Comparative GC-MS Based *In vitro* Assays of 5α-Reductase Activity Using Rat Liver S9 Fraction

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Abstract: 5α-Dihydrotestosterone (DHT) is the primary active metabolite of testosterone, catalyzed by 5α-reductase (5αR) in the skin, prostate, and liver. In this study, the 5αR activity in rat liver S9 fraction in the presence of a NADPH-generating system was evaluated and compared by gas chromatography-mass spectrometry (GC-MS)-based *in vitro* assays. Testosterone and a 5αR inhibitor, finasteride, were added to the S9 fractions and incubated at 37°C for 1 h. Both testosterone and DHT were quantitatively measured and compared with two different GC-MS-based steroid profiling techniques. DHT was not detected by conventional GC-MS analysis in the absence of finasteride when the concentration of testosterone in the S9 fraction was less than 0.2 μM, whereas the isotope-dilution GC-MS (GC-IDMS) system was able to evaluate the 5αR activity. Because the S9 fraction contains more reactive enzymes and is easier to collect from tissues compared with a microsomal solution, the combination of the S9 fraction and GC-IDMS technique may be a promising assay for evaluating the 5αR activity in large-scale clinical studies.

Key words: 5α-Reductase, Testosterone, Dihydrotestosterone, Isotope-dilution, GC-MS, Liver microsome

Introduction

5α-Dihydrotestosterone (DHT) is primarily produced from a major androgen testosterone by the enzyme 5α-reductase (5αR), which is present throughout the body in two forms, type 1 and 2 (Figure 1). The formation of DHT is associated with the development of prostate diseases and androgenic alopecia.[1-3] Finasteride, a selective inhibitor of 5αR type 2, has been medicated for both benign prostate hyperplasia (BPH) and male-pattern baldness (MBP) and suppresses serum DHT by about 70%.[4]

The quantitative analysis of steroid metabolites, including testosterone and DHT, and their interesting biological pathway have been used for physiological monitoring, toxicological evaluation, and disease diagnosis.[5,6] In routine clinical screening, immunoassays have advantages but they are not selective in small molecule analysis, which can result in overestimations owing to the cross-reactions of antibodies.[7,8] In contrast, gas chromatography-mass spectrometry (GC-MS)-based techniques show better quantitative reproducibility, and GC-MS profiling is widely used in steroid analysis.[9,10] Isotope-dilution mass spetrometry (IDMS) is the primary method, using an adjusted isotope labeled standard to minimize run-to-run variations.[11,14] In an isotope-dilution assay, both accuracy and precision are potentially improved against other calibration methods owing to compensation by identical losses and correcting matrix effects.[13]

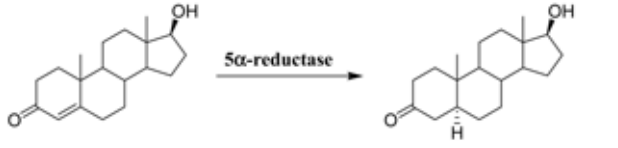

Figure **1.** Biosynthesis of 5α-dihydrotestosterone from testosterone catalyzed by 5α-reductase.

Liver S9 fraction is most frequently used in assays that measure the metabolism of xenobiotics, and it contains cytosol and microsomes, which are easier to prepare than purified microsomes.[15] Therefore, we compared two different steroid profiling techniques with GC-IDMS[14] and a traditional GC-MS method[16] for quantitative detection of testosterone and DHT, in the evaluation of 5α-reductase activity after incubation of the rat liver S9 fraction.

Experimental Section

Chemicals

Testosterone and DHT reference standards examined were obtained from Steraloids (Newport, RI, USA). The internal standards (ISTDs), 16,16,17-d_3-testosterone and

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16,16,17-d_3-DHT were purchased from NARL (Sydney, Australia). β-nicotinamide adenine dinucleotide phosphate hydrate (β-NADPH) and 40 mM potassium phosphate buffer and PBS buffer were obtained from Sigma (St. Louis, MO, USA) and T&I (Seoul, Korea), respectively. For solid-phase extraction (SPE), an Oasis HLB cartridge (3 mL, 60 mg) was supplied by Waters (Milford, MA, USA). Sodium phosphate monobasic, sodium phosphate dibasic, and potassium carbonate were acquired from Sigma. The derivatizing agents *N*-methyl-*N*-trifluorotrimethylsilyl acetamide (MSTFA), flophemesyl chloride, ammonium iodide (NH_4I), and dithioerythritol (DTE) were also purchased from Sigma. All organic solvents were of analytical or HPLC grade and purchased from Burdick & Jackson (Muskegan, MI, USA). Deionized water was prepared using a Milli-Q purification system (Millipore, Billerica, MA, USA).

Preparation of standard solution

The stock solutions of all the reference standards were dissolved in methanol at 100 μg/mL, and the working solutions were made up with methanol at concentrations of 0.01–10 μg/mL. Finasteride and testosterone used in the liver S9 fraction incubation system were made using DMSO at a concentration of 0.1 mM. All standard solutions were stored at −20°C until required.

Sample preparation of the liver S9 fraction

Male Sprague-Dawley (SD, 8-9 weeks) rats were obtained from Japan SLC, Inc. (Shizuoka, Japan). They were anesthetized with isoflurane before extraction of the liver. The liver was washed with PBS buffer and homogenized in five volumes of 20 mM potassium phosphate buffer containing 0.32 M sucrose and 1 mM DTT (pH 6.5). To remove the nuclei and other debris, the whole-liver homogenate was centrifuged at 1,000 g for 10 min. The liver S9 fraction was then obtained by centrifugation of the homogenate supernatant at 9,000 g for 30 min. The supernatant was then measured for protein content by Bradford's assay.

Incubation of finasteride and testosterone with rat liver S9 fraction

Incubation was performed as described previously, with a minor modification.[17-19] The incubation mixture consisted of 0.2 μM testosterone, a NADPH-generating system (0.5 μmol of $NADP^+$, 5 μmol of glucose-6-phosphate, 5 μmol of $MgCl_2$), 0.1 mM finasteride, and 500 μg of S9 fraction in a final volume of 0.5 mL of 40 mM potassium phosphate buffer (pH 6.5). The incubation was carried out at 37°C for 1 h. An incubation system without finasteride was used as a control.

Comparative GC-MS methods in evaluation of 5α-reductase activity

Both the GC-IDMS method and the GC-MS-based steroid assay for quantitative detection of testosterone and DHT were based on our previous reports.[14,16] Briefly, the microsomal fractions (0.5 mL) were spiked with 10 μL of ISTDs (d_3-testosterone; 1 μg/mL, d_3-DHT; 0.5 μg/mL) for isotope-dilution assay and 20 μL of ISTDs (d_3-T and d_4-17β-E2, 0.2 μg/mL; d_4-F and d_8-17α-OH-Prog, 1 μg/mL; and methyltestosterone and d_9-Prog, 2 μg/mL) for steroid assay. After the addition of 2.5 mL of 0.2 M sodium acetate buffer, the samples were extracted using the Oasis HLB™ SPE cartridges and the eluents were evaporated under a nitrogen stream. One milliliter of 0.2 M phosphate buffer (pH 7.2) and 500 μL of 5% potassium carbonate were then added, and the solution was extracted twice with 2.5 mL of methyl tert-butyl ether (MTBE) for the isotope-dilution assay and with ethyl acetate : *n*-hexane (2:3, v/v) for the steroid assay. The resulting extracts were evaporated using a N_2 evaporator at 40°C. Flophemesyl chloride (50 μL) was added to the dried residue and incubated at room temperature for 15 min. After evaporation of the reaction solution under nitrogen at 70°C, the residue was further derivatized with MSTFA/NH_4I/DTE (40 μL; 500:4:2, v/w/w) at 60°C for 20 min. In the steroid assay, chemical derivatization was done using MSTFA/NH_4I/DTE only. Finally, 2 μL of the resulting mixture was subjected to GC-MS in the selected-ion monitoring (SIM) mode.

GC-MS conditions

GC-IDMS analysis was performed using an Agilent 6890 Plus gas chromatograph interfaced with a single-quadrupole Agilent 5975 MSD at an electron energy of 70 eV. Each sample (2 μL) was injected in split mode (8:1) at 280°C with helium as the carrier gas at 234.42 kPa (0.8 mL/min) and at a constant flow-rate, and they separated through an Ultra-1 capillary column (25 m × 0.2 mm i.d., 0.33 μm film thickness; Agilent Technologies; Palo Alto, CA, USA). The GC oven temperature was initially set to 280°C. It was then increased to 320°C at 8°C/min; this temperature was maintained for 6 min, with an ion source temperature of 230°C. For quantitative analysis, the characteristic ions of each androgen were determined as their flophemesyl-TMS derivatives (Figure 2).

For steroid assay, the sample was injected in split mode (8:1) at 280°C and separated using an Ultra-1 capillary column. The GC oven temperature was initially set to 215°C, increased to 260°C at 1°C/min and then increased to 320°C at 15°C/min; this temperature was maintained for 1 min. The carrier gas was helium at a constant head pressure of 255.1 kPa. For quantitative analysis, the characteristic ions of each steroid were determined as their TMS derivatives (data not shown).

Results and Discussion

Extraction of rat liver S9 fraction

Various techniques have been tested for the extraction of

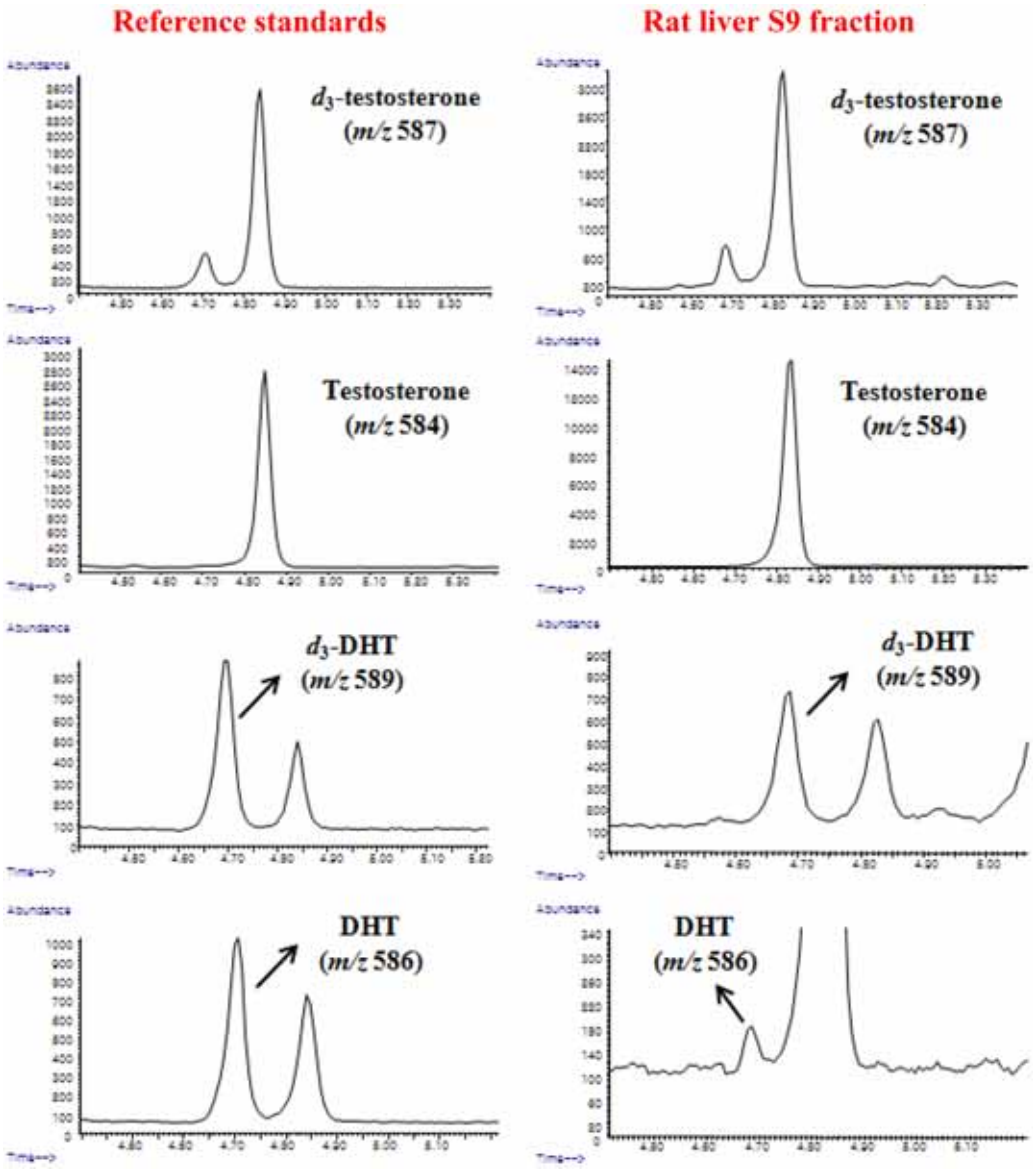

Figure **2.** GC-IDMS chromatograms of testosterone and DHT and their internal standards, obtained from the reference standards and the incubated rat liver S9 fraction.

Table 1. Extraction efficiencies of three different techniques for preparing rat liver fractions

	Finasteride (mM)	Concentration (ng/mL)		5α-reductase activity (DHT/T)
		Testosterone	DHT	
Homogenizer	0	54.16	5.70	0.105
	0.1	189.01	-	-
Tissue homogenizing kit	0	85.54	10.57	0.124
	0.1	176.62	-	-
TissueLyzer	0	134.63	4.05	0.030
	0.1	182.49	-	-

the rat liver S9 fraction such as homogenizer, tissue homogenizing kit, and TissueLyzer. The 5α-reductase activity of all three fractions was measured, and the former two methods showed relatively low efficacy than that of the TissueLyzer (Table 1). However, the simple homogenizing technique was selected because the tissue homogenizing kit is designed only for small scale incubation.

Measurement of protein amount in rat liver S9 fraction

Solubilized protein content in the obtained S9 fractions were measured with a simple and accurate Bradford assay procedure. This required five dilutions of a protein standard, in this case bovine serum albumin (BSA), ranging from 5 to 20 mg/mL. These dilutions were incubated with Coomassie® Brilliant Blue G-250 dye at room temperature for 5 min and were then measured for absorbance at 570 nm by using a UV spectrometer. Comparison of the absorbance of the extract of rat liver S9 fraction with the BSA standard curve provided a relative measurement of protein concentration.

Evaluation of 5α-reductase activity

To quantitatively detect testosterone and DHT, both GC-IDMS and steroid assay were tested. The metabolic ratio of DHT to testosterone, which reflects the 5α-reductase enzyme activity,[13,16] was evaluated. Owing to better selective and sensitive detection of DHT in the absence of finasteride when the concentration of testosterone was lower than 0.2 μM in the S9 fraction, the 5α-reductase activities could be comprehensively evaluated only by the GC-IDMS method.

Conclusions

Due to compensation by identical losses and correcting matrix effects in IDMS analysis, the devised GC-IDMS resulted in a precise and accurate quantification technique for evaluating 5α-reductase activity compared to the steroid assay. Owing to the easy preparation of the enzyme fraction, the combined method of GC-IDMS with rat liver S9 fraction assay can be useful for evaluating drug efficacy in large-scale clinical applications.

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