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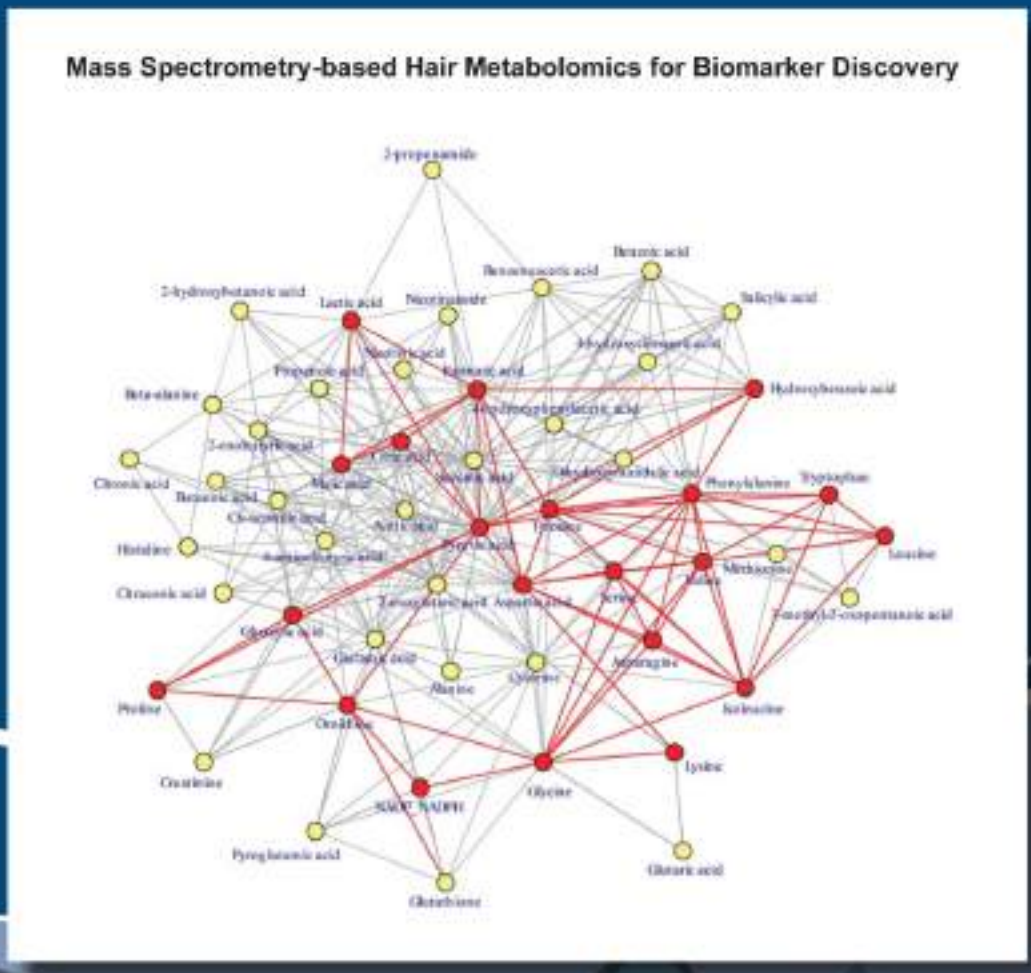

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Editorial Office
Administration office of the Korean Society for Mass Spectrometry
78 Daejeon Dunsan P.O.Box, 111 Dunsan-ro, Seo-gu, Daejeon, 35239, Republic of Korea
Contact: Yae Jin Joo
Homepage: www.ksms.org
E-mail: ksms@ksms.org
Tel: 82-70-8688-1696
Fax: 82-43-240-5159

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Aims and Scope

Mass Spectrometry Letters publishes brief letters (maximum length of 4 pages), technical notes, articles, reviews, and tutorials on fundamental research and applications in all areas of mass spectrometry. The manuscripts can be either invited by the editors or submitted directly by authors to the journal editors. Mass Spectrometry Letters topical sections are diverse, covering ion chemistry in a broad sense; gas-phase thermodynamics or kinetics; theory and calculations related with mass spectrometry or ions in vacuum; ion-optics; analytical aspects of mass spectrometry; instrumentations; methodology developments; ionization methods; proteomics and its related research; metabolomics and its related research; bioinformatics; software developments; database development; biological research using mass spectrometry; pharmaceutical research by mass spectrometry; food sciences using mass spectrometry; forensic results using mass spectrometry; environmental mass spectrometry; inorganic mass spectrometry; chromatography-mass spectrometry; tandem mass spectrometry; small molecule research using mass spectrometry; TOF-SIMS, etc. The scope of Mass Spectrometry Letters is not limited to the above-mentioned areas, but includes ever-expanding areas related directly or indirectly to mass spectrometry. Criteria for publication are originality, urgency, and reportable values. Short preliminary or proof-of-concept results, which will be further detailed by the following submission to other journals, are recommended for submission.

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Dr. Bong Chul Chung's Retirement

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EDITORIAL www.msletters.org | Mass Spectrometry Letters

Special Issue in Honor of Dr. Bong Chul Chung's Retirement

We would like to express our sincere appreciation to Dr. Bong Chul Chung as one of core members of the Study Group of Mass Spectrometry and the founder and third president (2008-2009) of the Korea Society for Mass Spectrometry (KSMS).

Dr. Chung received his Ph.D. in organic chemistry from the Korea Advanced Institute of Science and Technology (KAIST) in 1985. He was one of core members of the Doping Control Center at the Korea Institute of Science and Technology (KIST) and contributed to the successful Seoul Asian Games in 1986 and Seoul Olympic Games in 1988.

In 1992, during his post-doctoral training in Prof. Cedric Shackleton's Lab at Children's Hospital Oakland Research Institute (CA, USA), Dr. Chung published a distinguished article describing "the synthesis of deuterium-labeled intact steroid glucuronides," which was the pioneering report on the availability of isotope-labeled internal standards for steroid analysis.

Since returning to the KIST, he has been a prominent member of many organizations and societies, and he shared his expertise with his colleagues and mentees. Dr. Chung's broad research interests involve the development of chromatography–mass-spectrometry-based assays of steroid hormones and their biomedical applications. He has been the principal investigator or a co-investigator in research studies that applied metabolomic platforms, published 210 peer-reviewed articles, and contributed to the development of 118 patents.

The first article in this special issue is Dr. Chung's minireview on hair metabolomics for biomarker discovery. We would like to express our sincere gratitude to Dr. Bong Chul Chung for his passion for research and dedication to the KSMS and look forward to continuing to collaborate with him.

Man Ho Choi, Ph.D.
Guest Editor
Korea Institute of Science and Technology

Jeongkwon Kim, Ph.D.
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Mass Spectrometry Letters

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REVIEW www.msletters.org | Mass Spectrometry Letters

Mass Spectrometry-based Hair Metabolomics for Biomarker Discovery

Yu Ra Lee[1,2], Jongki Hong[2,3], and Bong Chul Chung[1,2]*

[1]*Molecular Recognition Research Center, Korea Institute of Science and Technology, Seoul 02792, Korea*
[2]*KHU-KIST Department of Converging Science and Technology, Kyung Hee University, Seoul 02447, Korea*
[3]*College of Pharmacy, Kyung Hee University, Seoul 02447, Korea*

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Abstract : Metabolomics makes it possible to analyze the interrelationships between various signaling molecules based on the metabolic pathways involved by using high-resolution devices. This approach can also be used to obtain large-scale metabolic information to identify the relevant pathways for disease diagnosis and prognosis and search for potential biomarkers. In the fields of medicine and forensics, hair analysis is used to detect various metabolites in the body. Hair can be harvested readily in a noninvasive manner and is easier to transport and store than blood and urine. Another advantage from a forensic viewpoint is that hair reflects all the components of body fluids. In addition, because of the unique coating structure of hair, it can be used for measurements without changing or destroying its adsorbed components. In this review, the pretreatments for hair analysis, instrumental conditions and clinical applications are discussed. Especially, the clinical use of hair metabolomics in the diagnosis of various diseases and the limitations of the technique are described.

Keywords : hair, metabolomics, biomarker, steroids

Introduction

Interest in metabolomic studies has grown rapidly in the past few years.[1] In addition, the analysis of human hair for genetic (DNA) and drug testing, which started in the 1960s, is a common technique these days, as hair is easy to collect and yields highly accurate results.

However, the hair roots are required for genetic and paternity testing. This is because the relevant biological information is present in the hair mother cells of the hair bulb. However, in the case of alcohol, tobacco, and narcotic consumption, the component of interest remains in the keratin protein layer of hair. Thus, these substances can be detected even in hair without roots.

Hair grows relatively slowly, and both endogenous compounds and those present in the environment are incorporated from the blood into the hair during its growth. This is reflected in the average chemical composition of hair over several months.[2] In particular, the analysis of the slow-growing hair matrix provides a suitable index for quantitatively evaluating the integrated hormone levels over several months.[3] Hair gradually forms a thick wall on its outer layer just before it is exposed to the outside environment and permanently records information related to the state of the minerals present in the human body. Normally, hair grows by 0.03 cm per day and 1 cm per month. Therefore, if one collects 3–4 cm of hair, one can obtain health-related information for the previous 3–4 months. In particular, hair stores 10–50 times more mineral information than blood and urine and can be analyzed more accurately.

Hair that grows 0.3 mm daily contains a wealth of information related to the levels of essential minerals, such as magnesium, potassium, iron, sodium, and calcium, in the body as well as heavy metals such as mercury, lead, and cadmium. The essential minerals found in hair are indispensable elements and play an important role in various physiological functions such cell generation and the stimulation of cell activity. Thus, by determining the contents of these minerals, it is possible to evaluate the metabolic rate, stress, immunity, and adrenal gland and thyroid gland status.

The medical conditions that can be prevented based on the results of hair examinations include chronic fatigue, obesity, diabetes, metabolic syndrome, atopy and skin

*Reprint requests to Bong Chul Chung
https://orcid.org/0000-0002-4449-0043
E-mail: bcc0319@kist.re.kr

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diseases, osteoporosis, arteriosclerosis, high cholesterol, and hypertension. These illnesses are caused by poor lifestyle and eating habits for long periods and vary from mental illnesses.

Hair analysis has also become increasingly important for detecting the presence of substances of abuse, both in clinical and forensic toxicology investigations.[4] Hair fiber offers several advantages over other biological matrices (blood and urine), including a larger window of detection, ease of collection, and sample stability.[5] Hair samples are extremely valuable for testing for long-term drug use. Moreover, as stated above, a key advantage of hair analysis is that hair samples are easy to obtain and can be acquired noninvasively. In addition, they do not have any additional storage requirements and can be kept at room temperature for long periods.

Hair is a strong matrix that is stable at room temperature, can be handled and transported with ease, cannot be tampered with readily during collection, can be collected noninvasively, and has a high resistance to decay in post-mortem cases.[6,7]

Metabolomics is a comprehensive technique to systematically analyze and quantify the changes in the behavior and secretion of metabolites within cells or tissues and reinterpret the metabolite network by linking the various metabolomic groups with their related physiological and pathological conditions. The main purpose of using metabolomics in current medicine is to determine the mechanism of drug action or disease; discover or measure biomarkers for diagnosis, prognosis, toxicity; and evaluate the therapeutic effects of drugs. Since human diseases and health disorders are caused by changes in the metabolism (metabolic pathways) of the body, metabolomics is increasingly being used for the discovery and identification of diagnostic biomarkers and therapeutic targets.[8,9] Metabolomics allows for a comprehensive analysis of metabolites and their associations with the metabolic processes related to various pathways.[10] Therefore, in the case of unknown metabolites, it is important to study the diseases they may cause as well as their correlations. Research in this direction has already begun recently.

Finally, it has been shown based on multivariate statistical analysis that it is possible to determine the gender of a person through the principal component analysis (PCA) and orthogonal partial least-squares discriminant analysis (OPLS-DA)[11] of hair.

In this review, we briefly introduce the pretreatments performed on hair samples prior to analysis and describe the clinical applications of hair metabolomics.

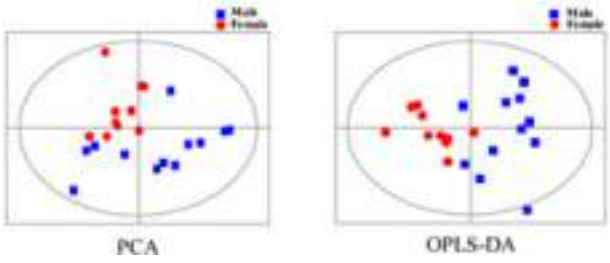

Figure 1. Score plots obtained from PCA and OPLS-DA of hair from healthy controls.

Sample Preparation

Washing for decontamination

An important step in the development of a decontamination procedure is to produce artificially contaminated specimens that be used for testing the efficiency of the washing process to remove the chemicals[12] and heavy metal ions[13] deposited on the surface of hair. The decontamination of hair is an important step that requires further examination.[14] Prior to analysis, the hair sample to be tested must be decontaminated by washing with a variety of solvents such as methanol, acetone,[15] and dichloromethane[16] to remove any oil or surface contaminants present.[17]

Alkaline hydrolysis

Alkaline hydrolysis, which completely dissolves the hair matrix, allows for the solubilization of all the drugs present in the hair sample.[18] For alkaline hydrolysis, 1 mL of 1 M NaOH is added to the hair sample and allowed to react at 80^{o}C for 1 h. The temperature can be kept at 70-90^{o}C. After the sample has cooled to room temperature, 1 mL of acetate buffer (pH 5.2) is added to the mixture, and its pH is adjusted to 5–6 by adding 0.1 mL of 2 M HCl. Finally, the mixture is extracted twice using 2.5 mL of hexane/ethyl acetate (3:2, v/v) by mechanical shaking for 10 min.[19]

Acidic hydrolysis

For acidic hydrolysis, 1 mL of 0.6 M HCl is added to the hair sample, and the mixture is incubated at 40^{o}C overnight. Alternatively, 1 mL of 1 M HCl is added to the hair sample, and the sample is kept overnight at 50^{o}C.[20] After heating, 0.9 mL of 2 M NaOH is used to adjust the pH of the mixture to 11–12. Then, derivatization[21] or extraction is performed using diethyl ether.[22] Another acidic hydrolysis method used to remove the protein components involves heating the hair sample with 6 M HCl at 110^{o}C for 16 h.[23] Acid hydrolysis could be used to extract basic metabolites due to protonation of the nitrogen atoms present in the molecules.[24]

Ultrasonication

Sonication is used to improve the digestion performance of enzymes. The most important parameters affecting the process are the cavitation frequency, ultrasound intensity, and type of solvent, bubbled gas, and external temperature used.[25] To maximize the extraction efficiency of the hair steroids, the effects of the treatment time (15, 30, 60, and

Table 1. Instrumental conditions for hair analysis using liquid chromatography system-mass spectrometer.

Sample preparation	Instrumental condition	Mobile phase	Analytes	Ref.
soak for 5 min or 5 h in a 1 mg/mL solution of cocaine base or HCl salt	Waters MALDI HDMS SYNAPT	A: methanol B: 10 mM ammonium bicarbonate, pH 10	cocaine	30
extracted with phosphate buffer and liquid-liquid extraction	Ultimate 3000 pump (Thermo Fisher, Les Ulis, France) and Orbitrap mass spectrometer (Q-Exactive)	A: 0.1% formic acid in water (ammonium formate 2 mmol/L) B: 0.1% formic acid in 1% water, ACN/MeOH, 50:50, v/v	untargeted screening	31
extracted with buffer:solvent mixture at 37°C for 18 h	Agilent 6550 QTOF coupled to a 1290 Infinity UHPLC system	A: 0.05% formic acid in 10 mM ammonium formate B: 0.05% formic acid in acetonitrile	7-amino-flunitrazepam, 7-amino-clonazepam, 7-amino-nitrazepam, acetylmorphine, alimemazine, alprazolam, amphetamine, etc.	32
under sonication at 45°C	A Quattro Premier tandem mass spectrometer (Waters)	A: 0.1% formic acid in water B: 0.1% formic acid in methanol	33 basic drugs (amphetamines, cocaine, opiates, opioids and metabolites)	33
added 3 mL NaOH 1N and incubated 95°C for 10 min	Acquity ultra-performance liquid chromatography (UPLC) system (Waters, Milford, MA, USA) coupled to a Micromass Quattro Ultima Platinum (Waters)	A: 5 mM formic acid in water B: 5 mM formic acid in acetonitrile	9-tetrahydrocannabinol	34
under sonication at 45°C	Agilent 6460 triple quadrupole mass spectrometer coupled to a 1290 Infinity UHPLC system	A: 5 mM ammonium formate containing 0.1% formic acid B: methanol/acetonitrile 1:1 with 0.1% formic acid	synthetic cannabinoids, synthetic cathinones, ketamine, piperazines and amphetamine	35
incubated with methanol for 24 h on a rolling mixer	UFLC system from Shimadzu coupled QTRAP® 6500 from SCIEX	A: 0.2 mM NH_4F in water/methanol 97:3, v/v B: 0.2 mM NH_4F in water/methanol 3:97, v/v	steroid hormone	36
under sonication at 40°C	Thermo ULTIMATE 3000 HPLC system coupled to a Thermo single-stage Orbitrap (Exactive) MS system	A: water with 5 mM ammonium formate and 0.1% formic acid B: methanol/acetonitrile 1:1 with 0.1% formic acid	drugs of abuse and pharmaceutical drugs	37
hydrolysis with 1 mL of 2.5 M NaOH at 60°C for 25 min	Ultimate 3000 LC system (Thermo Fisher, Les Ulis, France) coupled a Thermo Scientific Q Exactive mass spectrometer	A: 0.1% formic acid in water B: 0.1% formic acid in acetonitrile	cannabinoids and metabolites	38
extracted with methanol-HCl mixture	Agilent 1100 LC system coupled 3000 triple quadrupole mass spectrometer	A: 0.1% formic acid in water B: formic acid in methanol	methamphetamine, amphetamine, methylenedioxymetamphetamine, methylenedioxyamphetamine, ketamine, norketamine, dehydronorketamine, 6-acetylmorphine, morphine, codeine	39
soak in 300 uL of methanol	MDS Sciex hybrid quadrupole time-of-flight mass spectrometer	A: 0.1% formic acid in water B: 0.1% formic acid in acetonitrile	cannabinoids	40
incubated with methanol at 37°C for 16 h	Agilent 1200 HPLC system coupled 3200 QTRAP tandem mass spectrometer	A: mixture of methanol-water (20:80, v/v) containing ammonium acetate (2 mM) B: mixture of methanol-water (80:20, v/v) containing ammonium acetate (2 mM)	antiretroviral drugs	41

Table 1. Continued.

Sample preparation	Instrumental condition	Mobile phase	Analytes	Ref.
extracted with DW at 80°C for 30 min	Agilent 1100 LC system coupled 4000 triple quadrupole mass spectrometer	A: 2.5 mM ammonium formate/DW (25:75, v/v) B: 2.5 mM ammonium formate/methanol (25:75, v/v)	nicotine and cotinine	42
incubated with acidic aqueous buffer at 100°C for 1 h	Waters Acquity UHPLC coupled with a triple quadrupole mass spectrometer	A: 0.3% formic acid in acetonitrile B: 5 mM ammonium formate pH 3	hallucinogenic drugs	43
incubated with 3 mL of 0.1 M HCl at 53°C for overnight	Agilent 1100 HPLC system coupled Sciex Triple Quad 5500 mass spectrometer	A: 60% (v/v) methanol in water B: 0.1% formic acid in acetonitrile	methamphetamine	44
incubated with acidic aqueous buffer at 100°C for 1 h	Waters Acquity UHPLC coupled with a triple quadrupole mass spectrometer	A: 0.1% formic acid in acetonitrile B: 5 mM ammonium formate pH 3	antidepressant and anxiolytic drugs	45
incubated with 0.5 mL of a mixture acetic acid/methanol (20:80, v/v) at 38°C for 12 h	Waters Acquity UPLC H-Class LC system coupled triple quadrupole mass spectrometer	A: 0.1% ammonium in water B: 0.1% ammonium in methanol	21 endocrine disrupting chemicals	46
under sonication for 2 h	Shimadzu UFLC 20A LC system coupled IT-TOF mass spectrometer	A: 0.1% formic acid in water B: 0.1% formic acid in acetonitrile	untargeted screening	47
incubated with 2 mL of a mixture acetic acid/methanol (15/85, v/v) at 38°C for overnight	Agilent 1200 LC system coupled to a 6410 Agilent triple quadrupole mass spectrometer	A: methanol B: 5 mM ammonium acetate aqueous solution	endocrine-disrupting compounds	48
Incubated with potassium hydroxide at 54°C for 16 h	Waters UPLC coupled with a QTOF mass spectrometer	A: 0.5% formic acid in water B: acetonitrile	untargeted screening	49

Table 2. Instrumental conditions for hair analysis using gas chromatography system-mass spectrometer.

Sample preparation	Instrumental condition	Used column	Analytes	Ref.
incubated with 1 mL 2M NaOH at 38°C overnight	Agilent GC-MSD instrument	HP-5MS (30 m × 0.25 mm, 0.25 μm)	organic pollutants	50
incubated with 2 mL MeOH at 50 °C for 18 h	Agilent 6850 GC system coupled 5975 MSD	HP-5MS (30 m × 0.25 mm, 0.25 μm)	cocaine and its derivatives	51
incubated with 1 mL 1M NaOH at 70°C for 15 min	Agilent GC-MSD instrument	HP-5MS (30 m × 0.25 mm, 0.25 μm)	phenobarbital	52
incubated with 2 mL 1N NaOH at 50°C for 18 h	Agilent 7890A GC system coupled 7000C triple quadrupole mass spectrometer	HP-5MS (30 m × 0.25 mm, 0.25 μm)	cannabinoids	53
incubated with 1mL 1 M NaOH at 90°C for 15 min	Agilent 7890B GC system coupled 7000B triple quadrupole mass spectrometer	Zebron ZB-5MSi (30 m × 0.25 mm, 0.25 μm)	cannabinoids	54
incubated with Proteinase K enzyme at 37.5°C for 50 min	Agilent 7890A GC system coupled 5975 MSD	BP-X5 SGE Forte Capillary column (30 m × 0.25 mm, 0.25 μm)	cannabinoids	55
incubated with 1 mL 1M NaOH at 90°C for 15 min	Varian CP-3800 GC system coupled Saturn 4000 MS/MS ion trap mass detector	VF-5MS capillary column (30 m × 0.25 mm, 0.25 μm)	cannabinoids	56
extracted with 1mL 0.1M HCl at 37°C for 16 h (overnight)	Agilent 6890 GC system coupled Leco® Pegasus® IV time-of-flight (TOF) mass spectrometer	DB-5MS (30 m × 0.25 mm, 0.25 μm)	drug analysis	57
extracted with methanol in an ultrasonic bath (~5 h)	Varian CP-3800 GC system coupled Saturn 2200 MS/MS ion trap mass detector	Zebron capillary column (30 m × 0.25 mm, 0.25 μm)	external contamination	58
after pulverized, incubated with 1 mL of acetonitrile at 40°C for overnight	Agilent 7890A GC system coupled 7000A triple quadrupole mass spectrometer	HP-5MS (30 m × 0.25 mm, 0.25 μm)	pesticide analysis	59
extracted with methanol or methanol/dichloromethane in an ultrasonic bath at 40°C for 1 h	Agilent 7890 GC system coupled 5975 MSD	Ultra-1 capillary column (25 m × 0.2 mm, 0.33 μm)	steroid hormone	19

120 min at 25°C) and temperature (25, 40, and 60°C for 60 min) during sonication with methanol have been evaluated. The effect of the extraction solvent used during ultrasonication has also been evaluated. For this, hair samples were incubated with methanol (0.5 mL) or methanol/dichloromethane (1:2 or 2:1) for 60 min at 40°C.[19]

Enzymatic hydrolysis

For the enzymatic hydrolysis of hair, added 500 μL of a 1,4-dithiothreitol solution (12 mg/mL in tris(hydroxymethyl) aminomethane-hydrochloric acid buffer solution (0.1 M, pH 7.2)) is added. The mixture is then incubated at 40°C for 12 h.[26]

Instrumental Conditions

Most experimental instrument for hair analysis consists of the use of liquid chromatography-mass spectrometry. Here is one example we used to explain the instrumental conditions for untargeted profiling.[27-29] An ACQUITY™ ultra-performance liquid chromatography system (Waters, Milford, MA, USA) coupled to a Q-Tof Premier™ quadrupole/time-of-flight hybrid mass spectrometer system from Waters (Milford, MA, USA) were used. The gradient elution system consisted of solvent A (water with 0.1% formic acid) and solvent B (acetonitrile with 0.1% formic acid) and was controlled as follows: 0–3 min, 5% B; 3–10 min, 5–50% B; 10–11.5 min, 50–95% B; 11.5–12 min, 95–5% B. The gradient was then returned to the initial concentration (5% B) and held for 2 min before running the next sample. Except for previous results, instrument conditions used in other studies were showed in Table 1. also classically used gas chromatography-mass spectrometry method is also summarized in Table 2. It was confirmed that most of the GC-MS methods were analyzed using a column composed of 5% phenyl polysilphenylene-siloxane.

Applications of Hair Metabolomics

Forensic science

Forensic chemistry uses biological samples such as urine and hair to evaluate the authenticity of psychotropic and other drugs in order to determine whether they are suitable for use as well as to measure the contents (purities) of their various components.

Drug confirmation tests performed on hair samples obtained during drug crime investigations are not only used to complement urine analysis but also for diagnosing drug use and checking for chronic drug abuse.

Moreover, the duration of drug use can be determined several months after use based on the detection range. For example, methamphetamine is a highly addictive central nervous system stimulant, and the changes in human hair metabolites after excessive methamphetamine use have been analyzed. Through network analysis, it has been shown that the concentrations of glycosphingolipids, sphingolipids, glycerophospholipids, and ether lipids as well as the metabolism of amino acids (glycine, serine, and threonine; cysteine and methionine) are affected by methamphetamine use.[60]

In addition, from a forensic point of view, a method for simultaneously analyzing the drugs and steroids present in hair samples has been developed.[61]

However, there are a few limitations associated with hair analysis. The differences in the hair growth rate with age, gender, ethnicity, as well as the individual variations between subjects make it difficult to interpret the concentrations of metabolites in hair. In addition, it is also difficult to estimate the time and volume details from hair segment analysis, as the drug integration mechanism of the hair matrix is not yet fully understood.[62] In addition, hair samples can be manipulated through cosmetic treatments, and the drug concentrations can be altered, resulting in false negatives. In particular, the oxidative bleaching of hair samples under alkaline conditions has a significant effect on drug concentration. However, recently, a method to identify the metabolites altered by oxidative beauty therapies was developed based on nontargeted hair metabolomics analysis. [63]

Androgenic alopecia

Androgenic alopecia is a well-known condition that occurs because of increased male hormone secretion.[64] However, androgenic alopecia in females (female-pattern baldness) differs from that in males. The causes of female-pattern baldness are not as clear as those of male-pattern baldness. Therefore, in a previous study, we performed untargeted metabolomics to comprehensively analyze the

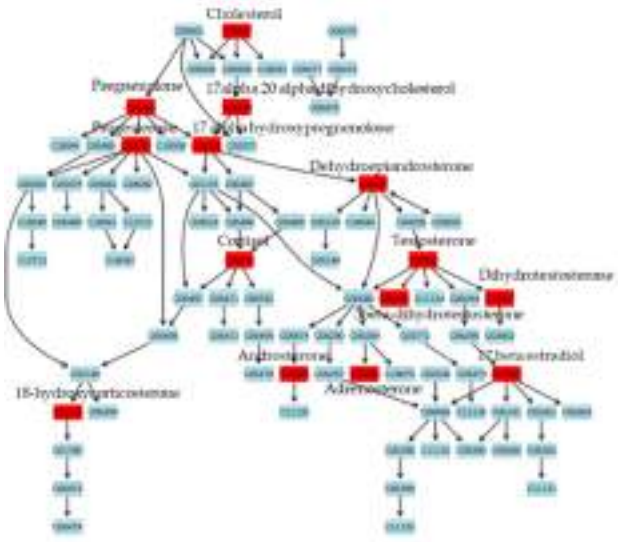

Figure 2. Steroid hormone biosynthesis with significant differences in male groups. Metabolites in red are significantly different for patients and controls.

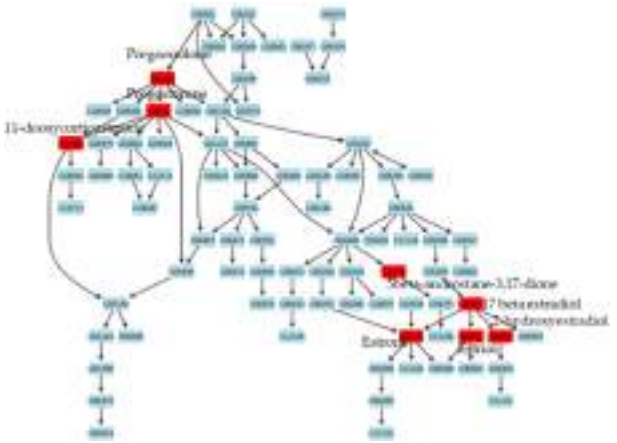

Figure 3. Steroid hormone biosynthesis with significant differences in female groups. Metabolites in red are significantly different for various groups.

metabolites and metabolic pathways of androgenic alopecia using human hair samples.[29] We were able to elucidate the extensive metabolic changes associated with androgenic alopecia based on sex.

In the case of the male groups, steroid hormone biosynthesis and the androgen metabolic pathways were significantly altered.

On the other hand, in the case of the female groups, steroid hormone biosynthesis and the estrogen metabolic pathways were significantly altered.

Hair follicles are among the most highly proliferative tissues. Therefore, we had also analyzed the levels of the polyamines and metabolites involved in cell proliferation in hair samples taken from patients with androgenic alopecia.[21,65]

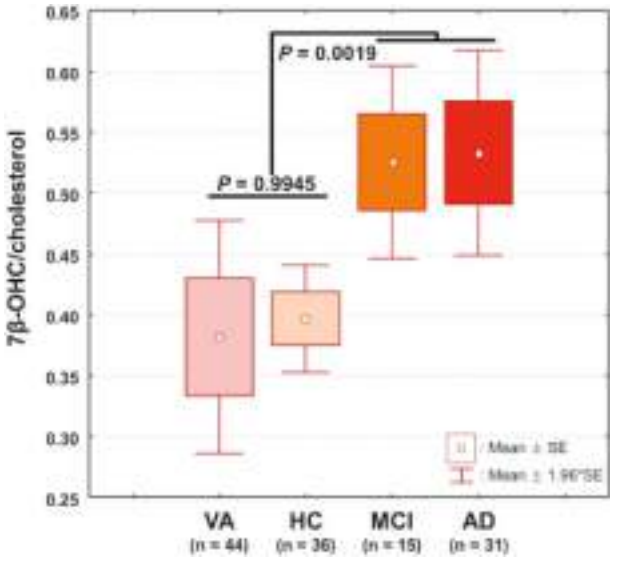

Figure 4. Box plot of hair metabolic ratios of 7β-hydroxycholesterol (7β-OHC) to cholesterol.

Neurodegenerative diseases

Neurodegenerative diseases are associated with several types of cognitive impairment, and the cortisol concentration in hair may reflect the level of chronic stress.[66] The metabolic ratio of 7β-hydroxycholesterol (7β-OHC) to cholesterol can be used as a predictable index for evaluating cognitive impairment. The ratios for the abnormal cognition (mild cognitive impairment (MCI) + Alzheimer's disease (AD)) and vasospastic angina (VA) groups were found to be significantly different.[67]

In addition, an analysis of the glucocorticoid levels in hair samples from patients with Parkinson's disease showed that the level of hair cortisone was significantly higher in the patients.[68]

Hair analysis during various trimesters of pregnancy

Hair metabolites are an important source of information in pregnancy research and are used to study the metabolic mechanisms and complications related to pregnancy.[2, 49, 69] The 40-week gestation period is divided into three trimesters. The period from the moment of confirmation of pregnancy to 13 weeks is called the first trimester, that from 14 weeks to 28 weeks is called the second trimester, and that from 28 weeks until delivery is called the third trimester. As pregnancy progresses, the concentrations of various metabolites change. The intermediates of glycolysis and the tricarboxylic acid cycle, such as pyruvic acid,

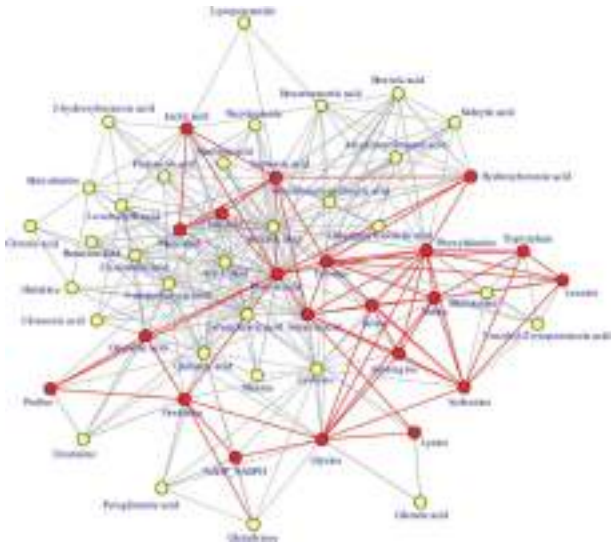

Figure 5. Metabolic network showing relationships between trimester-related metabolic pathways and various metabolites. Red circles represent metabolites that are significantly altered throughout pregnancy while yellow circles represent those that are not.

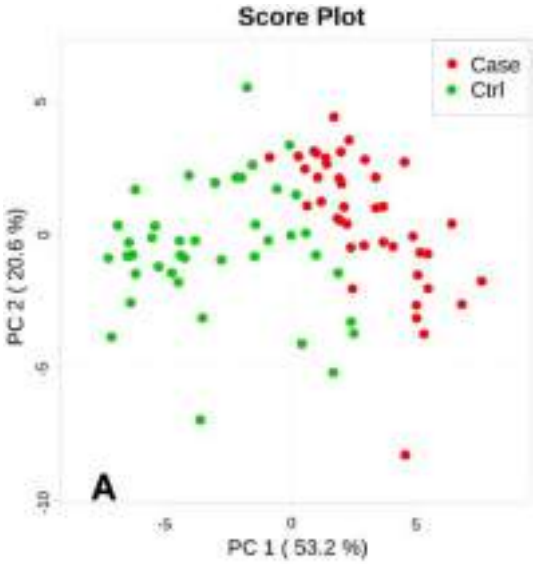

Figure 6. Principal component analysis (PCA) score plot. Red circles represent fetal growth restriction cases while green circles represent normal controls.

fumaric acid, citric acid, and malic acid, link the various metabolic pathways. In particular, carbohydrate metabolism is significantly altered during pregnancy.[49]

In addition, based on an analysis of hair samples, we were able to confirm that there is a difference in the PCA results for the group with fetal growth restriction and those for normal controls. In particular, there were significant differences in the lactate, levulinate, 2-methyloctadecanate, tyrosine, and margarate levels of these groups.[2]

The onset of intrahepatic cholestasis of pregnancy, a maternal liver disease, can lead to sudden consequences, including fetal death and stillbirth. Attempts are being made to predict this disease based on predictive biomarkers using maternal hair samples. However, the results obtained so far have been unsatisfactory, as the hair samples collected at the onset of the disease did not show metabolic changes, suggesting rapid development.[70]

Perspective and limitations

In this review, we discussed hair metabolomics, including the sample preparation techniques used and the clinical applications of the method. We believe this review provides insights for improving the currently used approaches for hair analysis. In the future, hair metabolomics will be used more widely not only in forensic science but also in many other fields.

From a forensic viewpoint, hair analysis should be a complementary approach rather than the primary technique. Since there is a possibility of false positive results owing to the external contamination of the hair sample, an efficient decontamination procedure is required.[71] Hair readily adsorbs contaminants even after short-term exposure. For example, environmental substances and shampoos adhere to its surface. In addition, the hair matrix may contain endogenous metabolites and other substances related to long-term exposure. Therefore, methods are being developed to profile both transiently exposed chemicals and endogenous metabolites in the same hair sample.[72]

A recent study confirmed that there are differences in the levels of metabolites with the hair color, suggesting that it is necessary to consider the hair color and hair segments in subsequent studies on hair metabolites.[73]

Thus, owing to their various advantages, metabolomics methods based on the noninvasive analysis of hair samples have been used in many clinical studies.

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References

1. Eisenbeiss, L.; Steuer, A. E.; Binz, T. M.; Baumgartner, M. R.; Kraemer, T. *Anal. Bioanal. Chem.* **2019**, 411, 3963, DOI: 10.1007/s00216-019-01873-4.
2. Sulek, K.; Han, T. L.; Villas-Boas, S. G.; Wishart, D. S.; Soh, S. E.; Kwek, K.; Gluckman, P. D.; Chong, Y. S.; Kenny, L. C.; Baker, P. N. *Theranostics* **2014**, 4, 953, DOI: 10.7150/thno.9265.
3. Gao, W.; Kirschbaum, C.; Grass, J.; Stalder, T. *J. Steroid Biochem. Mol. Biol.* **2016**, 162, 92, DOI: 10.1016/j.jsbmb.2015.12.022.
4. Pragst, F.; Balikova, M. A. *Clin. Chim. Acta* **2006**, 370, 17, DOI: 10.1016/j.cca.2006.02.019.
5. Vogliardi, S.; Tucci, M.; Stocchero, G.; Ferrara, S. D.; Favretto, D. *Anal. Chim. Acta* **2015**, 857, 1, DOI: 10.1016/j.aca.2014.06.053.
6. Kohler, I.; Guillarme, D. *Bioanalysis* **2014**, 6, 1255, DOI: 10.4155/bio.14.80.
7. Niu, Z.; Zhang, W.; Yu, C.; Zhang, J.; Wen, Y. *Trends Anal. Chem.* **2018**, 102, 123, DOI: 10.1016/j.trac.2018.02.005.
8. Sugimoto, M.; Kawakami, M.; Robert, M.; Soga, T.; Tomita, M. *Curr. Bioinform.* **2012**, 7, 96, DOI: 10.2174/157489312799304431.
9. Worley, B.; Powers, R. *Curr. Metabolomics* **2013**, 1, 92, DOI: 10.2174/2213235X11301010092.
10. Muthubharathi, B. C.; Gowripriya, T.; Balamurugan, K. *Mol. Omics* **2021**, 17, 210, DOI: 10.1039/D0MO00176G.
11. Dong, Z.; Wang, C.; Zhang, J.; Wang, Z. *Anal. Bioanal. Chem.* **2017**, 409, 4751, DOI: 10.1007/s00216-017-0419-2.
12. Duca, R. C.; Hardy, E.; Salquebre, G.; Appenzeller, B. M. *Drug Test Anal.* **2014**, 6 Suppl 1, 55, DOI: 10.1002/

dta.1649.
13. Zhang, H.; Carrillo-Navarrete, F.; López-Mesas, M.; Palet, C. *Water* **2020**, 12, 1263, DOI: 10.3390/w12051263.
14. Collins, S.; James, T.; Southworth, F.; Davidson, L.; Williams, N.; Orchard, E.; Marczylo, T.; Amlot, R. *Sci. Rep.* **2020**, 10, 20822, DOI: 10.1038/s41598-020-77930-1.
15. Janda, I.; Weinmann, W.; Kuehnle, T.; Lahode, M.; Alt, A. *Forensic Sci. Int.* **2002**, 128, 59, DOI: 10.1016/s0379-0738(02)00163-9.
16. Muller, A.; Iwersen-Bergmann, S. *Drug Test Anal.* **2020**, 12, 948, DOI: 10.1002/dta.2791.
17. Balikova, M. *Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub* **2005**, 149, 199.
18. Cirimele, V.; Kintz, P.; Mangin, P. *Biomed. Chromatogr.* **1996**, 10, 179, DOI: 10.1002/(SICI)1099-0801(199607)10:4<179::AID-BMC586>3.0.CO;2-N.
19. Jung, H. J.; Kim, S. J.; Lee, W. Y.; Chung, B. C.; Choi, M. H. *Rapid Commun. Mass Spectrom.* **2011**, 25, 1184, DOI: 10.1002/rcm.4975.
20. Kim, J. Y.; Jung, K. S.; Kim, M. K.; Lee, J. I.; In, M. K. *Rapid Commun. Mass Spectrom.* **2007**, 21, 1705, DOI: 10.1002/rcm.3010.
21. Lee, Y. R.; Lew, B. L.; Sim, W. Y.; Lee, J.; Hong, J.; Chung, B. C. *J. Dermatol.* **2019**, 46, 985, DOI: 10.1111/1346-8138.15063.
22. Choi, M. H.; Kim, K. R.; Chung, B. C. *J. Chromatogr. A* **2000**, 897, 295, DOI: 10.1016/S0021-9673(00)00800-1.
23. Ito, S.; Miyake, S.; Maruyama, S.; Suzuki, I.; Commo, S.; Nakanishi, Y.; Wakamatsu, K. *Pigment Cell Melanoma Res.* **2018**, 31, 393, DOI: 10.1111/pcmr.12673.
24. Ferreira, C.; Paulino, C.; Quintas, A. *Chem. Res. Toxicol.* **2019**, 32, 2367, DOI: 10.1021/acs.chemrestox.9b00301.
25. Bermejo, P.; Capelo, J.; Mota, A.; Madrid, Y.; Cámara, C. *Trends Anal. Chem.* **2004**, 23, 654, DOI:10.1016/j.trac.2004.06.007.
26. Miguez-Framil, M.; Moreda-Pineiro, A.; Bermejo-Barrera, P.; Lopez, P.; Tabernero, M. J.; Bermejo, A. M. *Anal. Chem.* **2007**, 79, 8564, DOI: 10.1021/ac071337n.
27. Lee, Y. R.; Im, E.; Kim, H.; Lew, B. L.; Sim, W. Y.; Lee, J.; Oh, H. B.; Paeng, K. J.; Hong, J.; Chung, B. C. *Metabolites* **2020**, 10, DOI: 10.3390/metabo10040131.
28. Lee, Y. R.; An, K. Y.; Jeon, J.; Kim, N. K.; Lee, J. W.; Hong, J.; Chung, B. C. *Metabolites* **2020**, 10, 487,DOI: 10.3390/metabo10120487.
29. Lee, Y. R.; Lew, B. L.; Sim, W. Y.; Hong, J.; Chung, B. C. *Metabolites* **2021**, 11, 178, DOI: 10.3390/metabo11030178.
30. Cuypers, E.; Flinders, B.; Boone, C. M.; Bosman, I. J.; Lusthof, K. J.; van Asten, A. C.; Tytgat, J.; Heeren, R. M. *Anal. Chem.* **2016**, 88, 3091, DOI: 10.1021/acs.analchem.5b03979.
31. Fabresse, N.; Larabi, I. A.; Stratton, T.; Mistrik, R.; Pfau, G.; Lorin de la Grandmaison, G.; Etting, I.; Grassin Delyle, S.; Alvarez, J. C. *Drug Test Anal.* **2019**, 11, 697, DOI: 10.1002/dta.2535.
32. Kronstrand, R.; Forsman, M.; Roman, M. *Forensic Sci. Int.* **2018**, 283, 9 DOI: 10.1016/j.forsciint.2017.12.001.
33. Fernandez Mdel, M.; Di Fazio, V.; Wille, S. M.; Kummer, N.; Samyn, N. *J. Chromatogr. B* **2014**, 965, 7, DOI: 10.1016/j.jchromb.2014.05.055.
34. Duvivier, W. F.; van Beek, T. A.; Pennings, E. J.; Nielen, M. W. *Rapid Commun. Mass Spectrom.* **2014**, 28, 682 DOI: 10.1002/rcm.6831.
35. Strano-Rossi, S.; Odoardi, S.; Fisichella, M.; Anzillotti, L.; Gottardo, R.; Tagliaro, F. *J. Chromatogr. A* **2014**, 1372C, 145, DOI: 10.1016/j.chroma.2014.10.106.
36. Gaudl, A.; Kratzsch, J.; Bae, Y. J.; Kiess, W.; Thiery, J.; Ceglarek, U. *J. Chromatogr. A* **2016**, 1464, 64, DOI: 10.1016/j.chroma.2016.07.087.
37. DiBattista, A.; Rampersaud, D.; Lee, H.; Kim, M.; Britz-McKibbin, P. *Anal. Chem.* **2017**, 89, 11853, DOI: 10.1021/acs.analchem.7b03590.
38. Montesano, C.; Simeoni, M. C.; Vannutelli, G.; Gregori, A.; Ripani, L.; Sergi, M.; Compagnone, D.; Curini, R. *J. Chromatogr. A* **2015**, 1406, 192, DOI: 10.1016/j.chroma.2015.06.021.
39. Chang, Y. J.; Chao, M. R.; Chen, S. C.; Chen, C. H.; Chang, Y. Z. *Anal. Bioanal. Chem.* **2014**, 406, 2445, DOI: 10.1007/s00216-014-7669-z.
40. Beasley, E.; Francese, S.; Bassindale, T. *Anal. Chem.* **2016**, 88, 10328, DOI: 10.1021/acs.analchem.6b03551.
41. Wu, Y.; Yang, J.; Duan, C.; Chu, L.; Chen, S.; Qiao, S.; Li, X.; Deng, H. *J. Chromatogr. B* **2018**, 1083, 209, DOI: 10.1016/j.jchromb.2018.03.021.
42. Inukai, T.; Kaji, S.; Kataoka, H. *J. Pharm. Biomed. Anal.* **2018**, 156, 272, DOI: 10.1016/j.jpba.2018.04.032.
43. Pichini, S.; Marchei, E.; Garcia-Algar, O.; Gomez, A.; Di Giovannandrea, R.; Pacifici, R. *J. Pharm. Biomed. Anal.* **2014**, 100, 284, DOI: 10.1016/j.jpba.2014.08.006.
44. Shu, I.; Alexander, A.; Jones, M.; Jones, J.; Negrusz, A. *J. Chromatogr. B* **2016**, 1028, 145, DOI: 10.1016/j.jchromb.2016.06.015.
45. Pichini, S.; Cortes, L.; Marchei, E.; Solimini, R.; Pacifici, R.; Gomez-Roig, M. D.; Garcia-Algar, O. *J. Pharm. Biomed. Anal.* **2016**, 118, 9, DOI: 10.1016/j.jpba.2015.10.016.
46. Rodriguez-Gomez, R.; Martin, J.; Zafra-Gomez, A.; Alonso, E.; Vilchez, J. L.; Navalon, A. *Chemosphere* **2017**, 168, 676, DOI: 10.1016/j.chemosphere.2016.11.008.
47. Xie, P.; Wang, T. J.; Yin, G.; Yan, Y.; Xiao, L. H.; Li, Q.; Bi, K. S. *J. Mol. Neurosci.* **2016**, 58, 93, DOI: 10.1007/s12031-015-0655-x.
48. Martin, J.; Santos, J. L.; Aparicio, I.; Alonso, E. *Anal. Chim. Acta* **2016**, 945, 95, DOI: 10.1016/j.aca.2016.10.004.
49. Delplancke, T. D. J.; de Seymour, J. V.; Tong, C.; Sulek, K.; Xia, Y.; Zhang, H.; Han, T. L.; Baker, P. N. *Sci. Rep.* **2018**, 8, 36, DOI: 10.1038/s41598-017-18317-7.
50. Martin, J.; Moder, M.; Gaudl, A.; Alonso, E.; Reemtsma, T. *Anal. Bioanal. Chem.* **2015**, 407, 8725, DOI: 10.1007/s00216-015-9026-2.
51. Pego, A. M.; Roveri, F. L.; Kuninari, R. Y.; Leyton, V.; Miziara, I. D.; Yonamine, M. *Forensic Sci. Int.* **2017**, 274,

83, DOI: 10.1016/j.forsciint.2016.12.024.
52. Roveri, F. L.; Paranhos, B. A.; Yonamine, M. *Forensic Sci. Int.* **2016**, 265, 75, DOI: 10.1016/j.forsciint.2015.12.033.
53. Rodrigues, A.; Yegles, M.; van Elsue, N.; Schneider, S. *Forensic Sci. Int.* **2018**, 292, 163, DOI: 10.1016/j.forsciint.2018.09.015.
54. Kieliba, T.; Lerch, O.; Andresen-Streichert, H.; Rothschild, M. A.; Beike, J. *Drug Test Anal.* **2019**, 11, 267, DOI: 10.1002/dta.2490.
55. Breidi, S. E.; Barker, J.; Petroczi, A.; Naughton, D. P. *J. Anal. Methods Chem.* **2012**, 2012, 907893, DOI: 10.1155/2012/907893.
56. Emidio, E. S.; Prata Vde, M.; Dorea, H. S. *Anal. Chim. Acta* **2010**, 670, 63, DOI: 10.1016/j.aca.2010.04.023.
57. Guthery, B.; Bassindale, T.; Bassindale, A.; Pillinger, C. T.; Morgan, G. H. *J. Chromatogr. A* **2010**, 1217, 4402, DOI: 10.1016/j.chroma.2010.04.020.
58. Pinho, S.; Oliveira, A.; Costa, I.; Gouveia, C. A.; Carvalho, F.; Moreira, R. F.; Dinis-Oliveira, R. J. *Biomed. Chromatogr.* **2013**, 27, 1003, DOI: 10.1002/bmc.2894.
59. Salquebre, G.; Schummer, C.; Millet, M.; Briand, O.; Appenzeller, B. M. *Anal. Chim. Acta* **2012**, 710, 65, DOI: 10.1016/j.aca.2011.10.029.
60. Kim, S.; Jang, W. J.; Yu, H.; Kim, J.; Lee, S. K.; Jeong, C. H.; Lee, S. *Int. J. Mol. Sci.* **2020**, 21, DOI: 10.3390/ijms21176041.
61. Shah, I.; Petroczi, A.; Uvacsek, M.; Ranky, M.; Naughton, D. P. *Chem. Cent. J.* **2014**, 8, 73, DOI: 10.1186/s13065-014-0073-0.
62. Wennig, R. *Forensic Sci. Int.* **2000**, 107, 5, DOI: 10.1016/s0379-0738(99)00146-2.
63. Eisenbeiss, L.; Binz, T. M.; Baumgartner, M. R.; Kraemer, T.; Steuer, A. E. *Analyst* **2020**, 145, 6586, DOI: 10.1039/d0an01265c.
64. Orentreich, N. *J. Soc. Cosmet. Chemists* **1960**, 11, 479.
65. Lee, Y. R.; Lee, J.; Lew, B. L.; Sim, W. Y.; Hong, J.; Chung, B. C. *Exp. Dermatol.* **2019**, 28, 1083, DOI: 10.1111/exd.13998.
66. Lee, D. Y.; Kim, E.; Choi, M. H. *BMB Rep.* **2015**, 48, 209, DOI: 10.5483/bmbrep.2015.48.4.275.
67. Son, H. H.; Lee, D. Y.; Seo, H. S.; Jeong, J.; Moon, J. Y.; Lee, J. E.; Chung, B. C.; Kim, E.; Choi, M. H. *J. Steroid Biochem. Mol. Biol.* **2016**, 155, 9, DOI: 10.1016/j.jsbmb.2015.09.024.
68. van den Heuvel, L. L.; du Plessis, S.; Stalder, T.; Acker, D.; Kirschbaum, C.; Carr, J.; Seedat, S. *Psychoneuroendocrinology* **2020**, 117, 104704, DOI: 10.1016/j.psyneuen.2020.104704.
69. He, X.; de Seymour, J. V.; Sulek, K.; Qi, H.; Zhang, H.; Han, T. L.; Villas-Boas, S. G.; Baker, P. N. *Acta Diabetol.* **2016**, 53, 119, DOI: 10.1007/s00592-015-0737-9.
70. de Seymour, J. V.; Tu, S.; He, X.; Zhang, H.; Han, T. L.; Baker, P. N.; Sulek, K. *Metabolomics* **2018**, 14, 79, DOI: 10.1007/s11306-018-1371-7.
71. Barroso, M.; Gallardo, E. *Bioanalysis* **2014**, 6, 1, DOI: 10.4155/bio.13.291.
72. Chen, Y.; Guo, J.; Xing, S.; Yu, H.; Huan, T. *Front. Chem.* **2021**, 9, 674265, DOI: 10.3389/fchem.2021.674265.
73. Eisenbeiss, L.; Binz, T. M.; Baumgartner, M. R.; Kraemer, T.; Steuer, A. E. *Metabolites* **2020**, 10, 381, DOI: 10.3390/metabo10100381.

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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*

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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

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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

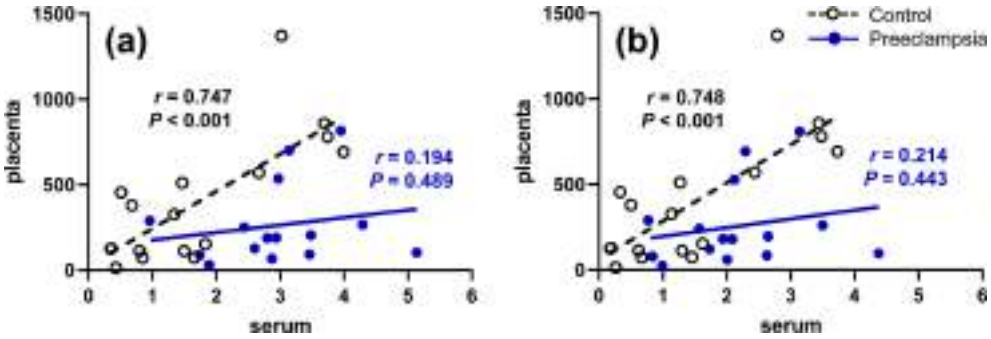

Figure 1. Scatter plots with linear fitted curves and Pearson correlations between serum and placental pregnenolone levels. Both (a) unweighted and (b) weighted regression models showed positive correlations between serum and placenta pregnenolone concentrations ($P < 0.001$ for both), whereas those 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

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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.

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Quantification of Fargesin in Mouse Plasma Using Liquid Chromatography-High Resolution Mass Spectrometry: Application to Pharmacokinetics of Fargesin in Mice

Min Seo Lee, Chang Ho Lim, Young Yoon Bang, and Hye Suk Lee*

Drug Metabolism & Bioanalysis Laboratory, College of Pharmacy, The Catholic University of Korea, Bucheon 14662, Republic of Korea

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Abstract : Fargesin, a tetrahydrofurofuranoid lignan isolated from Flos Magnoliae, shows anti-inflammatory, anti-oxidative, anti-allergic, and anti-hypertensive activities. To evaluate the pharmacokinetics of fargesin in mice, a sensitive, simple, and selective liquid chromatography-high resolution mass spectrometric method using electrospray ionization and parallel reaction monitoring mode was developed and validated for the quantification of fargesin in mouse plasma. Protein precipitation of 6 μL mouse plasma with methanol was used as sample clean-up procedure. The standard curve was linear over the range of 0.2–500 ng/mL in mouse plasma with the lower limit of quantification level at 0.2 ng/mL. The intra- and inter-day coefficient variations and accuracies for fargesin at four quality control concentrations including were 3.6-11.3% and 90.0-106.6%, respectively. Intravenously injected fargesin disappeared rapidly from the plasma with high clearance values (53.2-55.5 mL/min/kg) at 1, 2, and 4 mg/kg doses. Absolute bioavailability of fargesin was 4.1-9.6% after oral administration of fargesin at doses of 1, 2, and 4 mg/kg to mice.

Keywords : fargesin, LC-HRMS, mouse plasma, pharmacokinetics

Introduction

Flos Magnoliae (Chinese name: Xin-yi) has been traditionally used for the treatment of allergic rhinitis, sinusitis, and headaches.[1-3] Fargesin (Figure 1A), a tetrahydrofurofuranoid lignan isolated from Flos Magnoliae, shows therapeutic effects for allergy, inflammatory diseases, hypertension, osteoarthritis, and atherosclerosis in the experimental animals by attenuating inducible nitric oxide synthase,[4,5] lipoxygenase,[6] various signaling pathways such as MAPK, CDK2/Cyclin E, PKC-dependent AP-1, and NF-κB,[7-11] ORAI1 channel,[2] reverse cholesterol transport,[12] oxidative stress,[13,14] apoptosis,[13] lipid and glucose metabolism,[15,16] and melanin synthesis.[17]

*Reprint requests to Hye Suk Lee
https://orcid.org/0000-0003-1055-9628
E-mail: sianalee@catholic.ac.kr

Fargesin inhibited CYP2C9-catalyzed diclofenac 4′-hydroxylation (K_i, 16.3 μM), UGT1A1-mediated SN-38 glucuronidaton (K_i, 25.3 μM), and UGT1A3-mediated chenodeoxycholic acid 24-acyl-glucuronidation activities (K_i, 24.5 μM) and showed the mechanism-based inhibition of CYP2C19-catalyzed [*S*]-mephenytoin 4′-hydroxylation (K_i, 3.7 μM), CYP2C8-catalyzed amodiaquine *N*-deethylation (K_i, 10.7 μM), and CYP3A4-catalyzed midazolam 1′-hydroxylation (K_i, 23.0 μM) human liver microsomes.[18,19] For the *in vivo* prediction of fargesin-induced drug interaction potential from *in vitro* data, the information regarding fargesin pharmacokinetics in the animals or humans is necessary. However, there are a few reports on the pharmacokinetics of fargesin after oral administration of purified extract of Flos Magnoliae or fargesin in the rats using high-performance liquid chromatography (HPLC) with atmospheric pressure chemical ionization tandem mass spectrometry (LC-APCI-MS/MS)[18] or ultraviolet detection.[20,21]

We have developed a rapid, simple, and sensitive LC-high resolution mass spectrometric method (LC-HRMS) for the quantification of fargesin in mouse plasma samples using the least mouse plasma volume (6 μL) and successfully applied the method to evaluate the pharmacokinetics of fargesin after intravenous and oral administration of fargesin at 1, 2, and 4 mg/kg dose in male ICR mice.

Experimental

Materials

Fargesin (purity, 98%) was obtained from Tokyo Chemical Industry (Tokyo, Japan). Magnolin (purity, 98.9%; internal standard) were obtained from PhytoLab GmbH & Co. (Vestenbergsgreuth, Germany). Water and methanol (LC-MS grade) were supplied by Fisher Scientific Co. (Fair Lawn, NJ, USA). All other chemicals used were of the highest quality available.

Sample preparation

Standard stock solution was prepared separately by dissolving fargesin (1 mg) in 1 mL of dimethyl sulfoxide and was diluted with methanol for the preparation of standard solutions (2.4 to 6000 ng/mL). The internal standard (IS) working solution (magnolin, 10 ng/mL) was prepared by diluting an aliquot of the stock solution with methanol. All standard solutions were stored at 4°C in darkness for 4 weeks.

Mouse plasma calibration standards for fargesin were prepared at eight concentration levels: 0.2, 0.4, 1, 5, 25, 100, 250, and 500 ng/mL. QC samples for fargesin were prepared at the concentrations of 0.2, 0.6, 20, and 450 ng/mL in drug-free mouse plasma and stored at -80°C until analyzed.

A 6 μL aliquot of mouse plasma sample was mixed with 18 μL of magnolin (IS, 10 ng/mL) in methanol. The mixture was vortexed and centrifuged at 13,500 rpm for 5 min. An aliquot of each supernatant was transferred to autosampler vial, and 5 μL was injected in the LC-HRMS system for analysis.

LC-HRMS analysis

Plasma concentrations of fargesin were analyzed by an LC-HRMS system coupled with Nexera X2 UPLC (Shimadzu, Kyoto, Japan) and Q-Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). The separation was performed on a Halo C18 column (2.1 × 100 mm, 2.7 μm; Advanced Material Technology, Wilmington, DE, USA) using a gradient elution of 10 mM ammonium formate in 5% methanol (mobile phase A) and 95% methanol (mobile phase B), with flow rate of 0.3 mL/min: 20% mobile phase B for 0.5 min, 20 to 98% mobile phase B for 2.5 min, 98% mobile phase for 3 min, 98% to 20% mobile phase B for 0.2 min, 20% mobile phase B for 2.8 min. The column and autosampler were maintained at 40°C and 4°C, respectively. Heated electrospray ionization source settings in positive ion mode were spray voltage, 3.50 kV; sheath gas, 40 (arbitrary units); auxiliary gas, 10 (arbitrary units); capillary gas heater temperature, 250°C; and auxiliary gas heater temperatures, 200°C, respectively. Nitrogen gas (purity 99.999%) was used for higher-energy collision dissociation, and the collision energies for fragmentation of fargesin and magnolin (IS) were 25 and 40 eV, respectively. Parallel reaction monitoring (PRM) transitions were *m/z* 388.17547→135.04407 for fargesin and *m/z* 417.19022→219.10136 for the magnolin (IS). Xcalibur software (version 3.1.66.10, Thermo Fisher Scientific Inc.) was used for LC-HRMS system control and data processing.

Method validation

Method validation was performed according to the methods set out in the FDA Guidance on Bioanalytical Method Validation (https://www.fda.gov/media/70858/download). The intra- and inter-day precisions and accuracies were evaluated by analyzing batches of calibration standards and QC samples (0.2, 0.6, 20, and 450 ng/mL) in five replicates on three different days. Accuracy was defined as the proximity of the measured mean value to the theoretical value and precision was defined as the coefficient of variation (CV, %) of the measured concentrations. LLOQ value was defined as the lowest amount of fargesin in a mouse plasma sample that could be quantified as follows: signal-to-noise ratio, > 5; CV, < 20%; accuracy, 80-120%.

The stability of fargesin in mouse plasma was evaluated by analyzing low and high QC samples in triplicate: post-preparation sample stability in the autosampler at 4°C for 24 h; short-term storage stability following storage of plasma samples at room temperature for 2 h; three freeze–thaw cycles, and long-term storage stability following the storage for 28 days at -80°C.

The recovery of fargesin were determined by comparing the peak areas of the extract of fargesin-spiked plasma with those of fargesin-spiked post-extraction into six different blank mouse plasma extracts at 0.6, 20, and 450 ng/mL levels.

Pharmacokinetic study of fargesin in mice

Male ICR mice (8 weeks of age weighing 26.4 – 41.6 g) were purchased from Samtako Inc (Osan, Korea). All experimental procedures involving animal care were approved by the Institutional Animal Care and Use Committee of The Catholic University of Korea (approval number 2021-004-01). All mice were allowed unrestricted access to water and food before experiment. They were housed under suitable and standard housing conditions at a temperature of 23 ± 2°C, with relative humidity of 55 ± 10%, 12 h light/12 h dark cycle.

Fargesin in dimethylsulfoxide:propylene glycol:water (1:6:3, *v/v/v*) was administered by the bolus injection via tail vein of mice for intravenous study and using oral gavage for the oral study at doses of 1, 2 and 4 mg/kg (*n* = 6)(administration volume, 3 mL/kg). Blood sample (approximately 20 μL) was collected from the retro-orbital plexus under light anesthesia with isoflurane at 2 (intravenous study only), 5, 15, 30, 45 and 60 min and 1.5, 2, 3, 4, 6, 8, 10, and 24 h after drug administration. Plasma samples were harvested by centrifugation at 3000×g for 5 min and stored at -80°C until analysis.

Fargesin in dimethylsulfoxide:propylene glycol:water (1:6:3, *v/v/v*) was administered by bolus injection into the tail vein at 4 mg/kg dose ($n = 3$) and by oral administration at 4 mg/kg dose ($n = 3$) to male ICR mice. Mice were returned to metabolic cages and urine and feces samples were collected individually for 48 hours. Urine and feces samples were stored in -80°C until analysis.

Pharmacokinetic parameters, including the area under the plasma concentration-time curve during the period of observation (AUC_{last}), the area under the plasma concentration-time curve to infinite time (AUC_{inf}), the terminal half-life ($t_{1/2}$), clearance (*CL*), volume of distribution at steady state (V_{ss}), and mean residence time (MRT), were analyzed using noncompartmental analysis (Phoenix WinNonlin 6.3; Pharsight, Mountain View, CA, USA). C_{max} and the time to reach C_{max} (T_{max}) were obtained directly from the experimental data. The extent of absolute oral bioavailability (*F*) was estimated by dividing AUC_{last} at each oral dose by AUC_{last} at intravenous administration. Each value is expressed as the mean ± standard deviation (SD). Statistical comparisons of pharmacokinetic variables were performed by one-way ANOVA followed by Tukey test. The values were treated as statistically significant when *p-value* < 0.05.

Results and Discussion

LC-HRMS analysis

The positive electrospray ionization of fargesin formed $[M+NH_4]^+$ ion at *m/z* 388.17547 instead of $[M+H]^+$ ion, and therefore, $[M+NH_4]^+$ ion was selected as the precursor ion and produced the intense product ion at *m/z* 135.04410 (Figure 1A). Magnolin (IS) showed $[M+H]^+$ ion at *m/z* 417.19022 and the intense product ion at *m/z* 219.10136 in MS/MS spectra (Figure 1B). PRM mode was used for the quantification of the analytes due to the high selectivity

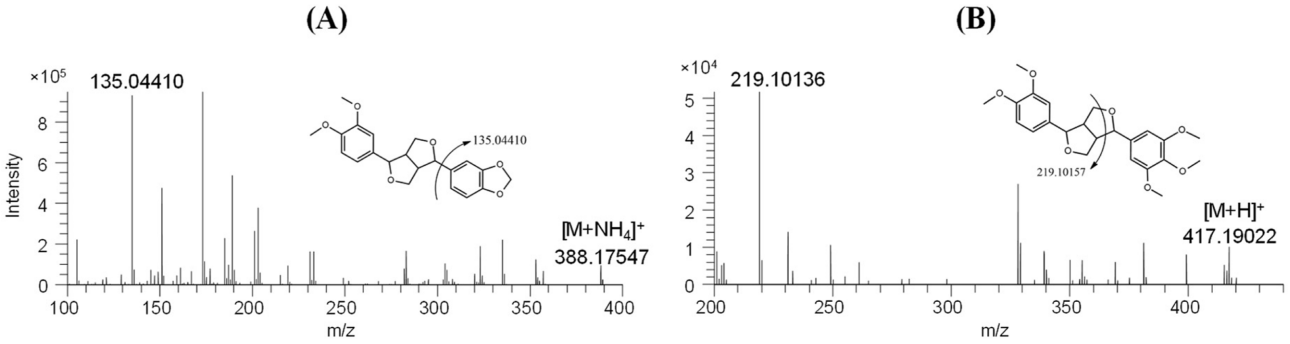

Figure 1. Product ion spectra of (A) fargesin and (B) magnolin.

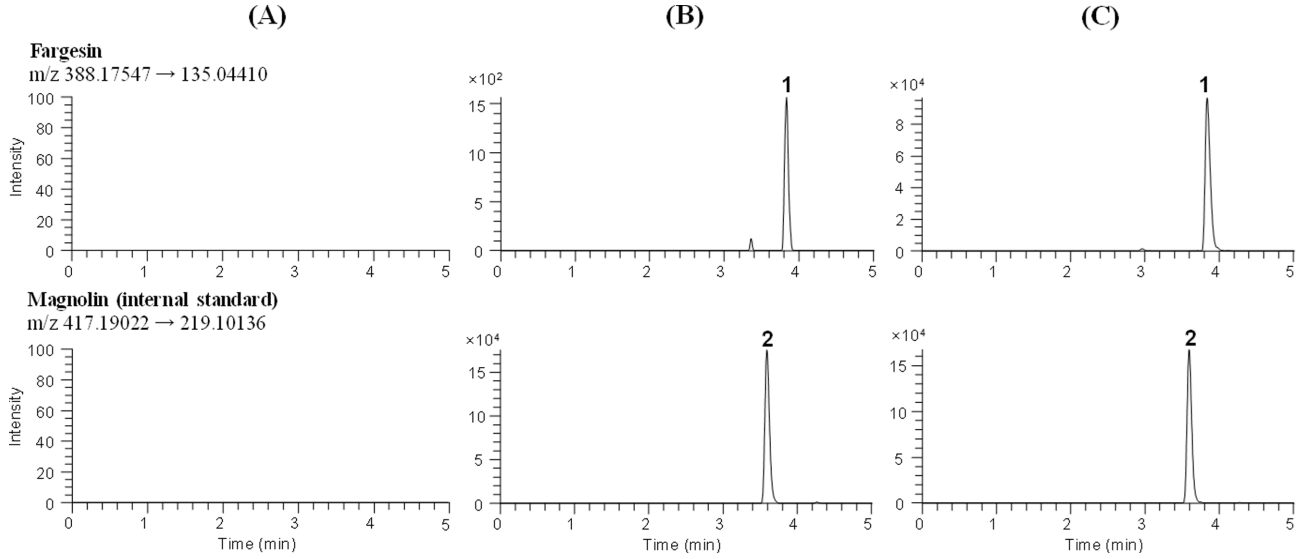

Figure 2. Representative parallel reaction monitoring chromatograms of (A) mouse blank plasma; (B) mouse plasma spiked with fargesin at LLOQ level (0.2 ng/mL); and (C) mouse plasma obtained 5 min after oral administration of fargesin at a dose of 1 mg/kg to a male ICR mouse. 1, fargesin (3.83 min); 2, magnolin (3.59 min, internal standard).

and sensitivity (Figure 2). Electrospray ionization mode yielded better sensitivity compared to APCI ionization[20] for the quantification of fargesin.

Analysis of blank plasma samples obtained from 40 mice revealed no significant interference peaks in the retention times of the analytes, indicating good method selectivity of the present method (Figure 2A). Figure 2B presents a typical PRM chromatogram of mouse plasma sample spiked with fargesin at 0.2 ng/mL. Figure 2C presents representative PRM chromatograms of a plasma sample obtained 5 min after intravenous administration of fargesin at a dose of 1 mg/kg in a mouse.

Method validation

Calibration curve for fargesin in mouse plasma was linear over the concentration ranges of 0.2–500 ng/mL with the coefficient of determination of 0.9977 using linear regression analysis with a weighting of 1/concentration (Table 1). The CV and accuracy of the calculated concentrations were 4.2% to 15.0% and from 95.0% to 103.6%, respectively, for eight calibration points. The CV value for the regression line slopes of fargesin was 0.7%, indicating good method repeatability.

The intra- and inter-day CV and accuracy values for fargesin in LLOQ, low, medium, and high QC samples ranged from 3.6% to 11.3% and from 90.0% to 106.6%, respectively (Table 2), indicating that the accuracy and precision of this method are acceptable.

Matrix effects of fargesin and magnolin (IS) were 91.7%-107.6% at 0.6, 20, and 450 ng/mL and 110.6%, respectively, indicating a little matrix effect (Table 3). The average recoveries of fargesin and magnolin (IS) in mouse plasma were 88.4%-98.1% at three concentrations and 95.1±3.2%, respectively (Table 3), indicating that the protein precipitation using methanol was suitable as sample preparation.

Table 1. Calculated concentrations of fargesin in calibration standards prepared with mouse plasma (n = 3).

Variables	Theoretical concentrations (ng/mL)								slope	r^2
	0.2	0.4	1	5	25	100	250	500		
Mean (ng/mL)	0.20	0.38	0.97	4.9	25.4	101.3	259.1	489.3	0.04655	0.9977
Accuracy (%)	100.0	95.0	97.0	98.0	101.6	101.3	103.6	97.9	-	-
CV (%)	15.0	13.2	12.0	4.7	7.8	7.2	4.7	4.2	0.7	0.3

Table 2. Precision (CV, %) and accuracy of fargesin in mouse plasma QC samples.

Variables	Intra-day (n = 5)				Inter-day (n = 15)			
QC (ng/mL)	0.2	0.6	20	450	0.2	0.6	20	450
Mean (ng/mL)	0.19	0.63	18.7	479.7	0.18	0.60	20.0	470.4
CV (%)	10.1	7.0	5.5	3.6	8.9	11.3	9.9	6.3
Accuracy (%)	95.5	105.6	93.7	106.6	90.0	100.0	100.0	104.5

Table 3. Matrix effects and recoveries of fargesin and magnolin (IS) in mouse plasma samples (n = 6).

Analytes (ng/mL)	Matrix effect (%)		Recovery (mean ± SD, %)
	Mean	CV	
Fargesin			
0.6	107.3	5.3	90.8 ± 4.1
20	107.6	3.4	88.4 ± 3.7
450	91.7	11.3	98.1 ± 4.4
Magnolin (IS, 10)	110.6	6.8	95.1 ± 3.2

Table 4. Post-preparation, short-term, long-term, and freeze–thaw stabilities of fargesin in mouse plasma QC samples (n = 3).

Stability conditions	fargesin concentration (ng/mL)			
	0.6		450	
	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)
Post-preparation for 24 h at 4°C	100.3	8.2	105.5	4.9
Short-term storage for 2 h at room temperature	93.5	9.0	96.9	0.6
Long-term storage for 28 days at -80°C	105.1	1.7	92.6	0.5
Three freeze–thaw cycles of –80°C to room temperature	99.6	4.3	89.6	4.0

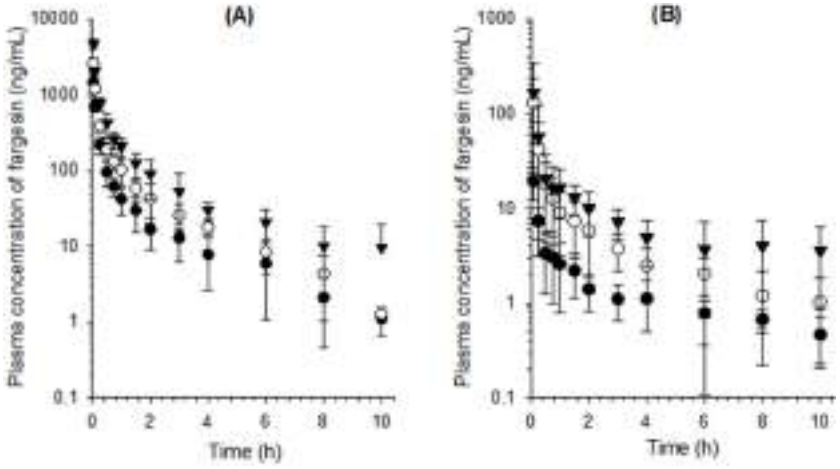

Figure 3. Mean plasma concentration-time profiles of fargesin after (A) an intravenous injection and (B) an oral administration at doses of 1 (●), 2 (○), and 4 (▼) mg/kg to male ICR mice. Data are represented as mean ± SD (n = 6).

Table 5. Mean pharmacokinetic parameters of fargesin after its intravenous injection and oral administration at 1, 2, and 4 mg/kg doses to male ICR mice. Data are represented as mean ± SD (n = 6).

Pharmacokinetic parameters	1 mg/kg	2 mg/kg	4 mg/kg
Intravenous injection			
C_0 (ng/mL)	2481.6 ± 1154.8	4461.0 ± 1007.7	8398.9 ± 1811.8
AUC_{last} (ng·min/mL)[a]	19776.0 ± 5038.4	37038.3 ± 6673.7	72486.9 ± 10273.8
AUC_{inf} (ng·min/mL)	19994.8 ± 5229.1	37943.2 ± 7706.0	73457.0 ± 10691.3
CL (mL/min/kg)	53.2 ± 15.0	54.5 ± 10.2	55.5 ± 8.8
V_{ss} (mL/kg)	2763.0 ± 516.2	3536.3 ± 588.6	3897.9 ± 1199.7
$t_{1/2}$ (min)	94.6 ± 21.2	119.2 ± 63.9	84.7 ± 16.7
MRT (min)	50.4 ± 16.2	53.9 ± 7.4	62.2 ± 11.7
Oral administration			
C_{max} (ng/mL) [a]	19.4 ± 7.2	130.3 ± 106.9	192.0 ± 178.0
T_{max} (min)	5.0	5.0	5.0
AUC_{last} (ng·min/mL) [a]	802.9 ± 240.7	3164.1 ± 1733.2	6953.8 ± 3847.1 [b]
AUC_{inf} (ng·min/mL)	877.6 ± 272.5	3553.8 ± 1581.3	7888.1 ± 3989.7
$t_{1/2}$ (min)	108.8 ± 57.4	140.0 ± 108.4	123.8 ± 60.2
F (%)	4.1 ± 1.2	8.5 ± 4.7	9.6 ± 5.3

[a] Dose normalized (1 mg/kg) AUC_{last} and C_{max} were compared for statistical analysis.
[b] Significantly different ($p < 0.05$) from 1 mg/kg.

Three freeze-thaw cycles, short-term storage at room temperature, long-term storage for 28 days at -80°C, and post-preparation stability for 24 h in 4°C autosampler showed negligible effect on the stability of fargesin (Table 4).

Pharmacokinetics of fargesin in male ICR mice

After intravenous injection of fargesin at doses of 1, 2, and 4 mg/kg to male ICR mice, the mean plasma concentration-time curves are shown in Figure 3A. The pharmacokinetics of intravenously injected fargesin showed a linear kinetics in the dose range of 1–4 mg/kg, which was evidenced by the dose proportional increase of AUC and dose independent CL (53.2-55.5 mL/min/kg), V_{ss} (2763.0-3897.9 mL/kg), and $t_{1/2}$ (84.7-119.2 min) (Table 4). The cumulative fecal excretion of fargesin for 48 h following its intravenous injection at 4 mg/kg was 0.014 ± 0.017% of the dose but it was not excreted in urine, indicating that high systemic clearance (53.2-55.5 mL/min/kg) of fargesin may result from the metabolism.

After oral administration of fargesin at doses of 1, 2, and 4 mg/kg to male ICR mice, the mean plasma concentration-time curves and pharmacokinetic parameters are shown in

Figure 3B and Table 5, respectively. Fargesin was rapidly absorbed after oral administration based on its T_{max} at the first blood sampling time point (5 min). The dose normalized C_{max} and $t_{1/2}$ (108.8-140.0 min) values of fargesin were comparable among three doses studied (Table 5). However, dose normalized AUC_{last} of fargesin at 4 mg/kg (1738.4 ± 961.8 ng·min/mL) was significantly larger than that at 1 mg/kg (802.9 ± 240.7 ng·min/mL). The absolute oral bioavailability of fargesin was 4.0-9.6% for oral dose examined. The cumulative fecal recovery of fargesin after its oral administration at 4 mg/kg dose was 0.089 ± 0.045% of the dose without urinary excretion. Based on these results, low F may be due to the extensive fargesin metabolism.

Conclusions

A sensitive, simple, and reproducible LC-HRMS method using protein precipitation as a sample clean-up procedure was developed for the determination of fargesin with LLOQ level of 0.2 ng/mL in 6 μL of mouse plasma. We evaluated the plasma concentrations of fargesin using this method and the pharmacokinetic parameters of fargesin after intravenous and oral administration of fargesin at doses of 1, 2, and 4 mg/kg to male ICR mice.

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References

1. Shen, Y.; Li, C. G.; Zhou, S. F.; Pang, E. C.; Story, D. F.; Xue, C. C. *Curr. Med. Chem*. **2008**, 15, 1616, DOI: 10.2174/092986708784911515.
2. Hong, P. T. L.; Kim, H. J.; Kim, W. K.; Nam, J. H. *Korean J. Physiol. Pharmacol*. **2021**, 25, 251, DOI: 10.4196/kjpp.2021.25.3.251.
3. Kim, H. J.; Nam, Y. R.; Nam, J. H. *Am. J. Chin. Med*. **2018**, 46, 1079, DOI: 10.1142/S0192415X18500568.
4. Baek, J. A.; Lee, Y. D.; Lee, C. B.; Go, H. K.; Kim, J. P.; Seo, J. J.; Rhee, Y. K.; Kim, A. M.; Na, D. J. *Nitric Oxide* **2009**, 20, 122, DOI: 10.1016/j.niox.2008.10.003.
5. Kim, J. S.; Kim, J. Y.; Lee, H. J.; Lim, H. J.; Lee, D. Y.; Kim, D. H.; Ryu, J. H. *Phytother. Res*. **2010**, 24, 748, DOI: 10.1002/ptr.3028.
6. Lim, H.; Son, K. H.; Bae, K. H.; Hung, T. M.; Kim, Y. S.; Kim, H. P. *Phytother. Res.* **2009**, 23, 1489, DOI: 10.1002/ptr.2783.
7. Pham, T. H.; Kim, M. S.; Le, M. Q.; Song, Y. S.; Bak, Y.; Ryu, H. W.; Oh, S. R.; Yoon, D. Y. *Phytomedicine* **2017**, 24, 96, DOI:10.1016/j.phymed.2016.11.014.
8. Yue, B.; Ren, Y. J.; Zhang, J. J.; Luo, X. P.; Yu, Z. L.; Ren, G. Y.; Sun, A. N.; Deng, C.; Wang, Z. T.; Dou, W. *Molecules* **2018**, 23, 1380, DOI:10.3390/molecules23061380.
9. Lu, J.; Zhang. H.; Pan. J.; Hu, Z.; Liu, L.; Liu, Y.; Yu, X.; Bai, X.; Cai, D.; Zhang, H. *Arthritis Res. Ther.* **2021**, 23, 142, DOI: 10.1186/s13075-021-02512-z.
10. Jun, A. Y.; Kim, H. J.; Park, K. K.; Son, K. H.; Lee, D. H.; Woo, M. H.; Chung, W. Y. *Invest. New Drugs* **2014**, 32, 1, DOI: 10.1007/s10637-013-9969-0.
11. Lee, G. E.; Lee, C. J.; An, H. J.; Kang, H. C.; Lee, H. S.; Lee, J. Y.; Oh, S. R.; Cho, S. J.; Kim, D. J.; Cho, Y. Y. *Int. J. Mol. Sci*. **2021**, 22, 2073, DOI: 10.3390/ijms22042073.
12. Wang, G.; Gao, J. H.; He, L. H.; Yu, X. H.; Zhao, Z. W.; Zou, J.; Wen, F. J.; Zhou, L.; Wan, X. J.; Tang, C. K. *Biochim. Biophys. Acta Mol. Cell. Biol. Lipids* **2020**, 1865, 158633, DOI: 10.1016/j.bbalip.2020.158633.
13. Wang, X.; Cheng, Y.; Xue, H.; Yue, Y.; Zhang, W.; Li, X. *Fitoterapia* **2015**, 105, 16, DOI: 10.1016/j.fitote.2015.05.016.
14. Sha, S.; Xu, D.; Wang, Y.; Zhao, W.; Li, X. *Can. J. Physiol. Pharmacol*. **2016**, 94, 900, DOI:10.1139/cjpp-2015-0615.
15. Lee, Y. S.; Cha, B. Y.; Choi, S. S.; Harada, Y.; Choi, B. K.; Yonezawa, T.; Teruya, T.; Nagai, K.; Woo, J. T. *Biofactors* **2012**, 38, 300, DOI:10.1002/biof.1022.
16. Choi, S. S.; Cha, B. Y.; Choi, B. K.; Lee, Y. S.; Yonezawa, T.; Teruya, T.; Nagai, K.; Woo, J. T. *J. Nat. Med*. **2013**, 67, 320, DOI: 10.1007/s11418-012-0685-4.
17. Fu, T.; Chai, B.; Shi, Y.; Dang, Y.; Ye, X. *J. Dermatol. Sci.* **2019**, 94, 213, DOI: 10.1016/j.jdermsci.2019.03.004.
18. Kim, J. H.; Kwon, S. S.; Jeong, H. U.; Lee, H. S. *Int. J. Mol. Sci*. **2017**, 18, 952, DOI: 10.3390/ijms18050952.
19. Park, R.; Park, E. J.; Cho, Y. Y.; Lee, J. Y.; Kang, H. C.; Song, I. S.; Lee, H. S. *Pharmaceutics* **2021**, 13, 187, DOI: 10.3390/pharmaceutics13020187.
20. Jeong, J. H.; Kim, D. K.; Ji, H. Y.; Oh, S. R.; Lee, H. K.; Lee, H. S. *Biomed. Chromatogr*. **2011**, 25, 879, DOI: 10.1002/bmc.1538.
21. Zhang, Z.L., X.; Cao, W.; Xiao, X.; Qu, J.; Zhao, M.; Li, X., *Curr. Pharm. Anal*. **2016**, 12, 379, DOI: 10.2174/1573412912666151211191600.

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<Books>

Bunker, P. R.; Jensen, P. Molecular Symmetry and Spectroscopy, 2nd ed.. NRC Research Press: Ottawa, 1998.

Zare, R. N. Angular Momentum, Wiley: New York, 1998.

<Software>

Werner, H. J.; Knowles, P. J.; Lindh, R.; Manby, F. R.; Schutz, M. MOLPRO, version 2006. 1., A package of ab initio Programs; See http://www.molpro.net.

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