Analytica Chimica Acta 671 (2010) 70–79

Contents lists available at ScienceDirect

Analytica Chimica Acta

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

Enzyme-linked immunosorbent assays for doping control of 5α-reductase inhibitors finasteride and dutasteride

Eva M. Brun[a], Ana Torres[a], Rosa Ventura[b], Rosa Puchades[a], Ángel Maquieira[a,*]

[a] Departamento de Química, Instituto de Reconocimiento Molecular y Desarrollo Tecnológico, Universidad Politécnica de Valencia, Camino de Vera s/n, 46071 Valencia, Spain
[b] Antidoping Control Laboratory, Grup de Recerca en Bioanàlisis, IMIM-Hospital del Mar, Doctor Aiguader 88, 08003 Barcelona, Spain

ARTICLE INFO

Article history:
Received 3 February 2010
Received in revised form 28 April 2010
Accepted 30 April 2010
Available online 7 May 2010

Keywords:
Finasteride
Dutasteride
5α-Reductase inhibitors
Doping control
Enzyme-linked immunosorbent assay

ABSTRACT

Finasteride and dutasteride are 5α-reductase inhibitors included in the World Anti-Doping Agency's list of banned substances. Two highly sensitive and selective ELISA assays were developed for these compounds. Polyclonal rabbit antibodies were raised using synthesized haptens and other commercial products. The best immunoassay obtained, based on an antibody-coated format, showed a limit of detection of 0.01 $\mu g\,L^{-1}$ and an IC_{50} of 0.75 $\mu g\,L^{-1}$ for finasteride (cross-reactivity with dutasteride < 4%). The second assay allowed finasteride and dutasteride determination, with limits of detection of 0.013 and 0.021 $\mu g\,L^{-1}$, and IC_{50} values 0.18 and 1.18 $\mu g\,L^{-1}$, respectively. Both assays were highly selective to a set of anabolic steroids, but they showed 37% and 30% cross-reactivity with the major urinary metabolite of finasteride, allowing its determination. The developed ELISA had better sensitivity than HPLC/MS/MS method and was applied as a screening technique to quantify dutasteride, finasteride, and its main metabolite in human urine without sample pre-treatment. Moreover, the analysis of dutasteride's excretion urines by ELISA was used to obtain its human excretion rate, essential to improve the analytical strategies about this type of drugs (permitted as medicines and prohibited in sport) and to establish an effective anti-doping policy.

1. Introduction

The synthetic 4-azasteroids finasteride, *N*-(1,1-dimethylethyl)-3-oxo-(5α,17β)-4-azaandrost-1-ene-17-carboxamide, and dutasteride, (5α,17β)-*N*-{2,5-bis(trifluoromethyl)phenyl}-3-oxo-4-azaandrost-1-ene-17-carboxamide (Table 1), are both inhibitors of 5α-reductase [1,2], the enzyme that converts testosterone to the more potent androgen 5α-dihydrotestosterone. This class of drugs is used therapeutically to treat benign prostatic hyperplasia [3], male baldness [4] and in the prevention of prostate cancer [5]. Recently, its use has been reported for the prevention or treatment of neurologically associated disorders, such as dementia, Parkinson, Alzheimer, schizophrenia and epilepsy [6].

The administration of these compounds with anabolic steroids confuses the evaluation of steroid profiles in sport drugs testing, since metabolic pathways of endogenous as well as synthetic steroids are influenced. In addition, the suppression of production and renal excretion of 5α-reduced metabolites of anabolic steroids may lead to false-negative doping-control results [7]. Therefore, finasteride and dutasteride were included as masking agents in the World Anti-Doping Agency's (WADA) prohibited list in 2005 [8,9]. Notable athletes who used hair loss treatments containing finasteride have been banned, including skeleton racers, bobsledders, footballers and ice hockey goaltenders [10].

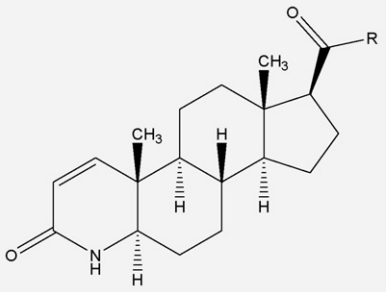
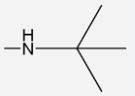
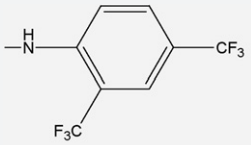
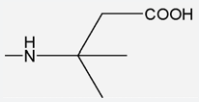
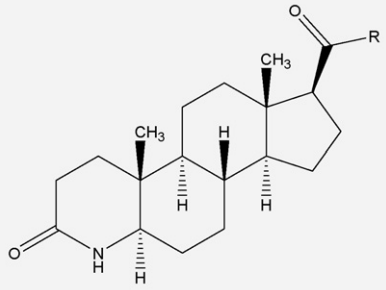
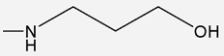
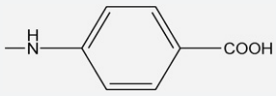
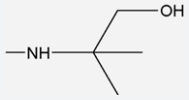
Doping-control analysis of athletes' biological fluids, mainly urine, is performed during competitions or training periods, in order to constrain the abuse of drugs for enhancing athlete performance [11]. The analyses are generally structured into a two-stage procedure: screening analyses, carried out on all the samples to be analyzed, and confirmatory analyses of those samples that have failed the screening analyses. The standard methods used in doping laboratories are based on chromatographic separation in combination with mass spectrometry, such as gas chromatography (GC/MS) and liquid chromatography (LC/MS) [12]. Urine samples must be hydrolyzed enzymatically to cleave the analytes and then liquid–liquid extracted. Moreover, derivatization of the analyte is a mandatory task before measurement by GC/MS. These chromatographic procedures permit the detection of prohibited substances at concentration levels below the minimum required performance limits (MRPLs) defined by WADA [9].

Liquid chromatography–tandem mass spectrometry methods for the determination of finasteride and its main metabolite (the corresponding ω-monocarboxylic acid [13–15]) have been reported [16–19], with limits of detection of 30 $ng\,mL^{-1}$ for finasteride [18]. However, dutasteride metabolites in urine have not been identified.

* Corresponding author. Tel.: +34 96 387 73 42; fax: +34 96 387 93 49.
E-mail address: amaquieira@qim.upv.es (Á. Maquieira).

0003-2670/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.aca.2010.04.066

Table 1
Chemical structure of finasteride, dutasteride and immunization haptens.

Structure	R	Compound
		Finasteride
		Dutasteride
		Finasteride metabolite
		Hapten **α1**
		Hapten **α2**
		Hapten **α3**
		Hapten **α4**
		Hapten **α5**

Alternatively, immunochemical methods have important advantages in doping, especially in sport field because the Olympic Committee rules to analyze numerous competitors' samples in short times. In this way, powerful screening high throughput techniques are very interesting, allowing the analysis of 100% of samples, being the positives confirmed subsequently by reference methods. Also, immunochemical assays can be developed with high or low selectivity to determine one compound or a family of them (for example, target and metabolites). This ability may help, in our case, in the identification of dutasteride metabolites in urine samples obtained after administration of the drug. In summary, immunochemical techniques are sensitive, high throughput and low-cost, appropriated for the *in situ* and rapid screening of doping agents [20], requiring minimum or no sample treatment to carry out the analysis.

This work presents for the first time the obtaining of antibodies and the necessary immunoreagents for finasteride and dutasteride determination. To this end, a pool of haptens was synthesized using several strategies, trying to always maintain the analytes' 4-azasteroid structure. These compounds were used to elicit polyclonal antibodies against finasteride and dutasteride. The obtained immunoreagents, sera and tracers, were used to develop a screening method for the determination of dutasteride, finasteride and/or its metabolite in urine samples without pre-treatment. This focuses the direct application on the analysis of urine, leading to the correspondent excretion curve, key information to make decisions on anti-doping policies.

2. Materials and methods

2.1. Chemicals and reagents

Finasteride, chemical reagents for hapten synthesis and protein conjugation purposes, bovine serum albumin (BSA), ovalbumin (OVA), lysozyme (LYS), hemoglobin (Hb), complete and incomplete Freund's adjuvant, *o*-phenylenediamine (OPD), Tween 20, horseradish peroxidase (HRP) and peroxidase labelled goat anti-rabbit immunoglobulins (GAR-HRP) were purchased from Sigma–Aldrich Química (Madrid, Spain). Dutasteride was purchased from AK Scientific (CA, USA), hapten α1 ((5α,17β)-3-oxo-4-azaandrost-1-ene-17-carboxylic acid) was from Steraloids Inc. (Newport, USA) and hapten α2 (3-oxo-4-aza-5α-androstane-17β-carboxylic acid) from Hangzhou Think Chemical Co. (Hangzhou, China). Keyhole limpet hemocyanin (KLH) was provided by Pierce (Rockford, IL). Blank urine, ref. 460, was obtained from BIO-RAD laboratories S.A. (Alcobendas, Madrid, Spain).

PBS (phosphate buffered saline) was 10 mmol L^{-1} sodium phosphate, 137 mmol L^{-1} NaCl, 2.7 mmol L^{-1} KCl, pH 7.4; PBS-T was PBS containing 0.05% Tween 20; CB (coating buffer) was 50 mmol L^{-1} sodium carbonate/bicarbonate buffer, pH 9.6. Detectabuse™ solid-phase extraction columns (XAD-2) were purchased from Biochemical Diagnostics Inc. (New York, NY, USA).

2.2. Instrumentation

^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were obtained with a 300 Varian spectrometer (300 MHz, Sunnyvale, CA). UV–vis spectra were recorded on an Agilent 8453 diode array spectrophotometer (Santa Clara, CA). Polystyrene 96-well microtiter plates were obtained from Costar (Cambridge, MA) and ELISA plate washer from Nunc MaxiSorp (Roskilde, Denmark). Well absorbances were read in a microtiter plate reader (Wallac, model Victor 1420 multilabel counter, Turku, Finland). Immunoassay competitive curves were mathematically analyzed by the Sigmaplot software package (Jandel Scientific, Erkrath, Germany).

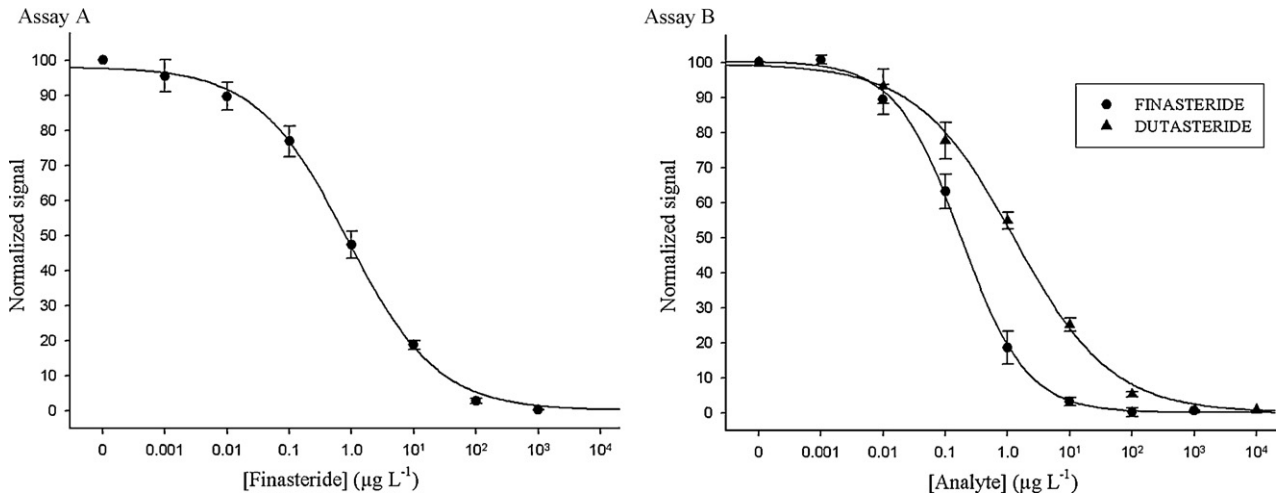


Fig. 1. Calibration curve for immunoassays A (finasteride) and B (finasteride and dutasteride). Mean values ± standard deviation ($n = 3$).

2.2.1. Liquid chromatography–tandem mass spectrometry conditions

Chromatographic separations were carried out with a Waters Acquity UPLC system, equipped with a quaternary pump system using an Acquity BEH C_{18} column (100 mm × 2.1 mm i.d., 1.7 μm particle size) (Waters Corporation, Milford, MA). The column temperature was set to 45 °C and the sample temperature was set to 10 °C. Separation was performed with a binary mobile phase at a flow rate of 0.6 mL min^{-1}. The mobile phase consisted of a mixture of deionised water with 0.01% formic acid (solvent A) and acetonitrile with 0.01% formic acid (solvent B). The gradient elution was as follows: from 0 to 0.6 min, 5% B; from 0.6 to 3.8 min, 90% B; during 0.2 min, 90% B; from 4 to 4.1 min, 5% B; from 4.1 to 5 min, 5% B. The mobile phases were filtered daily using 0.22 μm filters. The sample volume injected was 10 μL

The UPLC instrument was coupled to a Quattro Premier XE triple quadrupole mass spectrometer (Micromass, Waters Corp., Milford, MA, USA) with electrospray ionization source Z-spray working in positive ionization mode. Acquisition was performed in positive ion mode using multiple reaction monitoring (MRM) mode. Source conditions were fixed as follows: capillary voltage, 3 kV; lens voltage, 0.2 V; source temperature 120 °C; desolvation temperature, 450 °C; cone gas flow rate, 50 L h^{-1}; desolvation gas flow rate, 1200 L h^{-1}. High-purity nitrogen was used as a desolvation gas, and argon was used as a collision gas. The following transitions were monitored: 303 > 97, for methyltestosterone (cone voltage 35 V and collision energy 25 eV); and 529 > 461 and 529 > 511, for dutasteride (cone voltage 55 V and collision energy 40 eV).

2.3. Hapten synthesis

Five haptens, named **αn** ($n = 1$–5) were used in this study (Table 1). Haptens **α1** and **α2** are commercially available compounds. Haptens **α3**–**α5** were synthesized by the addition of different aminoalcohols or aminoacids to the acid chloride of hapten **α2**.

*Generation of the acid chloride of hapten **α2** (AC**α2**).* A suspension of commercial hapten **α2** (1.5 mmol, 478.5 mg) in toluene (6.5 mL) was treated at room temperature with oxalyl chloride (1.2 mL). Then, the mixture was stirred for 4 h at room temperature, the solvent was removed under reduced pressure and the acid chloride generated was re-dissolved in dichloromethane (8 mL).

*Hapten **α3***: 4α,6α-Dimethyl-2-oxo-hexadecahydro-indeno [5,4-*f*]quinoline-7-carboxylic acid (3-hydroxypropyl)-amide.

3-Amino-1-propanol (0.8 mL) was added to AC**α2** in dichloromethane, and the mixture was stirred for 1 h at room temperature. Then, water was added and the mixture extracted with dichloromethane. Hapten **α3** crystallized from the aqueous phase as a pure white solid (421 mg, 75%).

^{1}H NMR (DMSO-d_6, δ ppm): 0.57 (3H, s, CH_3), 0.78 (3H, s, CH_3), 3.0 (1H, m, CH_2–NH), 3.20 (1H, m, CH_2–NH), 3.40 (2H, m, CH_2–OH), 4.41 (1H, t, $J = 5.1$ Hz, OH), 7.00 (1H, brs, NH), 7.43 (1H, t, $J = 6.0$ Hz, NH–CH_2).

^{13}C NMR (DMSO-d_6, δ ppm): 11.6 (CH_3), 13.3 (CH_3), 20.7, 23.2, 24.1, 30.5, 31.4, 32.1, 32.7, 33.1, 34.3, 35.7, 37.7, 43.3, 51.4, 54.9, 55.6, 58.6 (CH_2–NH), 60.9, 92.2 (CH_2–OH), 156.8 (CO), 171.6 (CO).

*Hapten **α4***: 4-[(4α,6α-Dimethyl-2-oxo-hexadecahydro-indeno [5,4-*f*]quinoline-7-carbonyl)-amino]-benzoic acid.

4-Aminobenzoic acid (1.5 mmol, 205.7 mg) was added to AC**α2** in dichloromethane, and the mixture was stirred for 17 h at room temperature. Water was added and the mixture extracted with dichloromethane. The organic phase was dried with Na_2SO_4 and the solvent was evaporated. The residue was re-dissolved in a 2 mol L^{-1} NaOH solution and then H_3PO_4 was added dropwise. Hapten **α4** precipitated as a beige solid (397 mg, 60%).

^{1}H NMR (DMSO-d_6, δ ppm): 0.62 (3H, s, 19-CH_3), 0.78 (3H, s, 18-CH_3), 0.8–1.0 (cyclic CH), 1.10–1.80 (cyclic CH), 1.90–2.4 (cyclic CH), 3.0 (1H, dd, 5-CH), 7.23 (2H, s, 2 NH), 7.72 (1H, d, $J = 7.8$ Hz, CH_2), 7.86 (1H, d, $J = 7.8$ Hz, CH_2), 9.83 (1H, COOH).

*Hapten **α5***: 4α,6α-Dimethyl-2-oxo-hexadecahydro-indeno [5,4-*f*]quinoline-7-carboxylic acid (2-hydroxy-1,1-dimethylethyl)-amide.

2-Amino-2-methyl-1-propanol (0.5 mL) was mixed with AC**α2** in dichloromethane, and stirred for 1 h at room temperature. Water was added and the mixture was extracted with dichloromethane. The organic phase was dried with Na_2SO_4 and the solvent was evaporated. The crude was purified through a chromatographic column using hexane/acetone mixtures of increasing polarity as an eluant, leading to hapten **α5** as a white solid (427 mg, 73%).

^{1}H NMR ($CDCl_3$, δ ppm): 0.69 (3H, s, CH_3), 0.90 (3H, s, CH_3), 1.28 (3H, s, ω-CH_3), 1.30 (3H, s, ω-CH_3), 2.40 (2H, m, CH_2–CO), 3.05 (1H, dd, $J = 3.9$ and 11.6 Hz, 5-H), 3.57 (2H, s, CH_2–OH), 5.32 (1H, brs, NH), 5.76 (1H, brs, NH).

^{13}C NMR ($CDCl_3$, δ ppm): 11.4 (CH_3), 13.3 (CH_3), 21.1, 23.5, 24.3, 24.9, 25.2, 27.4, 28.6 (C1), 29.5, 33.4, 35.1, 35.8, 38.5, 44.2, 51.2, 55.7, 56.2, 57.3, 60.7, 71.0, 172.2 (CO), 173.5 (CO).

Table 2
Maximum absorbance values obtained in titration of sera (dilution 1/1000) using different coating conjugates (1 μg mL^{-1}).

Serum	Coating conjugate											
	OVA-α1	BSA-α1	OVA-α2	BSA-α2	OVA-α3	BSA-α3	OVA-α4	BSA-α4	OVA-α5	BSA-α5	Hb-α1	LYS-α1
BSA-α1 (I)	2.8		2.8		0.2		1.2		0.3		2.6	2.7
BSA-α1 (II)	2.9		2.8		0.3		1.0		0.3		2.3	1.9
KLH-α1 (I)	2.7	2.9	2.9	2.9	0.2	0.7	1.8	2.9	0.3	0.8	2.7	2.8
KLH-α1 (II)	2.6	2.9	2.7	2.9	0.2	0.3	1.7	2.9	0.2	0.5	2.8	2.8
BSA-α2 (I)	2.3		2.4		0.2		1.1		0.4		2.4	2.4
BSA-α2 (II)	2.5		2.8		0.3		1.0		0.3		2.0	2.0
KLH-α2 (I)	2.6	2.8	2.6	2.9	0.3	0.5	1.6	2.8	0.3	0.6	2.8	2.8
KLH-α2 (II)	2.6	2.7	2.8	2.8	0.2	0.5	2.0	2.9	0.4	0.9	2.8	2.5
BSA-α3 (I)	0.2		0.2		1.6		0.8		0.2		0.7	0.1
BSA-α3 (II)	0.2		0.2		1.6		0.8		0.2		0.4	0.1
KLH-α3 (I)	0.2	0.3	0.2	0.2	2.1	1.3	1.3	1.3	0.5	0.4	0.3	0.3
KLH-α3 (II)	0.2	0.4	0.2	0.2	1.7	0.8	1.0	1.0	0.4	0.3	0.3	0.2
BSA-α4 (I)	0.3		0.4		2.3		2.8		2.5		1.4	0.6
BSA-α4 (II)	0.3		0.3		1.2		2.7		2.1		1.1	0.6
KLH-α4 (I)	0.6	1.5	0.7	1.3	2.7	2.9	2.8	2.8	2.4	1.6	1.9	0.8
KLH-α4 (II)	0.8	1.9	0.7	0.9	2.1	2.9	2.8	2.8	2.8	1.6	2.0	1.3
KLH-α4 (III)	0.4	1.0	0.4	1.7	1.0	0.9	2.2	0.9	2.2	1.1	1.9	1.4
KLH-α4 (IV)	1.0	2.6	0.7	2.7	1.6	2.4	2.8	1.9	2.7	2.2	2.5	2.3
BSA-α5 (I)	0.5		0.3		0.3		0.5		0.5		2.8	0.9
BSA-α5 (II)	0.7		0.6		0.4		0.7		0.6		1.5	1.0
KLH-α5 (I)	1.2	2.5	2.1	2.1	1.7	2.8	2.3	1.6	2.7	1.3	2.7	2.7
KLH-α5 (II)	1.2	1.8	1.6	1.7	1.0	2.3	1.1	1.0	1.6	0.7	1.9	2.2

I–IV correspond to sera from different rabbits.

2.4. Immunoreagents preparation

Haptens **α1**, **α2** and **α4** were covalently attached through their carboxylic acid moieties to BSA and KLH for immunization purposes, to HRP for enzyme tracers preparation, and to OVA, LYS and Hb to obtain the coating conjugates, by means of the active ester method [21]. Briefly, a freshly prepared solution of *N*-hydroxysuccinimide (NHS) (75 μmol) and dicyclohexylcarbodiimide (DCC) (75 μmol) in 200 μL of anhydrous *N*,*N*′-dimethylformamide (DMF), was added to a solution of each hapten in the same solvent (200 μL). The reaction mixture was stirred at room temperature for 4 h. After centrifugation of the product, 145 μL of the supernatant was diluted to 200 μL with DMF and slowly added to a solution of protein (10 mg/1.5 mL for all proteins except HRP, 3 mg/1 mL) in carbonate buffer. The mixture was stirred at room temperature for 2 h. Finally, the conjugates were separated from uncoupled hapten by gel-exclusion chromatography on D-Salt dextran desalting columns (Pierce, Rockford, IL), eluted with PBS and stored at −20 °C until use.

Haptens **α3** and **α5** were also covalently linked to BSA, KLH, OVA, and HRP through their hydroxyl group, leading to a carbamate group [22]. Basically, alcohol (0.21 mmol) was dissolved in anhydrous acetonitrile (2 mL) under argon atmosphere, and cooled to 0 °C. Then, *N*,*N*′-carbonyldiimidazole (CDI) (0.25 mmol) was added, and stirred for 24 h at 4 °C. The solvent was evaporated under argon stream and redissolved in 2.8 mL of anhydrous DMF. A 40 molar excess of CDI derivative was incubated with protein (15 mg/3.8 mL) in CB and stirred for 2 days at 4 °C. The conjugates were purified by gel-exclusion chromatography as previously indicated.

The conjugation of each hapten to proteins was estimated by measuring the hapten:protein molar ratio according to UV absorbance. From the spectral data of the haptens, proteins and the corresponding conjugates, and assuming that the molar absorptivity of haptens is the same for the free and conjugated forms, apparent mean hapten:protein densities were 27 for BSA, 40 for Hb, 16 for OVA, 3 for LYS and 2 for HRP. Hapten densities of KLH conjugates were difficult to calculate due to the poor solubility of these conjugates.

2.5. Immunization and antiserum obtaining

The immunogens (0.20 mg in 0.5 mL of PBS) were suspended in 0.5 mL of Freund's complete adjuvant and injected intramuscularly into two or four female New Zealand white rabbits (I–IV). Later, animals were boosted four times more at 21-day intervals with the same immunogen suspended in 0.5 mL of Freund's incomplete adjuvant. Whole blood was collected and allowed to coagulate overnight at 4 °C. Then, serum was separated by centrifugation and stored at −80 °C in the presence of 0.02% NaN_3. A total of 22 sera were obtained.

To test sera recognition, optimal concentrations of coating conjugates, serum dilution and enzyme tracers were chosen by checkerboard titration [23]. For this purpose, two assay formats were studied: conjugate-coated and antibody-coated.

Table 3
Sensitivity reached for the best serum-coating conjugate combinations.

Serum	Coating conjugate	IC_{50} Finasteride (μg L^{-1})	IC_{50} Dutasteride (μg L^{-1})
KLH-**α3** (I)	OVA-**α3**	289	n.c.
KLH-**α4** (I)	BSA-**α2**	1100	464
KLH-**α4** (I)	BSA-**α1**	39	95
BSA-**α4** (II)	OVA-**α3**	189	n.c.
KLH-**α4** (III)	Hb-**α1**	15	27
KLH-**α5** (I)	LYS-**α1**	0.9	58
KLH-**α5** (I)	Hb-**α1**	1.5	110
KLH-**α5** (I)	BSA-**α1**	3	8
KLH-**α5** (II)	Hb-**α1**	0.2	1.6

n.c. – no competition.

Table 4
Effect of different variables on ELISA assay performances.

Variable	Assay A		Assay B	
	IC_{50} ($\mu g\,L^{-1}$)	A_0 (A.U.)	IC_{50} ($\mu g\,L^{-1}$)	A_0 (A.U.)
PBS (mmol L^{-1})				
5	3.78 ± 0.45	0.69 ± 0.02	1.68 ± 0.51	0.50 ± 0.03
10	0.92 ± 0.31	0.95 ± 0.02	0.24 ± 0.04	0.87 ± 0.03
20	0.75 ± 0.23	1.06 ± 0.03	0.21 ± 0.03	1.04 ± 0.04
40	1.39 ± 0.34	0.94 ± 0.02	0.23 ± 0.04	0.63 ± 0.04
pH				
6.2	0.97 ± 0.43	0.51 ± 0.02	1.61 ± 0.47	0.42 ± 0.02
7.4	0.75 ± 0.23	1.06 ± 0.03	0.21 ± 0.03	1.04 ± 0.04
8.2	1.23 ± 0.36	0.97 ± 0.03	0.46 ± 0.12	0.62 ± 0.02
Tween 20 (%)				
0.025	0.77 ± 0.24	1.09 ± 0.02	0.25 ± 0.04	0.87 ± 0.03
0.05	0.75 ± 0.23	1.06 ± 0.03	0.21 ± 0.03	1.04 ± 0.04
0.1	0.85 ± 0.39	0.90 ± 0.04	0.33 ± 0.04	0.78 ± 0.01
0.25	0.93 ± 0.40	1.00 ± 0.03	0.54 ± 0.16	1.03 ± 0.02
Time (min)				
5	0.29 ± 0.02	0.45 ± 0.01	0.07 ± 0.01	0.39 ± 0.04
15	0.41 ± 0.11	0.64 ± 0.03	0.12 ± 0.03	0.66 ± 0.02
30	0.53 ± 0.14	0.82 ± 0.01	0.19 ± 0.02	0.88 ± 0.03
60	0.75 ± 0.23	1.06 ± 0.03	0.21 ± 0.03	1.04 ± 0.04
[GAR-HRP]				
1/4000	0.75 ± 0.23	1.06 ± 0.03	0.19 ± 0.02	0.88 ± 0.03
1/2000	1.74 ± 0.38	1.76 ± 0.03	0.18 ± 0.03	1.43 ± 0.04

Assay A: pair KLH-**α5**(I)/LYS-**α1** (1:16,000/2 mg L^{-1}), PBS-T 20 mmol L^{-1}, 0.05% (v/v) Tween 20, pH 7.4, 1 h competition time, [GAR-HRP] 1/4000. Assay B: pair KLH-**α5**(II)/Hb-**α1** (1:2000/0.5 mg L^{-1}), PBS-T 20 mmol L^{-1}, 0.05% (v/v) Tween 20, pH 7.4, 30 min competition time, [GAR-HRP] 1/2000.

Table 5
Cross-reactivity of the developed assays.

Compound	Assay A		Assay B	
	IC_{50} ($\mu g\,L^{-1}$)	CR (%)	IC_{50} ($\mu g\,L^{-1}$)	CR (%)
Finasteride	2.16	100.00	0.65	100.00
Dutasteride	58.15	3.71	3.68	17.66
Finasteride metabolite	5.86	36.86	2.20	29.55
Boldenone metabolite 1	>10,000	<0.02	>10,000	<0.01
Epimetendiol	>10,000	<0.02	>10,000	<0.01
Metenolone	>10,000	<0.02	40.27	1.61
Oxandrolone	>10,000	<0.02	29.57	2.20
Epioxandrolone	>10,000	<0.02	>10,000	<0.01
3′-Hydroxystanolozol	>10,000	<0.02	>10,000	<0.01
4β-Hydroxystanozolol	>10,000	<0.02	>10,000	<0.01
16β-Hydroxystanozolol	>10,000	<0.02	>10,000	<0.01
Canrenone	818.54	0.26	>10,000	<0.01
Trenbolone	>10,000	<0.02	>10,000	<0.01
1-Testosterone	489.31	0.44	39.10	1.66
Salbutamol	>10,000	<0.02	>10,000	<0.01
Terbutaline	>10,000	<0.02	>10,000	<0.01
Androsterone	>10,000	<0.02	>10,000	<0.01
Etiocholanolone	>10,000	<0.02	>10,000	<0.01
5α-Androstanedione	>10,000	<0.02	>10,000	<0.01
Androstanolone (DHT)	>10,000	<0.02	100.60	0.65
Epitestosterone	>10,000	<0.02	>10,000	<0.01
Estradiol	>10,000	<0.02	>10,000	<0.01
Norandrosterone	>10,000	<0.02	>10,000	<0.01
Norandrosterone glucuronide	>10,000	<0.02	>10,000	<0.01
Noretiocholanolone	>10,000	<0.02	>10,000	<0.01
Cortisol	>10,000	<0.02	>10,000	<0.01
Tetrahydrocortisol	>10,000	<0.02	>10,000	<0.01
Pregnanediol	>10,000	<0.02	>10,000	<0.01
Pregnanetriol	>10,000	<0.02	>10,000	<0.01
Testosterone	676.84	0.32	118.33	0.55
Testosterone glucuronide	114.21	1.89	68.54	0.95
Androsterone glucuronide	>10,000	<0.02	>10,000	<0.01
Etiocholanolone glucuronide	>10,000	<0.02	>10,000	<0.01

2.6. ELISA optimization

Assay optimization was performed for the most sensitive assays using finasteride as the competitor analyte following Tijssen's protocol [24]. Standards were prepared in PBS-T from a stock solution in methanol. Antibody and tracer working solutions were prepared in PBS-T and mixed with an equivalent volume of standards in the plate.

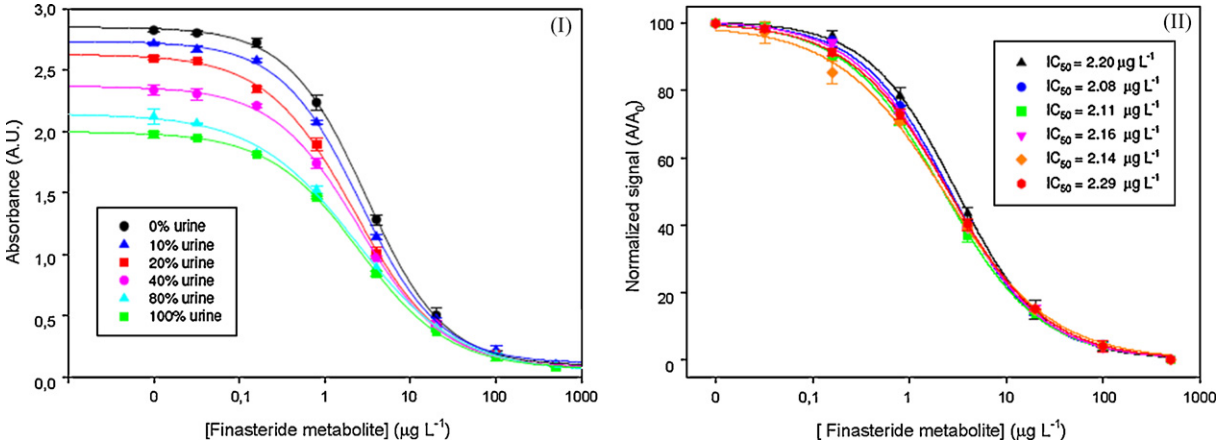


Fig. 2. Tolerance to urine of assay B. (I) Calibration curves with different percentages of urine. (II) Normalized calibration curves. Mean values ± standard deviation ($n = 3$).

The influence of several experimental parameters (ionic strength, pH, surfactant percentage, incubation time and concentration of GAR-HRP tracer) was examined in order to improve the sensitivity of the immunoassay. Criteria used to optimize the assay performances were sensitivity (IC_{50}), maximum absorbance (A_0), dynamic range (DR, established between the concentrations producing 20% and 80% inhibition) and limit of detection (LOD, estimated at 10% inhibitory concentration).

2.6.1. General procedure of the optimized assays

Assay A: Flat-bottomed polystyrene ELISA plates were coated overnight at 4 °C with 100 μL/well of coating conjugate LYS-**α1** (2 mg L^{-1} in CB). The following day, plates were washed six times with PBS-T. After that, a volume of 50 μL of standard in the optimized PBS-T (20 mmol L^{-1} PBS, pH 7.4 and 0.05% Tween 20) and 50 μL of serum KLH-**α5** (I) 1:16,000 in the same buffer were added to the coated plates and incubated for 1 h at room temperature. After washing, plates were incubated for 1 h with 100 μL/well of GAR-HRP (diluted 1:4000 in PBS-T). Once washed, peroxidase activity was determined by adding 100 μL/well of substrate solution (2 mg mL^{-1} OPD and 0.012% H_2O_2 in 25 mmol L^{-1} sodium citrate, 62 mmol L^{-1} sodium phosphate, pH 5.5). After 10 min, the enzymatic reaction was stopped by addition of 2.5 mol L^{-1} H_2SO_4 (50 μL/well), and the absorbance was read in a dual-wavelenght mode at 490/650 nm. The standard curve was fitted to a four-parameter logistic equation according to the following formula: $y = (A - B/[1 - (x/C)^D]) + B$, where A is the maximal absorbance, B is the minimum absorbance, C is the concentration producing 50% of the maximal absorbance, and D is the slope at the inflection point of the sigmoid curve.

Assay B: ELISA plates were coated overnight at 4 °C with 100 μL/well of coating conjugate Hb-**α1** (0.5 mg L^{-1} in CB). The following day, plates were washed with PBS-T. After that, a volume of 50 μL of standard in the optimized PBS-T (20 mmol L^{-1} PBS, pH 7.4 and 0.05% Tween 20) and 50 μL of serum KLH-**α5** (II) 1:2000 in the same buffer were added to the coated plates and incubated for 30 min at room temperature. After washing, plates were incubated for 1 h with 100 μL/well of GAR-HRP (diluted 1:2000 in PBS-T). Once washed, peroxidase activity was determined by adding 100 μL/well of substrate solution. After 10 min, the enzymatic reaction was stopped by addition of 2.5 mol L^{-1} H_2SO_4 (50 μL/well).

2.7. Urine samples

Standard curves were prepared using commercial blank urine. In addition, 20 human urine samples from different healthy volunteers were collected, centrifuged for 5 min at 3500 rpm and stored at −20 °C until analysis. Human excretion study was carried out by administering a single oral dose of 0.5 mg of dutasteride. Urine samples were collected for 72 h and frozen at −20 °C until analysis.

2.7.1. LC–MS/MS analysis of dutasteride: sample preparation procedure

Aliquots of urine samples (5 mL) were added to a methanolic solution of methyltestosterone (50 μL of a solution of 50 μg mL^{-1}),

Table 6
Finasteride metabolite recoveries from spiked blank human urine.

Urine (%)	[Finasteride metabolite] added (μg L^{-1})	[Finasteride metabolite] found (μg L^{-1})	Recovery (%)
100	15	14.4 ± 1.6	95.6
20	15	17.2 ± 3.5	114.7
	30	34.2 ± 5.9	114.1
	60	60.8 ± 9.4	101.3
	120	112.8 ± 0.3	94.0
10	15	14.9 ± 0.2	99.4
	30	31.5 ± 0.5	104.8
	60	61.7 ± 5.7	102.7
	120	119.5 ± 2.3	99.6
5	15	19.2 ± 6.1	128.1
	30	31.3 ± 0.1	104.5
	60	72.2 ± 16.5	120.4
	120	129.2 ± 4.8	107.7
2.5	15	37.4 ± 9.9	124.8
	30	52.3 ± 8.1	87.2
	60	163.0 ± 27.3	135.9

Table 7
Finasteride recoveries from spiked human urine samples.

Sample	[Finasteride] added ($\mu g\,L^{-1}$)	[Finasteride] found ($\mu g\,L^{-1}$)	Recovery (%)	CV (%)
MB1	10	7.6 ± 1.8	75.9	24.2
	30	31.7 ± 4.7	105.7	14.8
	60	42.6 ± 6.0	70.9	14.0
MB2	30	61.3 ± 9.7	204.3	14.7
MB3	10	10.2 ± 2.1	101.7	21.0
	30	24.5 ± 3.9	81.5	15.8
	60	42.1 ± 8.8	70.2	20.9
MB4	10	7.7 ± 1.9	76.9	25.0
	30	25.2 ± 2.1	84.0	8.2
	60	43.0 ± 5.1	71.6	11.9
MB5	30	70.6 ± 18.4	235.3	22.9
MB6	10	12.9 ± 1.5	129.0	11.7
	30	35.9 ± 4.8	119.8	13.3
	60	48.8 ± 5.3	81.4	10.9
MB7	10	9.1 ± 2.0	90.8	21.6
	30	32.2 ± 8.6	107.4	26.7
	60	41.5 ± 8.2	69.1	19.8
MB8	10	13.2 ± 2.8	131.6	21.0
	30	28.7 ± 3.7	95.6	12.8
	60	45.3 ± 6.9	75.4	15.2
MB9	10	8.8 ± 2.8	87.8	32.0
	30	29.9 ± 4.7	99.8	15.7
	60	48.6 ± 5.6	80.9	11.6
MB10	10	12.9 ± 2.7	129.3	20.6
	30	34.4 ± 5.4	114.9	15.8
	60	63.3 ± 6.9	105.5	10.9
MB11	10	10.5 ± 1.0	105.4	9.3
	30	24.0 ± 2.4	80.1	9.8
	30	49.7 ±3.6	82.8	7.3
MB12	10	14.7 ± 2.4	146.8	16.2
	30	22.8 ± 2.8	76.1	12.2
	60	49.1 ± 6.7	81.8	13.6
MB13	10	14.4 ± 0.8	144.3	5.6
	30	34.2 ± 4.6	114.0	13.4
	60	60.3 ± 4.2	100.6	6.9
MB14	10	8.1 ± 1.3	80.5	16.7
	30	25.9 ± 4.3	86.3	16.7
	60	55.9 ± 10.3	93.2	18.4
MB15	10	7.4 ± 1.9	74.1	25.3
	30	23.6 ± 2.0	78.5	8.3
	60	42.6 ± 3.5	71.0	8.2
MB16	10	9.4 ± 1.7	93.9	18.5
	30	23.3 ± 1.5	77.6	6.5
	60	64.8 ± 10.0	107.9	15.4
MB17	10	19.1 ± 2.5	190.8	13.0
	30	27.9 ± 6.1	93.0	21.8
	60	68.5 ± 6.8	114.1	10.0
MB18	10	24.1 ± 3.8	241.3	15.8
	30	30.1 ± 4.2	100.2	13.9
	60	61.6 ± 10.3	102.7	16.7
MB19	10	13.0 ± 1.7	129.5	13.0
	30	26.4 ± 2.3	87.9	8.8
	60	67.8 ± 5.1	113.0	7.4
MB20	10	11.9 ± 1.6	119.2	13.1
	30	26.5 ± 3.5	88.3	13.1
	60	67.3 ± 8.9	112.1	13.2

$n = 4$.

used as an internal standard. Samples were applied to Detectabuse columns previously conditioned with methanol (2 mL) and water (2 mL). The columns were washed with Milli Q water (2 mL) and eluted with methanol (2 mL). The methanolic extracts were evaporated to dryness under a nitrogen stream in a bath at 49 °C. The residues were reconstituted in 1 mL of sodium phosphate buffer (0.2 mol L^{-1}, pH 7), and subjected to enzymatic hydrolysis with β-glucuronidase from *E. coli* (30 μl) and incubation in a water bath at 55 °C for 1 h. 250 μL of 5% K_2CO_3 solution was then added and extracted with n-pentane (6 mL) by shaking at 40 rpm for 20 min. After centrifugation (3500 rpm, 5 min), the organic layers were evaporated to dryness under a nitrogen stream in a bath at 40 °C. The extracts were reconstituted with 100 μL of a mixture of deionised water:acetonitrile (90:10, v/v) and 5 μL aliquots were analyzed by UPLC–MS/MS.

3. Results and discussion

3.1. Hapten design and synthesis

Five different haptens were designed in order to raise polyclonal antibodies (Table 1). Finasteride differs from dutasteride only in D-ring amide substituent. Hapten **α1**, resulting from the amide hydrolysis, and **α2**, which lacks the double bond on ring A, were selected in order to maximize recognition of the common structure of these 4-azasteroids, both being commercially available. These haptens need a spacer arm in order to maximize

the compound exposition to the immune system. For this reason, hapten **α2** was chosen to synthesize new haptens by introducing alcohols or acids as D-ring amide substituents. To this end, treatment of hapten **α2** with oxalyl chloride led to the corresponding acid chloride, which on reaction with 3-aminopropan-1-ol, 4-aminobenzoic acid, and 2-amino-2-methyl-1-propanol yielded the haptens **α3**–**α5**, respectively. Hapten **α4** presents an aromatic spacer arm and is therefore the most similar to dutasteride, while hapten **α5** is the compound which preserves finasteride structure to a greater degree.

3.2. Sera screening

The obtained sera were titrated in antibody-coated format, but none of them showed enough titer to carry out competition assays. In general, results of sera screening in the conjugate-coated format showed high titers (Table 2). The combinations of serum/coating conjugates that showed specific recognition were used to carry out competitive assays in order to set up a sensitive assay for finasteride. Results are given in Table 3. The pairs KLH-**α5** (I)/LYS-**α1** (1:16,000/2 mg L^{-1}) (assay A) and KLH-**α**5 (II)/Hb-**α1** (1:2000/0.5 mg L^{-1}) (assay B), that provided the lowest IC_{50} values, were selected to set-up an ELISA.

3.3. ELISA optimization

Optimal assay performance for both assays was obtained with 20 mmol L^{-1} PBS, pH 7.4 and 0.05% Tween 20 (Table 4). Respect to the duration of the competition step, the best assay features (IC_{50}/A_0 ratio) were obtained with 60 min incubation time for assay A, and 30 min for assay B. Finally, the influence of secondary antibody (GAR-HRP) concentration was investigated. In assay A, a concentration of 1/2000 provided the worst IC_{50}, while assay B features were improved under these conditions.

After the optimization (Fig. 1) assay A exhibited an IC_{50} of 0.75 μg L^{-1}, a LOD of 0.01 μg L^{-1} and a dynamic range between 0.07 and 8.2 μg L^{-1} for finasteride. Assay B showed an IC_{50} of 0.18 μg L^{-1} and a LOD of 0.013 μg L^{-1} for finasteride, and an IC_{50} of 1.18 μg L^{-1} and a LOD 0.021 μg L^{-1} for dutasteride.

3.4. Cross-reactivity studies

Assay selectivity was evaluated against a set of endogenous and exogenous anabolic steroids, as well as the major finasteride metabolite present in urine. As original concentration of cross-reacting compounds (Table 5) in methanolic stock solutions was low (100 mg L^{-1}), all the calibration curves, including analytes, were carried out using 4% methanol in PBS-T as assay medium. This is why IC_{50} values for finasteride and dutasteride in Table 5 are higher than values reported above, obtained in PBS-T. The cross-reactivity values were calculated according to the following equation: CR = (IC_{50} [μg L^{-1}] Finasteride/IC_{50} [μg L^{-1}] compound) × 100. The interferences were negligible for most tested compounds in both assays (<0.02%), except for finasteride metabolite, which showed a CR of 37% in assay A and 30% in assay B, and for dutasteride in assay B (18%). The high cross-reactivity of finasteride metabolite for both assays can be explained on the basis of its chemical structure. On one hand, this compound is very similar to finasteride (Table 1), so it is logic that it would be recognized by high affinity finasteride antibodies. On the other hand, these antibodies have been obtained using hapten **α5** for immunization. It is, among the haptens used, the most similar to finasteride and finasteride metabolite and, therefore, this would be the reason of the high recognition obtained. This result makes these immunoassays a powerful tool to determine 5α-reductase inhibitors in human matrices, assay A being generic for finasteride and its metabolite, and assay B generic for finasteride, finasteride metabolite and dutasteride.

Table 8
Finasteride metabolite recoveries from spiked human urine samples.

Sample	[Met. Finasteride] added (μg L^{-1})	[Met. Finasteride] found (μg L^{-1})	Recovery (%)	CV (%)
MB11	12	12.2 ± 1.6	101.3	13.4
	36	31.0 ± 4.1	86.0	13.4
	72	73.0 ± 8.5	101.4	11.6
MB12	12	18.7 ± 1.9	156.2	20.2
	36	45.1 ± 7.9	125.2	17.5
	72	73.2 ± 11.0	101.6	15.0
MB13	12	19.4 ± 1.3	161.4	7.1
	36	41.4 ± 3.9	115.1	9.4
	72	78.7 ± 10.2	109.3	13.0
MB14	12	13.9 ± 1.5	116.2	10.5
	36	32.9 ± 4.9	91.4	15.0
	72	66.9 ± 8.1	92.9	12.2
MB15	12	14.6 ± 0.3	121.3	2.2
	36	39.8 ± 3.0	110.6	7.6
	72	67.3 ± 7.0	93.5	10.5
MB16	12	13.2 ± 1.9	109.7	14.5
	36	44.2 ± 4.0	122.8	9.0
	72	83.2 ± 9.6	115.6	11.5
MB17	12	17.6 ± 1.6	146.9	9.3
	36	44.0 ± 5.9	122.1	13.5
	72	81.3 ± 7.3	112.9	9.0
MB18	12	18.5 ± 1.5	153.9	8.1
	36	44.7 ± 7.9	124.2	17.6
	72	83.9 ± 9.1	116.5	10.9
MB19	12	14.8 ± 2.7	123.2	18.0
	36	33.0 ± 1.1	91.7	3.3
	72	86.6 ± 9.3	120.3	10.8
MB20	12	13.3 ± 1.7	110.8	12.5
	36	38.6 ± 8.0	107.1	20.7
	72	83.1 ± 12.8	115.3	15.4

$n = 4$.

Table 9
Dutasteride recoveries from spiked human urine samples.

Sample	[Dutasteride] added ($\mu g\,L^{-1}$)	[Dutasteride] found ($\mu g\,L^{-1}$)	Recovery (%)	CV (%)
21	12	17.2 ± 2.3	143.3	13.4
	36	46.1 ± 4.7	128.0	10.1
	72	68.4 ± 3.9	95.0	5.7
22	12	10.3 ± 1.2	85.8	11.7
	36	38.0 ± 5.2	105.5	13.8
	72	81.4 ± 6.8	113.0	8.4
23	12	11.8 ± 1.5	98.3	12.7
	36	30.4 ± 4.7	84.3	15.5
	72	73.4 ± 8.6	102.0	11.7
24	12	13.5 ± 1.9	112.5	14.1
	36	30.8 ± 4.3	85.5	13.8
	72	69.6 ± 7.5	96.6	10.7
25	12	14.3 ± 1.8	119.2	12.6
	36	31.8 ± 5.7	88.4	17.8
	72	74.7 ± 8.6	103.7	11.5
26	12	12.7 ± 1.4	105.8	11.0
	36	38.5 ± 7.5	106.9	19.6
	72	76.9 ± 9.6	106.8	12.5
27	12	16.7 ± 2.1	139.2	12.6
	36	39.2 ± 5.4	108.9	13.7
	72	77.2 ± 8.2	107.2	10.6
28	12	13.7 ± 1.7	114.2	12.4
	36	35.3 ± 4.7	98.0	13.3
	72	75.1 ± 8.5	104.3	11.3
29	12	13.6 ± 2.4	113.3	17.6
	36	42.1 ± 5.1	116.9	12.1
	72	67.2 ± 6.2	93.4	9.3
30	12	11.2 ± 1.3	93.3	11.6
	36	34.8 ± 4.8	96.8	13.8
	72	69.3 ± 7.6	96.3	11.0

$n = 4$.

3.5. Analysis of urine samples

The tolerance of assays A and B to human urine was studied, preparing the calibration curve with different percentages of blank human urine. Assay A only tolerated urine percentages lower than 4%, while assay B maintained both sensitivity and dynamic range, even in 100% urine (Fig. 2).

Subsequently, a blank urine sample was spiked with different concentrations of finasteride metabolite. Samples were diluted in 20 mmol L^{-1} PBS and analyzed by ELISA. Excellent recovery results were obtained, with values ranging from 87.2% to 135.9% (Table 6).

Because WADA rules that urine samples outside the established density range must be re-sampled from athletes, representative urines were studied. For this, 20 different human urine samples from volunteers (males and females with different life style, ages and builds) were collected and spiked with finasteride at three levels. Also, 10 urine samples were spiked with finasteride metabolite and dutasteride at similar levels. All samples were diluted at 20% in 20 mmol L^{-1} PBS and analyzed by the developed immunoassay (Tables 7–9). Blanks of these samples were analyzed to check that no matrix effects or interferents existed. Blanks 2 and 5 gave positive results in ELISA (values of 5.0 ± 1.3 and 9.5 ± 3.0 $\mu g\,L^{-1}$, respectively), so recovery of finasteride was over-estimated due to the interferences. All other samples were below the limit of detection, so good recoveries were obtained for finasteride (between 69.1% and 131.6%), finasteride metabolite (86.0–125.2%) and dutasteride (85.8–128.0%). However, samples 12, 13, 17 and 18 could not be well quantified at low analyte concentrations, also being over-estimated, both for finasteride and finasteride metabolite. The same occurred with samples 21 and 27 for dutasteride. Additional work will be carried out to discover the origin of this setback.

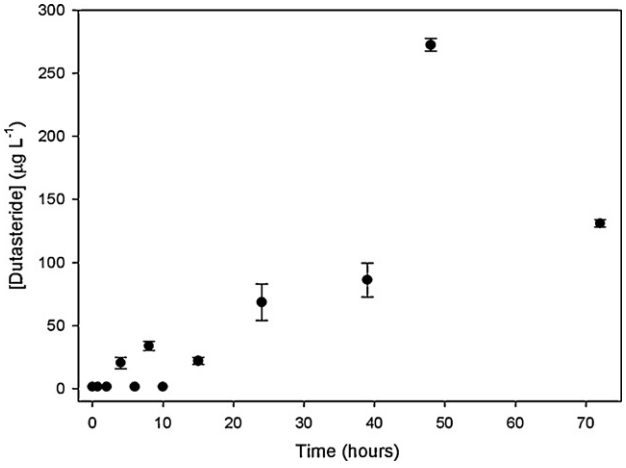


Fig. 3. Dutasteride human excretion kinetic in urine.

3.6. Dutasteride excretion study

Basal and excretion urines obtained over a period of 72 h were collected, extracted and analyzed by LC–MS/MS. Unfortunately, dutasteride was only detected at a concentration of 1 $\mu g\,L^{-1}$ in samples collected from 9 to 11 h after administration. These chromatographic results agree with bibliographical data [25,26], where it is reported that <0.1% of unchanged dutasteride is excreted in urine.

On the other hand, the same urine samples were diluted in 20 mmol L^{-1} PBS and analyzed by ELISA (Fig. 3). Results indicated that after 20 h of administration, dutasteride metabolites are excreted at a concentration of 50 $\mu g\,L^{-1}$, showing a maximum of 270 $\mu g\,L^{-1}$ after 48 h (sample 11). These results indicate that dutasteride is mainly excreted in urine as metabolites. These metabolites could be more easily recognized by the antibody than dutasteride itself. So, the immunoanalysis would provide a

higher concentration than real dutasteride level. Consequently, more pharmacokinetics work should be carried out to clarify these results, though it is not easy to obtain dutasteride metabolites and the compound recognized by the ELISA has not been identified yet. For this reason, molecular interaction model approaches will be also studied.

4. Conclusions

Novel haptens similar to finasteride and dutasteride have been synthesized. These haptens have led to the development of two competitive ELISAs with excellent sensitivity, which detect finasteride and dutasteride down to 0.01 and 0.02 $\mu g\,L^{-1}$, respectively. One of the assays, generic for finasteride, finasteride metabolite and dutasteride, maintained its sensitivity and dynamic range, even in 100% urine, while the other one only tolerated lower urine percentages. The developed generic ELISA performed very well, reaching high recoveries of dutasteride, finasteride and its metabolite from spiked human urine samples.

These methods compete very favorably with chromatographic techniques, because the sensitivity of the developed immunoassay is high considering also that it is a direct analysis. Also on rapidity, our proposal is highly attractive because it spends down to 2 h analyzing for duplicate 40 different urine samples per 96 wells ELISA plate. Furthermore, to this high working capacity it must be considered the simplicity and the *in situ* development. This means get the former results on competition even in presence of the tested sportsman, considering that frequently sportive events spend more time than the immunoassay development.

On the other hand, the assay was useful to establish an excretion study of dutasteride in human urine and it may help in the identification of dutasteride metabolites in this fluid. Knowledge about the excretion of prohibited substances is essential to assess anti-doping rules.

Acknowledgments

This work is financed by project DEP2006-56177-C03-03 (Spanish Ministerio de Ciencia e Innovación). Pilar Sáez's Technical Assistance (Grup de Recerca en Bioanàlisis, IMIM-Hospital del Mar) is gratefully acknowledged. The English revision of this paper was performed by the Language Centre of Universidad Politécnica de Valencia, Spain.

References

[1] G.H. Rasmusson, G.F. Reynolds, T. Utne, R.B. Jobson, R.L. Primka, C. Berman, J.R. Brooks, J. Med. Chem. 27 (1984) 1690–1701.
[2] H.G. Bull, M. Garcia-Calvo, S. Andersson, W.F. Baginsky, H.K. Chan, D.E. Ellsworth, R.R. Miller, R.A. Stearns, R.K. Bakshi, G.H. Rasmusson, R.L. Tolman, R.W. Myers, J.W. Kozarich, G.S. Harris, J. Am. Chem. Soc. 118 (1996) 2359–2365.
[3] T.T. Ashbum, K.B. Thor, Nat. Rev. 3 (2004) 673–683.
[4] A. Tosti, B.M. Piraccini, J. Am. Acad. Dermatol. 42 (2000) 848–849.
[5] C.A. Coltman, I.M. Thompson, P. Feigl, Eur. Urol. 35 (1999) 544–547.
[6] Soskic V, Schrattenholz A, PCT Int. Appl. WO 2006058781 (2006).
[7] H. Geyer, E. Nolteernsting, W. Schänzer, in: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck-Engelke (Eds.), Recent Advances in Doping Analysis, vol. 7, Sport und Buch Strauβ, Köln, 1999, pp. 71–80.
[8] The World Anti-Doping Code. The 2009 Prohibited List International Standard, World Anti-Doping Agency, Montreal, Canada, 2009, http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-Prohibited-list/WADA_Prohibited_List_2010_EN.pdf (accessed 18.01.10).
[9] WADA Technical Document TD2009MRPL (Minimum Required Performance Limits for Detection of Prohibited Substances), World Anti-Doping Agency, Montreal, Canada, 2009, http://www.wada-ama.org/rtecontent/document/MINIMUM_REQUIRED_PERFORMANCE_LEVELS_TD_v1_0_January_2009.pdf (accessed 18.01.10).
[10] T. Graf-Baumann, Br. J. Sports Med. 40 (2006) i55–i57.
[11] C.G. Georgakopoulos, C. Tsitsimpikou, M-H.E. Spyridaki, E. Lyris, E.G. Cookeas, TrAC Trends Anal. Chem. 18 (1999) 1–13.
[12] M. Tsivou, N. Kioukia-Fougia, E. Lyris, Y. Aggelis, A. Fragkaki, X. Kiousi, Ph. Simitsek, H. Dimopoulou, I.-P. Leontiou, M. Stamou, M.-H. Spyridaki, C. Georgakopoulos, Anal. Chim. Acta 555 (2006) 1–13.
[13] J.R. Carlin, P. Höglund, L.O. Eriksson, P. Christofalo, S.L. Gregoire, A.M. Taylor, K.E. Anderson, Drug Metab. Dispos. 20 (1992) 148–155.
[14] J.R. Carlin, P. Christofalo, B.H. Arison, R.E. Ellsworth, A. Rosegay, R.R. Miller, S.H.L. Chu, W.J.A. Vandenheuvel, Drug Metab. Dispos. 25 (1996) 100–109.
[15] Y. Ishii, H. Mukoyama, M. Ohtawa, Drug Metab. Dispos. 22 (1994) 79–84.
[16] M. Thevis, H. Geyer, U. Mareck, U. Flenker, W. Shänzer, Ther. Drug Monit. 29 (2007) 236–247.
[17] J.-O. Thörngren, F. Östervall, M. Garle, J. Mass Spectrom. 43 (2008) 980–992.
[18] M. Mazzarino, F. Botrè, Rapid Commun. Mass Spectrom. 20 (2006) 3465–3476.
[19] R. Ventura, M. Roig, N. Monfort, P. Sáez, R. Bergés, J. Segura, Eur. J. Mass Spectrom. 14 (2008) 191–200.
[20] M.A. Huestis, M.L. Smith, Drug Discov. Today 3 (2006) 49–57.
[21] J.J. Langone, H. van Vunakis, Methods Enzymol. 84 (1982) 628–640.
[22] H.L. Beasley, D.P. McAdam, K.A. Larkin, B.S. Ferguson, R.J. Bushway, J.H. Skerritt, Bull. Environ. Contam. Toxicol. 59 (1997) 375–382.
[23] E.M. Brun, M. Garcés-García, R. Puchades, A. Maquieira, J. Immunol. Methods 295 (2004) 21–35.
[24] P. Tijssen, in: R.H. Burdon, P.H. Knippenberg (Eds.), Laboratory Techniques in Biochemistry and Molecular Biology, vol. 15, Elsevier, Amsterdam, 1985, pp. 236–239.
[25] J. Segura, R. Ventura, J. Marcos, R. Gutiérrez, in: R. Smith, M. Bogusz (Eds.), Handbook of Analytical Separations, Elsevier, Loughborough, UK, 2007, pp. 699–744.
[26] S. Simoes, B. Vitoriano, C. Manzoni, X. de la Torre, in: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck-Engelke (Eds.), Recent Advances in Doping Analysis, vol. 13, Sport und Buch Strauβ, Köln, 2005, pp. 199–209.