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A high-throughput screening (HTS) immunochemical method for the analysis of stanozolol metabolites in cattle urine samples☆

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ABSTRACT

A high-throughput immunosorbent solid-phase extraction (HTS-IS-SPE) procedure coupled to enzyme-linked immunosorbent assay (ELISA) has been established for the analysis of stanozolol (St) and its main metabolite in cattle, 16β-hydroxy-stanozolol (16βOH-St), in cow urine samples. The chemical structure of the immunizing hapten 2′H-androst-2-eno[3,2-c]-pyrazol-17-hemiglutarate **5** (hapten A) has been designed to accomplish simultaneous detection of St and 16βOH-St. The antibodies obtained have been used to establish a microplate ELISA method able to detect these metabolites with IC_{50} values of 0.57 μg L^{-1} and 1.46 μg L^{-1}, respectively in PBST. Immunosorbents prepared by covalently attaching the antibodies to Sepharose, efficiently removed the matrix interferences caused by the cattle urine samples. Moreover, St and 16βOH-St were efficiently extracted from urine samples as demonstrated by LC–MS/MS analysis. The immunosorbents are filled on small mini-columns arranges on a 96-SPE-setup compatible with the microplate based ELISA methods. Samples and standards can be run in parallel which increment considerably the speed of the screening method. The recovery values of the whole HTS-IS-SPE-ELISA procedure has found to be 112 ± 10% and St can be detected in hydrolyzed urine samples with LOD of 1.26 ± 0.46 μg L^{-1} using just 1 mL of sample. As proof-of-concept the urinary excretion profile of St treated animals has been investigated by analyzing individual sampling points. Results from pooled urine samples have also been compared with the results obtained by GC–MS analysis demonstrating the StIR equiv. measured with the HTS-IS-SPE-ELISA protocol are in accordance with the St and 16βOH-St levels found with the chromatographic method. The analytical procedure is rapid, effective and the detectability achieved is below the MPRL (minimum performance required levels) recommended by CRL (Community Reference Laboratory) to the European Community.

1. Introduction

Stanozolol (17-methyl-2′H-androst-2-eno[3,2-c]-pyrazol-17-ol, St) is one of the most important synthetic anabolic steroids used as growth promoter in cattle, to increase athletic performance and in horseracing [1]. The use of substances having a hormonal action for growth promotion in farm animals was prohibited in 1981 by the European Union (Directive 81/602/EEC). This prohibition applies to Member States and imports from third countries alike (Directive 96/22/EC as amended by Directive 2003/74/EC). In contrast, six steroid hormones are currently approved for use in US livestock to speed muscle growth. More than 90% of US livestock are currently injected with these hormones. Both the Food and Drug Administration (FDA) and a joint committee of the Food and Agricultural Organization and World Health Organization (FAO/WHO) deemed in 1988 that certain levels of residues of these additives in meat are safe for consumption. In contrast, in the European Union, the Scientific Committee on Veterinary Measures relating to Public Health (SCVPH) after thoroughly evaluating the risks of hormone residues in bovine meat products to human health, concluded in 1999 that no acceptable daily intake (ADI) could be established for any of the hormones studied [2]. Having examined additional scientific data the SCVPH confirmed its opinion in 2000 and 2002 [3,4]. Regarding sports, the practice of enhancing performance through foreign substances or other artificial means is very old. Ancient Greek athletes are known to have used stimulating portions. It was in 1976, when the International Olympic Committee (IOC) introduced anabolic steroids to its list of prohibited substances. Similarly, the World Antidoping Agency (WADA) publishes every year a list of prohibited drugs [5], where anabolic steroids are included in the S1 group (Anabolic agents).

Food safety and accredited WADA laboratories use HPLC–MS and GC–MS as the most common analytical methods to routinely control the use of these substances [6–10]. The analysis of

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anabolic steroids present some difficulties related to their neutral and relatively non-polar nature, together with the very low levels that should be detected (below 2 $\mu g\,L^{-1}$) and the high levels of naturally occurring steroids usually present in the samples. Moreover, knowledge of the metabolic and excretion routes is necessary for an efficient control of these substances. Thus, 16β-hydroxy-stanozolol (16βOH-St) have been identified as the major urinary metabolite of St in cattle. Additionally, excretion usually takes place as glucuronide of sulfate conjugates, which complicate the analysis. Many laboratories have validated analytical protocols for the chromatographic detection of the parent compound, however detection of the corresponding metabolites has not always been implemented [1]. Thus, although, immunochemical extraction/determination of, St has been reported using antibodies available from few commercial sources (Abcam, Neogen Corporation, Randox Laboratories), information is limited regarding recognition of the metabolites. Moreover, published literature regarding hapten design and antibody characterization is scarce. Barret et al. [11] designed a hapten for the specific recognition of St introducing the spacer arm for the OH_{17} of norstanozolol (NorSt), however the paper does not provide information on the antibody features and the immunoassay specificity. Hungerford et al. [12] described a generic immunoassay for 17α alkyl anabolic steroids in equine urine by using 16β-hydroxymestanolone as immunizing hapten. The polyclonal antibodies raised recognized very well several 16β,17β-dihydroxy-17α-methyl steroids, including 16βOH-St, while St was barely recognized.

A screening method recognizing both St and 16βOH-St with sufficient detectability to analyze these substances in urine samples would improve the efficiency of the inspections. Antibody-based detection methods offer rapid, simple and cost-effective alternatives for analytical measurements. Performed in 96-well microtiter plates allow performing many analysis at the same time. In addition, appropriate hapten desing allow raising antibodies with tailored features and cross-reactivity patterns which makes possible registering both, parent molecules and their metabolites, simultaneously. Thus, regarding androgenic anabolic steroid analysis we have recently reported the simultaneous detection of St and the main human metabolite 3′-hydroxy-stanozolol using antibodies raised against a hapten maximizing recognition of the common chemical moiety of these two substances [13]. However, in spite of the significant number of immunoassays reported to analyze urinary metabolites (i.e, [14–17], etc.), sample preparation is often the bottleneck of the screening methods. Mostly, the high detectability of those methods allows avoiding this effect by just simple dilution of the biological sample. However, urine is a quite intricate and complex sample since the components causing the interferences are different from every individual, and even for a single one the composition changes depending on the collection time, the health status and other external features related to habits or type of food ingested by the animal. Moreover, the need to hydrolyze the sample to release the drug from the conjugated form produces an even more complex sample, which makes frequently inevitable the need to introduce clean-up steps prior the immunochemical analysis. The use of solid-phase extraction (SPE) methods as clean-up procedures of complex samples before chromatographic analysis or automated analytical systems is common; although it is much more seldomly used prior bioanalytical methods. In this context, the idea of attaching antibodies to inert supports to prepare immunosorbents (IS) has afforded several advantages over conventional stationary phases for trace-analysis of low-molecular weight analytes in complex matrices [18–20]. Hence, based on the specific molecular antigen–antibody recognition, IS provide higher selectivity and at the same time the possibility of targeting the parent compound and the metabolites thanks to the antibody cross-reactivity. A wide range of IS-SPE applications have been developed in both, on-line or off-line formats, followed by HPLC, GC or capillary electrophoresis (CE) [21–23], but also coupled to immunochemical methods [24,25]. Thus, in this context our group has reported the efficiency of immunosorbents for trace residue analysis of biocides in coastal seawaters [26,27] or for biomonitoring human exposure to organochlorinated substances by analyzing thrichlorophenol metabolites in urine [28,29].

With these precedents, the objective of this work has been to establish an efficient immunochemical method to control illegal use of St in cattle by analyzing urine samples. The significant non-specific interferences caused by the matrix demanded for developing a sample treatment method compatible with the immunochemical procedure. With the objective to avoid tedious, manually performed serial immunoaffinity urine extractions and to meet the ever-increasing demands for higher sample throughput, it is crucial to streamline all aspects of the process, from sample treatment to analyte measurement. Therefore, a parallel IS-SPE method coupled with ELISA has been developed and evaluated to analyze urine samples for St and St metabolites detection. There are only few reports on 96- or 384-parallel HTS-IS-SPE-ELISA [29,30] and the present paper demonstrates that these type of setups can offer significant benefits on food safety control and animal inspections

2. Experimental procedures

2.1. General methods and instruments

Thin layer chromatography was performed on 0.25 mm, precoated silica gel 60 F254 aluminum sheets (Merck, Gibbstown, NJ) and the separations of different compounds from reactions was done by column chromatography with silica 60 A C.C. 35–70 μm SDS. ^{1}H and ^{13}C NMR spectra were obtained with a Varian Inova-500 (Varian Inc., Palo Alto, CA) spectrometer (500 MHz for ^{1}H and 125 MHz for ^{13}C). Infrared spectra were measured on a Bomem MB120 FT-IR spectrophotometer (Hartmann & Braun, Québec, Canada). The MALDI- MS (matrix assisted laser desorption ionization mass spectrometer) used for analyzing the protein conjugates was a time-of-flight (TOF) mass spectrometer Bruker Biflex III (Bruker, Kalsruhe, Germany) equipped with a laser unit that operates at a wavelength of 337 nm and the maximum output of 6 mW. Analytes were detected in the MRM mode using an LC/MS/MS system Waters 2695 equipped with autosampler and quaternary pump. A triple quadrupole mass spectrometer Micromass Quattro-micro (Waters Corp.) equipped with electrospray source was used to obtain the MS and MS/MS data. Experimental procedure and conditions of ionization and chromatographic behavior is collected in the Supporting information. The pH and the conductivity of all buffers and solutions were measured with a pH meter 540 GLP and a conductimeter LF 340, respectively (WTW, Weilheim, Germany). Polystyrene microtiter plates were purchased from Nunc (Maxisorp, Roskilde, Denmark). Washing steps were carried out using a SLY96 PW microplate washer (SLT Lab instruments GmbH, Salzburg, Austria). Absorbances were read using a SpectramaxPlus (Molecular Devices, Sunnyvale, CA) at a single wavelength mode of 450 nm. The competitive curves were analyzed with a four parameter using the software SoftmaxPro v4.7 (Molecular Devices) and GraphPad Prism v4.00 (GraphPad Software Inc., San Diego, CA). Unless otherwise indicated, data presented correspond to the average of at least two well replicates. The C18 (100 mg) cartridges were purchased from Varian Inc. (Palo Alto, CA). Immunochemicals such as Bovine serum albumin (BSA), Horseradish peroxidase (HRP), Horseshoe Crab Hemocyanin (HCH) and Anti-rabbit IgG Peroxidase (Anti-IgG-HRP) were obtained from Sigma Chemical Co. (St. Louis, MO). Stanozolol (St), Boldenone (B), Dihydrotestosterone (DHT) and Methylboldenone (MB)

were purchased from Sequoia Research Products, Ltd. (Oxford, UK). Testosterone-3D was purchased by Sigma–Aldrich (St. Louis, MO). 16β-OH-Stanozolol and 3′-OH-Stanozolol were kindly given from Dr. R. Stephany, RIVM, Netherlands. Other chemical reagents were purchased from Aldrich Chemical Co. (Milwaukee). The assay As147/8BSA was described in a previous work [13]. VersaPlate 96-Well SPE System (Varian, Palo Alto, CA) consists of a 96-well baseplate, removable 96 empty columns, and a vacuum manifold set. The vacuum manifold was connected to a water pump. The vacuum controller (mechanical gauges) was purchased from Hucoa-Erlöss (Barcelona, Spain). VersaPlate accessories, such as disposable waste reservoir, cartridge removal tool, 20 μm pore frits, 96 glass vials (0.75 mL) in a collection rack, 96-well microplate Teflon-coated silicone rubber seal, sealing tape pads, and sealing caps, were purchased from Varian. The purification of conjugated proteins was carried out by dialysis that consist on three washes on PBS 0.5 mM and the last one on water. Hi-Trap desalting columns were purchased from Amersham Bioscience (Uppsala, Sweden).

2.2. Buffers

PBS is 10 mM phosphate buffer, 0.8% saline solution, and unless otherwise indicated the pH is 7.5. Borate buffer is 0.2 M boric acid/sodium borate pH 8.7. Coating buffer is 50 mM carbonate–bicarbonate buffer pH 9.6. PBST is PBS with 0.05% Tween 20. PBST I is PBS 10 mM with 0.005% Tween 20. PBST II is PBS 25 mM with 0.001% Tween 20. Citrate buffer is a 40 mM solution of sodium citrate pH 5.5. The substrate solution contains 0.01% TMB (tetramethylbenzidine) and 0.004% H_2O_2 in citrate buffer. The coupling buffer used in the immunosorbent preparation was 0.2 M $NaHCO_3$, 0.5 M NaCl, pH 8.3. Capping buffer A was 0.5 M ethanolamine buffer (0.5 M NaCl, pH 8.3). Capping buffer B was 0.1 M acetate (0.5 NaCl, pH 4).

2.3. Urine samples

Animal treatments were performed at the University College of Dublin (Ireland) under the direction of Prof. Mark Crowe. Six animals (numbered 0619, 0635, 0539, 0150, 0,022, 0643) were used for this study. Three animals (0150, 0,022, 0643) were treated with a single dose of St (200 mg) by intramuscular injection and three others (0619, 0635, 0539) were used as controls. Urine samples were collected after 1, 3, 6, 9, 12 and 24 h and 2, 4, 6, 8 and 10 days after the treatment in both groups of animals. Negative control bovine urine samples were obtained by pooling urines from three non-treated cows (P1–5). Pooled T-samples (P16–P20) from treated animals were also prepared by pooling urines from selected sampling time points and for the three animals. The urine samples were stored at −80 °C and shipped frozen in aliquots as blind samples. Prior using them the samples were melt at room temperature. Hydrolysis of the conjugates was accomplished adding few drops of acetate buffer pH 4.6 1 M to 1 mL of cow urine and 5 μL of β-glucurodinase (Sigma Chemical Co., St. Louis, MO). The mixture was incubated at 37 °C overnignt.

2.4. Molecular modelling and theoretical calculations

Molecular modelling was performed using the Hyperchem 6.03 software package (Hyperube Inc, Gainesville, FL). Theoretical geometries and electronic distributions were evaluated for stanozolol, 16β-hydroxystanozolol, 3′-hydroxystanozolol and potential hapten (as amide derivative) using semiempirical quantum mechanics MNDO and PM3 models. All the calculations were performed using standard computational chemistry criteria.

2.5. Hapten synthesis

2.5.1. 2′H-androst-2-eno[3,2-c]-pyrazol-17-ol NorStanozolol, **3** [31]

Ethyl formate (2.76 mL, 34.26 mmol) followed by sodium methoxide (0.56 g, 10.3 mmol) were added to a solution of the dihydrotestosterone (DHT, 1.5 g, 5.16 mmol) in dry pyridine (30 mL) kept under nitrogen. The mixture was vigorously stirred overnight at room temperature .The reaction was followed by TLC (hexane:AcOEt, 1:1). After 12 h, the mixture was poured into a cold solution of glacial acetic acid (15 mL) and water (150 mL). The precipitate formed was extracted with CH_2Cl_2 (2 × 50 mL), washed with water (2 × 25 mL) and extracted with a 2% KOH aqueous solution (3 × 30 mL). The combined basic extracts were washed with ether (2 × 25 mL), acidified with glacial acetic acid (6 mL) and extracted again with CH_2Cl_2 (3 × 25 mL). Finally, the organic layer was washed with of water (2 × 25 mL), dried with anh. $MgSO_4$, filtered and evaporated to dryness under reduced pressure to obtain 2-hydroxymethyleneandrostan-17b-ol-3-one(**2**) (1.14 g, 70% yield). ^{1}H NMR (500 MHz, $CDCl_3$); δ: 8.62 (1H, s, OCH =), 3.65 (1H, dd, C_{17}H, *J* 9.10 8.25 Hz), 2.36–0.74 (H20, m), 0.77 (H3, s, $C_{19}H_3$), 0.75 (H3, s, $C_{18}H_3$). Hydrazine hydrate (0.35 mL, 7.2 mmol) was added to a solution of **2** (1.1 g, 3.34 mmol) in 0.5% of glacial acetic acid–ethanol (35 mL). The mixture was refluxed for 2 h according to TLC (hexane:AcOEt, 1:1) analysis. The resulting solution was evaporated to dryness under reduced pressure and the solid obtained redissolved in CH_3Cl (30 mL) and washed with water (2 × 20 mL). The organic layer was dried with anh. $MgSO_4$, filtered and evaporated to dryness under reduced pressure to obtain the desired product **3** (1.1 g, 95% yield). ^{1}H NMR (500 MHz, $CDCl_3$); δ: 7.31 (1H, s, $C_{3'}$H), 3.71 (1H, t, C_{17}H, *J* 8.50 Hz), 2.66–0.70 (H20, m), 0.81 (H3, s, $C_{18}H_3$), 0.78 (H3, s, $C_{19}H_3$)

2.5.2. N-trityl-2′H-androst-2-eno[3,2-c]-pyrazol-17-ol (**4**)

Trytyl chloride (0.45 g, 1.6 mmol) was added to a solution of **3** (0.50 g, 1.6 mmol) in anh. dioxane. (15 mL) in the presence of Et_3N (0.25 ml, 1.8 mmol) and under N_2 atmosphere. The reaction mixture was stirred overnight at 90 °C. The reaction was followed by TLC (hexane:AcOEt, 1:1). When the reaction was finished the mixture was evaporated to dryness and then redissolved in of AcOEt (20 mL) and washed with water (2 × 10 mL). The organic layer was dried with anh.$MgSO_4$, filtered and evaporated to dryness under reduced pressure. The crude product was chromatographed on a silica gel column using 3:1 hexane:AcOEt as mobile phase to isolate **4** (0.50 g, 58% yield). ^{1}H NMR (500 MHz, $CDCl_3$); δ: 7.34 (9H, m, $H_{o,p}$Trityl), 7.21 (6H, m, H_mTrityl), 7.02 (1H, s, $C_{3'}$H), 3.72 (1H, t, C_{17}H, *J* 8.44 Hz), 2.68–0.70 (H20, m), 0.82 (3H, s, $C_{18}H_3$), 0.79 (3H, s, $C_{19}H_3$).

2.5.3. 2′H-androst-2-eno[3,2-c]-pyrazol-17-hemiglutarate (**5**) Hapten type A

A solution of glutaric anhydride (0.22 g, 1.92 mmol) in anh. THF (2 mL) and pyridine (2.5 mL) were added to a solution of **4** (0.50 g, 0.89 mmol) in the same solvent (6 mL). Following a solution of dimethylaminopyridine (DMAP, 0.25 g, 2 mmol) in anh. THF (2 mL) was also added. The mixture was kept overnight at reflux. The reaction was followed by TLC (hexane:AcOEt, 1:1) until the complete disappearance of the starting material. The solvent of the crude mixture was evaporated to dryness, redissolved 10% of conc. HCl in acetone, and then kept during 3 h at reflux according to TLC (hexane:AcOEt, 1:3) analysis. The crude was poured into a solution of 1N HCl (50 mL) and extracted with AcOEt (3 × 25 mL). The organic layer was dried with anh. $MgSO_4$, filtered and evaporated to dryness. The crude product was purified by column chromatography on silica gel using 50:50:1 $CHCl_3$:AcOEt:HAcO as mobile phase to isolate the desired product **5** (0.10 g, 26%). ^{1}H NMR (500 MHz, $CDCl_3$); δ: 7.30 (1H, s, $C_{3'}$H), 4.67 (1H, t, C_{17}H, *J* 8.25 Hz), 2.63–0.70 (H20, m), 2.42

(2H, t, $HO_2C–CH_2$, *J* 7.51 Hz), 2.41 (2H, t, $O_2C–CH_2$, *J* 7.51 Hz), 1.98 (2H, q, CH_2, *J* 7.21 Hz), 0.88 (3H, s, $C_{18}H_3$), 0.81 (3H, s, $C_{19}H_3$)

2.6. Preparation of the protein conjugates

2.6.1. Active ester method (5HCH, 5BSA and 5HRP)

Hapten **5** (30 μmol) was reacted with NHS (*N*-hydroxysuccinimide, 17.25 mg, 150 μmol) and DCC (dicyclohexylcarbodiimide, 61.89 mg, 300 μmol) in 600 μL of anh. DMF and added in equal volumes (200 μL) drop wise to HRP (2 mg), BSA (10 mg) and HCH (10 mg). The conjugates were purified were purified by Hi-Trap™ Desalting column, and the conjugates stored freeze-dried at −80 °C. Working aliquots were prepared at 1 mg mL^{-1} in PBS and stored at 4 °C. Characterization of the protein conjugates was performed by MALDI-TOF-MS by comparing the observed molecular weight of the prepared conjugates and the intact protein. The spectra were obtained by mixing 2 μL of the matrix (*trans*-3,5-dimethoxy-4-hydroxycinnamic acid, 10 mg mL^{-1} in CH_3CN/H_2O 70:30, 0.1% TFA) with 2 μL of a solution of the conjugates or proteins (5 mg mL^{-1} in MilliQ water). Due to the fact that the HRP have only 1–2 free accessible lysines and that the difference in the molecular weight is so small, it was measured the molecular weight of the conjugates and the non-conjugated HRP using BSA as a internal standard

2.7. Polyclonal antisera

Two female New Zealand white rabbits weighing 1–2 kg were immunized with 5HCH according to the immunization protocol previously described [32] to obtain As145 and As149, respectively. Evolution of the antibody titer was assessed by measuring the binding of serial dilution of the antisera to microtiter plates coated with the homologous BSA conjugate. After an acceptable antibody titer was observed, the animals were exsanguinated and the blood collected on vacutainer tubes provided with a serum separation gel. The antiserum was obtained by centrifugation and stored at −80 °C in the presence of 0.02% NaN_3. For the preparation of the immunosorbents the IgG fraction was isolated from the antisera by precipitation with 40% $(NH_4)_2SO_4$ precipitation. The IgG fraction was restored with 10 mM PBS and dialyzed against 0.5 mM PBS (4 × 5 L) and Milli Q water (1 × 5 L).

2.8. Competitive ELISAs

The appropriate dilutions of the antisera and the enzyme tracer and/or coated antigen were established after 2D checkerboard titration assays performed as described in Ref. [32].

2.8.1. ELISA As145/5HRP (for St/16βOH-St)

Microtiter plates were coated with the antiserum As145 (1/8000 in coating buffer, 100 μL/well) overnight at 4 °C. The following day the plates were washed four times with PBST and solutions of the different concentrations of the standards, cross-reactants, or samples (prepared in PBST II) were added (50 μL/well) followed by the solution of the enzyme tracer **5**HRP (1/16000 in PBST II, 50 μL/well). After 30 min at room temperature, plates were washed again four times with PBST. The substrate solution was added (100 μL/well) and the enzymatic reaction stopped after 30 min at room temperature with 4N H_2SO_4 (50 μL/well). The absorbances were measured at 450 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 difference between the maximal absorbance and the minimum absorbance, and *D* is the slope *t* the inflection point of the sigmoid curve.

2.8.2. ELISA As147/8BSA (for St)

St was analyzed using the protocol previously described [13]. The immunoassay has an IC_{50} of 0.16 μg L^{-1} and a LOD of 0.022 μg L^{-1} with a dynamic range from 0.045 to 0.60 μg L^{-1} and a slope of 1.2. The most important cross-reactants are the following steroids: Methylboldenone (11%), 16β-hydroxy-stanozolol (1.6%), 3′-hydroxy-stanozolol (1.1%), and Norstanozolol (10%)

2.9. Cross-reactivity determinations

Stock solutions of different steroidal compounds were prepared (10 mM in DMSO) and stored at 4 °C. Standard curves were prepared in the same buffer for each ELISA format in the same range that the reference analyte (St or 16βOH-St) and each IC_{50} determined in the competitive experiment described above. The cross-reactivity values were calculated according to the following equation: (IC_{50} Stanozolol/IC_{50} steroidal compounds) × 100.

2.10. Accuracy

This parameter was assessed by preparing 10 different blind spiked samples in PBST and measuring them in the ELISA. Measurements were performed in triplicate and in three separate plates run on different days.

2.11. Matrix effect of cow urine samples

Non-specific interferences caused by the cattle urine were assessed on the As145/**5**HRP (St and 16βOH-St) and As147/**8**BSA (St) ELISAs by preparing serial dilutions of the urine in the assay buffer and using them to prepare standard curves. Matrix effect was evaluated by comparing the parallelism of the standard curves with that prepared in the assay buffer.

2.12. High-throughput immunosorbent SPE (HTS-IS-SPE)

2.12.1. Immunosorbent (IS) preparation

Antibody immobilization was performed by covalently coupling the obtained IgGs (Ab145) to NHS-activated Sepharose 4 Fast Flow as follows: A suspension (1 mL) of the Sepharose was washed with cold 1 mM HCl (12 mL). The drained gel (0.5 mL) was transferred to a tube and mixed with the solution of the purified IgG (10 mg mL^{-1}, 0.5 mL) in coupling buffer. The suspension was left shaking (100 rpm) for 3 h at room temperature and finally washed with coupling buffer (3 mL). The non-reacted NHS groups were blocked for 1 h at room temperature with capping buffer A. After three repetitive cycles of washing alternating the capping buffers A and B (3 × 1 mL), the gel was washed with 10 mM PBS buffer (3 mL). Finally, it was reconstituted to the initial suspension volume (1 mL) and stored at 4 °C in 0.05 M Na_2HPO_4 (pH 7) containing 0.1% NaN_3 until needed. Antibody coupling efficiency to the Sepharose gel was estimated measuring the concentration of the total IgG initially loaded and the IgG and after coupling by UV at λ = 280 nm. The coupling efficiency to Sepharose gel was 80%. Previously, the collected solution after coupling of IgG to the Sepharose gel was purified from the co-eluting NHS groups using a HiTrap™ desalting column prepacked with Sephadex® G-25 Superfine.

2.12.2. Preparation of the 96-IS-SPE Rack

A set of 96 VersaPlate empty columns was assembled in the 96-format SPE vacuum manifold (VersaPlate System, Varian). The Ab145 derivatized Sepharose suspension (0.4 mL) was loaded on each column, packed by suction under vacuum and washed with PBS (10 mL) to obtain immunosorbent columns with 0.2 mL of bed volume. The theoretical binding capacity of the columns was estimated according to the amount of IgG coupled, the bivalent binding

capabilities of the IgG molecules, and on the assumptions that the IgG fraction contains about 10% of specific IgGs and that around 50% of the antibody molecules may not be accessible due to steric hindrance or to a random antibody orientation.

2.12.3. General HTS-IS-SPE procedure

HTS-IS-SPE was performed with the VersaPlate 96-well SPE system, which consists of vacuum manifold set equipped with a vacuum controller and a water pump. The solutions (sample application, washes, and elution) were passed through the immunosorbent under low vacuum maintaining the flowrate in the range of 1–2 mL min^{-1}. All liquid loadings were performed using an 8-channel electronic pipette (Eppendorf). Waste from sample loading, washing, and regeneration steps was collected in a disposable reservoir. Eluted fractions were collected in a rack of 96 glass vials (0.75 mL). When not in use, the VersaPlate 96-well IS-SPE assembly was stored sealed with caps at 4 °C in PBS containing 0.1% NaN_3. Each HTS-IS- SPE cycle consisted of sample loading, washing, eluting, and regenerating. The columns were brought to room temperature, washed with PBS (5 mL), and preconditioned with 80% EtOH (1 mL) and PBS (1 mL). After loading the sample (spiked PBS or urine, 1 mL) the columns were washed with PBS or 10% EtOH-PBS (1 mL). The bound analyte was eluted with 80% EtOH-PBS (0.6 mL). In order to ensure a efficient wash was loaded 1 mL of EtOH-PBS (80%) to the IAC mini-columns, before regeneration. Subsequently, the columns were regenerated with PBS (4 mL). Eluted fractions were analyzed by ELISA as described below.

2.13. ELISA analyses

Unless otherwise indicated, the fractions eluted after HTS-IS-SPE clean-up were diluted 10 times with PBS and Tween 20 was added to reach a concentration of 0.005%. Subsequently, the samples were measured by ELISA following the protocol described previously using St standards prepared in 8% EtOH-PBST 0.005%. Unless otherwise indicated, data presented correspond to the average of at least two well replicates.

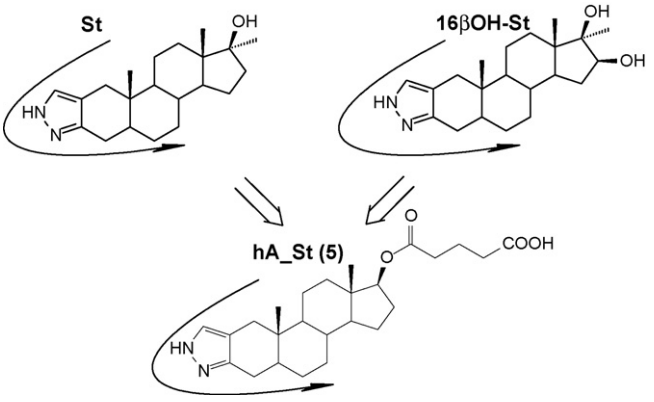

Fig. 1. Chemical structures of hapten A, St and 16βOH-St. The arraow indicate the expected recognition moieties of these chemical structures.

2.14. MS analyses

Pooled samples P1–P5 were analyzed by GC–MS at the European Reference Laboratory placed at RIVM (Bilthoven, the Netherlands) under the supervision of Prof. R. Stephany. The LC–MS/MS measurements were done following the LC and MS conditions as described by Rubies et al. [10]. The conditions are described in the Supporting Information.

3. Results and discussion

Computing-modelling studies have proven to be very helpful for evaluating the suitability of a particular chemical structure to raise antibodies with certain affinity and selectivity features [33–36]. In this work, we addressed the production of antibodies with capability to recognize St, but also 16βOH-St, the main metabolite found in cow. With this objective, we performed comparative studies around the physico–chemical properties of St, its metabolites and the proposed hapten. Hapten type A (hA_St, product **5**) was designed to maximize the common region of St and 16βOH-St chemical structures by introducing a spacer arm at posi-

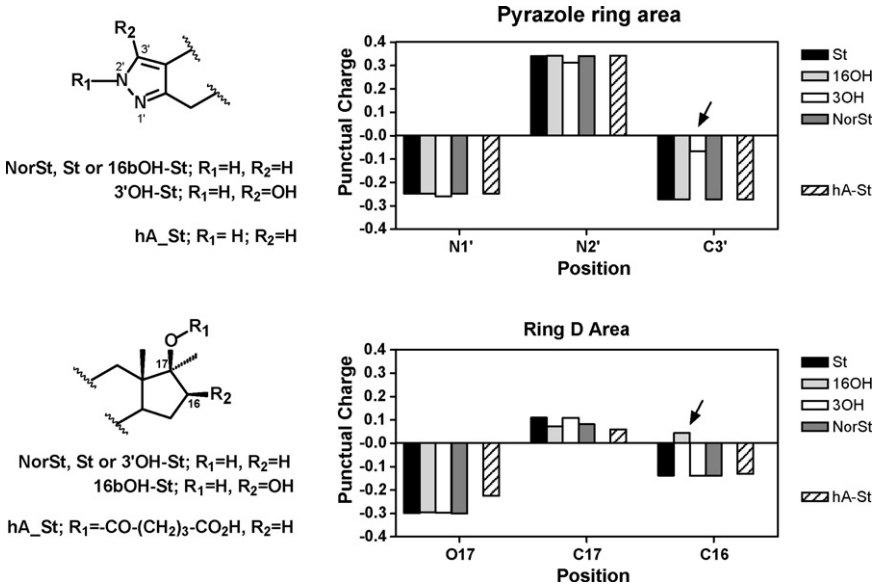

Fig. 2. Punctual charges of St, 16βOH-St, 3′OH-St, NorSt and haptens type A and B calculated for the pyrazol ring (top) and ring D (bottom) using molecular modelling tools. The arrows show that the greater differences are found at position C-3′ (3′OH metabolite) and C-16 (16βOH metabolite) when comparing with haptens type A and B, respectively.

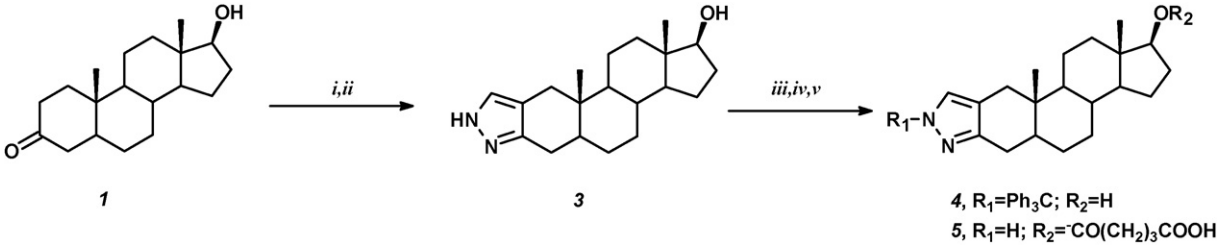

Fig. 3. Synthetic pathway used to synthesize the immunizing hapten type A (product **5**). Synthetic procedures and purification protocols are described in Section 2. (i) DTH, HCO_2Et, CH_3ONa in pyr. (ii) **2**, H_4N_2 in HAcO/EtOH 0.5%. (iii) **3**, Ph_3CCl, Et_3N in dioxane. (iv) **4**, glutaric anhydride, pyr, DMAP in THF. (v) HCl:acetone.

tion OH_{17}, exposing the pyrazole ring to the immune system (see Fig. 1). The root mean square error (RMSE) value calculated by overlapping the backbone of chemical structures to be compared was used to assess the geometric differences between the chemical structures. In respect to the proposed hapten, hA_St, a RMSE of 0.019 and 0.022 Å for St and 16bOH-St was calculated, which indicated that the introduction of the spacer arm into the target analyte does not represent a significant variation on the backbone. Similarly, the electronic properties did not change significantly, according to the punctual charges recorded on both sides of the molecule. As it can be observed in Fig. 2, the differences in the area of the pyrazole ring for St, 16βOH-St and the hapten were not significant. Only a slight difference was observed in ring D, for the position 16, holding the OH group of 16βOH-St, however we did not expect a big influence since this area is close to the location of the spacer arm, and therefore probably more hided due to the shielding effect of the protein. The punctual charges of the human metabolite, 3′-hydroxystanozolol (3′OH-St) were also calculated, and as expected the greater difference was observed at the 3′ position in the pyrazol ring due to additional hydroxyl group. Since, this hapten A addresses recognition to this area, it could be predicted that this analyte would not be recognized, or recognized in a much less extent.

With these premises, we addressed preparation of hapten A (product **5**) using St as starting material using the 17β-hydroxy group to introduce the linker through the formation of a stable ether group. However, unexpectedly all attempts were unsuccessful, probably due to the steric hindrance produced by the methyl groups at C18 and C17α. In order to circumvent this effect, we attempted to perfom the synthesis using Norstanozolol (NorSt), lacking the methyl at C17α position,as a starting material. Synthesis of NorSt **3** was performed from dihydrotestosterone (DHT) **1** following the procedure described by Christiansen et al. [37] (see Fig. 3). Reaction of the hydroxyl group at C17 required to previously protects the nitrogen of the pyrazole ring since its reactivity is greater, as demonstrated previously [13]. Thus, NorSt was reacted with trityl chloride to form the protected derivative **4** and subsequently it was reacted with glutaric anhydride in the presence of pyridine and dimethylaminopyridine (DMAP). Finally, the hydrolysis of the trityl group lead to the desired hapten with a moderate yield.

The preparation of the antigens and immunogens was accomplished by coupling hapten **5** to HCH, BSA and HRP using the active ester method [13]. The hapten densities of the BSA conjugates according to MALDI-TOF-MS analysis were around 9–10 (**5**BSA). The hapten density of the HCH conjugates could not be recorded due to its high molecular weight (>2000 kDa), however since the BSA conjugates were prepared simultaneously from the same batch of activated hapten, it was assumed a suitable degree of conjugation to use the HCH conjugates as immunogens.

Polyclonal antisera As145 and As149 were obtained by immunizing rabbits with **5**HCH. Both antisera showed high antibody titers and a high recognition of the different protein conjugates according to the results of the 2D-checkerboard titration experiments. Recognition of St and 16βOH-St were assessed through competitive experiments in direct and indirect ELISA formats using **5**HRP and **5**BSA as competitors, respectively. While the indirect format provided assays with high background noise, detectability and signal-to-noise ratio of the direct format were more suitable (see Table 3 in Supporting Information). Although further experiments, introducing a blocking step could have solved the problem of the background noise, the excellent features of the direct format prompted us for continuing our investigations using this format. Thus, an IC_{50} of 5.7 nM (1.87 $\mu g\,L^{-1}$) for St was already reached without optimizing the assay (30 min competitive step, 10 mM PBS pH 7.5, 0.05% Tween). Evaluating the effect of some physico–chemical parameters we did found that a decrease in the concentration of Tween 20 down to 0.001% increased the assay detectability reaching IC_{50} values of 2.30 nM (0.75 $\mu g\,L^{-1}$). On the same way, increasing the conductivity of the assay buffer until 34 m S cm^{-1} (25 mM PBS) also produced an increase in the detectability without affecting the maximum absorbance of the assay. Thus, an IC_{50} value of 1.75 nM (0.57 $\mu g\,L^{-1}$) could be reached by varying these parameters and using St as standard. Moreover, the assay was found to perform appropriately between pH values ranging from 6.5 and 8.5 (See Fig. 1 in Supporting information for the influence of the concentration of Tween 20, ionic strength and pH in the IC_{50} of the assay). Fig. 4 shows the calibration curve and the immunoassay parameters defining this assay can be found in Table 1. As expected (see Table 2), As145/**5**HRP recognized very well Nor St (IC_{50} of 1.02 nM; 0.32 $\mu g\,L^{-1}$) and 16βOH-St could be detected with an IC_{50} of 4.11 nM (1.46 $\mu g\,L^{-1}$, 41% cross-reactivity in respect of St). As predicted by the molecular modelling studies the cross-reactivity of 3′OH-St, the main metabolite found in

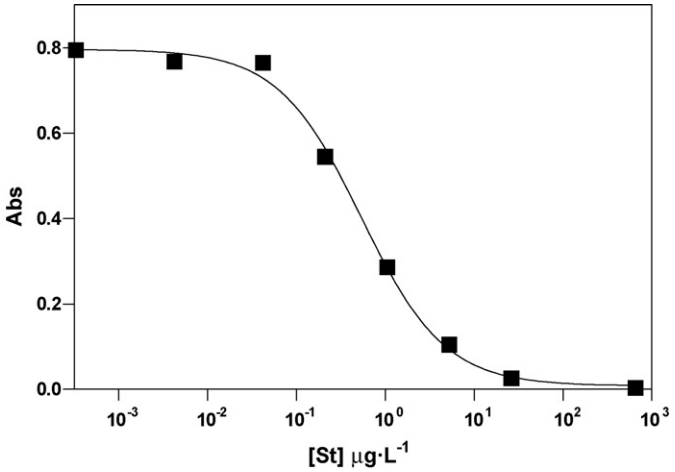

Fig. 4. Calibration curve of the As145/5HRP ELISA developed. The data presented correspond to the average and the standard deviation of an assay run on three different days. The curves were run using triplicates for each concentration. See Table 2 for the features of these ELISAs.

Table 1
Features of the ELISAs obtained for the analysis of St in cattle[a].

Assay	
	As145/5HRP
Amax	0.80 ± 0.04
Amin	0.03 ± 0.01
LOD, ($\mu g L^{-1}$)	0.036 ± 0.009
IC_{50}, ($\mu g L^{-1}$)	0.55 ± 0.04
Dynamic range, ($\mu g L^{-1}$)	0.104 ± 0.01 to 2.72 ± 0.06
Slope	−0.90 ± 0.02
R^2	0.996

[a] The parameters are extracted from the four-parameter equation used to fit the standard curve. Each curve was built using two-well replicates. The data presented correspond to the average of three calibration curves run in three different days.

Table 2
Recognition of structurally related chemicals, expressed by their IC_{50} and the percentage of crossreactivity[a] in respect to Stanozolol.

Assay		
Compounds	As145/5HRP	
	IC_{50}, (nM)	%CR
Stanozolol	1.65	100
Norstanozolol	1.02	166
16β-hydroxystanozolol	4.11	41
3′-hydroxystanozolol	141.6	1.2
Testosterona	283.3	0.6
Boldenone	154.5	1.1
Methylboldenone	106.2	1.6
Dihydrotestosterone	340	0.5
Cholesterol	>2000	<0.08
Estrone	>2000	<0.08
Progesterone	>2000	<0.08
Pregnenolone	>2000	<0.08
Dexamethasone	>2000	<0.08

[a] Cross-reactivity is expressed as % of the IC_{50} of Stanozolol/IC_{50} steroidal compound. Shadow boxes indicate recognition of St and the corresponding metabolites by each assay.

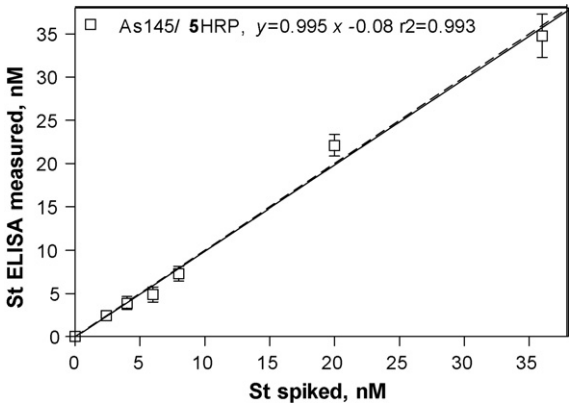

Fig. 5. Correlation between the spiked and the measured concentration values of PBS spiked samples using the As145/5HRP ELISA. The data correspond to the average of three well replicates. The dotted line correspond to a perfect correlation (slope = 1). Each point is the average and standard deviation of analyses made on three different days.

human, was negligible (1%) as well as other steroids tested. Thus, the assay was fulfilling the requirements to control illegal treatment of cattle with St since both the parent compound and the metabolite were detected with IC_{50} values below the MRPLs. On the other hand, on the sport field Norstanozolol, administrated as Prostanozol [38], is a substance prohibited by the WADA, which indicates that this assay could also be suitable for this purpose. Finally, the ELISA As145/**5**HRP was assessed for accuracy by measuring during different days 10 blind samples prepared in buffer. As it is shown in Fig. 5, the measured values match very well the spiked values as it is demonstrated by the slope, 0.995, and the regression coefficients R^2 0.993.

With the ELISA developed and characterized, we addressed its application to the analysis of stanozolol and its main metabolite,

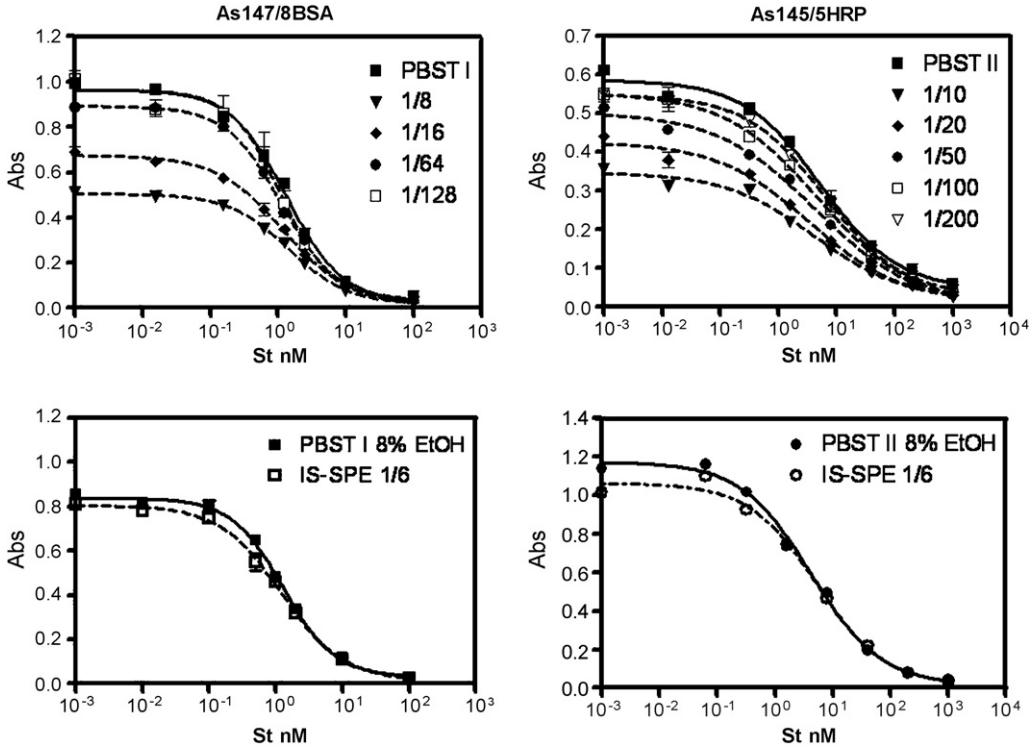

Fig. 6. Graphs showing performance of the As 147/8BSA and As145/5HRP ELISAs in hydrolyzed cattle urine samples before (top) and after IS-SPE extraction (bottom). Calibration curves were constructed in raw hydrolyzed urine samples serially diluted in the assay buffer before extraction. After IS-SPE the fraction eluted with 80% EtOH-PBS was diluted 10 times with the assay buffer and used to prepared the standard curve.

Table 3
Results from the IS-SPE recovery studies performed with different structurally related chemicals[a].

Compound	Spiked conc., (nM)	Measured conc., (nM)	% Recovery.
St	400	393.5	98
16bOH-St	400	376.1	94
3′OH-St	400	318.9	80
NorSt	400	376.3	94
MB	400	203.0	51
B	400	192.0	48

[a] Solutions (1 mL) of the steroids were loaded into the column, washed with 10% EtOH-PBS and eluted with 80% EtOH-PBS and analyzed by HPLC–MS/MS to quantify the concentration of the eluted steroids

Table 4
Characteristic parameters of HTS-IS-SPE ELISAs for As147/8BSA and As145/5HRP[a].

Cow urine	As147/8BSA	As145/5HRP
LOD (μg/L)	0.19 ± 0.06	1.26 ± 0.48
Working range (μg/L)	0.48 ± 0.11	3.45 ± 0.99
	11.68 ± 1.7	110 ± 13.11
IC_{50} (μg/L)	2.36 ± 0.41	19.70 ± 3.60

[a] The concentration values shown are calculated for St.

16βOH-St in cow urine, in parallel, with the As147/**8**BSA assay developed before and very specific for St. The combination of both assays would help on knowing the excretion ratio of both analytes. For this purpose, a blank cow urine sample prepared as pool of urines from control animals was used. The urine was hydrolyzed using β-glucuronidase/sulfatase as described before [28]. The non-specific interferences caused by the hydrolyzed urine on both ELISA were very strong (see Fig. 6). A dilution factor of more than 100 times had to be applied to avoid non-specific effects, which dropped the LOD values far below of the MRPLs values. Moreover, the extent of the matrix interferences was different for each urine sample (data not shown).

Immunosorbents have demonstrated to provide clean extracts. Previous studies performed in our group demonstrated the high efficiency of this type of selective stationary phases and the possibility to use them on a 96-setup format allowing simultaneous clean-up of multiple urine samples. Combined with ELISA on a microtiter plate format provides high sample throughput analytical capabilities [29]. Thus, the antibodies isolated from As145 were covalently coupled, through their free amino groups of the accessible lysines, to NHS activated Sepharose. A set of 96 mini-columns (200 μL drained gel bed) placed on a rack were packed with this immunosorbent. A theoretical maximum binding capacity of 480 ng (1.45 nmol) of St was estimated for each minicolumn attending the amount of antibody immobilized.

First experiments were addressed to evaluate performance of the mini-columns loading PBS solutions (1 mL) containing different St concentrations proved that the Stanozolol was retained and quantitatively eluted using 70% EtOH (1 mL). Mean recovery values were 112 ± 10%. Washing solutions with different EtOH content were evaluated to find out the maximum organic solvent that could be used to completely remove the matrix interferences caused by the urine. Thus, PBS spiked samples (1 mL, 10 nM) were loaded into the mini-columns, washed with PBS containing different percentages of EtOH (0%, 10%, 20%, 30%; 1 mL) and eluted with PBS containing 70% of EtOH (1 mL). All fractions were analyzed by ELISA after diluting the samples with PBS to accomplish organic solvent concentration values below 7%. The results showed that the mini-columns could be washed with up to 20% of EtOH without affecting the recovery of the elution step. Elution was accomplished with percentages greater than 70%, however, narrow elution bands were accomplished by increasing the ethanol content. Therefore the final protocol consisted of (1) Sample loading 1 mL sample; (2) Washing: 1 mL of 10%EtOH-PBS; (3) Elution: 0.6 mL of 80% EtOH-PBS, (4) Cleaning: 1 mL 80% EtOH-PBS and (5) Regeneration: 4 mL of PBS.

The selectivity of the immunosorbents was evaluated by loading PBS samples spiked with St, 16bOH-St, 3′OH-St, NorSt, MB and B at 400 nM and analyzing the eluted fractions by LC–MS/MS as described [10]. The results obtained are summarized in the Table 3. As expected the most important human and cow St urinary metabolites and NorSt are completely retained in the column under these conditions, while the recovery for MB and B is lower. Even though 3′OH-St was poorly recognized by ELISA with the same antibody, this metabolite was retained by the immunosorbent. The lower selectivity of the immunosorbents under non-competitive conditions in respect to the ELISA has already been reported by Grant and Sporns [39], which on the other side is very useful since often extraction of structurally related substances is desired. The explanation of this behaviour is that the As145 was raised with the hapten 5 which was designed to detect simultaneously St, 16bOH-St and NorSt.

With this information, the HTS-IS-SPE-ELISA protocol described above was applied to the analysis of hydrolyzed urine samples with the objective to evaluate the capability to remove matrix interferences. After elution, the fractions were diluted 10 times in PBS and used to prepare standard curves in order to evaluate the interferences of the extracts in the two ELISAs (As145/5HRP for St and 16bOH-St, and As147/8BSA for St) comparing the response with standard curves prepared, with 8% of EtOH in PBST I or II depending on the assay. As it can be observed in the Fig. 6, the protocol rendered very clean extracts with the urine diluted just six times. Considering this dilution factor, cow urine samples can be analyzed using the HTS-IS-SPE-ELISA with a LOD of 0.19 ± 0.06 for As147/8BSA and 1.26 ± 0.48 μg L^{-1} for As145/5HRP expressed in StIR equiv. Table 4 summarizes the detectability using both assays and demonstrate that St can be analyzed in urine samples and that the method reported here meets the analytical requirements to analyze these susbstances, attending to the recommended MRPLs for Stanozolol.

Table 5
Measurements of P1–5 and P16–20 samples with the As147/8BSA i As145/5HRP assays expressed in equivalents of immunoreactivity of St i16bOH-St.

Sample	As147/8BSA[a]	As145/5HRP[a]	GC–MS[c]	As145/5HRP[a]	GC-MS[c]
	St	St	St	16βOH-St[b]	16βOH-St
P16	1.180 ± 0.61	16.14 ± 2.78	0.22	33.85 ± 5.81	25
P17	2.31 ± 0.26	24.39 ± 3.21	0.46	51.17 ± 6.75	93
P18	0.82 ± 0.20	17.84 ± 2.29	0.16	37.42 ± 4.80	71
P19	0.42 ± 0.11	4.06 ± 0.97	–	8.52 ± 2.04	9
P20	0.43 ± 0.26	8.30 ± 2.22	–	17.42 ± 4.65	19
P1–5	<LOD	<LOD	–	<LOD	–

[a] Results in μg/L, mean value and standard deviation from three replicates in different days.
[b] Results in immunoequivalents of 16bOH-St, based on cross-reactivity values of 16bOH-St in As145/5HRP with 8%EtOH (50%).
[c] Results in μg/L, samples measured by RIVM reference laboratory.

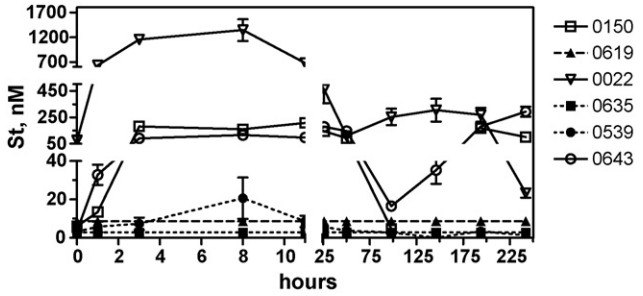

Fig. 7. Excretion profile of 16βOH-Stanzolol measured with the HTS-IS-SPE-ELISAAs145/5HRP and using 16βOH-Stanozolol as reference. Results are expressed as 16βOH-StIR equiv. Results shown correspond to the average and standard deviation of analyses made by two replicates. The eluted fractions were analyzed in three well replicates.

Finally, the HTS-IS-SPE-ELISA protocol was applied to the analysis of the urine samples collected from St treated animals at several intervals. A first approach consisted of measuring the control samples (P1–5) and the so-called "pooled T-samples" (P16–17), previously analyzed by GC–MS at the European Reference Laboratory at RIVM, but which concentration values were not known by us. Each sample was processed in duplicate and analyzed simultaneously with both assays using St and 16βOH-St as references. The data obtained are summarized in the Table 5. Samples P1–P5 were found to be negative in both assays while samples P16–P20 were positive in also in both assays. The greater StIR equiv. values obtained in the As145/5HRP ELISA (for St and 16βOH-St) indicated a higher concentration of 16βOH-St in the samples, since the values obtained using the As147/8BSA ELISA (only St) were very low. This conclusion was in accordance to the concentration values found by GC–MS as it can be observed in Table 5. By applying the cross-reactivity of 16βOH-St in the As145/5HRP ELISA a second set of values expressed as 16βOH-StIR equiv. were obtained that match quite close those of the GC–MS, which points to the value of the to provide reliable results when used as screening method to detect animals treated with St.

Subsequently, analyses were performed with individual samples to determine the excretion profile using the HTS-IS-SPE-ELISA As145/5HRP, since as demonstrated before the 16βOH-St was the main substance found in urine. Six samples were collected within the first 24 h after treatment and then sampling was performed once a day on days 2, 4, 6, 8 and 10. The results of the analysis showed different excretion profiles (see Fig. 7). The urine from non-treated animals analyzed as blind samples gave zero response in all sampling points. The urine from treated animals showed maximum excretion levels 2 h after treatment and the levels remained high the first 2 days. After this period the levels decreased significantly and on day 8 a second increase was observed. The same pattern was observed for animals 0150 and 0643, while the excretion levels of cow 0022 were significantly much higher during the first 48 h. It is not within the aim of this paper to provide pharmacological significance to these results, but just to exemplify the applicability of the methodology developed. Additional treatments would be necessary to establish a representative excretion profile and the results should have to be validated by a reference chromatographic method.

4. Conclusions

A HTS-IS-SPE-ELISA method has been established using antibodies raised against an immunizing hapten addressing recognition of both St and 16βOH-St, the main urinary metabolite found in cattle. The procedure uses hydrolyzed urine and the metabolites are selectively extracted by using small mini-columns filled with immunosorbent and arranged on a 96-well setup compatible with the microplate based ELISA used to analyze the extracts. The immunosorbents efficiently remove the matrix interferences caused by the hydrolyzed urine and shows good recovery. As demonstrated by comparison of the results obtained analyzing the pooled T-Samples by GC–MS, the HTS-IS-SPE-ELISA method is able to provide results that match very well with those obtained by the reference method. Just 1 mL of sample is sufficient to perform the analysis and the detectability achieved is below the MRPLs. Moreover, the system is able to perform many analyses in parallel, which makes it suitable for improving the efficiency of the actual methods of control if used as screening method. In this paper we have shown applicability of the protocol to the analyzed of hydrolyzed urine samples. The method developed can find applicability to the analysis of St and its main metabolites in centralized laboratories as a screening method to CRLs or similar have to carry out. However, the method can find applicability to the analysis of other complex samples of interest for the food safety field of for pharmacologic/toxicity studies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2009.08.027.

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