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Simultaneous quantitation of nine hydroxy-androgens and their conjugates in human serum by stable isotope dilution liquid chromatography electrospray ionization tandem mass spectrometry

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ABSTRACT

Castration resistant prostate cancer (CRPC), the fatal form of prostate cancer, remains androgen dependent despite castrate levels of circulating testosterone (T) and 5α-dihydrotestosterone (DHT). To investigate mechanisms by which the tumor can synthesize its own androgens and develop resistance to abiraterone acetate and enzalutamide, methods to measure a complete androgen profile are imperative. Here, we report the development and validation of a stable isotope dilution liquid chromatography electrospray ionization tandem mass spectrometric (SID-LC-ESI–MS/MS) method to quantify nine human hydroxy-androgens as picolinates, simultaneously with requisite specificity and sensitivity. In the established method, the fragmentation patterns of all nine hydroxy-androgen picolinates were identified, and [$^{13}C_3$]-5α-androstane-3α, 17β-diol and [$^{13}C_3$]-5α-androstane-3β, 17β-diol used as internal standards were synthesized enzymatically. Intra-day and inter-day precision and accuracy corresponds to the U.S. Food and Drug Administration Criteria for Bioanalytical Method Validation. The lower limit of quantitation (LLOQ) of nine hydroxy-androgens is 1.0 pg to 2.5 pg on column. Diols which have been infrequently measured: 5-androstene-3β, 17β-diol and 5α-androstane-3α, 17β-diol can be determined in serum at values as low as 1.0 pg on column. The method also permits the quantitation of conjugated hydroxy-androgens following enzymatic digestion. While direct detection of steroid conjugates by electrospray-ionization tandem mass spectrometry has advantages the detection of unconjugated and conjugated steroids would require separate methods for each set of analytes. Our method was applied to pooled serum from male and female donors to provide reference values for both unconjugated and conjugated hydroxy-androgens. This method will allow us to interrogate the involvement of the conversion of 5-androstene-3β, 17β-diol to T, the backdoor pathway involving the conversion of 5α-androstane-3α, 17β-diol to DHT and the inactivation of DHT to 5α-androstane-3β, 17β-diol in advanced prostate cancer.

1. Introduction

Prostate cancer (CaP) is the most frequently diagnosed cancer and the second leading cause of cancer death in males in the United States [1]. Pathophysiological studies have shown that the development of CaP is initially dependent on androgens and mediated by the androgen receptor (AR) signaling axis [2–4]. Thus, androgen deprivation therapy (ADT) has been the primary clinical treatment for localized advanced or metastatic CaP [5,6]. However, progression of CaP occurs within 1–2 years in almost all patients receiving ADT despite castrate levels of circulating androgens (e.g. T and DHT), and is defined as castration resistant prostate cancer (CRPC), the fatal form of CaP [7,8]. CRPC is now treated with new drugs that either target androgen biosynthesis or antagonize the AR. Abiraterone acetate, which inhibits the activities of cytochrome P450 17α-hydroxylase/17,20-lyase (*CYP17A1*) to block the

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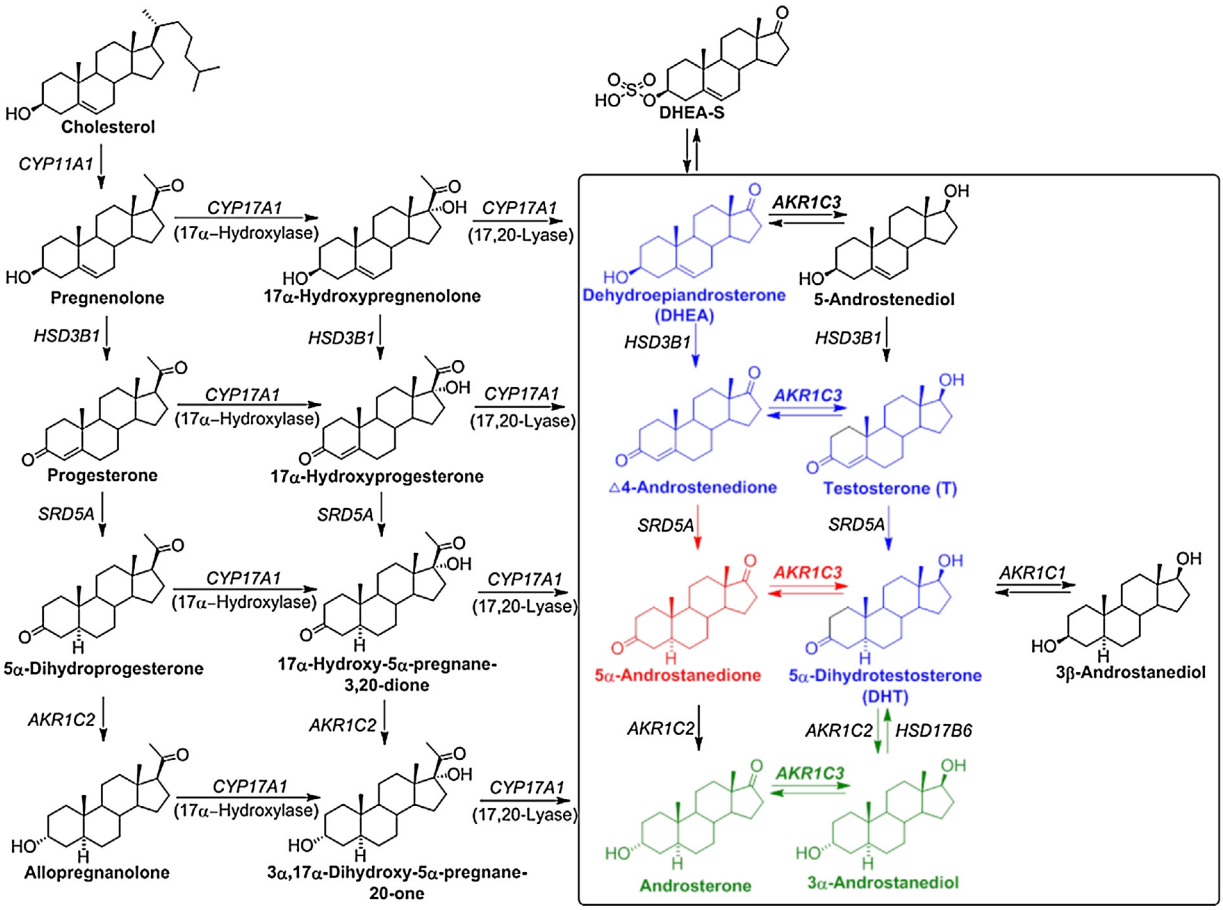

Scheme 1. Intracrine androgen biosynthesis. Intraprostatic androgen metabolism is shown in the rectangle. Blue: classical pathway; Red: alternative pathway; Green: backdoor pathway. 3α-androstanediol: 5α-androstane-3α, 17β-diol; 3β-androstanediol: 5α-androstane-3β, 17β-diol; 5-androstenediol: 5-androstene-3β, 17β-diol; 5α-androstanedione: 5α-androstane-3,17-dione; Δ^4-androstenedione: Δ^4-androstene-3,17-dione; *AKR1C1*: 20α-hydroxysteroid dehydrogenase; *AKR1C2*: type 3 3α-hydroxysteroid dehydrogenase; *AKR1C3*: type 5 17β-hydroxysteroid dehydrogenase; *CYP11A1*: cytochrome P450 11A1; *CYP17A1*: cytochrome P450 17A1; DHEA-S: DHEA-sulfate; *HSD3B1*: type 1 3β-hydroxysteroid dehydrogenase; *HSD17B6*: type 6 17β-hydroxysteroid dehydrogenase; *SRD5A*: 5α-reductase. Enzymes are identified by gene names in italics.

conversion of pregnenolone to DHEA (Scheme 1), significantly reduces circulating androgens, improves overall survival in CRPC patients and has been approved by FDA [9–11]. Enzalutamide, a second generation of AR antagonist, also offers efficacious treatment and survival benefit for patients with advanced prostate cancer [12,13]. The positive results obtained with these two agents in clinical trials indicate that CRPC remains androgen driven by the reactivation of AR signaling due in part to intratumoral androgen biosynthesis [5,14,15]. However, resistance to abiraterone and enzalutamide has been reported due to an elevated expression level of *CYP17A1*,*AKR1C3*, *AR* gene amplification or the emergence of AR splice variants which are constitutively active etc. [15–18]. In order to investigate the efficacy of new drug treatments, understand mechanisms of drug resistance and create precision treatment for CRPC, clinical chemistry requires methods to measure serum and intratumoral androgen levels with the requisite specificity, sensitivity, accuracy and precision.

T and DHT can be synthesized via four different pathways, where the enzymes involved in the prostatic androgen biosynthesis are shown in Scheme 1. The classical pathway involves DHEA → Δ^4-androstenedione → T → DHT. An alternative pathway bypasses the formation of T and converts Δ^4-androstenedione to 5α-androstanedione which is further reduced to DHT. Another pathway to DHT, also known as the backdoor pathway, converts androsterone to 3α-androstanediol which is subsequently oxidized to DHT. In addition, a potential route to testosterone involves the conversion of DHEA to 5-androstenediol and its subsequent dehydrogenation and isomerization to T [19]. The central role of AKR1C3 is also shown.

Several analytical methods exist to measure T and DHT and their precursors involved in intracrine androgen biosynthesis such as immunoassay, gas chromatography tandem mass spectrometry (GC–MS/MS) and liquid chromatography tandem mass spectrometry (LC–MS/MS) [20,21]. In comparison to traditional immunoassays such as radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA), mass spectrometry does not suffer from cross reactivity problems that can plague antibody based methods. In addition, LC–MS/MS provides accurate structural information of analytes (e.g. it can distinguish between different regio- and stereo-isomers), and can measure many analytes simultaneously using the selected reaction monitoring (SRM) mode and thus reduces the sample size of the biospecimen [21–29]. However, LC–MS/MS methods can be limited by insufficient sensitivity from poorly ionized steroids using soft ionization sources (e.g. ESI) [30,31]. To circumvent this problem, chemical derivatization techniques have been introduced to form easily ionized analytes prior to LC–MS/MS analysis [32]. Girard-T/P reagents targeting carbonyl groups (e.g. keto-androgens) have been successfully applied to improve detection sensitivity. LC-ESI–MS/MS coupled with Girard-T derivatization has been used by us to systematically quantify the keto-androgen profile in patients with CaP and CRPC [30,33–35]. Steroids which contain both a keto group