Steroids 164 (2020) 108732

Contents lists available at ScienceDirect

Steroids

journal homepage: www.elsevier.com/locate/steroids

Measurement of steroid hormones by liquid chromatography-tandem mass spectrometry with small amounts of hair

Yoshitaka Hobo [1,*], Jun Nishikawa, Yoshimichi Miyashiro, Akira Fujikata

ASKA Pharmaceutical Medical Co., Ltd., 5-36-1 Shimosakunobe, Kawasaki Takatsu-ku, Kanagawa 213-8522, Japan

ARTICLE INFO

Keywords:
Steroid hormones
Hair strands
LC-MS/MS

ABSTRACT

Steroid hormone levels in hair reflect the integrated values (average values) of hormone secretion over the past few months. We have used a method to evaluate diseases and chronic stress, discrimination of banned drug use, and so on. In contrast, the hair analysis methods reported so far required at least 10 mg (about 50 to 100 hair strands) of hair to analyze multiple steroid hormones from the same sample. Here, we developed a new method for measuring steroid hormones in hair by liquid chromatography-tandem mass spectrometry, which identifies multiple steroid hormones from 5 to 10 (about 1 mg) hair strands. Ten steroid hormones (cortisol, cortisone, testosterone, dihydrotestosterone, dehydroepiandrosterone, androstenedione, progesterone, pregnenolone, androstenediol and estradiol) covering from sex hormones to stress hormones were derivatized and measured by four different measuring systems. The method showed good linearity for all steroids with correlation coefficients of 0.999 or more. The accuracy and precision of intra- and inter-assay ranged from 96.0 to 106.4% and 4.8 to 8.1% for intra-assay, and from 96.9 to 104.9% and 6.9 and 10.6% for inter-assay, respectively. A mixed solution containing 0.1 M trifluoroacetic acid and 50% acetonitrile was used to extract hair and to enhance the cortisol extraction efficiency approximately twice compared to the previously reported extraction with methanol. This method has the potential to clarify the relationship between steroid hormone levels and diseases that show alopecia such as chronic stress and androgenetic alopecia.

1. Introduction

Steroid hormones are biologically active substances having important roles in sexual maturation, sugar metabolism, and maintenance of homeostasis. Since the endogenous concentrations of steroid hormones reflect various physiological and pathological conditions, they are also used as a biomarker [1–3]. In most studies, blood (plasma, serum) saliva [4,5] and urine [6] were used frequently as samples for measurement. Recently, the number of studies on steroid hormones in hair has increased. Because the concentrations in a hair show the integrated values (average values) of hormone secretion over the past period [7,8] hair samples are highly useful compared with the conventional samples such that they cannot be influenced by a circadian rhythm of secretion [9] and can be collected non-invasively.

While the analysis of steroid hormones in hair was initially investigated as a method of measuring androgen concentrations for sports doping [10–12] now was subsequently applied for evaluating chronic stress by measuring of glucocorticoids in hair [13–15]. The most famous study was the report by Manenschijn et al., which showed that cortisol in hair was significantly correlated with cardiovascular diseases [16] and occupational stress [17].

Besides, the relationship between diseases that show symptoms of hair loss, including androgenetic alopecia (AGA) and steroid hormone concentrations in hair has not been fully clarified [18,19]. To elucidate the pathology of such diseases, it is necessary to measure multiple steroid hormones simultaneously covering from sex hormones (testosterone, dihydrotestosterone, and estradiol) to stress hormones (cortisol, cortisone).

In previous studies [16,17,20–23], 10 mg of hair was used for measurements. This is equivalent to about 50 to 100 hair strands when using 3 cm of them from the scalp [21]. Although hair is a non-invasive sample, 100 hair strands could be a burden for the participants. To minimize the burden, reducing the sampling amount to 1/10 of the previous research is desirable.

To overcome such problems, we have established a method that can simultaneously measure multiple steroid hormones even in a small

* Corresponding author.
E-mail address: hobou@ap-med.co.jp (Y. Hobo).
[1] ORCID ID: 0000-0002-5921-4287.

https://doi.org/10.1016/j.steroids.2020.108732
Received 26 December 2019; Received in revised form 24 August 2020; Accepted 15 September 2020
Available online 22 September 2020

amount of a hair sample. The method comprised the combination of the following three parts: (1) use of a novel, highly efficient extraction solvent instead of methanol [22], (2) derivatization of steroids with picolinic acid and fusaric acid, and (3) high-resolution and high-sensitivity analysis using 2D-LC.

Here, for evaluating chronic stress and AGA, we developed a new method for measuring steroid hormones in hair by liquid chromatography-tandem mass spectrometry (LC-MS/MS) of which the required sample amount was 5 to 10 hair strands (about 1 mg).

2. Material and methods

2.1. Chemicals and reagents

Testosterone (T), dihydrotestosterone (DHT), cortisone (E), dehydroepiandrosterone (DHEA), progesterone (P4), androstenedione (A-dione), pregnenolone (P5), and estradiol (E2) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cortisol (F) was purchased from NIST (Maryland, USA). Both androstenediol (Δ5-A-diol) and pentafluoropyridine were purchased from Acros Organics (Geel, Belgium). T-$^{13}C_3$, DHT-$^{13}C_3$, and E-$^{13}C_3$ were purchased from ISO SCIENCE (King of Prussia, PA, USA). F-d_4 and P5-d_4 were purchased from C / D / N Isotopes Inc. (Quebec, Canada). E2-$^{13}C_4$ and P4-$^{13}C_3$ were obtained from Hayashi Pure Chemical Industries Ltd. (Osaka, Japan). DHEA-$^{13}C_3$, A-dione-$^{13}C_3$, and Δ5-A-diol-d4 were synthesized at ASKA Pharmaceuticals Co. Ltd. (Kanagawa, Japan). 4-dimethylaminopyridine (DMAP), 2-methyl-6-nitrobenzoic anhydride (MNBA), picolinic and fusaric acids were obtained from Tokyo Chemical Industry (Tokyo, Japan). Triethylamine, trifluoroacetic acid (TFA), methanol, 1 M sodium hydroxide solution (1 M NaOH) and acetonitrile were purchased from FUJIFILM Wako Pure Chemical Industries (Osaka, Japan). Pyridine and acetic acid were purchased from Nacalai tesque (Kyoto Japan). OASIS MAX cartridge (60 mg, 3 mL) was purchased from Waters (Milford, MA, USA). HyperSep SI cartridge (500 mg, 3 mL) was purchased from Thermo Fisher Scientific (Waltham, MA, USA). All other reagents used for doing experiments were of a special grade or its equivalent.

2.2. Instrumentation

NEXERA LC-30AD (Shimadzu, Kyoto, Japan) and Agilent 1290 Infinity LC (Agilent, Santa Clara, CA), both were used for the HPLC system. API5000 and API4000 (AB SCIEX, Framingham, MA, USA) were used for the triple quadrupole tandem mass spectrometer. The system was controlled by Analyst ver.1.6 (AB SCIEX). The analytical columns were used: Kinetex C18 (1.7 μm, 150 × 2.1 mm id) from Phenomenex (Torrance, CA, USA), CAPCELL CORE PFP (2.7 μm, 50 × 2.1 mm id), CAPCELL CORE ADME (2.7 μm, 150 × 2.1 mm id), CAPCELL CORE C18 (2.7 μm, 150 × 3.0 mm id), and CAPCELL PAK ADME (2 μm, 100 × 2.1 mm id) from Osaka Soda (Osaka, Japan), and Cortecs C18 (1.6 μm, 150 × 2.1 mm id) from Waters (Milford, MA, USA).

2.3. Chromatographic conditions

2.3.1. F and E

An analysis was performed with heart-cutting 2D-LC-MS/MS using Agilent 1290 Infinity LC for HPLC and API5000 for MS/MS system. CAPCELL PAK ADME (2 μm, 100 × 2.1 mm i.d.) was used for the first separation, while CAPCELL CORE C18 (2.7 μm, 150 × 3.0 mm i.d.) was used for the second separation. The mobile phase had a gradient elution of 0.1% formic acid solution and acetonitrile/methanol (1:1) mixture (for the 1st), and a gradient elution of 0.1% formic acid solution and methanol (for the 2nd). The flow rates were 0.45 mL/min (1st) and 0.55 mL/min (2nd), respectively. The column temperature was set to 40 °C. The injection volume was 20 μL.

2.3.2. T, DHT, DHEA, P4 and A-dione

An analysis was performed with heart-cutting 2D-LC-MS/MS using Agilent 1290 Infinity LC for HPLC and API5000 for MS/MS system. CAPCELL CORE ADME (2.7 μm, 150 × 2.1 mm i.d.) was used for the first separation, and Cortecs C18 (1.6 μm, 150 × 2.1 mm i.d.) was used for the second separation. The mobile phase had a gradient elution of 0.1% formic acid solution and acetonitrile (for the 1st), and a gradient elution of 1 mM acetic acid solution and acetonitrile (for the 2nd). The flow rates were 0.55 mL/min (1st) and 0.5 mL/min (2nd), respectively. The temperature of the column was set to 50 °C while the injection volume was 15 μL.

2.3.3. P5 and Δ5-A-diol

An analysis was done with LC-MS/MS using NEXERA LC-30AD (for HPLC) and API4000 (for MS/MS system) for measuring P5 and Δ5-A-diol in hair. The analytical column was a Kinetex C18 (1.7 μm, 150 × 2.1 mm i.d.) used at 50 °C. The mobile phase had a gradient elution of 0.1% formic acid solution and acetonitrile. The flow rates were 0.55 mL/min with an injection volume of 10 μL.

2.3.4. E2

An analysis was performed with heart-cutting 2D-LC-MS / MS using Agilent 1290 Infinity LC for HPLC and API5000 for MS/MS system. CAPCELL CORE PFP (2.7 μm, 50 × 2.1 mm i.d.) was used for the first separation while Cortecs C18 (1.6 μm, 150 × 2.1 mm i.d.) was used for the second separation. The mobile phase had a gradient elution of 0.1% formic acid solution and acetonitrile for both the analysis. The flow rates were 0.55 mL/min (1st) and 0.5 mL/min (2nd), respectively. The column temperature was set to 50 °C and the injection volume was 20 μL.

2.4. Sample preparation

2.4.1. Hair collection

Hair samples were collected from 8 healthy male volunteers. After being given sufficient explanations about the study protocol, all participants provided written consent. The hair strands in the posterior vertex of them were cut as close to the scalp as possible. The standard calibration curve, Inter- and Intra-day variation and Matrix interference are discussed in Sections 2.5.1, 2.5.2 and 2.5.3, respectively. The collected hair strands were cut at a length of 3 cm from the scalp and used for the measurement. 3 cm length of hair with five to ten strands was weighed and washed twice with 0.5 mL 2-propanol. The solvent for extracting steroids, the total lengths of 400 hairs (without the limitation of 3 cm from the scalp) were collected from 3 male volunteers as presented in Section 3.6. The washing procedure was performed twice with 10 mL 2-propanol.

2.4.2. Extraction procedure

Washed hair samples were collected and crushed into powder with a ball mill (ShakeMaster® NEO, Biomedical Science Co., Ltd., Tokyo, Japan), and an internal standard was added to them (F-d_4, E-$^{13}C_3$: 1000 pg, DHT-$^{13}C_3$, DHEA-$^{13}C_3$, Δ5-A-diol- d_4: 100 pg, P4-$^{13}C_3$, P5- d_4, A-dione-$^{13}C_3$: 200 pg, T-$^{13}C_3$: 300 pg, E2-$^{13}C_4$: 10 pg). Zero-point 5 mL of 0.1 M trifluoroacetic acid and 50% acetonitrile solution was added to the pulverized hair and incubated at 40 °C for 1 h. After adding 4 mL of methyl *tert*-butyl ether to the extract, the mixture was shaken. The organic layer was then separated and dried with a centrifugal evaporator. To the residue, 0.5 mL of methanol and 1 mL of water were sequentially added to get it dissolved which was applied into the OASIS MAX cartridge, a mixed-mode type solid-phase extraction (SPE) column. The SPE column was washed with 1 mL of 1% acetic acid solution, 1 mL of 45% methanol solution, and 1 mL of 1 M sodium hydroxide solution. Androgen, progestogen and glucocorticoid fraction was then eluted with 1 mL of methanol (a). The further washing procedure was performed with 1 mL of 1% acetic acid solution and 1 mL of 1% pyridine in 60% methanol. After that estradiol fraction was eluted with 1 mL of 1%

Table 1
Mass spectrometric conditions for the steroids measured.

Compound	Derivatization	MRM (*m/z*)	DP (V)	EP (V)	CE (eV)	CXP (V)
F	PI[a]	468.3 → 309.3	180	10	30	16
F-d_4	PI[a]	472.2 → 454.1	180	10	30	16
E	PI[a]	466.3 → 448.3	160	10	29	14
E-$^{13}C_3$	PI[a]	469.3 → 451.3	160	10	29	14
T	PI[a]	394.2 → 147.2	80	10	35	25
T-$^{13}C_3$	PI[a]	397.2 → 147.2	80	10	35	25
DHT	PI[a]	396.4 → 255.2	101	10	25	14
DHT-$^{13}C_3$	PI[a]	399.3 → 258.1	80	10	25	14
DHEA	PI[a]	394.2 → 175.1	71	10	31	28
DHEA-$^{13}C_3$	PI[a]	397.2 → 256.2	71	10	27	14
A-dione	none	287.2 → 97.0	96	10	31	16
A-dione-$^{13}C_3$	none	290.3 → 100.2	96	10	31	16
P4	none	315.2 → 97.1	106	10	33.4	18
P4-$^{13}C_3$	none	318.3 → 100.2	106	10	33.4	18
P5	PI[a]	422.4 → 281.3	46	10	21	8
P5-d_4	PI[a]	426.3 → 285.3	46	10	21	10
Δ5-A-diol	PI[a]	501.3 → 255.4	50	10	23	14
Δ5-A-diol-d_4	PI[a]	505.4 → 259.3	50	10	23	14
E2	tfpy-FU[b]	583.2 → 308.1	141	10	39	17
E2-$^{13}C_4$	tfpy-FU[b]	587.2 → 311.8	141	10	39	17

[a] Picolynoil acid derivatization.
[b] 3-tetrafluoropyridyl-17-Fusaric acid derivatization.

pyridine in 90% methanol (b).

Dried with a centrifugal evaporator, the fraction of (a) was derivatized with picolinic acid, and then purified on the HyperSep SI cartridge, which is a normal phase SPE column. After the eluent was evaporated, the residue was dissolved in 100 μL of 40% acetonitrile solution. From 100 μL, 20 μL (3.3.1F, E), 15 μL (3.3.2 T, DHT, DHEA, P4, A-dione), and 10 μL (3.3.3 P5, Δ5-A-diol) of sample solutions were sequentially subjected to the quantitation in LC-MS/MS. The detailed conditions of derivatization were reported elsewhere [24].

Dried with a centrifugal evaporator, the fraction of (b) was derivatized sequentially with pentafluoropyridine and fusaric acid, and then purified with the HyperSep SI cartridge. After the eluent was evaporated, the residue was dissolved in 100 μL of 80% acetonitrile solution. From 100 μL, 20 μL of sample solution was used for quantitating in LC-MS/MS. The detailed derivatization conditions were reported earlier [25].

2.5. Validation parameters

2.5.1. Standard calibration curve

The standard calibration curves were prepared with 8 or 9 samples including zero concentration. For the standard samples, purified water was used as a surrogate matrix. After adding 50 μL of standard solutions (solvent:methanol) corresponding to each defined concentration, the same procedure was performed (same as Section 2.4.2). For zero concentration, 50 μL of methanol was added. For preparing calibration curves, the equation $Y = aX + b$ with 1/X weighting was used, where X is the compound concentration and Y is the ratio of the compound peak area to the internal standard peak area. The slope and the Y-intercept were determined by the least-square method from the curve.

2.5.2. Inter- and Intra-day variation

Intra-day variation was determined by calculating accuracy and precision estimates (A&P) for hair and surrogate samples with $n = 5$ replicates. Inter-day variation was also determined by calculating A&P with the same assays on three separate days. For the lower limit of quantification (LLOQ) and low concentration quality control (LQC), the A&P was calculated using purified water as the surrogate matrix. For medium concentration quality control (MQC) and high concentration quality control (HQC), a standard solution was spiked to the hair sample and the A&P was calculated based on the recovery value. The recovery value (%) was determined by using the formula: Re (%) = {(Result of the spiked sample (pg)-Result of the unspiked sample (pg))/spike added (pg)} × 100

2.5.3. Matrix interference

To evaluate the matrix interference of LC-MS/MS response by hair samples, two assays were performed. First, absolute and relative matrix effects were calculated. Since the effect of endogenous concentration was not negligible, we used stable isotopes (internal standards) as surrogate analyte for the evaluation. Absolute matrix effects were expressed as the ratio of the mean peak area of surrogate analyte in 6 post-extraction spiked samples to the mean corresponding area in standard solutions. Relative matrix effects were expressed as the coefficient variation (CV) of the peak area in 6 post-extraction spiked matrix samples.

Second, the recovery test and the dilution parallelism test were assessed. Standard solutions were spiked to the hair samples of 6 individuals, and the corresponding recovery was calculated. As zero material, 50 μL of methanol was added instead of the standard solution. The calculating formula of the recovery was the same as stated in Section 2.5.2. Besides, the hair extracts prepared by adding a high concentration of steroid hormones to three individual hair samples were diluted in three stages with a surrogate matrix (purified water), and the dilution parallelism was evaluated.

2.6. Selection of solvent for steroids extraction

In the previous reports, the 'gold-standard' method was used to extract cortisol from the hair samples incubating with methanol overnight (16–18 h) [22]. Here, but chose an alternate method of extracting solvents and examined the optimal extracting condition. The hair samples obtained in Section 2.4.1 were crushed into powder by a ball mill and stirred well to prepare a pool pulverized hair sample. Using 20 mg and 1 mg of each pulverized pool hair sample, incubation was performed with four extraction solvents (a mixture of 0.1 M trifluoroacetic acid and 50% acetonitrile solution, methanol, ethanol, and acetonitrile). The incubation time was used as 5 points counting with 0, 0.5, 1, 2, and 16 (h) while the extraction rate was evaluated by the quantitative value at that particular time.

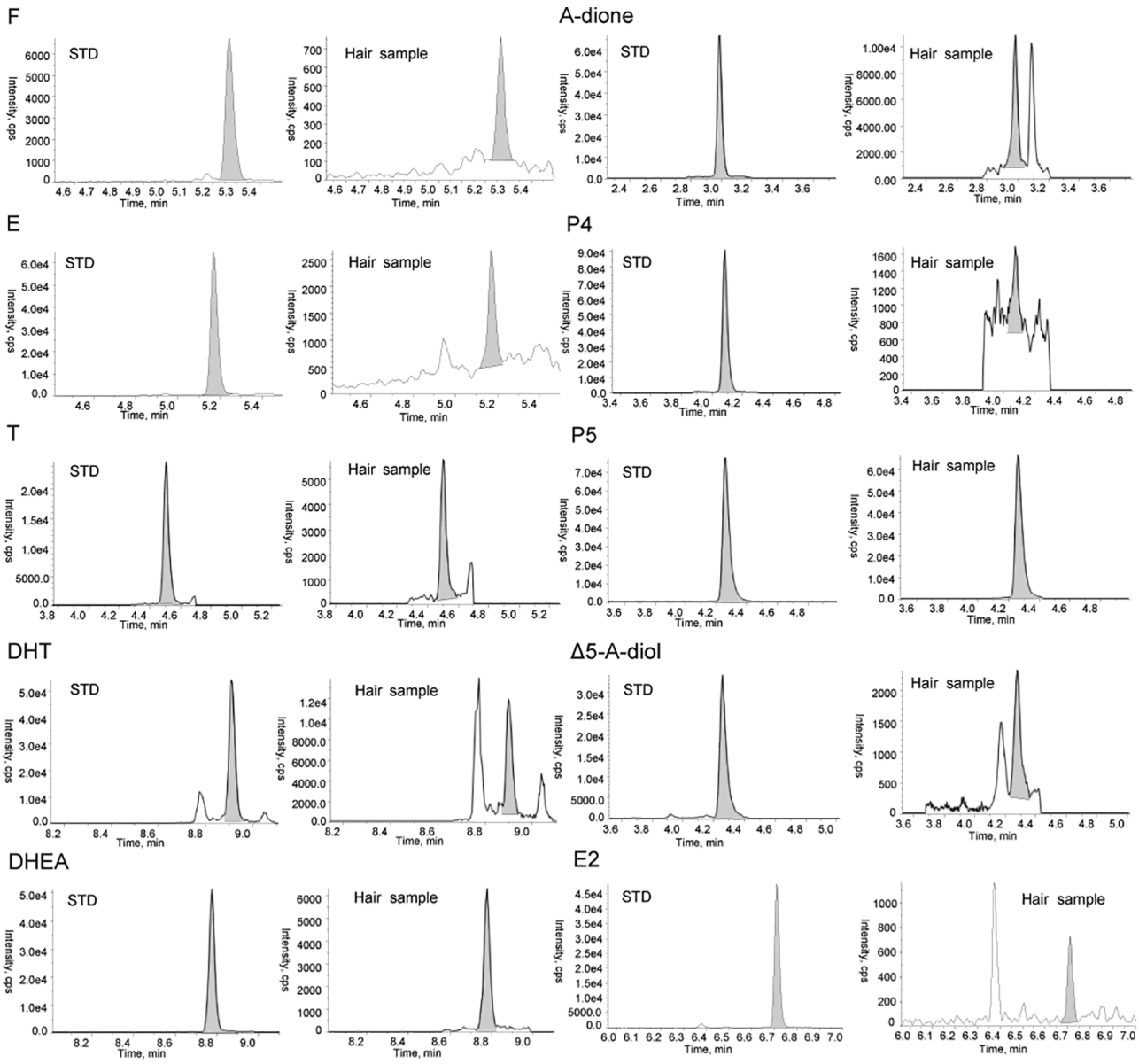

Fig. 1. The typical chromatogram of each steroids (calibration standard sample and actual hair sample). The concentration is as follows; F:STD = 100 pg/mg, F:Hair sample = 7.74 pg/mg, E:STD = 500 pg/mg, E:Hair sample = 32.70 pg/mg, T:STD = 50 pg/mg, T:Hair sample = 12.41 pg/mg, DHT:STD = 12.5 pg/mg, DHT:Hair sample = 3.12 pg/mg, DHEA:STD = 100 pg/mg, DHEA:Hair sample = 16.12 pg/mg, A-dione:STD = 100 pg/mg, A-dione:Hair sample = 14.10 pg/mg, P4:STD = 100 pg/mg, P4:Hair sample = BLQ, P5:STD = 100 pg/mg, P5:Hair sample = 70.90 pg/mg, Δ5-A-diol:STD = 50 pg/mg, Δ5-A-diol:Hair sample = 2.63 pg/mg, E2:STD = 1 pg/mg, E2:Hair sample = 0.0208 pg/mg. STD: calibration standard sample, BLQ: Below Lower limit of Quantification. Hair sample usage: 1.00 mg.

Table 2
Linearity, calibration range, and intra-&inter-day variation of all steroids.

Compound	Calibration Curve	Linearity	Calibration range	Intra-assay		Inter-assay	
		(*r*)	(pg/mg)	%Re[a]	%CV[b]	%Re[a]	%CV[b]
F	y = 1.15x + 0.00253	0.9997	2–1000	98.2	6.5	104.0	10.6
E	y = 4.33x + 0.0115	0.9998	10–5000	101.0	6.0	100.8	7.1
T	y = 5.01x + 0.000882	0.9998	0.5–500	101.7	7.3	104.4	7.9
DHT	y = 1.35x-0.000828	0.9990	0.25–125	100.7	5.5	102.2	6.9
DHEA	y = 0.137x + 0.000495	0.9996	2–1000	106.4	4.8	104.9	8.7
A-dione	y = 6.34x + 0.0141	0.9994	2–1000	103.6	8.1	103.2	9.2
P4	y = 4.59x + 0.0256	0.9996	2–1000	100.5	4.7	99.8	7.1
P5	y = 1.66x + 0.00394	0.9996	1–500	96.0	7.4	97.4	10.5
Δ5-A-diol	y = 0.461x + 0.00163	1.0000	1–500	99.6	5.6	96.9	7.9
E2	y = 0.717x + 0.000259	0.9999	0.005–50	99.8	7.9	101.8	8.3

[a] Accuracy is expressed as the mean values of data obtained from LLOQ, LQC, MQC and HQC.
[b] Precision is expressed as the mean values of data obtained from LLOQ, LQC, MQC and HQC.

Table 3
Matrix effects of all steroids.

Compound	Surroage analyte		Matrix Effect		Method used
	Stable isotope	added (pg)	%AME[a]	%CV[b]	
F	F-d_4	1000	106.7	2.0	2.3.1
E	E-$^{13}C_3$	1000	136.4	4.6	2.3.1
T	T-$^{13}C_3$	300	78.9	4.1	2.3.2
DHT	DHT-$^{13}C_3$	100	76.0	12.1	2.3.2
DHEA	DHEA-$^{13}C_3$	100	86.1	7.1	2.3.2
A-dione	A-dione-$^{13}C_3$	200	91.1	2.3	2.3.2
P4	P4-$^{13}C_3$	200	88.5	2.9	2.3.2
P5	P5- d_4	200	76.3	2.2	2.3.3
Δ5-A-diol	Δ5-A-diol- d_4	100	76.0	2.9	2.3.3
E2	E2-$^{13}C_4$	10	75.8	7.2	2.3.4

[a] Absolute matrix effects are calculated by comparing the mean peak area in 6 post-extraction spiked samples to the mean peak area in standard solutions.
[b] Relative matrix effects are expressed as the coefficient variation (C.V.) of the peak area in 6 post-extraction spiked matrix samples.

3. Result

3.1. Mass spectrometric conditions

The LC eluent was introduced into the mass spectrometer through an ESI ion source. Multiple reactions monitoring (MRM) mode was utilized for the detection of target compounds. The mass spectrometer was operated in the positive ionization mode and the working conditions are summarized in Table 1. The typical chromatogram of each steroid is shown in Fig. 1.

3.2. Linearity, calibration range, and inter-&intra-day variation

A linear response in the peak area ratios was observed in the standard samples for all steroids. Table 2 lists the detailed summary of the calibration curves. The correlation coefficients of the calibration curve are r = 0.999 or more for all steroids, showing good linearity. The results of intra- and inter-day variation are also shown in Table 2. The accuracy and precision of the QC samples remain between 96.0 and 106.4% and 4.8–8.1% for intra-day, respectively, and between 96.9 and 104.9%, 6.9–10.6% for inter-day, respectively. The accuracy and precision of

Table 4
Additional recovery and dilution linearity of all steroids.

Compound	Recovery test (n = 6)				Dilution test (n = 3)					
	Added	Detected[a]	Recovery	CV	Dilution	Observed[b]	Recovery	*r*		
	(pg)	(pg)	(%)	(%)	factor	(pg)	O/E[c] (%)			
F	0	5.55 ± 1.63	–	–	×8	41.41 ± 3.06	101.3	0.9993	~	0.9995
	100	99.62 ± 1.55	94.1	2.2	×4	87.36 ± 9.04	106.6			
	400	403.72 ± 8.50	99.5	2.0	×2	166.20 ± 8.13	101.6			
					×1	327.35 ± 17.9	–			
E	0	18.88 ± 7.14	–	–	×8	246.55 ± 24.6	101.3	0.9977	~	0.9999
	500	522.87 ± 19.5	100.8	2.6	×4	476.37 ± 33.7	106.6			
	2000	2009.81 ± 108	99.5	5.7	×2	915.77 ± 79.5	101.6			
					×1	1856.59 ± 112	–			
T	0	6.95 ± 3.30	–	–	×8	21.05 ± 2.46	96.5	0.9998	~	1.0000
	50	58.36 ± 3.36	104.6	2.7	×4	43.16 ± 3.08	99.1			
	200	217.83 ± 3.01	100.5	1.8	×2	84.97 ± 4.00	97.6			
					×1	174.13 ± 7.84	–			
DHT	0	1.76 ± 0.778	–	–	×8	4.90 ± 0.246	97.4	0.9999	~	1.0000
	12.5	13.55 ± 1.10	94.4	3.9	×4	10.02 ± 0.621	99.6			
	50	50.20 ± 1.09	96.9	2.5	×2	20.18 ± 1.33	100.3			
					×1	40.24 ± 1.8	–			
DHEA	0	20.14 ± 11.2	–	–	×8	48.20 ± 2.61	94.9	0.9983	~	0.9999
	100	128.39 ± 15.5	108.3	5.7	×4	102.94 ± 5.47	101.4			
	400	425.80 ± 29.8	101.4	6.4	×2	200.17 ± 7.73	98.8			
					×1	406.48 ± 21.1	–			
A-dione	0	8.77 ± 2.99	–	–	×8	39.27 ± 2.31	97.5	0.9994	~	1.0000
	100	109.4 ± 5.99	100.6	5.0	×4	81.47 ± 7.05	101.0			
	400	408.27 ± 23.0	99.9	5.3	×2	162.91 ± 16.2	100.9			
					×1	322.48 ± 20.8	–			
P4	0	BLQ[d]	–	–	×8	41.05 ± 1.50	98.5	0.9997	~	0.9999
	100	100.47 ± 3.91	100.1	4.1	×4	84.36 ± 7.64	100.9			
	400	398.99 ± 10.3	99.7	2.6	×2	165.26 ± 8.62	99.0			
					×1	334.18 ± 23.9	–			
P5	0	58.55 ± 35.0	–	–	×8	28.49 ± 7.56	101.3	0.9997	~	0.9999
	50	105.61 ± 35.0	94.1	3.5	×4	57.76 ± 13.3	103.1			
	200	254.53 ± 35.6	98.0	1.8	×2	113.30 ± 28.6	100.8			
					×1	224.46 ± 53.9	–			
Δ5-A-diol	0	2.31 ± 1.11	–	–	×8	21.62 ± 1.62	102.2	0.9999	~	0.9999
	50	50.55 ± 1.97	96.5	2.1	×4	43.35 ± 4.46	102.3			
	200	196.55 ± 6.21	97.1	2.8	×2	85.64 ± 7.45	101.1			
					×1	169.34 ± 14.2	–			
E2	0	0.018 ± 0.00820	–	–	×8	2.76 ± 0.258	98.9	0.9988	~	0.9999
	1	0.99 ± 0.0445	97.1	4.4	×4	5.88 ± 0.639	104.9			
	10	10.0881 ± 0.473	100.7	4.7	×2	11.71 ± 1.49	104.4			
					×1	22.47 ± 3.12	–			

[a] Detected (pg) is expressed as the mean values of 6 samples ± standard deviation.
[b] Observed (pg) is expressed as the mean values of 3 samples ± standard deviation.
[c] O/E:Observed/Expected ratio.
[d] BLQ:Below Lower limit of Quantitation.

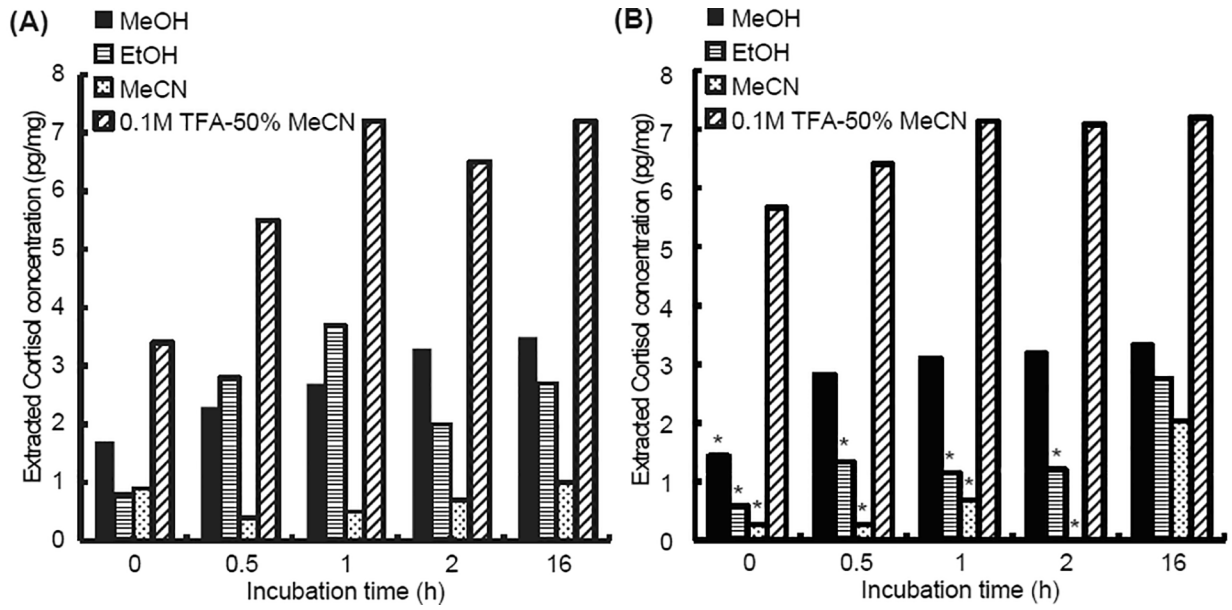

Fig. 2. Comparison of the extraction efficiencies of hair steroids according to time and extraction solvent. Pulverized sample was incubated with 0.5 mL of four different solvents (MeOH. EtOH, MeCN, and 0.1 M TFA-50% MeCN) to determine the effects of time (0, 0.5, 1, 2, and 16 h at 40 °C) using (A) a 20 mg of hair; (B) a 1 mg of hair. MeOH, methanol; EtOH, ethanol; MeCN, acetonitrile; 0.1 M TFA-50% MeCN; 0.1 M trifluoroacetic acid and 50% acetonitrile solution. The asterisk denoted that the value is below the lower limit of quantitation (BLQ).

Table 5
Steroid hormone concentrations in 6 individual hair samples.

Sample	F	E	T	DHT	DHEA	A-dione	P4	P5	Δ5-A-diol	E2
#1	5.46	15.78	3.81	1.12	7.76	5.94	BLQ[a]	34.97	1.19	BLQ[a]
#2	7.74	32.70	12.41	3.12	16.12	14.10	BLQ[a]	70.90	2.63	0.0208
#3	6.15	16.77	9.15	2.08	14.04	9.93	BLQ[a]	28.13	1.53	0.0228
#4	2.70	14.19	3.99	0.99	16.47	6.53	BLQ[a]	123.38	4.20	0.0157
#5	5.72	20.08	6.09	1.72	27.61	8.73	BLQ[a]	44.39	1.65	0.0212
#6	5.50	13.77	6.24	1.50	38.81	7.40	BLQ[a]	49.52	2.68	0.0289

All concentrations are reported in pg/mg.
[a] BLQ:Below Lower limit of Quantitation.

individual QC samples are suited for FDA Bioanalytical Method Validation GUIDANCE (accuracy: 85–115%, LLOQ 80–120%, precision: within 15%, LLOQ within 20%).

3.3. Matrix interference

The absolute and relative matrix effects are provided in Table 3. The absolute matrix effect of steroids ranged from 75.8 to 136.4%. The relative matrix effects ranged from 2.0 to 12.1%. The results of the recovery test for 6 individuals and the dilution parallelism test for the hair extracts of 3 individuals are listed in Table 4. The mean recoveries for each hair matrix are between 94.1 and 108.3%, and the precision is 1.8 to 6.4%. The dilution curves of each extracted hair had good linearity in which the correlation coefficients are found to be over 0.9977.

3.4. Selection of solvent for steroids extraction

Fig. 2 presents the changes in cortisol extracted value with time by using (A) a 20 mg and (B) a 1 mg of hair sample. Compared with the previous extraction with methanol (16 h), the present extraction with a mixed solution containing 0.1 M trifluoroacetic acid and 50% acetonitrile yielded about twice extraction efficiency. Besides, the extracted value of this solvent reached a plateau in 1 h. In contrast, extraction with an organic solvent alone did not improve the extraction efficiency even with the change of solvent. In the case of other steroid hormones, we found similar results i.e. a mixed solution of 0.1 M trifluoroacetic acid and 50% acetonitrile obtained a higher extraction efficiency than methanol.

3.5. Analysis of hair samples

To further, evaluate the usefulness of the developed LC-MS/MS analysis, we applied this method for analyzing the concentration of steroid hormone in hair samples of 6 individuals (healthy male volunteers). The results for each steroid hormone are shown in Table 5. Eight steroids, excluding P4 and E2, were measured in all six patients. For E2, the measured value was below the lower limit of quantification (BLQ) by one person, but the other five persons obtained concentrations above LOQ. P4 was below the lower limit of quantification in all cases.

4. Discussion

We have developed a method by analyzing the sample in LC-MS/MS that can measure 10 steroid hormones in only 5 to 10 hair strands (about 1 mg) from the same sample. It was a combination of following the three techniques to establish a sensitive analysis method: (1) use of a novel, highly efficient solvent extraction instead of methanol; (2) derivatization of steroids with picolinic acid and fusaric acid; (3) high-resolution and high-sensitivity analysis using 2D-LC.

The solvent which was used for the extraction was a mixed solution containing 0.1 M trifluoroacetic acid and 50% acetonitrile, enhancing the extraction efficiency twice as high as the conventional methanol

method (16 h incubation) in 1 h. The proposed method has significantly reduced the time required for the assay and also reduced the hair sample required for the assay.

The analytical method had the robustness and the calibration curve had linearity over a wide quantitative range. In the test of intra- and inter-day variation, good accuracy, and precision were obtained (Section 3.2).

Steroids, except F and E, showed negative matrix effect behavior that ranged from 75.8 to 91.1%. This suggested a weak suppression of ionization for these steroids in hair samples. On the contrary, E showed positive matrix effects which was 136.4%, suggesting an enhancement of ionization. In the recovery test, the recoveries and precision of 6 individuals at 2 points showed a good result, and in the dilution parallelism test, good dilution linearity was obtained in 3 individuals (Section 3.3). This implied that there was almost no matrix interference on the measurement in this method despite the existence of the matrix effects. Additionally, it was considered as an appropriate method for using purified water as a surrogate matrix instead of a hair sample for calibrating curve samples or low concentration QC samples (LLOQ and LQC) [26].

In Section 3.5, 6 individual male hairs were measured and we could obtain values with sufficient differences between each individual. The F, E and DHEA concentrations (2.70–7.74 pg/mg, 14.19–32.70 pg/mg and 7.76–38.81 pg/mg, respectively) did not deviate from previous reports [16,21,29]. In the previous reports, sufficient P4 concentrations were obtained in male hair [27], but the results of P4 in this assay was below the lower limit of quantification in all cases. This was thought to be due to the reduced sample volume. As P4 is a female sex hormone and its concentration will be higher in female hair [28], expecting this method to be useful in measuring P4 in female hair.

The previous report provided a high-sensitivity analysis including F, E, and T in hair [29], although the sensitivity of DHEA was lower than that of steroid hormones. Besides, a method for measuring DHT in hair with high sensitivity has not yet been reported. Hence, to analyze DHEA and DHT with higher sensitivity, we used the derivatization method in our earlier report [30,31]. In the present study, the DHEA concentrations in hair were comfortably above the LOQ but the DHT concentrations were quite low. To accurately measure DHT concentrations in hair from a small amount of sample, the present method (a combination of three methods) will be useful.

While DHT is the most important sex hormone in males, E2 plays an important role in female sex hormones. The present method is capable to measure both DHT and E2 with high sensitivity. Earlier measurement of E2 was assessed in female hair, but not in males [32]. Here, the concentrations of E2 in male hair ($\leq$0.0289 pg/mg) is extremely low compared to the reported value for female hair (4.5 $\pm$ 1.2 pg/mg), reflecting the differences in sex. The present study dealt with only male hair although could be a powerful tool for studying sex steroid hormones in female hair.

5. Conclusion

Herein, we reported the highly sensitive and low-invasive quantitation method for 10 steroid hormones in hair, covering from sex hormones to stress hormones. There have already been several reports on the assessment of chronic stress by measuring the steroid hormone concentrations in hair samples. However, the number of hair samples for the assay (50 to 100 hair strands = about 10 mg) would be a burden for the participants and be unrealistic when applied to large-scale cohort studies, examinations, and diagnosis. The novel method we have developed achieved a realistic hair usage (5 to 10 hair strands = about 1 mg) for cohort studies and hair diagnosis, so it will contribute to the convenience of future research. In future research, we hope that this method will clarify the relationship between steroid hormone levels and diseases that show alopecia such as chronic stress and AGA.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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