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Development of a new gas chromatography–mass spectrometry (GC–MS) methodology for the evaluation of 5α-reductase activity

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ABSTRACT

The androgens testosterone (T) and dihydrotestosterone (DHT) play a key role in the function and integrity of prostate tissue, but are also implicated in prostate cancer and benign prostatic hyperplasia (BPH). The reduction of androgen levels can be achieved by the inhibition of 3-oxo-5α-steroid-4-dehydrogenase (5α-reductase), which is responsible for the irreversible conversion of T into its more active metabolite DHT. In fact, the use of 5α-reductase inhibitors (RIs), like finasteride, can be a valuable strategy for the treatment of BPH and in chemoprevention of prostate tumors. In this work a new method based on a dispersive liquid–liquid microextraction (DLLME) procedure, followed by gas chromatography–mass spectrometry (GC–MS), to evaluate the 5α-reductase activity, by measuring the conversion percentage of T into DHT was optimized and validated. Enzymatic assays were carried out in human prostate microsomes, using T as substrate. T and DHT were extracted by the developed DLLME technique and quantified, after silylation, by GC–MS. Variables affecting the extraction efficiency and derivatization of T and DHT were evaluated. The optimized method showed good linearity (with correlation coefficients over 0.9994 for T and 0.9995 for DHT), good recoveries (higher than 80%), and good intra- and inter-day precision (below 13%, 3 levels, $n=6$). The detection limits for T and DHT were 0.5 nM and the limits of quantification were 5 nM. The new GC–MS method is a good alternative to the already described methods, to evaluate 5α-reductase activity, since it avoids the use of radioactive compounds and corresponds to a fast and sensitive methodology with a good extraction efficiency, accuracy and high recovery. As this method allows the evaluation of 5α-reductase activity, also permits the study of inhibitory efficacy of new molecules as potential RIs.

1. Introduction

The androgens, testosterone (T) and dihydrotestosterone (DHT), play a key role in prostate development, cell proliferation and growth, but also in prostate diseases, particularly benign prostate hyperplasia (BPH) and prostate cancer [1,2]. Testosterone is converted to DHT by the 3-oxo-5α-steroid-4-dehydrogenase (5α-reductase; 5α-R) enzyme, being DHT the main androgen in the prostate, responsible for differentiation and growth of the prostatic tissues. Both T and DHT bind to androgen receptor (AR), inducing conformational change of AR and activating transcription of AR-regulated genes (ARRG), involved in cell growth, proliferation and survival [1,3,4]. Although both androgens bind to AR in the same manner, DHT presents 2–5 times higher binding affinity to AR and dissociates more slowly than T. The DHT is also responsible for a 10-fold higher potency of AR signaling than T [5–7].

Abbreviations: T, testosterone; DHT, dihydrotestosterone; BPH, benign prostate hyperplasia; 5α-R, 5α-reductase; AR, androgen receptor; RIs, 5α-reductase inhibitors; DLLME, dispersive liquid–liquid microextraction; GC–MS, gas chromatography–mass spectrometry; TLC, thin layer chromatography; HPLC, high performance liquid chromatography; LC/APCI-MS, liquid chromatography/atmospheric pressure chemical ionization-mass spectrometry; LC/ESI-MS, liquid chromatography/electrospray ionization-mass spectrometry; SIM, selected ion monitoring; DTT, dithiothreitol; DMSO, dimethyl sulfoxide; NADPH, membrane-bound nicotinamide dinucleotide; MeCN, acetonitrile; MeOH, metanol; $C_2H_3Cl_3$, 1,1,1-trichloroethane; C_2HCl_3, trichloroethylene; C_6H_5Cl, chlorobenzene; C_2Cl_4, tetrachloroethylene; MSTFA, N-methyl-N-(trimethylsilyl)trifluoroacetamide; BSTFA, N, O-bis(trimethylsilyl)trifluoroacetamide; HFBA, heptafluorobutyric anhydride; NH_4I, Ammonium iodide; DTE, 1,4-dithioerythritol; EI, electron ionization

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Testosterone is irreversibly converted into DHT by the enzyme 5α-R, that reduces the double bond between carbons 4 and 5 ($\Delta^{4,5}$) inserting an hybride ion (H-) to the α face of the steroid at C-5 position and a proton to the β face at C-4 position [2]. There are three isoforms of the 5α-R enzyme, the isoenzymes type 1 (5α-R1), type 2 (5α-R2) and type 3 (5α-R3). The isoenzymes 5α-R1 and 5α-R2 are NADPH-dependent and membrane-associated (microsomal) proteins [4]. The 5α-R1 is mainly expressed in skin and liver but is also present in epithelial cells of prostate tissue. The 5α-R2 is expressed predominantly in stromal and basal epithelial cells of the prostate and other genital tissues and the 5α-R3 is expressed in androgenic and non-androgenic tissues [3,8,9]. The 5α-R2 has higher affinity to substrate and is present in higher concentrations in prostatic tissue than 5α-R1 [10,11]. However, in prostate cancer, 5α-R1 expression is increased compared to normal and BPH tissue, whereas 5α-R2 expression and activity is decreased or unchanged [12–14]. Thus, besides the role of DHT, the development of prostate diseases is also associated to the expression of 5α-R isoenzymes. Androgens can be antagonized by acting directly at the receptors or by preventing their biosynthesis, by the inhibition of 5α-R, blocking the irreversible conversion of T into DHT [2,8,15]. Finasteride and dutasteride are known steroidal 5α-reductase inhibitors (RIs) [16,17]. Nowadays, the inhibition of 5α-R with these RIs corresponds to a better target for the treatment of BPH, while the efficacy in chemoprevention of prostate cancer is still debated.

Traditionally, prostatic 5α-R activity has been evaluated using radiolabeled substrates, in human prostate cells [18] and also in human or in rat prostatic microsomes [19–22]. Prior to the scintillation counter a chromatographic separation is mandatory, which can be accomplished by thin layer chromatography (TLC) [19,20,23] or, more efficiently, by high performance liquid chromatography (HPLC) [21,22,24]. However, as these methods, besides being too expensive, are not advised for routine analysis due to the human health risks posed by the radiolabeled compounds, other methodologies were developed.

In order to prevent the use of radiolabeled compounds, Mitamura et al. developed a liquid chromatography/atmospheric pressure chemical ionization-mass spectrometry (LC/APCI-MS) method that quantify 5α-DHT by an absolute calibration curve, which was applied to study the enzyme activity in rat prostatic tissue [25]. More recently, Abe et al. proposed an liquid chromatography/electrospray ionization-mass spectrometry (LC/ESI-MS) method to study 5α-R activity in rat liver, using dexamethasone as internal standard for quantitative purposes [26]. However, these methods lack detailed validation parameters which hinders their thoroughly evaluation. Additionally, LC–MS instruments are still inaccessible for most of laboratories worldwide.

Despite GC–MS is the reference technique for measuring androgens in biological samples [27], only a few number of authors have reported its use to quantify T and DHT in human lysate prostatic tissues [28,29]. Moreover, as far as our knowledge there are no reports of the application of GC–MS in assays dedicated to study human microsomal prostatic 5α-R activity. This work reports the development and validation of a new and very sensitive GC–MS method to evaluate 5α-R activity in human prostate microsomes, by measuring the conversion percentage of T into DHT (ratio DHT/T), using isotopic analogous as internal standards. It is based on a fast, simple, and very effective procedure of dispersive liquid–liquid microextraction (DLLME) of the analytes followed by a silylation step and GC–MS quantification in selected-ion monitoring (SIM) mode. Several variables affecting extraction efficiency and selectivity were optimized, including (i) nature and amount of extractive and dispersive solvents in DLLME, (ii) nature and amount of derivatizing reagent and (iii) derivatizion conditions in order to minimize the presence of coextractives and allow the analysis of trace amounts of T and DHT in human prostate microsomes. The developed method was validated in what respects linearity, limits of detection (LOD) and limits of quantification (LOQ), precision, and accuracy.

The development of this new simple and fast methodology for T and DHT analysis provides a valuable tool to evaluate new compounds as potential RIs, in order to further study structure-activity relationships (SAR), which may lead to new and potent RIs to be introduced in BPH treatment and in prostate cancer chemoprevention.

2. Material and methods

2.1. Reagents and standards

Testosterone (T, 99% purity grade) and dihydrotestosterone (DHT, 99% purity grade) were obtained from Fluka (Neu-Ulm, Germany) and Sigma-Aldrich (Chemie GmbH, Steinheim, Germany), respectively. Internal standards testosterone-d_3 solution (T-d_3, 98 atom% D) and dihydrotestosterone $^{13}C_3$ solution (DHT-$^{13}C_3$, 99 atom% ^{13}C) were also obtained from Fluka and Sigma-Aldrich, respectively. Individual stock internal solutions of T-d_3 (3.47 μM) and DHT-$^{13}C_3$ (3.44 μM) were prepared in acetonitrile (MeCN). All the solutions were stored at −20 °C when not in use.

Dithiothreitol (DTT), dimethyl sulfoxide (DMSO) and NADPH were obtained from Sigma-Aldrich. Bradford assay kit was from Bio-Rad (Laboratories Melville, NY, USA). MeCN and methanol (MeOH) both HPLC grade were obtained from Fluka. Isooctane and acetone both HPLC grade were obtained from Sigma-Aldrich. Extractive solvents: 1,1,1-trichloroethane ($C_2H_3Cl_3$), trichloroethylene (C_2HCl_3), chlorobenzene (C_6H_5Cl) and tetrachloroethylene (C_2Cl_4) were high purity solvents for GC analysis obtained from Fluka. Derivatization reagents: N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA, 98.5% purity grade), N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA, 99% purity grade) and heptafluorobutyric anhydride (HFBA, 99% purity grade) were obtained from Fluka. Ammonium iodide (NH_4I, 99% purity grade) and 1,4-dithioerythritol (DTE, 99% purity grade) were obtained from Sigma-Aldrich. Finasteride was obtained from Sequoia Research Products Ltd. (Pangbourne, UK).

Ultrahigh purity helium (99.9999%) for GC–MS and nitrogen for solvent evaporation were obtained from Gasin (Maia, Portugal).

2.2. Apparatus and GC–MS conditions

The analyses were performed on an Agilent (Little Falls, DE, USA) gas chromatograph 6890 equipped with an electronically controlled split/splitless injection port and an inert 5973 mass selective detector with electron ionization (EI) chamber. The injection was made in pulsed splitless mode at 280 °C (pulsed pressure 32 ml/min, held 1 min). The GC separation was conducted with a DB-5 MS (30 m × 0.25 mm i.d. × 0.25 μm film thickness of 5% phenyl, 95% dimethyl arylene siloxane) column using helium as carrier gas and a flow rate of 1.0 ml/min. The GC oven was held at 105 °C for 1 min and subsequently ramped at 20 °C/min to 320 °C held for 3.25 min. Total run time was 15 min. The MS transfer line was held at 280 °C.

Mass spectrometric parameters were set as follows: EI with 70 eV energy; ion source temperature, 230 °C, MS quadrupole temperature, 150 °C and solvent delay 4.5 min. The MS system was routinely set in selective ion monitoring (SIM) mode and each analyte was quantified based on peak area using one target and three qualifier ion(s). Complete SIM parameters and retention times of the analytes are shown in Table 1. Agilent Chemstation was used for data collection/processing and GC–MS control.