Rapid Immunoassays for Detection of Anabolic Nortestosterone in Dietary Supplements

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Abstract

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An enzyme immunoassay (ELISA) and an immunochromatographic strip were designed for a rapid detection of nortestosterone in dietary supplements. Two polyclonal antibodies and two types of nortestosterone-protein coating conjugates were tested to develop the most appropriate method. Under optimal experimental conditions, the most sensitive ELISA achieved the IC_{50} and the limit of detection values of 6.41 and 0.09 ng/ml, respectively. The assay specificity was tested measuring cross-reactivity of several steroids. The interference with the assay was negligible (< 0.1%), except for cross-reactivity with another frequently abused steroid testosterone (23%). The optimised gold particle-based immunochromatographic strip provided in semi-quantitative test a visual detection limit of 1 ng/ml. None of these methods showed the interference using a filtrate of the suspension of non-contaminated sample. After the validation for particular matrices, the ELISA and the strip test could be useful tools for a rapid analysis of nortestosterone in crude extracts of dietary supplements.

Keywords: 19-nortestosterone; ELISA; colloidal gold immunoassay; strip test

Dietary supplements with strength and muscle bulk enhancing effects contain usually amino acids, proteins, prohormones and creatine as active components. A lot of these dietary supplements are freely available through numerous internet sites which causes an expansion in the use of these supplements (Václavíková & Kvasnička 2013). Some studies have shown that many supplements contain banned substances that are not declared as ingredients and present risks for the consumer (Mikulcikova *et al*. 2008). The most common are anabolic steroids such as testosterone precursors, 19-nortestosterone, methandienone, or boldenone (Petroczi *et al.* 2011). These undeclared substances can cause health risks to consumers and may lead to positive results in sports doping control, especially with the nandrolone metabolite norandrosterone. 19-Nortestosterone (NTS) (17β-hydroxy-19-norandrost-4-en-3-one), also named nandrolone, is an anabolic steroid which occurs naturally in the human body, but only in tiny quantities. It is very similar in structure to the male hormone testosterone, and has many of the same effects in terms of increasing muscle mass. Ergogenic use for this steroid in sports, racing, and bodybuilding is controversial because of its adverse effects and the potential to gain an advantage. Its use is referred to as doping and is banned by all major sporting organisations.

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The analysis of nutritional supplements for anabolic steroids has proven to be rather difficult due to the different matrices in various products. Traditional methods for the analysis of NTS such as LC/MS and GC/MS (Martello *et al.* 2007; Stepan *et al.* 2008) are highly sensitive and reliable. However, they involve multiple steps in the sample preparation and analysis, require expensive equipment and skilled analysts, and are therefore unsuitable for routine analysis of a large number of samples or on-site determinations. In contrast, immunoassays could be portable and cost-effective, with adequate sensitivity, high selectivity, and a simple sample extraction process. Therefore, immunotechniques have become popular and are increasingly considered as alternative/complementary methods for the residue analysis (Popii & Baumann 2004).

During the last decade, several immunoassays based on the enzyme-linked immunosorbent immunoassay (ELISA) for the detection of NTS in veterinary areas of animal health control have been described (Lu *et al.* 2006; Xu *et al.* 2006; Jiang *et al.* 2011b, 2012). Moreover, the strip immunoassays for the visual detection of NTS residues in unit of ng/ml in buffer or animal urine have been published by Liu *et al.* (2007), Tian *et al.* (2009), and Jiang *et al.* (2011a). To our best knowledge, no use of immunoassay for the detection of NTS in dietary supplements has been reported up to now. That is why we present here experiments leading to the design of ELISA and immunochromatographic test for these purposes.

MATERIAL AND METHODS

Chemicals and immunoreagents. The rabbit polyclonal antibodies (RAbs) specific for NTS were prepared in the Biotest s.r.o. (Konarovice, Czech Republic). Bovine serum albumin (BSA), Tween 20, polyethylene glycol (PEG, MW 3350), and 3,3',5,5'-tetramethylbenzidine (TMB) were purchased from Sigma-Aldrich Inc. (St. Louis, USA). The NTS-3-ovalbumin (NTS-3-OVA) and NTS-3-BSA conjugates were synthesised using 19-NTS-3-karboxymethyloxime in a reversed micellar system (Lapčík *et al.* 2004). Donkey anti-goat antibody (DAG), goat anti-rabbit antibody (GAR) as well as the goat anti-rabbit antibody with horseradish peroxidase (GAR/Px) were obtained from Nordic Immunological Laboratories (Tilburg, the Netherlands). All antibodies were supplied as purified IgG fractions of polyclonal antiserum. Gold colloid nanoparticles (an average diameter of 40 nm) were from BB International (Cardiff, UK). GAR labelled with gold nanoparticles was prepared according to the procedure described for carbon nanoparticles (Holubová-Mičková *et al.* 2010) with a slight modification. Steroids standards were from Steraloids Inc. (Newport, USA). Individual stock standard solutions containing 1 mg/ml of each compound were prepared by dissolving accurately weighed amounts in ethanol which was stored in darkness at 4°C. Working standard solutions were freshly prepared by serial dilutions in buffers. Other common chemicals were of the highest purity available and purchased from Sigma-Aldrich. Various types of nitrocellulose membranes were from Whatman GmbH, Dassel, Germany (PRIMA 80, PRIMA 125, AE 98 FAST, AE 98, Protran BA 79, Protran BA 83, Protran BA 85), Millipore Ltd., Praha, Czech Republic (Immunopore FP, Immunopore RP, HiFlow Plus HF 135, HiFlow Plus HFB180, HiFlow Plus 090), and Sartorius Stedim Biotech S.A., Aubagne Cedex, France (Unisart 140). Vinyl backing ARcare® 7823 and the absorbent pad CFSP both was from Millipore Corp. (Glen Rock, USA). ELISA polystyrene microtitre-plates Costar (catalogue No. 9018) were obtained from Corning Inc. (Corning, USA).

Instrumentation. ELISA plates were washed with Labsystem Multiwash (New York, USA) and the absorbance was read in Labsystem Multiscan MCC/340 (Helsinki, Finland). The data were processed using Microsoft Excel software (Microsoft Corporation, Tulsa, USA). For preparation of an immunochromatographic test the Linomat V (Camag AG, Muttenz, Switzerland) and a programmable strip cutter (Economic Cutter ZQ2000; Shanghai Kinbio Tech Co., Ltd., Shanghai, China) were used. Epson Perfection V700 Photo Scanner (Seiko Epson Corporation, Nagano, Japan) and the TotalLab TL100 software (Nonlinear USA Inc., Durham, USA) were used for the quantification of the colour intensity of lines on strips.

ELISA. The ELISA of NTS was carried out in an indirect competitive format as follows: the microplates were coated with the NTS-3-OVA conjugate solution in 0.05M carbonate-bicarbonate buffer, pH 9.6 (100 µl/well), left to incubate for 2 h at 37°C, and then washed four times with PBS containing Tween 20 (0.05%, v/v). The aliquots (50 µl/well) of NTS or others steroid standards, diluted in PBS, and the aliquots (50 µl/well) of RAbs were pipetted into the wells. Incubation for 2 h at 37°C and wash-

ing as described above. Subsequently, GAR/Px was added (100 µl/well), left to interact for 1.5 h at 37°C and washed. Peroxidase substrate in the reaction buffer was added to each well (100 µl/well). After 10 min incubation at 24°C, the enzyme reaction was terminated by adding 50 µl/well of 2.5M sulphuric acid, and the absorbance at 450 nm was measured.

To construct the standard curve, the absorbance values were fitted to a four-parameter logistic equation. The IC_{50} values represented the concentration of NTS that produced 50% inhibition of antibody binding to the hapten conjugate. The detection limit was defined as the lowest concentration of NTS that exhibits a signal of 10% inhibition. The linear working range was calculated as the concentrations of NTS providing a 20–80% inhibition rate of the maximum signal. The specificity of antibodies was expressed as cross-reactivity (CR) value with structurally related steroids. The CR was calculated as: (IC_{50} of NTS)/(IC_{50} of competitors) × 100.

Design of immunochromatographic strip. The strips were prepared similarly as in the procedure described previously (Holubová-Mičková *et al.* 2010), using nitrocellulose membrane sheet, vinyl backing, NTS-3-OVA (or NTS-3-BSA) and DAG solutions (5mM borate buffer, pH 8.8), Linomat V and cutter (Figure 1). Then, the strips were stored in sealed bags under dry conditions at laboratory temperature until used.

Procedure of immunochromatographic assay. In a microplate well, 50 µl of NTS standard solution in buffer (or filtrate of sample suspension) was mixed with 50 µl of the running buffer (0.2M borate buffer, pH 8.8), 20 µl of GAR-gold conjugate, and 1 µl of an anti-NTS antibody (1 µg/ml). Subsequently, the membrane strip was dipped into this reactant mixture vertically. The solution migrated toward the absorbent pad and after 10 min, the test result was observed. The colour intensity of test line could be assessed visually with naked eyes. For the quantification of the colour intensity, grayscale densitometry was used after the strip was fully air-dried and the intensity of the test lines was quantified using the TotalLab TL100 software.

Table 1. Significant parameters of optimised ELISA

Parameter	Chosen as optimal for antibody	
	No. 102	No. 120
Concentration of nortestosterone-3-OVA (µg/ml)	0.125	0.25
Concentration of antibody (µg/ml)	0.02	0.025
Additives in reaction buffer	0.1% BSA	0.1% gelatin
IC_{50} (ng/ml)	5.17 ± 0.2	6.41 ± 0.4
Linear working range (ng/ml)	1–32	1–67
Detection limit (ng/ml)	0.12 ± 0.03	0.09 ± 0.02

RESULTS AND DISCUSSION

Six rabbits were immunised with NTS-3-BSA and the antisera obtained were tested for reactivity with NTS. Checkerboard titrations were performed. Only two antibodies marked No. 102 and No. 120 were selected and with the NTS-3-OVA used for the development of ELISA.

The optimal reagent concentrations of the immunoreagents used were determined when the maximum absorbance ranged from 1.0 to 1.5, and the dose-response curve pursued the lowest IC_{50} values. Because inert proteins (such as BSA, gelatin, milk powder) and surfactants (such as Tween-20) are commonly used in ELISA to reduce nonspecific interactions, their influence on the assay performance was examined. It was observed that the addition of 0.1% BSA and 0.1% gelatin in PBS for the dilution of antibodies No. 102 and No. 120 had a positive effect on the curve parameters,

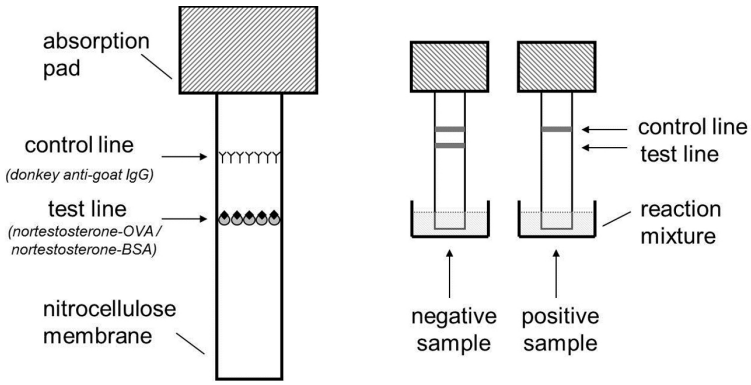

Figure 1. Schematic illustration of the strip test design

Table 2. Cross-reactivities (CR) of related structural or functional analogues in the nortestosterone ELISA

Analogue	CR (%) for antibody	
	No. 102	No. 120
Nortestosterone	100	100
Testosterone	9	23
DHEA	1	5
5-β-Androstane-3,17-dione	< 0.01	< 0.01
Epitestosterone	< 0.01	< 0.01
Progesterone	< 0.01	0.3
Cortisone	< 0.01	< 0.01
4-Androstene-11-β-ol-3,17-dione	< 0.01	< 0.01
17-OH progesterone	< 0.01	0.08
Cortikosteron	< 0.01	< 0.01
Aldosterone	< 0.01	< 0.01
17-β-Estradiol	0.06	0.02
Estriol	< 0.01	< 0.01
4-Androstene-3,17-dione	< 0.01	< 0.01
Cortisol	< 0.01	< 0.01
11-Deoxycortikosteron	< 0.01	< 0.01
21-Deoxycortisol	0.02	0.1

DHEA – dehydroepiandrosterone

respectively. Optimal combinations of concentrations are summarised in Table 1.

Figure 2 shows the representative calibration curves obtained using the optimised ELISA. The central section was accepted as the assay working range. In Table 1, the analytical parameters of the assay are shown. The limit of detection (LOD) was about 0.10 ng/ml. It is comparable with that obtained by other authors (Jiang *et al.* 2011b). From the metabolic fate of NTS in humans is it clear that the method used to analyse dietary supplements should have a detection limit of about 20 μg/g (De Cock *et al.* 2001). We can deduce that converted to solution (filtrate of suspended sample), the LOD value achieved with ELISA is sufficient in abundance.

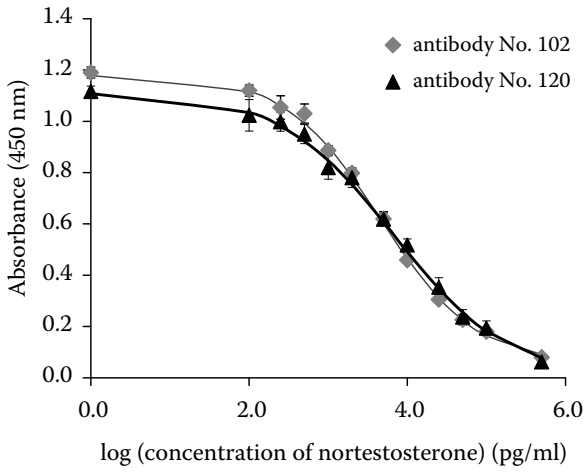

Figure 2. Standard curves of ELISA

Subsequently, the specificity of the described ELISA was evaluated by the determination of cross-reactivity (CR) based on the IC_{50} values of the individual chemicals. This was undertaken by adding various structurally or functionally related analogues instead of using NTS. When using antibody No. 120 the method exhibited slight cross-reactivity with structurally similar testosterone (23%) and DHEA (5%). Antibody No. 102 also interacted weakly (9%) with testosterone. The interferences by other structurally related analogs were negligible (≤ 0.1%), thus proving that the developed assay is highly specific for NTS (Table 2).

In the immunochromatographic method, the colloidal particles, which are indirectly conjugated to the analyte-specific antibody, serve as the label for the immunoassay. The principle of the test is as follows: The target analyte in the reactant mixture is bound by colloid-antibody (RAb-GAR-gold) and migrates due to capillary effects along the membrane (Figure 1). The test line, impregnated with NTS-protein conjugate, captures any free colloid-antibody as a narrow pink coloured band which is visible by the eye. Therefore, the samples free of NTS will result in just free colloid antibodies in the reaction mixture, which will give a pink coloured test line of maximum intensity. Alternatively, analyte-positive samples, resulting in binding to the colloid-antibody, are identified by a decrease or the absence of colour intensity in the test line. The control line acts as a positive control to assure that the labelled secondary Ab migrated through the system.

Most of the components necessary for the immunochromatographic strip design as well as the experimental conditions have a key influence on both the performance and final sensitivity. The concentrations of the reagents were preliminary optimised to satisfy the following assay criteria: good sensitivity, minimum immunoreagent consumption, and the appearance of the test line with good colour intensity and sharpness (for negative sample). Therefore, checkerboard titration experiments were carried out. Several amounts of NTS-protein conjugate immobilised on the membrane (25–400 ng per strip) against different amounts of primary antibody (0.25–4 μg per strip) were investigated

in the assays of the standard solutions containing NTS at concentrations of 0, 5, and 500 ng/ml. The same experiments were performed for different concentrations of gold-labelled GAR. Preliminary optimised concentrations of reagents were finally specified after the following optimization of other assay conditions. Then, some other factors affecting the strip test performance were also evaluated: the type of membrane and the type of running buffer. Thirteen various types of nitrocellulose membrane were tested. The type of membrane influenced the flow time and sharpness of the detection lines. Also, the composition of the running buffer markedly affected mainly the character of the reactant flow, level of background colour, sharpness, and intensity of the test line. Phosphate buffer and borate buffer of various pH values and ion concentrations were tested. Moreover, the effects of additives such as BSA, PEG, sucrose, surfactants and their combinations were tested to improve the test performance. In all these experiments, two anti-NTS antibodies (No. 102 and No. 120) and two NTS-protein conjugates (NTS-3-OVA and NTS-3-BSA) were used, alternatively. The qualitative evaluation of the lines intensity found visually was quantified using scanner and computer software. The optimised conditions found for the strip test are summarised in Table 3.

The sensitivity of the strip test was determined by testing the NTS standard samples in the concentration range from zero to 2000 ng/ml. The details of the test line intensity on the selected strips are shown in Figure 3. The visual LOD of the assay was defined here as the minimum NTS concentration producing

Table 3. Design parameters of optimised immunochromatographic strip test

Parameter	Chosen as optimal with antibody No. 102 and nortestosterone-3-BSA
Type of nitrocellulose membrane	Prima 85
Amount of nortestosterone-3-BSA conjugate in test line	50 ng
Amount of DAG in control line	200 ng
Amount of primary antibody per strip	1 μg
Amount of GAR-gold conjugate per strip	1 μg
Composition of running buffer	0.1M borate buffer, pH 8.8 (0.1% BSA, 1% PEG, 0.1% Tween)

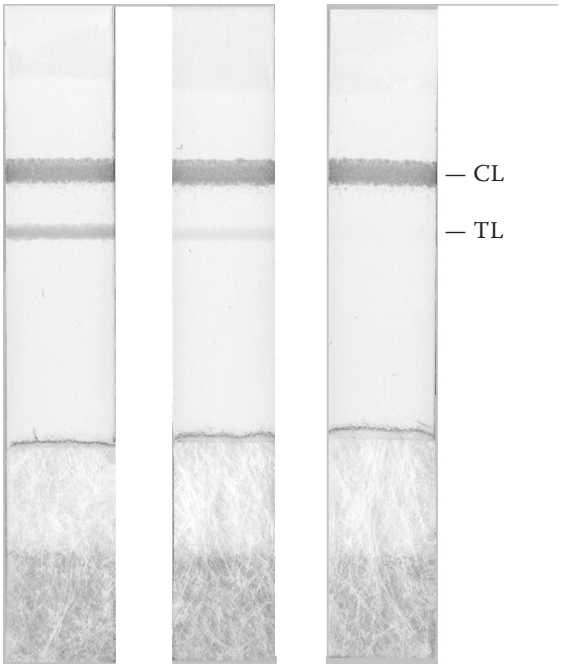

Figure 3. Typical strip tests after assay procedure of standard (Nortestosterone concentrations: (**A**) negative control, (**B**) 5 ng/ml, and (**C**) 500 ng/ml (TL – test line; CL – control line)

the colour density of the test line significantly weaker than that at zero concentration (it means absolute agreement between the result assessments of three repetitions of the same test by five observers). The NTS concentration of 1.0 ng/ml caused a slight but visually distinguishable difference in the test line intensity compared to the negative control. Thus, 1.0 ng/ml of NTS was considered to be the visual LOD for the semi-quantitative test. It is sufficient value for assaying suspended samples of dietary supplements. At concentrations of NTS > 100 ng/ml, the test line was invisible.

CONCLUSION

This study successfully demonstrated the potential of using the rapid immunoassays for sensitive detection of NTS in nutritional supplements. First, the quantitative ELISA assay was designed. Highly specific rabbit polyclonal antibody and the NTS-3-OVA conjugate were used for its construction. Under optimised experimental conditions, the LOD was 0.09 ng/ml. Moreover, the same antibody and conjugate NTS-3-BSA were used also for designing the strip test of optimal parameters that provides visual LOD of 1 ng/ml. Both developed assays are suf-

ficiently sensitive. However, the semi-quantitative strip test is easier to perform, allowing for non-specialised personnel on-site application without requirements for intensive labour and any device for sample analysis. This property makes it useful for incorporation into monitoring programs for the control of food supplements contamination. Additional investigations would be needed to the evaluate this assay as fit-for-purpose.

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