.placenta.2018.07.004.
9. Uddin, M. N.; Horvat, D.; Jones, R. O.; Beeram, M. R.; Zawieja, D. C.; Perger, L.; Sprague, D. C. C.; Kuehl, T. J. *J. Matern. Fetal Neonatal Med.* **2015**, 28, 1296, DOI: 10.3109/14767058.2014.951627.
10. Vianna, P.; Bauer, M. E.; Dornfeld, D.; Chies, J. A. B. *Med. Hypotheses* **2011**, 77, 188, DOI: 10.1016/j.mehy.2011.04.007.
11. McCalla, C. O.; Nacharaju, V. L.; Muneyyirci-Delale, O.; Glasgow, S.; Feldman, J. G. *Steroids* **1998**, 63, 511, DOI: 10.1016/s0039-128x(98)00056-7.
12. Kosicka, K.; Siemiątkowska, A.; Szpera-Goździewicz, A.; Krzyścin, M.; Bręborowicz, G. H.; Główka, F. K. *Endocrine* **2018**, 61, 125, DOI: 10.1007/s12020-018-1586-4.
13. Jayasuriya, N. A.; Hughes, A. E.; Sovio, U.; Cook, E.; Charnock-Jones, D. S.; Smith, G. C. S. *J. Clin. Endocrinol. Metab.* **2019**, 104, 2355, DOI: 10.1210/jc.2018-02312.
14. Keevil, B. G. *Clin. Biochem*. **2016**, 49, 989, DOI: 10.1016/j.clinbiochem.2016.04.009.
15. Lee, C.; Kim, J. H.; Moon, S. J.; Shim, J.; Kim, H. I.; Choi, M. H. *J. Steroid Biochem. Mol. Biol.* **2020**, 198, 105615, DOI: 10.1016/j.jsbmb.2020.105615.

16. Moon, J. Y.; McNamara, K. M.; Lee, J. J.; Chung, B. C.; Sasano, H.; Choi, M. H. *J. Steroid Biochem. Mol. Biol.* **2018**, 178, 185, DOI: 10.1016/j.jsbmb.2017.12.012.
17. Lee, S. H.; Lee, N.; Hong, Y.; Chung, B. C.; Choi, M. H. *Anal. Chem*. **2016**, 88, 11624, DOI: 10.1021/acs.analchem.6b03183.
18. Moon, J. Y.; Moon, M. H.; Kim, K. T.; Jeong, D. H.; Kim, Y. N.; Chung, B. C.; Choi, M. H. *J. Steroid Biochem. Mol. Biol*. **2014**, 139, 182, DOI: 10.1016/j.jsbmb.2013.02.014.
19. Perez-Sepulveda, A.; Monteiro, L. J.; Dobierzewska, A.; España-Perrot, P. P.; Venegas-Araneda, P.; Guzmán-Rojas, A. M.; González, M. I.; Palominos-Rivera, M.; Irarrazabal, C. E.; Figueroa-Diesel, H.; Varas-Godoy, M.; Illanes, S. E. *PLoS One* **2015**, 10, e0139682, DOI: 10.1371/journal.pone.0139682. eCollection 2015.
20. Loco, J. V.; Elskens, M.; Croux, C.; Beernaert, H. *Accred. Qual. Assur*. **2002**, 7, 281, DOI: 10.1007/s00769-002-0487-6.
21. Irony, I.; Biglieri, E. G.; Perloff, D.; Rubinoff, H. *J. Clin. Endocrinol. Metab*. **1987**, 65, 836, DOI: 10.1210/jcem-65-5-836.
22. Biglieri, E. G.; Kater, C. E. *Clin. Chem*. **1991**, 37, 1843, DOI: 10.1093/clinchem/37.10.1843.
23. Marques, P.; Tufton, N.; Bhattacharya, S.; Caulfield, M.; Akker, S. A. *Endocrinol. Diabetes Metab. Case Rep*. **2019**, 18-0164, DOI: 10.1530/EDM-18-0164.
24. Walker, B. R.; Williamson, P. M.; Brown, M. A.; Honour, J. W.; Edwards, C. R. W.; Whitworth, J. A. *Hypertension* **1995**, 25, 626, DOI: 10.1161/01.hyp.25.4.626.
25. Shah, D. M.; Gomez-Sanchez, C. E.; Knuppel, R. A.; Vaughn, W. K. *Am. J. Perinatol*. **1987**, 4, 102, DOI: 10.1055/s-2007-999749.
26. Lenders, J. W. M.; Williams, T. A.; Reincke, M.; Gomez-Sanchez, C. E. *Eur. J. Endocrinol*. **2018**, 178, R1, DOI: 10.1530/EJE-17-0563.
27. Shin, Y. Y.; Jeong, J. S.; Park, M. N.; Lee, J. E.; An, S. M.; Cho, W. S.; Kim, S. C.; An, B. S.; Lee, K. S. *Mol. Med. Rep*. **2018**, 17, 2681, DOI: 10.3892/mmr.2017.8165.
28. Wang, G.; Huang, Y.; Hu, T.; Zhang, B.; Tang, Z.; Yao, R.; Huang, Y.; Fan, X.; Ni, X. *FASEB J*. **2020**, 34, 15379, DOI: 10.1096/fj.202001003RR.
29. Aufdenblatten, M.; Baumann, M.; Raio, L.; Dick, B.; Frey, B. M.; Schneider, H.; Surbek, D.; Hocher, B.; Mohaupt, M. G. *Pediatr. Res*. **2009**, 65, 198, DOI: 10.1203/PDR.0b013e31818d6c24.
30. Mohammed, N. A.; Ali, A. H.; El-Tahawy, N. F. G.; Rifaai, R. A. *J. Cytol. Histol*. **2018**, 9, 1, DOI: 10.4172/2157-7099.1000496.
31. Jackson, J. A.; Albrecht, E. D. *Biol. Reprod*. **1985**, 33, 451, DOI: 10.1095/biolreprod33.2.451.
32. Warshaw, M. L.; Johnson, D. C.; Khan, I.; Eckstein, B.; Gibori, G. *Endocrinology* **1986**, 119, 2642, DOI: 10.1210/endo-119-6-2642.
33. Escobar, J. C.; Patel, S. S.; Beshay, V. E.; Suzuki, T.; Carr ,B. R. *J. Clin. Endocrinol. Metab*. **2011**, 96, 1385, DOI: 10.1210/jc.2010-2504.
34. Lim, J. H.; Kim, S. Y.; Kim, D. J.; Park, S. Y.; Han, H. W.; Han, J. Y.; Lee, S. W.; Yang, J. H.; Ryu, H. M. *Pharmacogenet. Genom*. **2010**, 20, 605, DOI: 10.1097/FPC.0b013e32833df033.
35. Hou, B.; Jia, X.; Deng, Z.; Liu, X.; Liu, H.; Yu, H.; Liu, S. *Hum. Genom*. **2020**, 14, 33, DOI: 10.1186/s40246-020-00286-0.