Clinica Chimica Acta 476 (2018) 15–24

Contents lists available at ScienceDirect

Clinica Chimica Acta

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

The Simultaneous measurement of serum testosterone and 5α-dihydrotestosterone by gas chromatography–mass spectrometry (GC–MS)

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ARTICLE INFO

Keywords:
Testosterone
Dihydrotestosterone
Gas chromatography–mass spectrometry
Reference intervals
Polycystic ovary syndrome (PCOS)

ABSTRACT

Background: Simultaneous measurement of testosterone (T) and 5α-dihydrotestosterone (DHT) is important for diagnosing androgen deficiency states and hyperandrogenism in males and females, respectively. However, immunoassays used for T and DHT determination suffer from inadequate specificity and sensitivity, while tandem mass spectrometry is expensive and demanding in use.

Methods and results: We developed a selective gas chromatography–mass spectrometry (GC–MS) method for parallel T and DHT measurement. The assay showed a linear response up to 46.5 nmol/L, intra- and interassay imprecision and inaccuracy < 15% and recoveries in spiked samples > 90% for both analytes. The limit of quantitation was 0.117 nmol/L for T and 0.168 nmol/L for DHT. Comparison with immunoassays revealed good agreement for T in males, but a bias in favour of immunoassays at low concentrations for T in females and DHT in both sexes. We established reference ranges for T and DHT and suggest interval partitioning for T according to age in men and menstrual cycle in women. Assay validation in a clinical setting suggests that measuring DHT or T/DHT ratio may help identify patients with polycystic ovary syndrome.

Conclusion: We developed a selective, simple and inexpensive GC–MS method for parallel measurement of T and DHT with potential use in the clinical laboratory.

1. Introduction

Testosterone (T) and 5α-dihydrotestosterone (DHT) are two naturally occurring androgens crucial to the development of male reproductive organs and the maintenance of male phenotype in adults. DHT is a metabolite of T produced by the activity of the NADPH-dependent enzyme 5α-reductase, which is expressed in the skin, liver, adipose tissue, blood cells and reproductive organs (testes, ovary) [1,2]. Although the androgenic potency of DHT is 3- to 10-fold greater than T due to its higher affinity towards the androgen receptor, its concentration in plasma amounts to approximately one-tenth of the T level in men and one-third to one-quarter in women (T in men: 9.2–33.7 nmol/L; T in women: 0.33–2.02 nmol/L; DHT in men: 0.47–2.65 nmol/L; DHT in women: 0.09–0.91 nmol/L) [1,3]. The parallel measurement of T and DHT is a cornerstone in the diagnostics and treatment of male hypogonadism and androgen deficiency states [1,4–6]. In addition, DHT and/or T/DHT ratio are used for the monitoring of androgen replacement therapy and 5α-reductase inhibitor treatment of benign prostate hyperplasia [7,8]. In women, T/DHT ratio has been proposed as an auxiliary parameter aiding the diagnosis of polycystic ovary syndrome (PCOS) [9].

In the routine hospital laboratory, T and DHT are traditionally determined using immunometric techniques such as radioimmunoassays, chemical luminescence or enzyme immunoassays (RIA, CLIA, EIA). Although fast, cost-effective and amenable to automation, immunoassays suffer from unsatisfactory sensitivity and specificity due to cross-reaction with structurally related steroids and matrix effects [3,10–18]. Because of these limitations, the utility of immunoassays for the determination of T and DHT in women, pre-pubertal children and male patients with hypoandrogenism has been questioned and the use of gas or liquid chromatography combined with mass spectrometry has been advocated [19]. Several chromatographic-tandem mass spectrometric protocols allowing for simultaneous assessment of T, DHT and other pathophysiologically relevant androgens have been reported in

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https://doi.org/10.1016/j.cca.2017.10.030
Received 4 June 2017; Received in revised form 9 October 2017; Accepted 29 October 2017
Available online 06 November 2017
0009-8981/

recent years [3,16,20–28]. Although characterized by an exquisite analytical performance permitting reliable measurement of T and DHT, these approaches require complex and expensive instrumentation and are demanding with respect to assay development, implementation and interpretation of results. In the present study, we developed and analytically and clinically validated for the first time a simple, but qualitatively precise and accurate method for simultaneous T and DHT determination in plasma based on an inexpensive bench-top GC–MS equipment, as an alternative to advanced tandem mass spectrometric protocols. The analytical performance of the method reported here allowed us to define generally applicable reference ranges for T and DHT in well characterized male and female populations and to assess the utility of DHT and T/DHT ratio for discrimination between PCOS patients and healthy subjects. In addition, our results argue strongly against the use of traditional immunoassays for the determination of DHT in clinical practice.

2. Material and methods

2.1. Study subjects and blood collection

For estimation of reference intervals, serum samples were collected from 295 healthy subjects as described previously [29]. Female subjects (n = 81) of reproductive age (23–43 years) were recruited at the University Hospital Münster Reproductive Medicine Unit during 2013 and 2014. They were anamnestically free of acute or chronic diseases, had regular menstrual cycles (25–35 days with < 4 days variation from cycle to cycle), and had not taken regular medication including hormonal contraceptives for at least 3 months. The health status was confirmed by physical examination and standard laboratory procedures. The follicular and luteal phases were defined as days 3–5 and 21–23 after onset of menstruation, respectively. Female postmenopausal subjects (n = 41, aged 55–66) and male subjects (n = 173, aged 20–68) were recruited from voluntary donors attending the blood donation center at the University Hospital Münster. Anamnestic, physical and laboratory examination of the subjects excluded acute or chronic disease, and they had not received regular medication for at least 6 months. The postmenopausal phase was defined as spontaneous amenorrhea for at least 12 months.

Patients with PCOS (n = 41) and their healthy counterparts (n = 43) were recruited among subjects attending the Reproductive Medicine Unit between 2013 and 2016. The PCOS group was defined according to the ESHRE/ASRM consensus and compared with a control group not fulfilling the PCOS criteria. The following parameters were recorded: age, weight, body mass index (BMI), menstrual history, cycle length and the presence of clinical androgenization (hirsutism). After cessation of any hormonal medication for 2 months, an endocrine status encompassing serum estradiol, luteinizing hormone (LH), follicle stimulating hormone (FSH), testosterone, dehydroepiandrosterone sulphate (DHEAS) and steroid hormone-binding globulin (SHBG) was assessed in the early follicular phase (day 3–5) of the menstrual cycle. Basal 17-hydroxyprogesterone (17OHP), prolactin and thyroid stimulating hormone (TSH) were assessed to exclude other causes of anovulation. The diameter of a dominant follicle and the presence of polycystic ovaries were evaluated with a transvaginal scan on day 12–14. Serum progesterone was determined on day 20–24 of the menstrual cycle. Blood samples were collected in the morning (8–12), centrifuged, and sera underwent immediate analysis for routine parameters or were frozen in small aliquots at − 80 °C for mass spectrometry. Samples used for reference interval estimation were analysed up to 18 months after collection. All other samples were analysed up to 3 months after collection. Due to long-lasting stability of androgens in frozen plasma [30], the storage time is not expected to influence the results of the present study.

2.2. Reagents, internal standards and calibrators

HPLC-grade acetonitrile and methanol as well as chlorotrimethylsilane (TMCS) were purchased from Merck (Darmstadt, Germany). *N*-methyl-*N*-trimethylsilyltrifluoracetamide (MSTFA) and *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) were obtained from abcr GmbH (Karlsruhe, Germany). T (17β-hydroxy-4-androsten-3-one) and DHT (17β-hydroxy-5α-androstan-3-one) (both > 99% pure), O-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride (PFBHA, Florox-Reagent) and all other reagents were from Sigma-Aldrich (Deisenhofen, Germany), and were of highest purity available. 2,3,4-deuterated T (D_3-T) and 2,3,4-$^{13}C_3$-DHT ($^{13}C_3$-DHT) (both > 98% pure, Sigma-Aldrich) were used as internal standards for T and DHT measurements, respectively. Stock solutions of each steroid were prepared separately by weighing out material on a four-point scale balance and dissolving it in methanol to give a concentration of 1.0 mg/mL. A working profile calibration solution was prepared in a volumetric flask (10 mL) by mixing appropriate volumes of individual steroid stock solution with methanol to give final concentrations of 100 ng/mL or 10 ng/mL for both T and DHT. An internal standard solution was prepared in an analogous manner and added to the protein precipitating reagent ($ZnSO_4 \cdot 7H_2O$, 25.0 mmol/L in methanol) to give the final concentration of 10.4 nmol/L for both D_3-T and $^{13}C_3$-DHT. Zinc sulphate has been selected for protein precipitation as it is the most efficient reagent for this purpose [31]. Calibrators containing both steroids were prepared by spiking the serum pool with known T and DHT concentrations with appropriate amounts of calibration solutions. The typical six-point calibration set was 0.35, 0.80, 1.73, 4.96, 14.3 and 46.5 nmol/L for T and 0.02, 0.48, 1.40, 4.60 and 45.8 for DHT including zero-calibrators. Zero-calibrator values were calculated using the standard addition method conforming to the German Industry Standard (DIN32633) [32]. Calibration curves were constructed from the known calibrator concentration and the ratio of the analyte peak area to the area of the internal standard peak.

2.3. Sample preparation

Aliquots (0.75 mL) of serum, calibrators or quality controls were mixed with an equal volume of precipitation reagent containing internal standards. Samples were vortexed for 1 min, centrifuged (14,000 rpm, 4 min, 4 °C), and the supernatant was decanted and subjected to an in-house developed solid-phase extraction method [33] using RP18 columns (Strata X, Phenomenex, Utrecht, The Netherlands) that had been preconditioned with acetonitrile and methanol prior to sample application, washed twice with methanol in water (50% v/v) and eluted with acetonitrile. Part of the eluate (0.3 mL) was transferred to silanized glass vials and male steroids (T, DHT, D_3-T and $^{13}C_3$-DHT) were derivatized for 30 min at 80 °C with Florox-Reagent (1.0% (w/w) in methanol, final concentration 10 mg/mL) as described by Fitzgerald and Herold with minor modifications [34]. Samples were then evaporated under vacuum (30 min, 60 °C), incubated for 30 min at 80 °C with 0.025 mL silanizing reagent (99% MSTFA with 0,01% (v/w) TMCS as catalyst) and used for further analysis. Preliminary experiments showed that MSTFA combined with TMCS produces more effective silanization than BSTFA as originally used by Fitzgerald and Herold [34].

2.4. GC–MS assay

Analysis was performed using a Shimadzu QP2010 ultra-GC–MS-system with negative chemical ionization (NCI) equipped with split/splitless-injector and autosampler. The GC system was fitted with a Rtx5MS-column (15 m × 0.25 mm × 0.1 μm, Restek, Bad Homburg, Germany). Helium was used as the carrier gas at a constant linear velocity rate of 56.0 cm/s (column flow rate 1.06 mL/min). The column oven temperature was set at 160 °C for 1 min and then gradually increased (25 °C/min) to 300 °C. The injection volume was 1.0 μL