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Enzyme-linked immunosorbent assays for the synthetic steroid gestrinone

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ABSTRACT

Gestrinone is a synthetic steroid hormone with anti-estrogenic and anti-progesterone properties. It is used to treat endometriosis, shrink uterine fibroids and reduce menorrhagia; besides, it has been investigated for use as contraceptive. Also, due to its anabolic effects, it has been included in the banned list of performance enhanced drugs in sport. Polyclonal antibodies raised against bovine serum albumin coupled to gestrinone 3-carboxymethyloxime (3OCMO-G) were used to develop two highly sensitive and specific enzyme-linked immunosorbent assays for gestrinone. One of them, based on direct format, shows a detection limit (LD) of 0.09 ± 0.03 ng L^{-1}. The second assay, hapten-protein coating format, can detect until (LD) 0.14 ± 0.05 ng L^{-1}. Both immunoassays were also highly specific, showing negligible or no cross-reactivity to other anabolic steroids. The developed ELISAs detected lower amounts of gestrinone than those determined by the reference chromatographic HPLC/MS/MS methods. The direct format was applied to quantify this steroid in spiked human urine without sample pre-treatment, with recovery values between 76 and 122%.

1. Introduction

The synthetic steroid gestrinone, (17α)-13-ethyl-17-hydroxy-18,19-dinorpregna-4,9,11-trien-20-yn-3-one, has anti-estrogenic and anti-progesterone properties. It has been shown several pharmacological applications such as uterine volume reduction, shrink uterine fibroids and stop bleeding [1]. Gestrinone was originally developed as an oral contraceptive for women [2], showing no significant advantages over other contraceptives [3], and it is currently used to treat endometriosis [4]. Due to gestrinone anabolic effects, it is the only marketed progestin included in the banned list of performance enhanced drugs in sport [5].

Gestrinone has a long half-life (28 h) when given orally, being metabolized by the liver and excreted by urine and feces [6]. The excretion of this drug and its metabolites has been extensively studied [7]. Two unidentified gestrinone metabolites were detected in human urine as well as the parent drug as glucuronide conjugates [8,9]. Based upon the mass spectrometric data from an *in vitro* study, four potential metabolites were identified [10]. One of them was in agreement with a glucuronide adduct of gestrinone, while the others were all hydroxylated and glucuroconjugated metabolites. The major one was identified as the glucuronide of 18-hydroxygestrinone. In another study of the metabolism of gestrinone in guinea pig bile [6], 2ζ,16β-dihydroxygestrinone was found as the major metabolite, as well as gestrinone and 6α-hydroxygestrinone (Fig. 1).

Analytical procedures usually applied to the determination of gestrinone and its main metabolites in human urine are gas chromatography–mass spectrometry (GC–MS) [9,11] and liquid chromatography–mass spectrometry (LC–MS) [9]. These methods require a pre-treatment of urine samples, with an enzymatic hydrolysis to cleave the steroid conjugates, followed by liquid–liquid extraction. Moreover, derivatization of the analyte is a mandatory task before measurement by GC–MS. Steroids with a 3-keto-4,9,11-trien ring system like gestrinone, trenbolone and tetrahydrogestrinone, show problems in the derivatization step, giving more than one peak by tautomerism [11]. Due to this inconvenience and the high sensitivity that can be achieved in the electrospray ionization mode, in recent years LC–MS–MS has become popular in the analysis of these compounds in biological fluids. Using this methodology, limits of detection of 0.8 and 1 ng mL^{-1} have been described for the determination of gestrinone in human serum [12] and urine [13], respectively.

Despite the excellent performance of chromatographic methods, immunoanalytical techniques are a very interesting alternative, as they combine: sensibility, selectivity, throughput and rapidity; besides they are presented in different formats as plate, dipstick, particles, microarray and biosensors. These techniques have proved to be suitable tools for the screening of anabolic steroids [14–16] and other prohibited substances [17], requiring little or no sample pre-treatment in most cases, although no immunological method has been reported for gestrinone yet.

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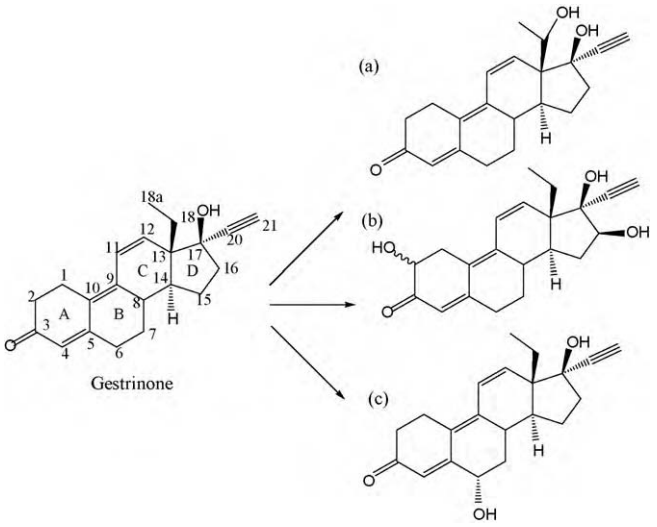

Fig. 1. Metabolism of gestrinone to (a) 18-hydroxy-gestrinone, (b) 2ζ,16β-dihydroxygestrinone and (c) 6α-hydroxygestrinone.

The availability of immunoreagents is the first step to develop this type of assays. In this respect, this paper reports the synthesis of immunoreagents and the production of antibodies for gestrinone determination. With this aim, the hapten gestrinone 3-carboxymethyloxime was synthesized and coupled to bovine serum albumin to elicit polyclonal antibodies. This conjugate was previously used [16] as coating hapten in the assessment of an ELISA for tetrahydrogestrinone. The obtained immunoreagents were used to develop, for the first time, two enzyme-linked immunoassays for the determination of gestrinone in urine without sample pre-treatment.

2. Materials and methods

2.1. Chemicals

Chemical reagents for hapten synthesis and protein conjugation purposes, bovine serum albumin (BSA), hemoglobin (Hb), complete and incomplete Freund's adjuvant, *o*-phenylenediamine (OPD), Tween 20, horseradish peroxidase (HRP) and peroxidase labeled goat anti-rabbit immunoglobulins (GAR–HRP) were purchased from Sigma–Aldrich Química (Madrid, Spain). Gestrinone was purchased from WaterStone Technology, LCC (Carmel, IN). Keyhole limpet hemocyanin (KLH) was obtained from Pierce (Rockford, IL). Standard human urine, Ref. 460, was provided by BIO-RAD laboratories S.A. (Alcobendas, Madrid, Spain).

2.2. Instrumentation

Thin-layer chromatography was performed on 0.25 mm, precoated silica gel 60 F_{254} aluminum sheets (Merck, Darmstat, Germany). Column chromatography was carried out on silica gel (0.063–0.2 mm particle size, 70–230 mesh), also from Merck. ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were obtained with a 300 Varian spectrometer (Sunnyvale, CA). UV–vis spectra were recorded on an Agilent 8453 diode array spectrophotometer (Santa Clara, CA). The centrifuge was Labofuge 400 from Heraeus Instruments (Hanau, Germany). Polystyrene 96-well microtiter plates were obtained from Costar (Cambridge, MA). Washing steps were carried out using a microplate washer from Nunc MaxiSorp (Roskilde, Denmark). Well absorbances were measured at 490 and 650 nm by means of a microtiter plate reader (Wallac, model Victor 1420 multilabel counter, Turku, Finland). Immunoassay competitive curves were mathematically analyzed with a four parameter equation using the Sigmaplot software package (Jandel Scientific, Erkrath, Germany).

2.3. Buffers

PBS (10 mmol L^{-1} sodium phosphate, 137 mmol L^{-1} NaCl, 2.7 mmol L^{-1} KCl, pH 7.5), PBS-T (PBS containing 0.05% (v/v) Tween 20); coating buffer (50 mmol L^{-1} sodium carbonate/bicarbonate buffer, pH 9.6) and citrate buffer (25 mmol L^{-1} sodium citrate and 62 mmol L^{-1} sodium phosphate, pH 5.5) were used. The substrate solution contains 2 mg mL^{-1} OPD and 0.012% (v/v) H_2O_2 in citrate buffer.

2.4. ELISA standards

A stock solution of gestrinone (10 mg mL^{-1}) was prepared in methanol and kept at 4 °C. Working standard solutions of 2 mg L^{-1} were prepared daily. The calibration curves were developed by serial aqueous dilution at concentrations ranging from 1×10^{3} to 1×10^{-3} μg L^{-1} (in plate) for each analyte.

2.5. Immunoreagents preparation

The synthesis of the gestrinone oxime hapten was prepared as described [18] with a 97% of yield.

A set of hapten-protein conjugates (BSA-3OCMO-G, Hb-3OCMO-G and HRP-3OCMO-G) was prepared using the mixed anhydride or the active-ester method. Conjugation to KLH and BSA was carried out by the mixed anhydride procedure [16]. Coupling to Hb and HRP was performed as follows: Basically, for activation, a freshly prepared solution of NHS (50 μmol) and DCC (50 μmol) in 150 μL of anhydrous dimethylformamide (DMF), was added to a solution of hapten (50 μmol) in the same solvent (200 μL). The reaction mixture was stirred at room temperature for 5 h. After centrifugation of the product, 11 μL of the supernatant was diluted to 100 μL with DMF and slowly added to a solution of carrier protein (3.6 mg of HRP or 10 mg of Hb) in 0.9 mL of 50 mmol L^{-1} sodium carbonate, pH 9.6. The mixture was stirred for 2 h at room temperature and overnight at 4 °C. Finally, the conjugates were purified by gel-exclusion chromatography on D-Salt dextran desalting columns (Pierce, Rockford, IL) eluted with PBS, and stored at −20 °C until use. BSA-hapten conjugate was used as immunogen, Hb-hapten as coating conjugate and HRP-hapten as enzyme tracer.

2.6. Immunization and antiserum obtaining

The immunizing conjugate BSA-3OCMO-G (0.20 mg in 0.5 mL of PBS) was suspended in 0.5 mL of Freund's complete adjuvant and injected intramuscularly into two female New Zealand white rabbits. Animals were boosted at 21-day intervals with the same immunogen suspended in 0.5 mL of Freund's incomplete adjuvant. Ten days after each boost, blood was obtained by bleeding the ear vein of the rabbit. When no titers enhancement was observed, whole blood was collected and allowed to coagulate overnight at 4 °C. Then, serum was separated by centrifugation and stored at −80 °C. Aliquots of sera As-I and As-II were stored at 4 °C in 50% ammonium sulphate.

2.7. Screening of sera, enzyme tracer, and coating conjugate

Optimal concentrations of coating conjugates, sera dilution, and enzyme tracers were chosen to produce absorbance values of approximately 0.5–1.2 units in the absence of analyte. First, the avidity of sera against different coating antigens was determined

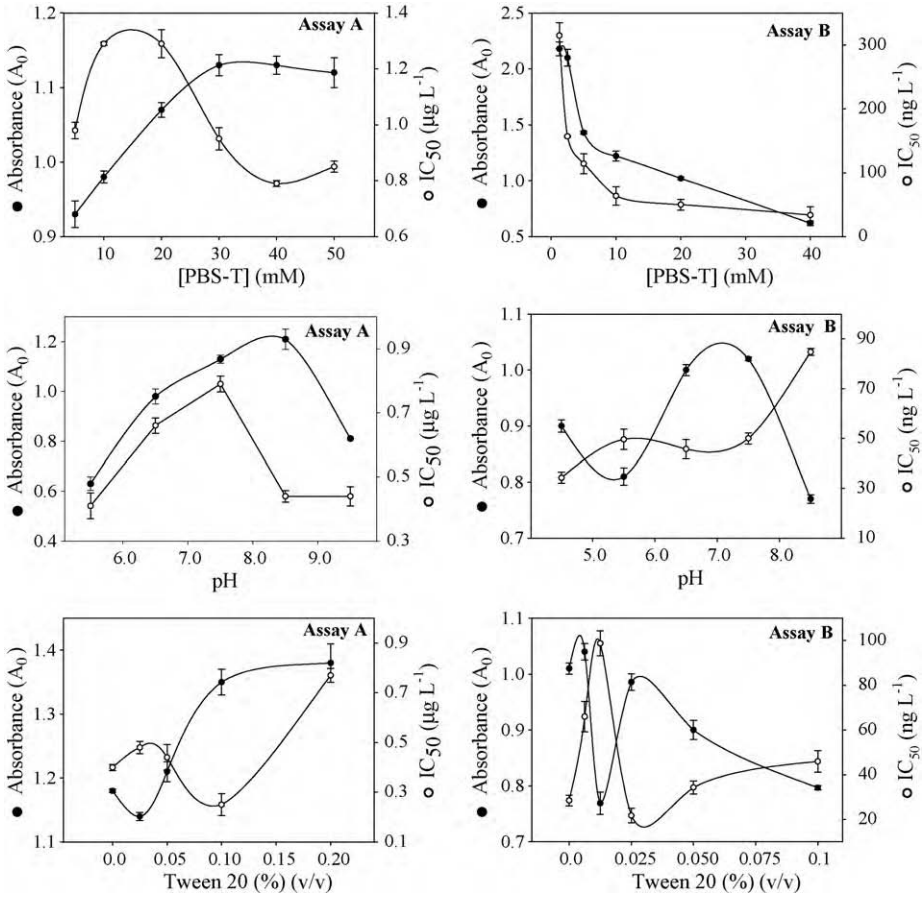

Fig. 2. Influence of buffer concentration (PBS), pH and Tween 20 on the maximum signal (A_0) and assay sensitivity (IC_{50}) in the gestrinone immunoassays A and B.

on a non-competitive indirect ELISA format using different antiserum (serial dilutions from 1/1000 to 1/64,000) and conjugate concentrations (from 0.001 to 1.0 mg L^{-1}). Similarly, their avidities against enzyme tracers were tested on a conjugate-coated ELISA format using several dilutions of enzyme tracers (serial dilutions from 0.001 to 1.0 mg L^{-1}) and serum (from 1/500 to 1/512,000).

2.8. Conjugate-coated format

Flat-bottomed polystyrene ELISA plates were coated dispensing 100 μL/well of the appropriate concentration of Hb-3OCMO-G conjugate solution in coating buffer and incubated overnight at 4 °C. The following day, plates were washed six times with PBS-T. For competitive assays, a volume of 50 μL of standards in distilled water and 50 μL of the appropriate sera dilution (in 2-fold concentrated PBS-T containing 6% of Hb as blocking protein) were mixed in plate wells and incubated for 1 h at room temperature. After washing, plates were incubated for 1 h with GAR-HRP diluted 1:2000 in PBS-T (100 μL/well). 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 M H_2SO_4 (50 μL/well), and the absorbance was read in dual-wavelength mode (490 nm as test wavelength and 650 nm as reference wavelength).

2.9. Antibody-coated format

Polystyrene ELISA plates were coated overnight at 4 °C with 100 μL/well of the appropriate dilution of antibody in coating buffer. The following day, plates were washed six times with PBS-T.

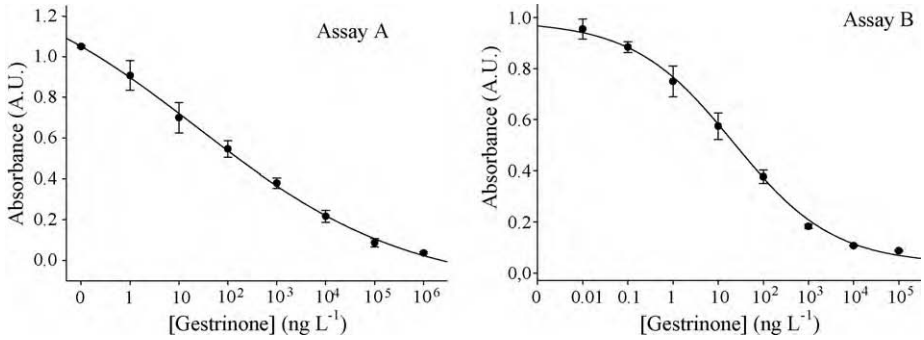

Fig. 3. Calibration curve for immunoassays A (antibody-coated) and B (conjugate-coated). Mean values ± standard deviation ($n = 3$).

Table 1
Features of the optimized antibody-coated and conjugate-coated ELISAs ($n = 3$).

Assay	Sera	Tracer/Coating conjugates	A_{max}	A_{min}	IC_{50} (ng L^{-1})	Slope	r^2
A	As-II	HRP-3OCMO-G[a]	1.22 ± 0.35	0.010 ± 0.005	23.4 ± 4.4	0.20 ± 0.04	0.999
B	As-II	Hb-3OCMO-G[b]	0.98 ± 0.23	0.06 ± 0.02	21.7 ± 3.2	0.39 ± 0.08	0.997

Data obtained from the four parameter logistic curve fit.
[a] Direct format. Tracer.
[b] Indirect format. Coating conjugate.

Then, 50 μL of standards in water, followed by 50 μL of HRP-hapten in 2-fold concentrated PBS-T were added and incubated for 1 h at room temperature. After washing, the HRP tracer activity was determined as above.

3. Results and discussion

3.1. Hapten design and synthesis

The initial and decisive step in the development of an immunoassay lies on the selection of appropriate haptens. A suitable hapten must preserve the basic analyte structure, avoiding the modification of the main functional groups present in the molecule. Gestrinone differs from other 3-keto-4,9,11-trien-steroids in ring D substituents, sharing the rest of the structure. In order to elicit specific antibodies against this compound, the hapten should link to proteins at a distal position of ring D (Fig. 1).

The main strategies in the synthesis of haptens for steroids are: (1) the introduction of a spacer arm through the hydroxyl group of ring D by reaction with succinic anhydride, and (2) the formation of an oxime through the carbonyl group of ring A, thus exposing ring D to the animal immune system. The first is a convenient approach to elicit generic antibodies, as the common structure of the majority of steroids is preserved. On the other hand, the synthesis of (carboxymethyl)oximes (OCMOs), routinely used as steroid haptens [18], is more appropriate to obtain specific assays, because antibodies are raised against a particular moiety of a selected steroid. Salvador et al. [16] developed a specific ELISA for tetrahydrogestrinone synthesizing as the immunizing hapten the corresponding oxime. In this work gestrinone oxime was obtained and used as coating hapten. The assay showed a cross-reactivity with gestrinone of only 6%.

As our objective was to develop a specific immunoassay for gestrinone, this second strategy was considered the most convenient for this purpose, and hapten 3OCMO-G was chosen to prepare all the immunoreagents: immunization conjugates, coating conjugate and tracer. At first, two proteins usually employed to produce sensitive polyclonal antibodies, BSA and KLH, were selected to prepare the immunizing conjugates. However, KLH conjugate was not successfully obtained due to precipitation of the protein and only BSA-hapten conjugate was used as immunogen.

3.2. Sera screening

The polyclonal sera obtained were titrated using the antibody-coated and the conjugate-coated formats. Serum As-I showed

Table 2
Cross-reactivity of the developed assays.

Compound	Assay A		Assay B	
	IC_{50} (nM)	CR (%)	IC_{50} (nM)	CR (%)
Gestrinone	0.07	100	0.07	100
Tetrahydrogestrinone	>3200	<0.002	13.70	0.51
Trenbolone	367.35	0.02	57.77	0.12
Methyltrienolone	>3516	<0.002	145.01	0.05
17β-Hydroxy-5β-androst-1-en-3-one	>3491	<0.002	>3491	<0.002
Epimetendiol	>3140	<0.002	>3140	<0.002
Metenolone	>3306	<0.002	>3306	<0.002
Oxandrolone	>3263	<0.002	>3263	<0.002
Epioxandrolone	>3263	<0.002	>3263	<0.002
3′-Hydroxystanozolol	>2903	<0.002	>2903	<0.002
4β-Hydroxystanozolol	>2903	<0.002	>2903	<0.002
16β-Hydroxystanozolol	>2903	<0.002	>2903	<0.002
Canrenone	>2937	<0.002	>2937	<0.002
1-Testosterone	>3467	<0.002	>3467	<0.002
Salbutamol	>4179	<0.002	>4179	<0.002
Terbutaline	>4439	<0.002	>4439	<0.002
Androsterone	>3443	<0.002	>3443	<0.002
Etiocholanolone	>3443	<0.002	>3443	<0.002
5α-Androstanedione	>3467	<0.002	>3467	<0.002
Androstanolone (DHT)	>3443	<0.002	>3443	<0.002
Epitestosterone	>3467	<0.002	>3467	<0.002
Estradiol	>3671	<0.002	>3671	<0.002
Norandrosterone	>3618	<0.002	220.65	0.03
Norandrosterone glucuronide	>2210	<0.003	>2210	<0.003
Noretiocholanolone	>3618	<0.002	>3618	<0.002
Cortisol	>2759	<0.003	>2759	<0.003
Tetrahydrocortisol	>2729	<0.003	>2729	<0.003
Pregnanediol	>3120	<0.002	>3120	<0.002
Pregnanetriol	>2972	<0.002	>2972	<0.002
Testosterone	>3467	<0.002	>3467	<0.002
Testosterone glucuronide	>2153	<0.003	>2153	<0.003
Androsterone glucuronide	>2143	<0.003	>2143	<0.003
Etiocholanolone glucuronide	>2143	<0.003	>2143	<0.003

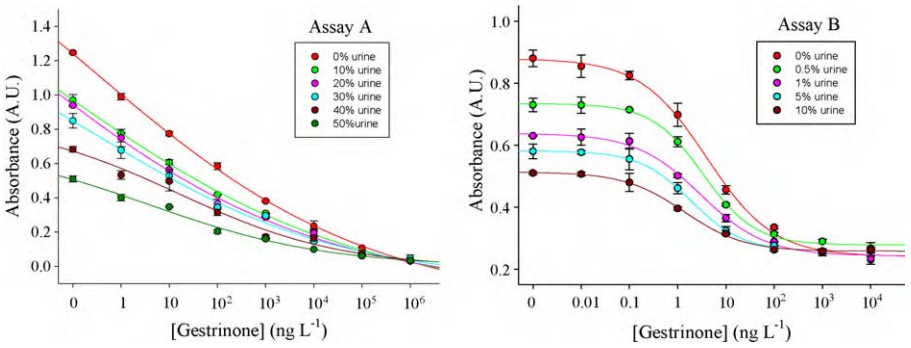

Fig. 4. Tolerance to urine of assays A and B. Calibration curves with different percentages of urine. Mean values ± standard deviation ($n = 3$).

low titers in both formats, while As-II afforded enough titers to carry out competitive assays. In the conditions usually used (PBS-T 10 mM, pH 7.5, 0.05% (v/v) Tween 20, 1 h competition time), the antibody-coated format showed inferior sensitivity (IC_{50} 1.29 μg L^{-1}) than the conjugate-coated one (IC_{50} 0.06 μg L^{-1}). Nevertheless, it is worth mentioning the good sensitivity reached by these immunoassays before optimization, since the minimum required performance levels for detection of anabolic steroids range between 2 and 10 μg L^{-1}.

The pairs As-II/HRP-3OCMO-G (1:1000/1 mg L^{-1}) (assay A) and As-II/Hb-3OCMO-G (1:8000/0.125 mg L^{-1}) (assay B) were selected to set-up a specific immunoassay.

3.3. ELISA optimization

Assay optimization was performed using gestrinone as the competitor analyte. A set of experimental parameters (ionic strength, pH, Tween 20 concentration, and competition time) was studied via a 1 h step reaction to improve the performances (sensitivity, maximum absorbance, dynamic range, and limit of detection) of both ELISA formats, at room temperature (Fig. 2).

To study the influence of the ionic strength, different concentrations of PBS, ranging from 0.125 to 5.0-fold of the original PBS buffer concentration (0.05% (v/v) Tween 20, pH 7.4) were tested. For immunoassay A, signal (A_0) increased gradually with salt concentration, showing low IC_{50} values between 30 and 50 mM. Therefore, 40 mM PBS was chosen as optimal ionic strength. The behaviour of assay B was completely different, loosing signal with the increment of ionic strength. In this case, 10 mM PBS was the optimum concentration, selected as a compromise between signal and sensitivity.

The effect of the medium pH for both assays is shown in Fig. 2. The best signal/sensitivity ratio was clearly obtained at pH 8.5 for assay A. In general, the sensitivity reached by assay B was very high. The selected option was pH 4.5, which provided the best IC_{50} value with good signal.

Using the established conditions, the effect of Tween 20 as surfactant on signal and sensitivity was studied. Percentages of 0.10% (v/v) Tween 20 for assay A and 0.025% (v/v) for assay B afforded the best analytical parameters.

Briefly, optimal assay performances were 40 mM PBS, pH 8.5 and 0.10% (v/v) Tween 20 for assay A, and 10 mM PBS, pH 4.5 and 0.025% (v/v) Tween 20 for assay B.

As assay A showed a high maximum signal, it was possible to reduce the competition time in order to improve the selectivity, so this step duration was studied. Times of 30, 40, 50 and 60 min were tested at room temperature, affording an important sensitivity enhancement with minor competition times, together with reduction of maximum signal. So, lower competition times (5, 10 and 20 min) were assayed at 30 °C in order to improve both signal and sensitivity, achieving the best IC_{50} value carrying out the assay at 30 °C during 5 min and leaving the enzymatic reaction time for 15 min. Under these conditions, competitive calibration curves were prepared. Fig. 3 shows the calibration curves of the optimized assays for gestrinone determination, corresponding to data obtained from three assays. Assay A exhibited an IC_{50} of 23.4 ± 4.4 ng L^{-1}, a detection limit (calculated as the concentration corresponding to 10% inhibition of the maximum signal) of 0.09 ± 0.03 ng L^{-1} and a dynamic range (established between the concentrations producing 20 and 80% inhibition) from 0.57 ± 0.18 to 7350 ± 1547 ng L^{-1}. Assay B showed an IC_{50} of 21.7 ± 3.2 ng L^{-1}, a detection limit of 0.14 ± 0.05 ng L^{-1} and a dynamic range between 0.90 ± 0.34 and 1350 ± 270 ng L^{-1} (Table 1). Both assays afforded comparable sensitivities, which were really high, despite the assays being homologous (the same hapten used to obtain antibodies and immunoreagents).

3.4. Cross-reactivity studies

Due to their similar structures, endogenous steroids present in urine can interfere in the immunochemical analysis, especially if they are present in a high concentration. It should be borne in mind that the concentration of urinary steroids such as testosterone and epitestosterone varies highly between individuals (for example, female or male, Asian or Caucasian) and also depends upon the specific gravity of the urine sample. Consequently, the knowledge of steroids cross-reactivity in the ELISA is crucial information to avoid potential interferences.

Therefore, assays selectivity was evaluated against a set of endogenous and exogenous anabolic steroids (Table 2). It would be also desirable to evaluate gestrinone metabolites as cross reactants, but it has been not possible to obtain some of these compounds, despite our interest.

Standards were prepared in PBS-T from a stock solution in methanol, and run in the ELISA following the protocols described before. The cross-reactivity values were calculated according to the following equation: (IC_{50} [nM] gestrinone/IC_{50} [nM] compound) × 100.

Assay A was more specific, while assay B showed interferences with other steroids like trenbolone, tetrahydrogestrinone, methyltrienolone and norandrosterone. Nevertheless, the cross-reactivity was negligible for all the tested compounds in both assays (<0.5%), due to the high affinity of the elicited antibodies against gestrinone. This result makes these immunoassays a powerful tool to determine gestrinone.

3.5. Application to urine analysis

3.5.1. Standard human urine

As an example of a real sample, the concentration of gestrinone in urine was determined by the developed ELISAs. With this aim, the

Table 3
Gestrinone recoveries from spiked standard human urine.

[Gestrinone] added ($\mu g\,L^{-1}$)	Assay A		Assay B	
	[Gestrinone] found ($\mu g\,L^{-1}$)	Recovery (%)	[Gestrinone] found ($\mu g\,L^{-1}$)	Recovery (%)
5	5.0 ± 0.3	100.4	4.8 ± 0.6	100.4
10	9.9 ± 0.6	99.0	10.1 ± 1.3	100.7
30	27.4 ± 1.5	91.2	24.7 ± 4.4	82.3
60	61.4 ± 6.6	102.3	55.5 ± 10.4	92.4
120	107.0 ± 11.1	89.1	103.8 ± 5.4	86.5

$n = 4$.

tolerance of assays A and B to human urine was studied, preparing the calibration curve with different ratios of standard urine. Assay A tolerated percentages lower than a 30% of urine in plate, maintaining both sensitivity and dynamic range (Fig. 4). However, assay B tolerated only percentages of urine under 0.5% in plate. The low tolerance showed by this assay can be bypassed, since the good sensitivity of the assay allowed high dilutions of a urine sample, even when it contained a low concentration of gestrinone.

Subsequently, a standard urine was spiked with different concentrations of gestrinone. The samples were diluted in PBS and analyzed by the two developed ELISAs. Excellent results were obtained in both formats, with recoveries ranging from 82 to 102% (Table 3).

3.5.2. Miscellaneous human urine

The composition of human urine is very complex, and depends of several factors, even on the food taken prior to sampling. Putative interfering compounds from urine, such as salts, fatty acids, amino acids, organic bases and other xenobiotics (e.g., vitamins, drugs) have been reported [19]. For that reason, representative urines were studied. Ten different human urine samples from volunteers (males and females of different life style, age and body complexion) were collected, centrifugated for 5 min at 3500 rpm and stored at −20 °C. Samples were analyzed to check that no matrix effects or interferents existed. All samples were diluted at 1% (v/v) in PBS (0.5% in plate) and analyzed by the developed immunoassays, preparing the calibration curve with 1% (v/v) of a standard human urine (0.5% in plate). Samples were below the limit of detection in assay A, but gave positive results in assay B, with analyte concentrations from 1.0 ± 0.4 to $26.2 \pm 1.5\,\mu g\,L^{-1}$. The origin of this interference may be the cross-reactivity with endogenous steroids present in urine, although the observed cross-reactivity with a set of these compounds was not significant (Table 2). To find out possible interferences, a competitive curve with other compounds present in urine, such as creatinine and human serum albumin, was carried out. The cross-reactivity of these compounds was low, but a sum of interactions could be the origin of the positive values showed by assay B in unspiked urine samples.

Urine samples were spiked with gestrinone at three levels and analyzed by the immunoassays A and B. Due to the interferences, gestrinone was over-estimated by assay B (data not shown). Nevertheless, assay A reached very good recoveries, between 76.6 and 122.2% (Table 4), providing a screening method to detect gestrinone at low concentration in human urine.

Table 4
Gestrinone recoveries from miscellaneous spiked human urine samples using assay A.

Sample	[Gestrinone] added ($\mu g\,L^{-1}$)	[Gestrinone] found ($\mu g\,L^{-1}$)	Recovery (%)	CV (%)
1	10	11.1 ± 0.5	114.2	4.0
	30	36.3 ± 6.7	120.8	18.5
	60	46.0 ± 2.6	76.6	5.7
2	10	9.9 ± 1.7	99.0	17.0
	30	31.9 ± 4.0	106.3	12.6
	60	63.3 ± 11.2	106.6	17.7
3	10	9.1 ± 1.3	91.3	14.7
	30	36.7 ± 4.9	122.2	13.3
	60	68.0 ± 7.0	113.3	10.3
4	10	9.2 ± 1.8	91.9	20.2
	30	24.4 ± 3.6	81.4	14.9
	60	67.4 ± 9.5	112.4	14.1
5	10	9.9 ± 0.9	98.5	8.7
	30	25.1 ± 4.1	83.6	16.4
	60	57.4 ± 4.0	95.6	7.0
6	10	10.5 ± 1.7	105.0	15.9
	30	24.3 ± 3.4	81.0	14.0
	60	60.1 ± 6.6	101.1	11.1
7	10	8.6 ± 0.9	86.0	10.1
	30	25.2 ± 3.3	83.9	13.0
	60	57.0 ± 5.7	95.0	10.0
8	10	9.0 ± 1.2	89.8	13.2
	30	26.6 ± 4.7	88.7	17.8
	60	69.0 ± 11.2	115.0	16.3
9	10	10.9 ± 1.0	109.2	9.2
	30	24.1 ± 2.5	80.4	10.5
	60	64.7 ± 9.6	107.9	14.9
10	10	9.3 ± 1.1	92.7	12.3
	30	25.8 ± 3.8	85.8	14.7
	60	57.1 ± 6.8	95.1	11.9

$n = 4$.

4. Conclusions

Novel enzyme-linked immunosorbent assays for gestrinone have been assessed. These immunoassays present excellent sensitivity, detecting gestrinone down to 30 ng L^{-1}, with limits of detection (IC_{10}) lower than 0.15 ng L^{-1}. Both assays were also highly specific, showing negligible or no cross-reactivity with other anabolic steroids. Assay A maintained its sensitivity and dynamic range, even in 30% urine, while assay B only tolerated low urine percentages. Nevertheless, the high sensitivity of both assays allowed the analysis of samples at high urine dilution. The developed ELISAs have been applied for the screening of gestrinone, determining this compound in spiked standard human urine samples with very good recoveries. In the case of miscellaneous urines, interferences were detected with assay B, leading to false positives. In this case, assay A did not show problems to quantify gestrinone at low levels, being also very rapid, since results are achieved in only 20 min. This immunoassay can be useful in order to detect human or animal sport doping, illegal animal fattening, or to develop pharmacological excretion studies.

High throughput, no sample-treatment and rapidity are the main advantages of immunoassay against chromatographic techniques. Although chromatography provides a doubtless tool to carry out multianalyte assays, the development of specific immunoreagents for different abuse substances is the basis to the future development of biosensors, microarraying based assays and dipsticks.

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