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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.

Table 1
MS conditions for the GC–MS analysis of DHT, T and I.S. derivatized (time windows and ions selected in SIM mode, quantification ions are in bold).

Analyte	t_R (min)	Time windows (min)	Data acquisition rate (scans/s)	SIM ions (*m*/*z*)
DHT-$^{13}C_3$	11.54	4.50–11.61	2.6	**437**, 407, 379, 346,146
DHT	11.55			**434**, 405, 343, 377, 143
T	11.81	11.61–15.00	3.17	**432**, 417, 327, 342
T-d_3	11.82			**435**, 420, 330, 345

2.3. Sample preparation

2.3.1. Preparation of prostate microsomes

Human prostate tissues, kindly provided by a local hospital after patient informed consent, were collected after surgery and placed in cold 20 mM sodium phosphate buffer (pH 6.5), washed and stored at −80 °C before use. Human prostate microsomes were prepared as previously described [19,20,30] with some modifications. The prostatic tissues were weighed and homogenized in a Polytron homogenizer with a solution of 20 mM sodium phosphate buffer (pH 6.5) containing 0.32 M sucrose and 0.1 mM DTT (1:1, w/v). The homogenate was centrifuged at 5000 × *g* for 30 min. After centrifugation, the supernatant was centrifuged at 20,000 × *g* for 30 min and then at 54,000 × *g* for 45 min. The microsomal pellet was washed and ressuspended in 20 mM sodium phosphate buffer (pH 6.5) containing 0.32 M sucrose, 0.1 mM DTT and glycerol and stored at −80 °C. All the procedures were carried out at 4 °C. The protein content was evaluated by the Bio-Rad protein assay using bovine serum albumin (BSA) as standard.

2.3.2. 5α-Reductase assay

To evaluate 5α-reductase (5α-R) activity on human prostatic tissue it was used a modification of the method previously described [19,20,25,31]. Briefly, for the reaction mixture it was used 50 μg of prostate microsomal protein, 500 nM of T, 1 mM DTT and 25 μl of DMSO in 40 mM sodium phosphate buffer (pH 6.5), in a final volume of 0.5 ml. The enzymatic reaction was initiated by the addition of 250 μM of NADPH and incubations were performed in a shaking water bath at 37 °C for 60 min. The enzymatic reaction was stopped by the addition of 500 μl of MeCN in ice. Then the steroids, T and DHT, were extracted by DLLME. To the enzymatic mixture with MeCN it was added 50 μl of T-d_3 (175 nM) and DHT-$^{13}C_3$ (175 nM) and 50 μl of C_2HCl_3. This mixture was transferred to a conical tube with 3 ml of H_2O, the tubes were closed, hand-shaken and centrifuged at 5000 × *g* for 3 min. After centrifugation, 30 μl of the lower phase, that contained the extracted steroids, was transferred to a new vial and dried using a gentle nitrogen stream at room temperature. Finally, the steroids were silylated by addition of 30 μL of MSTFA+NH_4I+DTE (500:4:2 vol/wt/wt) during 5 min in a household microwave (600 W), and 1 μL of the extract was injected in the GC–MS system.

As a control of the 5α-R activity it was used as reference compound, the RI finasteride. Finasteride was dissolved in DMSO and diluted in 40 mM sodium phosphate buffer (pH 6.5) before assay. To screen the ability of finasteride to inhibit 5α-R activity, using this new method, the enzymatic reaction was performed with 1 μM of finasteride. To determinate the IC_{50} value it was used different concentrations of finasteride (0.01–1 μM). All experiments were carried out in triplicate.

The ratio between T/T-d_3 and DHT/DHT-$^{13}C_3$ allows the quantification of T and DHT produced after 5α-R reaction and permits to estimate the 5α-R activity, by comparing the amount of DHT formed after the enzymatic reaction.

2.4. Validation procedure

The analytical method validation was performed according to the guidelines of the FDA [32]. The parameters studied were linearity, limit of detection (LOD) and limit of quantification (LOQ), precision, accuracy and recovery.

3. Results and discussion

3.1. Optimization of sample preparation

3.1.1. Optimization of DLLME

The aim of the present work was to develop a simple and highly sensitive DLLME method to allow the accurate quantification of T and DHT and the evaluation of the biochemical activity of potential RIs in human prostatic microsomes. In general, DLLME consists in the formation of a cloudy solution promoted by the fast addition of an extractive and a dispersive solvent to an aqueous sample. The analytes are jointed in the sedimented phase by centrifugation, allowing great enrichment factors and low detection limits. In this procedure, the equilibrium is achieved in few seconds due to the large contact surface between tiny droplets formed and the sample [33]. The technique has been successfully used as extractive step for the determination of compounds present in trace amounts in different matrices, including biological matrices [34]. Beyond the fact that it is the first time that a DLLME method is applied to this kind of matrix, the novelty of the proposed method is the use of the extract obtained by the previous enzymatic reaction as a dispersive solvent in the subsequent DLLME procedure. To achieve the optimum DLLME conditions, several parameters were taken into account including nature and amount of the extractive solvent as well as the nature and amount of dispersive solvent.

Generally, the extractive solvent in a DLLME method must fulfill the following requirements: higher density than water, immiscibility with water, good extraction capability of the analyte(s) and chromatographic compatibility. According to these properties some chlorinated solvents such as $C_2H_3Cl_3$ (density: 1.32, insoluble in water), C_2HCl_3 (density: 1.46; water solubility: 1.28 g/l), C_6H_5Cl (density: 1.11; water solubility: 0.5 g/l) and C_2Cl_4 (density: 1.62; water solubility: 0.17 g/l), were tested in this study in triplicate. Hence, 500 μl of the MeCN containing 175 nM of both T and DHT was mixed with 50 μl of each extractive solvent and rapidly injected to 3 ml of deionised water, and 30 μL of sedimented phase was collected. The average peak areas shows that C_2HCl_3 was the best extractive solvent for the target analytes as can be seen in Fig. 1.

In the next step of the optimization, the extractive solvent volume was evaluated. It is well known that lower volumes enhance the enrichment factor of the DLLME process, although reducing the volume of the sedimented phase. For the purpose of the present study, two replicates were investigated using 1 ml of enzymatic reaction extract (obtained from a spiked human microsome sample) added with three different volumes of C_2HCl_3: 50 μl, 100 μl and 150 μl. Lower volumes than 50 μl, tested at an early stage, resulted in a very small volume of sedimented phase and subsequently in a decrease of reproducibility of the method. The increment of the volume of C_2HCl_3 from 50 to 150 μl resulted in an increase volume of sedimented phase from 30 to 90 μl with the subsequent decrease of enrichment factor and detection limits. Thus, 50 μl was selected as an optimum volume of extractive solvent.

In the optimization of the proposed method it was also evaluated the nature of the dispersive solvent. Usually, these kinds of enzymatic reactions are stopped combining temperature and the addition of a selected organic solvent. Here, the organic solvent was chosen to make possible its use as dispersive solvent in DLLME. Taking into account the main requirements of a dispersive solvent in DLLME procedure, such as (i) miscibility with both sample phase and extractive solvent and (ii) capacity to decrease the interfacial tension of extractive solvent in order to make the droplet size smaller, MeOH and MeCN were tested. Initially triplicate experiments were performed in extracts of human prostate microsomes spiked with 175 nM of both T and DHT, subjected to the enzymatic reaction (described in Section 2.3.2) which was stopped with immersion of tubes in ice and by adding 500 μl of MeOH or 500 μl of MeCN. Then, 50 μl of C_2HCl_3 were added and the extracts were promptly transferred for a \conical tube containing 3 ml of deionized water. After centrifugation, 30 μl of sedimented phase was collected, evaporated, silylated and analyzed by GC–MS. The results showed that MeCN provided not only a cleaner sedimented phase with higher density of droplets, but also a higher analytical signal than those obtained by MeOH. Therefore, MeCN was chosen as dispersive solvent. In the next step the effect of dispersive solvent volume was tested using 500 μl and 1000 μl of MeCN. The use of 500 μl was chosen because it was sufficient to denature the enzyme and thereby to stop the enzyme's catalytic activity. In addition, the volume of sedimented phase collected was identical to the one obtained with 1000 μl, providing a maximum analytical response for the target compounds.

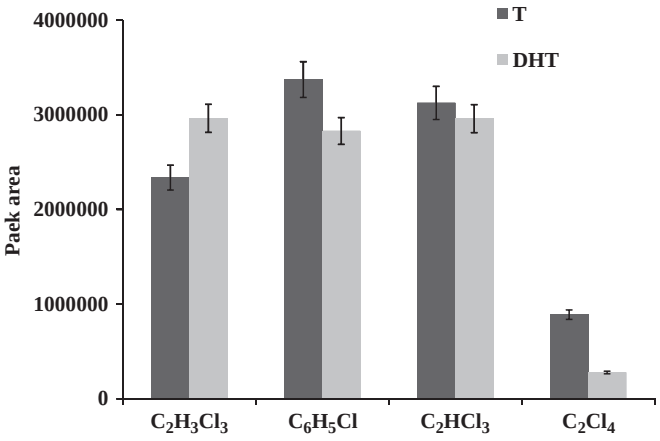

Fig. 1. Comparison of peak area response using different extractive solvents.

3.1.2. Optimization of the derivatization conditions

To improve both the selectivity of the analysis and the efficiency of the chromatography, as well as to enhance the detectability of GC–MS, steroid compounds must be derivatized. Both silylation and acylation reactions have been used to derivatize the hydroxyl and keto groups present in the molecular structure of T and DHT [35]. In this study, the results obtained using different silylation reagents [BSTFA with 1% TMCS; MSTFA and the mixture of MSTFA with NH_4I as catalyst and DTE as antioxidant 1000:8:4 (v/w/w)] and an acylation reagent (HFBA) were compared to select the most appropriate derivatization

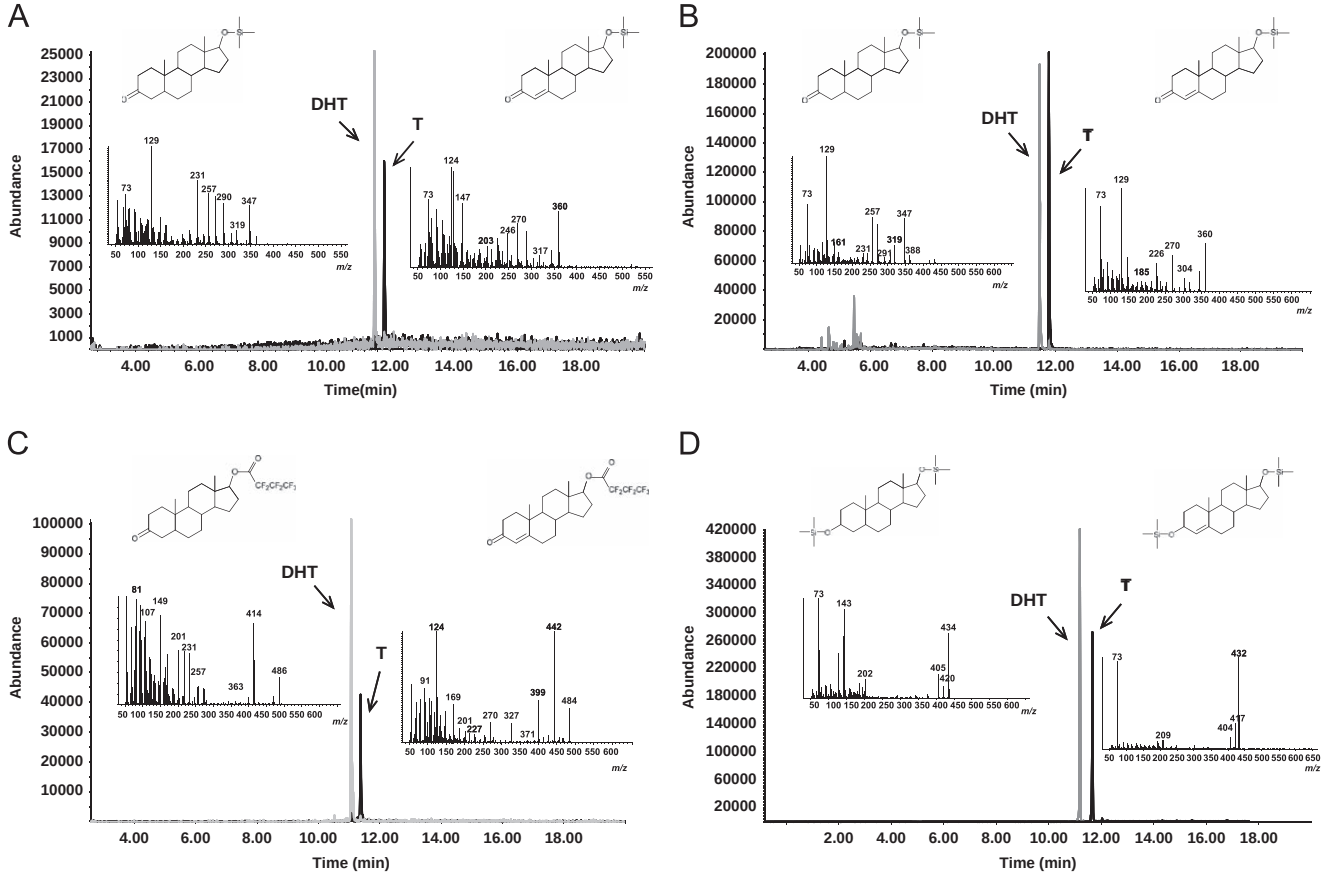

Fig. 2. Total ion chromatograms (TIC) of a standard solution of T and DHT at 175 nM. Individual full-mode spectra of each analyte. Each steroid was derivatized with (A) BSTFA with 1% TMCS, (B) MSTFA, (C) HFBA:acetone and (D) MSTFA+NH_4I+DTE.

mixture. Initially, a standard solution of T and DHT, at 175 nM each, was previously evaporated to dryness and further derivatized by adding 100 μl of the above mentioned silylating reagents or 50 μl of an acylating mixture of HFBA:acetone (1:4 v/v). All the reactions were performed at 70 °C for 30 min. After cooling, the derivatized solutions were directly injected into the GC–MS. The silylation with MSTFA or with the mixture of BSTFA with 1% TMCS provides monosilyl derivatives obtained by the replacement of the active hydroxylic hydrogen of T and DHT by a trimethylsiyl (TMS) group, resulting in the formation of mono-O-TMS derivatives (Fig. 2). With the mixture of MSTFA+NH_4I+DTE, a simultaneous silylation of the C-17 hydroxyl and the C-3 keto groups was achieved generating both TMS ether and TMS enol ether derivatives. The mass spectra of the TMS derivatives of the studied steroids, its structures, as well as the full chromatograms for each silylating reagent tested are shown in Fig. 2. As can be seen, the mixture of MSTFA+NH_4I+DTE (Fig. 2D) provided derivatives with higher selective and specific *m*/*z* ion fragments for both T and DHT when compared to other silylating reagents. The acylation with HFBA converted both compounds into the respective heptafluorobutyrate esters (Fig. 2C), which, despite the higher mass increment given, the analytical peak areas were lower than those obtained with the silyl derivatives (Fig. 2A, B and D). Consequently, the mixture of MSTFA+NH_4I+DTE was chosen as the derivatization reagent.

To lessen the time required for the derivatization step, a household microwave was tested, as previously described in literature [36]. The effect of derivatization time ranging from 1 to 7 min, with intervals of 1 min, was examined at 600 W irradiation power. Between 1 and 4 min, 60% of the derivatives of both T and DHT detected were C-17 mono-TMS derivatives instead of the C-3 and C-17 di-TMS derivatives expected. From 5 to 7 min only di-TMS derivatives were achieved and all with similar analytical signals. These analytical signals were similar to those obtained with the standard conditions of 70 °C/30 min without microwave. Hence, 5 min was chosen as the optimal derivatization time, which was shorter than the 30 min required when the react heating block was used. The microwave power of 600 W was compared with 1200 W using 3 min of derivatization. However, the use of 1200 W did not provide higher analytical signal for T and DHT than the 600 W, indeed lower reproducibility between vials was observed. Thus, 600 W was chosen as microwave power.

The effect of volume of derivatizing reagent used was studied using a dryness extract of human prostate microsomes spiked with 175 nM of both T and DHT, which was obtained by the DLLME procedure. Three different volumes of 20 μl, 30 μl and 50 μl were tested in triplicate. Experiments with 20 μl provided a good analytical signal, but the RSD was very high (25%), thus it was not chosen. The increment of the volume of silylating reagent from 30 to 50 μL did not result in an increased peak area, so 30 μL was selected as the optimum volume of derivatizing reagent.

Table 2
Calibration data, lower limits of detection and quantification of T and DHT in the presence or in the absence of prostate microsomes.

Analyte	Without microsomes			With microsomes		
	r^2	LOD (nM)	LLOQ (nM)	r^2	LOD (nM)	LLOQ (nM)
T	0.9947	1	5	0.9994	0.5	5
DHT	0.9940	1	5	0.9995	0.5	5

3.2. Method performance

3.2.1. Linearity

The linearity of the method was evaluated by analyzing six concentrations (between 5 and 500 nM for both T and DHT) in standard solutions and in human prostate microsomes, using the described 5α-reductase (5α-R) assay. Calibration curves were constructed by plotting the analyte/IS ratio obtained against the concentration of each analyte. The method showed to be linear in both standards and microsomes extracts presenting a coefficient

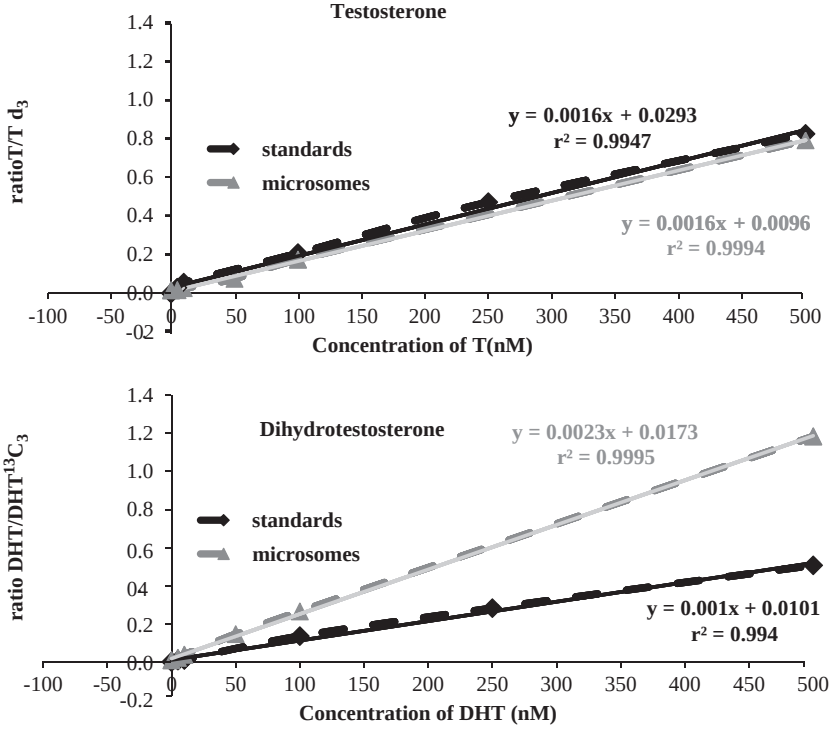

Fig. 3. Linearity of the method evaluated by calibration curves of T and DHT with or without prostate microsomes.

of determination (r^2) for T of 0.9947 and 0.9994, respectively, and for DHT of 0.9940 and 0.9995, respectively (Table 2 and Fig. 3). Moreover, comparing the calibration curves of standard solutions and microsomes, the method demonstrated to be more sensitive for DHT when microsomes extracts were used, since the ratio values (steroids/internal standards) were higher in microsomes. For T, both calibration curves were similar. The enhancement in response was observed previously in other works [33], depending on the properties of the analyte itself and the presence of other ionizable compounds present in the extract. The linearity of the method was tested several times using matrix-matched calibration.

The method allows the quantification of the intraprostatic levels of T and DHT in prostate microsomes, since we could measure the basal levels of androgens, as observed in matrix-matched calibration curves.

3.2.2. Lower limit of detection (LOD) and lower limit of quantification (LLOQ)

The lower limit of detection (LOD) and the lower limit of quantification (LLOQ), that allows the evaluation of the sensitivity of the method, were determined by successive analysis of samples with decreasing amounts of T and DHT. The LODs, which corresponds to the lowest tested concentrations, where the signal-to-noise ratio was higher than 3:1, were 1 nM for T and DHT in standard samples without microsomes and 0.5 nM with microsomes (Table 2). The LLOQ corresponds to the lowest concentration of steroids that was measured with acceptable accuracy and precision (relative standard deviation (%RSD) < 20%). For T and DHT the LLOQs were 5 nM in standard samples and in human prostate microsomes (Table 2).

3.2.3. Precision and accuracy

The intra- and inter-assay precision was determined using samples with or without prostate microsomes spiked with three different concentrations of T and DHT, 5 nM, 50 nM and 500 nM. Intra-assay precision was evaluated by performing the extraction and analysis of six spiked samples in the same day while inter-assay precision was determined by analysis of triplicate spiked samples in three different days for a period of 3 weeks. The relative standard deviation (%RSD) obtained was below 13% for both intra- and inter-assay precision (Table 3), which is within the normal criteria accepted for bio-analytical methods validation.

3.2.4. Extraction efficiency

The method's recovery was evaluated by analyzing samples with and without microsomes spiked with three known concentrations of T and DHT, 5 nM, 50 nM and 500 nM. Extraction efficiency was evaluated considering the following formula: $R=(SS-NS)\times 100/As$ (where SS is the value obtained from spiked sample, NS from not spiked sample and As the amount added). Extraction efficiency was higher than 80% for T and DHT (Table 3), which suggested a good efficiency of the extraction procedure used.

3.3. Enzymatic reaction

To control the ability of the method to evaluate the activity of 5α-R, it was used the RI finasteride at 1 μM. The percentage of 5α-R activity is calculated by comparing the production of DHT in the sample with or without finasteride.

The RI finasteride presented an inhibition of 5α-R activity of approximately 81.70% ± 2.43, which is in accordance with the values previously described in human prostate [25,37]. Moreover, in this study finasteride presented an IC_{50} of 0.096 μM (Fig. 4). An IC_{50} for finasteride of 237 nM [25], of 470 nM [38] and of 147 nM [39] were also reported. Our results confirmed that our method can be used for the evaluation of the anti-5α-R activity of new compounds as potential RIs, by measuring the conversion percentage of T into DHT.

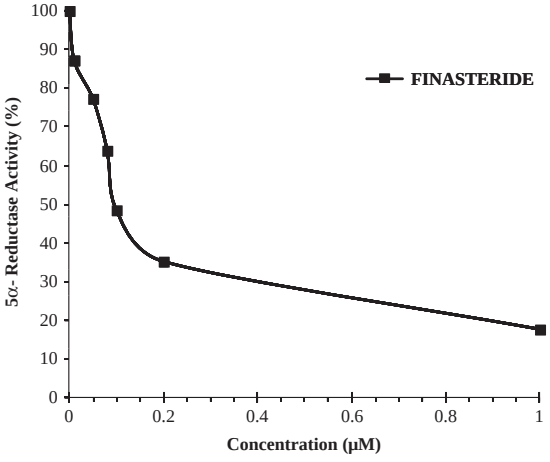

Fig. 4. The 5α-reductase activity of finasteride in human prostate microsomes.

Table 3
Intra- and inter-assay precision and recovery for each androgen in a solution with and without prostate microsomes.

Analyte	Concentration 5 nM				Concentration 50 nM				Concentration 500 nM			
	Intra-assay		Inter-assay		Intra-assay		Inter-assay		Intra-assay		Inter-assay	
	RSD[a] (%)	Extraction efficiency[a] (%)	RSD[b] (%)	Extraction efficiency[b] (%)	RSD[a] (%)	Extraction efficiency[a] (%)	RSD[b] (%)	Extraction efficiency[b] (%)	RSD[a] (%)	Extraction efficiency[a] (%)	RSD[b] (%)	Extraction efficiency[b] (%)
Without prostate microsomes												
T	11	85.07	3	87.71	6	86.97	10	85.24	3	96.77	7	96.99
DHT	10	87.52	6	82.04	8	88.11	11	87.94	3	96.6	6	94.71
With prostate microsomes												
T	9	92.02	7	95.75	6	93.74	13	81.87	6	94.62	10	85.57
DHT	4	92.71	2	89.48	9	91.32	8	85.04	8	94.86	11	82.14

[a] $n=6$.
[b] $n=3$.

4. Conclusions

An isotopic dilution DLLME-GC/MS method to evaluate the activity of 5α-reductase (5α-R), by measuring the conversion percentage of T into DHT, was developed and validated. The method showed to be very sensitive, accurate and precise, with a good linearity, allowing the simultaneous separation, unequivocal identification and quantification of T and DHT in prostatic microsomes.

The use of DLLME to extract androgens from the microsomal matrix was a relatively simple and fast sample preparation procedure, requiring only basic laboratory equipment (centrifuge), small amount of reagents and allowing the attainment of clean and enriched extracts. The DLLME is a fast and a low cost technique, that allows enhanced extraction efficiency and high recovery [40]. Basically, after the enzymatic reaction, the target analytes in the acetonitrile solution (dispersive solvent) were enriched in trichloroethylene, which was found to work better as extractive solvent than 1,1,1-trichloroethane, chlorobenzene or tetrachloroethylene. Then, the analytes were silylated by a microwave-accelerated procedure, permitting an efficient and rapid derivatization, avoiding the use of increased temperatures, which surpasses the main disadvantage pointed to the GC–MS. The accurate GC–MS determination of T and DHT was achieved by employing T-d_3 and DHT-$^{13}C_3$ as internal standards, which allowed compensation of target analyte losses and enhancement or suppression matrix effects. The presented GC–MS method also use a lower concentration of NADPH (250 μM) and microsomal protein (50 μg) in a smaller final reaction volume (500 μl) when compared with the TLC, HPLC and LC–MS methods already described (1000 μl of reaction volume, 1–3 mM of NADPH and 100–600 μg of protein) [25,31,41–47]. Moreover, this method avoids the use of radioactive compounds which corresponds to an important advantage to evaluate the activity of 5α-R.

The measurement of T and DHT and the resulting evaluation of 5α-R activity used to study potential RIs, to be introduced in BPH or prostate cancer treatment, as also for the diagnosis of 5α-R deficiency, can be efficiently achieved by the method proposed. The results obtained with finasteride, a steroidal enzymatic inhibitor, confirmed that this method allows the evaluation of 5α-R activity, and can be applied to study the biochemical activity of new potential RIs.

It should be emphasized that, to our best knowledge, none of the until now published GC–MS methods, enables such rapid, accurate and sensitive measurements of intraprostatic levels of T and DHT as well as the evaluation of the 5α-R activity in prostate tissue, as the method introduced in this study. The evaluation of 5α-R activity in human prostate microsomes allows the study of potential 5α-R inhibitors that effectively inhibit DHT production responsible for the development of some androgen-dependent prostate diseases, such as BPH and prostate cancer. Moreover, we are convinced that these measurements can be generically employed for other biological tissues and fluids.

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