

Diagnostic Evaluation of Enzyme Activity Related to Steroid Metabolism by Mass Spectrometry-Based Steroid Profiling

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Abstract: Gas chromatography-mass spectrometry (GC-MS) methods have been used extensively in clinical steroid analyses. Evaluating the metabolic ratios of precursors to products by accurate quantification of individual steroid levels in biological samples can reveal the activities of enzymes associated with steroid metabolism. This review article discusses the impact of GC-MS-based steroid profiling on our understanding of the biochemical role of steroids and their metabolic enzymes in hormone-dependent diseases, such as congenital adrenal hyperplasia (CAH), cortisol-mediated hypertension, apparent mineralocorticoid excess (AME), male-pattern baldness, and breast and thyroid cancers. Steroid profiling is a comprehensive analytical technique that can be applied whenever the highest specificity is required and may be a reasonable initial diagnostic approach.

Keywords: steroid, metabolite profiling, biomarker, clinical diagnosis, steroidogenesis

Introduction

The expression and activity of the enzymes associated with steroid metabolism have major implications in the development and progression of various hormone-related diseases in humans, including congenital adrenal hyperplasia (CAH), hypertensive events, androgenic alopecia, and various cancers.¹⁻⁸ In addition, divergent expression and/or activity of key steroid enzymes, such as 7-reductase, 24-reductase, and cytochrome P450 side-chain cleavage enzyme, eventually lead to differential accumulation of cholesterol in steroidogenesis.⁹⁻¹¹ Abnormal cholesterol metabolic pathways are associated with neurodegenerative diseases.¹²⁻¹⁴ Steroid hormones regulate various body functions and are components of the endocrine system, which coordinates the physiological responses to various biochemical stresses. Many endocrine disorders can be attributed to defects in these enzymes, which lead to hormonal imbalances and have serious consequences.

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The biochemical expression of steroid hormones depends on the rate of metabolism of cholesterol (Figure 1),¹⁵ which is biosynthesized via the mevalonate pathway. The mevalonate pathway leads to the production of lanosterol, which can then be converted to cholesterol through either the Bloch pathway, via desmosterol, or the Kandutsch-Russell pathway, via 7-dehydrocholesterol (Figure 2).¹⁶ Quantitative analysis of steroid hormones is crucial for predicting the overall biological impact that steroids have on peripheral target tissues and their potential role in the development and progression of hormone-dependent diseases.

In general, biologically active steroids are present at very low concentrations in the biological specimens, such as urine, blood, and tissue, used in clinical applications. Steroids are categorized as sterols, progestins, corticoids, androgens, and estrogens. All steroids have similar chemical structures, but different chemical and physical properties, especially estrogens, which have aromatic resonance in their A-ring (Figure 1). They also exhibit some behaviors (e.g., free forms, conjugated forms, and protein-binding forms); therefore, their different chemical properties should be considered when developing analytical methods. Due to the high sensitivity and selectivity, mass spectrometry-based steroid profiling combined with gas chromatography and liquid chromatography (GC-MS^{1,4,10,15} and LC-MS^{2,9,17,18}, respectively) is widely used to analyze metabolites and their precursors in steroid metabolism and determine the ratio of steroid metabolites to their precursors as an indicator of enzyme activity.

This review presents an overview of the function and expression of the steroidogenic enzymes that are associated

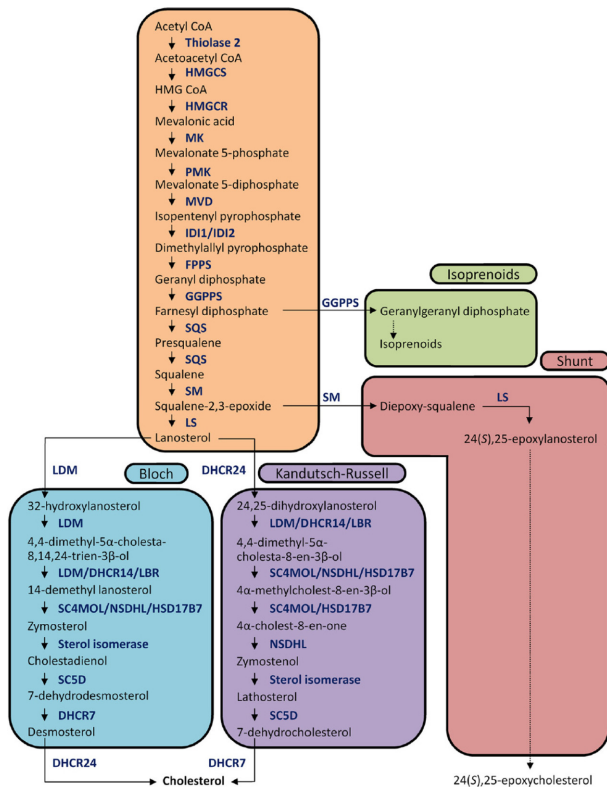


Figure 2. Cholesterol biosynthetic pathways. The mevalonate pathway produces lanosterol, which can be converted to cholesterol by either the Bloch pathway, via desmosterol, or the Kandutsch-Russell pathway, via 7-dehydrocholesterol. Two other branches also diverge from the mevalonate pathway. The intermediates and enzymes in this shunt pathway have not yet been fully elucidated; however, they are presumed to follow the Kandutsch-Russell pathway. Abbreviations: MK, mevalonate kinase; PMK, phosphomevalonate kinase; MVD, diphosphomevalonate decarboxylase; FPPS, farnesyl pyrophosphate synthase; SQS, squalene synthase; LDM, lanosterol 14 α -demethylase; SC5D, sterol C5-desaturase. Data are from reference #16.

has the advantage of high sample throughput, making it attractive for clinical applications. In addition, it has been successfully applied to measure 10 adrenal steroids, including 17-OHP, in the serum of CAH patients.²⁸ The high speed of analysis and overall analytical sensitivity of MALDI-MS make it an attractive alternative to the traditional methods used for steroid analysis.

11 β -HSD isoenzymes in corticoid metabolism

Adrenal cortisol regulates carbohydrate metabolism, enhances vasoconstrictor response to catecholamines, and increases water clearance at normal physiological concentrations.²⁹ The major metabolic pathways of cortisol (F) include interconversion of F to cortisone (E) through the

activity of 11 β -hydroxysteroid dehydrogenase (11 β -HSD) isoenzymes and the reduction of the double bond in the A-ring by either 5 β - or 5 α -reductase to yield 5 β -tetrahydrocortisol (THF) or 5 α -THF (allo-THF; Figure 1). Homozygous inactivating mutations in the 11 β -HSD type 2 isoenzyme result in cortisol-mediated hypertension or apparent mineralocorticoid excess (AME).³⁰ Patients with AME excrete lower amounts of cortisone metabolites, i.e., tetrahydrocortisone (THE), than cortisol metabolites, i.e., THF and allo-THF.^{31,32} When GC-MS-based steroid profiling was applied to rule out the possibility of AME in two patients with hypokalemic hypertension, the results showed low levels of plasma renin activity and aldosterone, normal urinary excretion of glucocorticoid metabolites, and elevated ratios of allo-THF+THF/THE and F/E, although the levels of THE and E were slightly low (Figure 4). These results differentiate AME syndrome from other hypertensive diseases with clinical features indicative of mineralocorticoid excess.⁴

Blood vessels are target tissues for both glucocorticoids and mineralocorticoids. The pathogenesis of hypertension is closely associated with excess sodium intake. Because steroid hormones modulate renal sodium retention, it is likely that variations in 11 β -HSD type 2 activity are responsible for the sensitivity of blood pressure to dietary salt.³³

There are seasonal variations in many endocrine systems, as well as in the prevalence of cardiovascular events. Glucocorticoid activity was assessed in healthy men, as measured by the plasma levels of F, E, THF, allo-THF, and THE in different seasons.³⁴ The results showed that plasma cortisol levels and tissue sensitivity to glucocorticoids are higher in winter, but cortisol production rate is lower. These variations are associated with 11 β -HSD type 1 activity and may offer a possible reason why increased glucocorticoid activity contributes to the increased prevalence of disease during the winter.

5 α -Reductase in male-pattern baldness

The growth of axillary, pubic, and beard hair is stimulated by androgens; however, the responses to androgens in the scalp are variable, which shortens the anagen phase of the hair growth cycle and may accelerate hair loss.³⁵ Hair follicles contain predominantly follicular epithelial cells that can metabolize testosterone to dihydrotestosterone (DHT; via 5 α -reductase type 2), the most biologically potent androgen. The degree of baldness is not correlated with the density of hair on the trunk and limbs. This indicates that the normal levels of circulating testosterone present after puberty are sufficient for maximal production of DHT and suggests that hair loss is associated with quantitative differences in the number of androgen receptors (ARs) and 5 α -reductase activity in the scalp.³⁶ Because both testosterone and DHT bind to the same AR, understanding the mechanism of androgen action in human hair follicles associated with male-

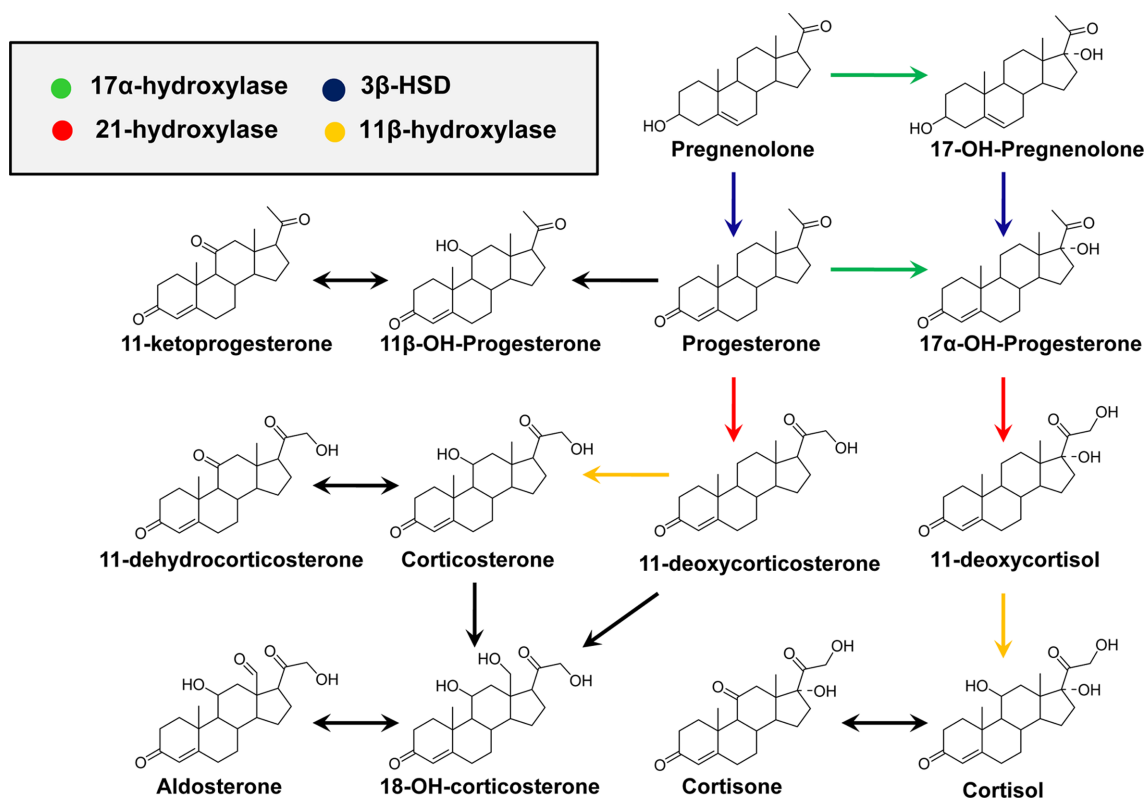


Figure 3. An overview of cortisol metabolism catalyzed by various metabolic enzymes in congenital adrenal hyperplasia (CAH). The enzymes that mediate each conversion are indicated by different colored arrows. Defects in the four main enzymes required for the synthesis of cortisol from cholesterol in the adrenal cortex can lead to CAH, including 3β-hydroxysteroid dehydrogenase (3β-HSD) and three hydroxylases, CYP17A1 (17α-hydroxylase), CYP21A2 (21-hydroxylase), and CYP11B1 (11β-hydroxylase).

pattern baldness (MPB) is of great interest.³⁷

Although many studies have provided details about the amounts of ARs and/or 5α-reductase in hair follicle tissues associated with androgenetic alopecia,^{38,39} the etiology of MPB is not clear, and no correlation has yet been found between androgen plasma levels.⁴⁰ Therefore, a better understanding of androgen levels and metabolism at the pilosebaceous level may help understand the process involved in alopecia. Based on hair androgen levels measured by GC-MS,⁴¹ the levels of DHT, testosterone, and epitestosterone in the vertex hair of the balding group (balding fathers [aged 28-55 years] and their sons [aged 8-16 years]) were compared to those in the hair of a control group comprising age/sex-matched non-balding subjects.⁶ The results showed a representative chromatographic window to screen for potential baldness by GC-MS analysis of these three measured steroids in hair (Figure 5). This chromatogram provided a fairly complete distinction between the balding group and the non-balding group. In the hair of balding subjects, an abundant amount of DHT was found, whereas in the control group, a noticeably high level of epitestosterone was detected. In contrast, there was no significant difference in testosterone in the mass chromatogram, although the level was slightly higher

in the hair of the balding subjects.

Although the androgen-associated mechanism of hair loss in MPB has been well established, there is little information on the racial differences in the expression of steroids in hair follicles. Based on a heat-map of visualized steroid signatures in hair,⁴² racial differences in steroid metabolism were examined.⁴³ In contrast to serum levels of testosterone and DHT,^{44,45} the hair levels of both of these steroid hormones were higher in the Korean group than in the Caucasian group. Although the hair levels of these MPB effectors were significantly higher in Koreans, clinically apparent MPB is more common in Caucasians than in Asians.⁴⁶ The higher levels of DHT detected in the Korean group, which has a lower incidence of MPB, suggest that the Caucasian population has higher affinity androgen receptors. In addition, the lower levels of epitestosterone, an anti-androgen, in Caucasians could also explain the difference in incidence. The different androgenic potential was not only indicated by the hair levels of these individual steroids but also by the balance of their metabolic enzymes, mainly 5α-reductase, 3β-HSD, and 17β-HSD, which are associated with androstenedione, a precursor of testosterone (Figure 6).

MS-based diagnostic evaluation of steroidogenic enzymes

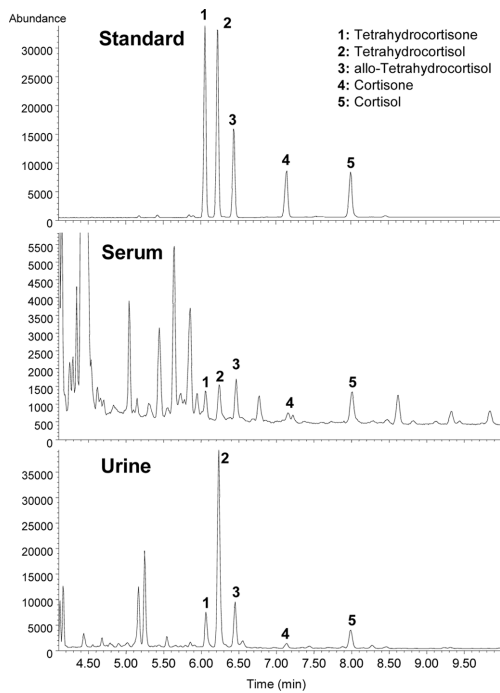


Figure 4. GC-MS total ion chromatograms of cortisol and cortisone and their metabolites (10 pg each in the standards) in two extracts, that is, human serum and urine samples. Data are from reference #4.

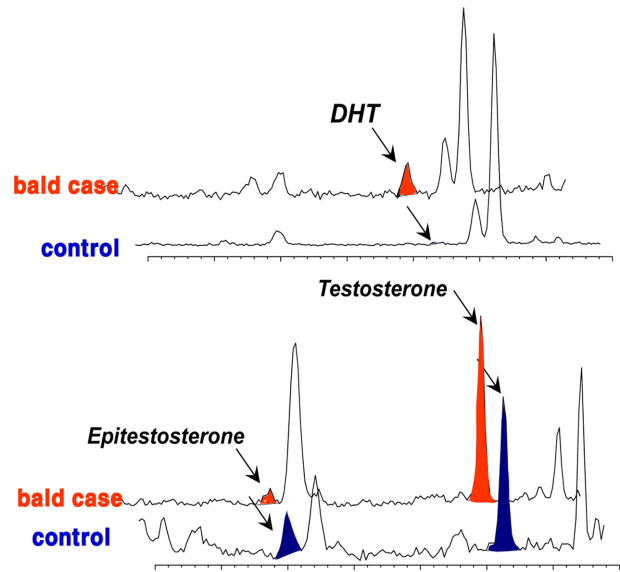


Figure 5. Reconstructed ion chromatogram (RIC) for the determination of DHT, epitestosterone, and testosterone in hair. Comparison of bald and control subject hair samples by selected-ion monitoring and chromatographed on an Ultra-1 (17 m × 0.2 mm i.d. × 0.11 mm film thickness) capillary column. The selected ion for DHT ($t_R = 12.78$, *a*) was m/z 586, and the ions for epitestosterone ($t_R = 11.60$, *b*) and testosterone ($t_R = 13.22$, *c*) were m/z 584. Data are from reference #41.

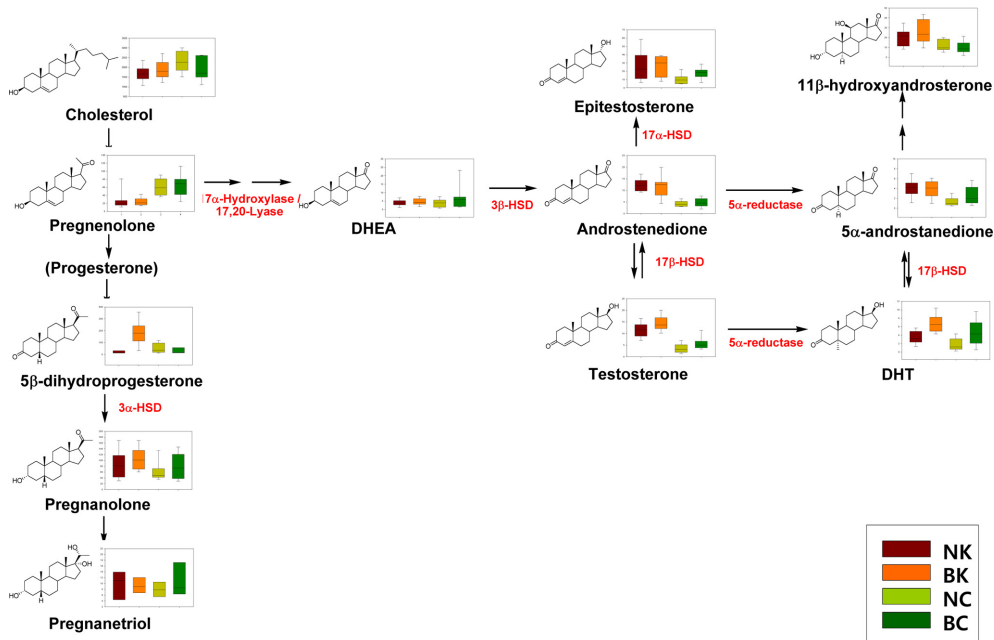


Figure 6. Steroid metabolism differences in balding and normal Koreans and Caucasians. The line within the box represents the median, the lower boundary of the box indicates the 25th percentile, and the upper boundary of the box indicates the 75th percentile. The whiskers above and below indicate the maximum and minimum steroid levels, respectively. The dots above and below indicate the plot outliers with the 10th and 90th percentiles, respectively. Abbreviations: 3α-HSD, 3α-hydroxysteroid dehydrogenase; 17α-HSD, 17α-hydroxysteroid dehydrogenase; 17β-HSD, 17β-hydroxysteroid dehydrogenase; NK, normal Korean; BK, balding Korean; NC, normal Caucasian; BC, balding Caucasian. Data are from reference #43.

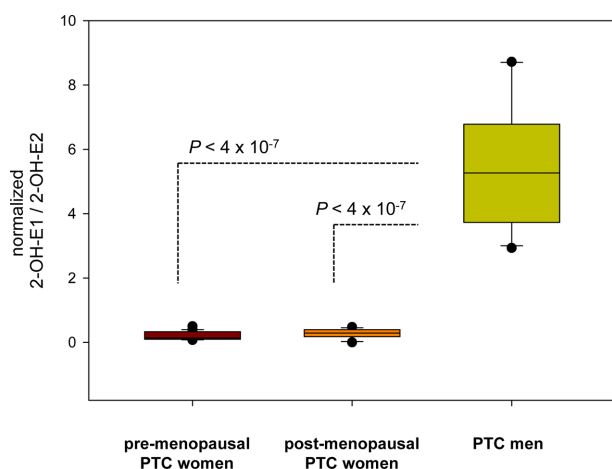


Figure 7. Sex-based differences in the 2-hydroxyestrone to 2-hydroxy-17 β -estradiol metabolic ratio. This metabolic ratio could help determine 17 β -hydroxysteroid dehydrogenase activity in estrogen metabolism. The line within the box represents the median, the lower boundary of the box indicates the 25th percentile, and the upper boundary of the box indicates the 75th percentile. The whiskers above and below indicate the maximum and minimum steroid levels, respectively. The dots above and below indicate the plot outliers with the 10th and 90th percentiles, respectively. Data are from reference #52.

Hydroxylation of estrogens in women

Although estrone and 17 β -estradiol are primarily responsible for female reproduction, their metabolites cause many of the positive and negative effects of estrogens. Modulation of estrogen hydroxylation is essential, as some of the other metabolites increase the risk of breast and other hormone-related cancers. Oxidation of estrogens occurs at the C-2, C-4, and C-16 positions to yield 2-hydroxy (2-OH) estrogen, 4-hydroxy (4-OH) estrogen, and 16 α -OH-estrone, respectively. It has been proposed that a shift toward the 2-hydroxyestrone pathway from the 16 α -hydroxyestrone metabolic pathway may be inversely associated with breast cancer risk because 2-hydroxyestrone is thought to be less genotoxic and estrogenic than 16 α -hydroxyestrone. However, there is no supporting evidence for an association between the circulating levels of 2-OH-estrone and 16 α -OH-estrone and the risk of breast cancer among postmenopausal women.^{47,48} Microsomes in normal tissue obtained from either breast cancer patients or patients who underwent reduction mammoplasty operations expressed comparable estradiol 2- and 4-hydroxylase activities. Therefore, an elevated ratio of 4/2-hydroxyestradiol in neoplastic mammary tissue may be a useful marker of malignant breast tumors and suggests a mechanistic role for 4-hydroxyestradiol in tumor development.⁴⁹ Individual hydroxyestrogens may play distinct roles in the onset and clinical progression of breast cancer.⁵⁰

Estrogen metabolism might also be associated with the physiopathological development of papillary thyroid carcinoma (PTC). To evaluate the differences in estrogen metabolism between benign and malignant PTC, GC-MS-based estrogen profiling was applied to urine samples from postmenopausal patients diagnosed with benign tumors and malignant stage I and stage III/IV PTCs. The results showed that increased 16 α -hydroxylation, a decreased 2/16 α -ratio, and increased reductive 17 α -HSD could be used as potential biomarkers.⁵¹ Quantitative steroid profiling combined with GC-MS was used to measure the urinary concentrations of 84 steroids in premenopausal and postmenopausal female and male patients with PTC. Statistically significant differences were found in the ratio of the estrogen metabolites 2-hydroxyestrone and 2-hydroxy-17 β -estradiol, which could indicate that there are 17 β -HSD differences between women and men with PTC (Figure 7).⁵²

Conclusion

Biochemical tests have been the basis for studies on disorders affecting steroid hormones and their metabolic enzymes. In the future, MS-based profiling may offer improvements over the current assays in terms of improved specificity and accuracy that will aid in the differential diagnosis of related diseases, help elucidate the causes of common clinical problems, and further our understanding of the developmental milestones in hormone-dependent diseases.

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