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ELISA ASSAY FOR STANOZOLOL

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ABSTRACT

Current methods for detection of stanozolol employ GC and HPLC (Delbeke and Debackere 1992). However, their complexity and cost make these methods unsuitable for high volume screening of samples. A straight forward ELISA should prove a valuable screening method for the racing/veterinary industry (Tobin *et al.* 1988). Therefore, an ELISA was developed for the detection of stanozolol in equine urine samples.

A stanozolol-specific antisera was produced in rabbits by injecting a protein:hapten immunogen. The drug hapten was also conjugated to horseradish peroxidase to form an enzyme:drug conjugate. Antisera coated onto microwells was optimised with the enzyme conjugate using a TMB substrate system. Standard curves were generated by competing the conjugate with known levels of stanozolol in buffer. The I_{50} (measure of assay sensitivity) was optimised to 4.0 ng/ml.

Background studies revealed that some negative urine samples interfered with the assay. This interference was eliminated by diluting the urine samples 1:4 in assay buffer.

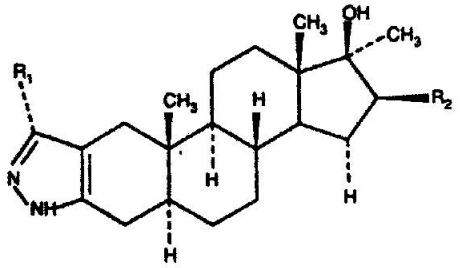
In addition, standard curves were generated using 4 common sample matrices (equine plasma, equine serum, equine urine and canine urine). The I_{50} was 17.3 ng/ml in equine plasma, 15.4 ng/ml in equine serum, 10.7 ng/ml in diluted equine urine and 14.2 ng/ml in diluted canine urine.

Cross-reactivities for several steroids, illegal drugs, therapeutic drugs, masking agents, vitamins and drug vehicles were determined. Specific cross-reactivity was seen with the stanozolol metabolite, 3'-Hydroxystanozolol.

Samples were collected after a single 1 g dose of stanozolol im. Stanozolol equilvalents were detectable in urine at least 12 days after administration. These samples were also treated with ß-glucuronidase from bovine liver to deconjugate any glucuronic acid metabolites of stanozolol. Days 1, 2, and 3 showed no change in the amount of apparent stanozolol concentration after ß-gluctironidase treatment. However, by Days 5, 6 and 7, there was an apparent increase in the stanozolol concentration. This may indicate that the formation and excretion of stanozolol metabolite conjugates lag behind the excretion of the free drug and free metabolites.

INTRODUCTION

Stanozolol (17α-methyl-2'H-androst-2-eno[3,2-c]pyrazol-17b-ol) is a member of a unique group of heterocyclic anabolic steroids (see Fig 1). Anabolic steroids are synthetic derivatives of testosterone possessing similar protein anabolic activity, but with minimal androgenic effects. Stanozolol is prescribed to promote weight gain, increase appetite and increase strength and vitality. Specifically in horses, stanozolol increases nitrogen and mineral retention, reverses tissue-depleting processes and promotes better use of dietary proteins. A typical im dosage is 0.55 mg/kg weekly for up to 4 weeks (Merck 1998). Stanozolol is classified as a Class 4 drug in the RCI Uniform Classification Guidelines of Foreign Substances, 1998.



Stanozolol:R_1=H, R_2=H
3'-Hydroxystanozolol:R_1=OH, R_2=H
16β-Hydroxystanozolol:R_1=H, R_2=OH

Fig 1: Stanozolol and metabolite structures.

TABLE 1: Cross reactivity study

3'-Hydroxystanozolol	63%	Dianabol	0.37%	Estradiol	0.03%
Anadrol	5.52%	Boldenone	0.05%	Progesterone	0.03%
Anavar	1.75%	Testosterone	0.05%	Testosterone Benzoate	0.03%
Metandren	1.74%	Estrone	0.04%	Aldosterone	0.02%
16ß-Hydroxystanozolol	1.32%	Nandrolone	0.04%	Estrone-3-Sulfate	0.02%
Methandriol	0.72%	Androstenedione	0.03%	Testosterone-17-ß-cypionate	0.02%

All compounds listed below had less than 0.01% cross reactivity:

Acepromazine	Furosemide	Phenylbutazone
ε-Amino-n-Caproic Acid	L-Glutamic Acid	Polyethylene Glycol
Amitriptyline	Glycopyrrolate	Prednisolone
Ascorbic Acid	Hordenine	Pregnisoline
Azaperone	Hydrocortisone	Procaine
Benzocaine	Ibuprofen	Promazine
Bupropion	Imipramine	Pyrantel
Cholecalciferol	Isoxsuprine	Pyridoxine
Clenbuterol	Lidocaine	Pyrilamine
Clomipramine	Maprotiline	Pyrimethamine
Desipramine	Medadione	Retinoic Acid
Dexamethasone	Sodium Bisulfite	Ritodrine
Diclofenac	Metaproterenol	Salbutamol
Dimethyl Sulfoxide	Methamphetamine	Salicylamide
Dipyrone	Methylene Blue	Salicylic Acid
Doxepin	Naproxen	Thiamine
Estriol	Niacinamide	(±)-α-Tocopherol
Fenfluramine	Orphenadrone	Tranzodone
Flumethasone	Oxyphenbutazone	Trimethoprim
Flunixin	Pentoxifylline	Trimipramine
Folic Acid	Perphenazine	Uric Acid
Folinic Acid	Phenothiazine	

MATERIALS AND METHODS

Reagents

N-hydroxysuccinimide (NHS), 1-Ethyl-3-(Diamino-propyl)Carbodiimide(EDC), ß-glucuronidase from bovine liver, and other buffer salts were purchased from Sigma Chemical Company. Horseradish peroxidase (HRP) was purchased from Boehringer Mannheim. Conjugate purification was performed by liquid chromatography using Pierce desalting columns. Protein A was purchased from Repligen, and 96 well plates were purchased from Costar.

Antibody and conjugate production

A hapten was formed by introducing a carboxylic acid group to stanozolol. Using NHS and EDC, the hapten was linked by active ester chemistry to BSA (Grabarek and Gergely 1990). Rabbits were inoculated with the hapten:BSA complex, boosted monthly and plasma was collected monthly. The plasma was allowed to clot and serum were collected by centrifugation. The rabbit antisera were stored at -20°C until screening.

The hapten produced was also used to make the drug:HRP conjugate. It was linked by active ester chemistry to the enzyme. The conjugates were purified using Pierce desalting columns.

Plate selection

The coating of anti-stanozolol antibodies on Costar plates was examined in each of 4 methods. The antisera was coated:

a) directly to the microwells;

b) on microwells pre-coated with protein A;

c) on microwells pre-coated with goat anti-rabbit IgG antisera;

d) on microwells pre-coated with sheep anti-rabbit IgG antisera.

Microwells pre-coated with protein A yielded optimal colour. These secondary antibody plates were used for all assay development. Antisera and

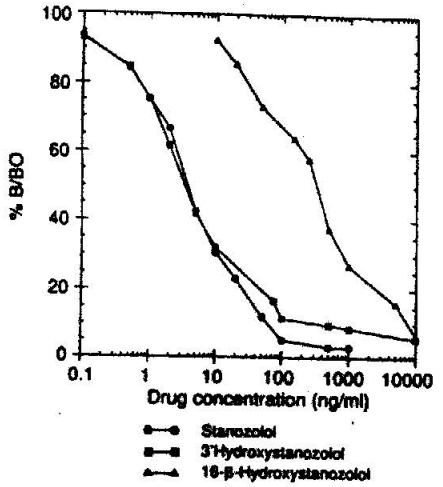


Fig 2: Standard curves for stanozolol and its metabolites.

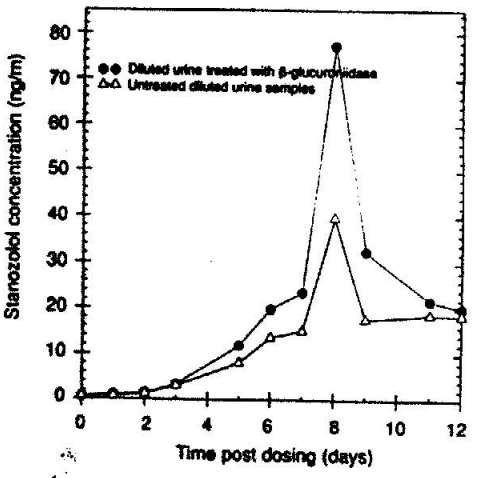


Fig 3: Duration of detection for β-glucuronidase treated and untreated diluted equine urine.

conjugate dilutions were optimised to yield the best standard curve performance.

ELISA test protocol

The ELISA was performed using the following procedure:

a) Incubate 20 µl of control or sample with 180 µl of stanozolol:HRP conjugate for 45 min (free drug competes with the drug:enzyme conjugate for a limited number of antibody binding sites);
b) Aspirate contents of assay wells and wash 3 times with 300 µl assay wash buffer;
c) Tap wells mostly dry and add 150 µl of Neogen K-Blue (tetramethybenzidine, TMB) Substrate;
d) Incubate for 30 min;
e) Measure absorbance at 650 nm (the amount of absorbance is inversely proportional to the amount of free drug present).

Treatment of equine urine

Equine urine samples were treated with ß-glucuronidase to increase free stanozolol concentrations by cleaving glucuronic acid from stanozolol. Urine (1 ml) was adjusted to pH 4.0-5.0 (if needed), and spiked to a concentration of 100 units of enzyme ml. The spiked urines were incubated for 4 h at 37°C. Samples were then diluted 1:4 in assay buffer and evaluated.

RESULTS AND DISCUSSION

Sample matrix effects

Thirty-two post race neat equine urine samples, and 40 post race canine urine samples were assayed. Some of the neat urines had significant matrix effects, seen as increased assay background. The samples were assayed after a 1:4 dilution in assay buffer. This dilution eliminated assay interference. Forty equine plasma samples were also assayed, and showed no significant matrix effects.

Sensitivity

Standard curves were generated with known levels of stanozolol in assay buffer. The colour of the zero standard was optimised to approximately 1.0 OD, and the I_{50} (measure of assay sensitivity) was 4.0 ng/ml (Fig 2). The assay sensitivity was also evaluated in 4 common sample matrices: equine plasma, equine serum, equine urine and canine urine. The urine was diluted 1:4 in assay buffer prior to use. The I_{50} was 17.3 ng/ml in equine plasma, 15.4 ng/ml in equine serum, 10.7 ng/ml in diluted equine urine and 14.2 ng/ml in diluted canine urine.

Cross reactivity

Cross reactivities were determined for several steroids, illegal drugs, therapeutic drugs, masking agents, vitamins and drug vehicles (Table 1). A total of 84 compounds were screened. Significant cross-reactivity was seen only with the stanozolol metabolite, 3'-Hydroxystanozolol. Standard curves were generated for this metabolite in assay buffer and the 4 common sample matrices. The I_{50} was 6.3 ng/ml (Fig 2) in assay buffer, 21.5 ng/ml in equine plasma, 15.1 ng/ml in equine serum, 34.0 ng/ml in diluted equine urine and 31.9 ng/ml in diluted canine urine. Another stanozolol metabolite,

16ß-Hydroxystanozolol, was a minor cross reactant (Fig 2). Three other 17a-methyl steroids were also minor cross reactants (see Table 1).

Administration testing

One gram of stanozolol im was given to a Standardbred mare. Urine and plasma were collected on Days 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 post injection and stored at -20°C until assayed. Stanozolol equilvalents was detected in urine as early as Day 2. Apparent stanozolol levels in the urine increased from Day 2, reaching a maximum concentration of 39.72 ng/ml by Day 8. However, treatment with ß-glucuronidase increased the apparent stanozolol concentration of the samples from Days 5 to 9, whereas the apparent stanozolol concentration of Days 1, 2 and 3 remained unchanged. This indicates that the formation and subsequent excretion of the conjugated metabolites lag behind unconjugated drug and metabolites (Fig 3).

No stanozolol was detected in the plasma samples collected following the stanozolol administration.

Conclusion

A rapid, easy to use ELISA for the detection of stanozolol was developed. It is sensitive, and specific for stanozolol or its 3'-hydroxy metabolite.

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