Reprint from

RECENT ADVANCES IN DOPING ANALYSIS (7)

W. Schänzer
H. Geyer
A. Gotzmann
U. Mareck-Engelke
(Editors)

Sport und Buch Strauß, Köln, 1999

R. ARMSTRONG, S. BIDDLE, E. HOUGHTON:
Highly Specific, Low Level Detection of Stanozolol and Metabolites using Solid Phase Extraction and ELISA Technology
In: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck-Engelke (eds.) Recent advances in doping analysis (7). Sport und Buch Strauß, Köln, (1999) 329-334

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Highly specific, low level detection of stanozolol and metabolites using solid phase extraction and ELISA technology.

H.F.L. Newmarket, Cambridgeshire, U.K.

Introduction

The analysis of urine samples for anabolic steroids can involve a number of complex procedures. Some steroids may cause more analytical problems than others: stanozolol is one such compound. The composition of the pyrazole ring gives the compound and some of its metabolites considerable basic functionality, distinguishing them from other steroids both structurally and in extraction behaviour.

The IOC desires the detection of 3'-OH-stanozolol (a major metabolite in man[1,2]) at a level of 2ng/ml in urine. Whilst this is achievable by GC/MS/MS and GC/HRMS analytical techniques[3], such procedures are time consuming and expensive. GC/MS systems also invariably need 'optimising' for the analysis of stanozolol metabolites, due to problems with chromatography and sensitivity.

This is true of 16β-OH-stanozolol, another major metabolite of stanozolol in man[1,2], which may be present in equivalent or greater amounts than 3'-OH-stanozolol for the duration of their excretion[2].

Therefore, initial detection of stanozolol abuse could be achieved by detecting the presence of 16β-OH-stanozolol at low levels (1-2ng/ml).

This presentation suggests a solid phase extraction followed by an ELISA, highly specific for this purpose. Those samples giving positive results can be followed up with a high degree of confidence for stanozolol and metabolites using established procedures.

Materials and methods

Extraction:

Individual blank human urine samples were used for the analyses. Pooled blank human urine was used for the spiked samples.

Samples were spiked using standards supplied by Radian International , Austin , Texas.

SPE cartridges used for the extraction were supplied by Varian, Harbor City, USA.

Samples were extracted as shown in **Figure 2.**

Extraction recovery was determined by LC/MS using an LCQ system, Finnigan Matt, Hemel Hempsted, U.K.

Immunoassay:

The antiserum was raised in sheep to 20nor-17,O^{17}-dehydro-stanozolol-17-CMO-HSA.

Microtitre plates were coated with a streptavidin-biotin-stanozolol complex.

Control samples for the immunoassay were 15ng/ml and 50ng/ml stanozolol in synthetic urine. Synthetic urine was used as the blank.

Cross-reactivity data for the antiserum was obtained using synthesized metabolites. (Metabolites courtesy of Institut für Biochemie, Deutsche Sportochschule, Cologne.)

Immunoassay was performed on a Tecan RSP5052, (Tecan Ltd., Reading, U.K.) as shown in **Figure 3.**

Results and discussion

From the data shown in **Fig.1** it is apparent that the antibody is recognising the pyrazole/A-ring moiety of the molecule, as any structural modification in this region results in a significant loss of cross-reactivity. For this reason, 16α-OH stanozolol is also expected to have a good degree of cross-reactivity but has not been assayed due to limited availability.

Compound	Cross-reactivity (%)
Stanozolol	100
16β-OH stanozolol	48.84
17-epi-stanozolol	22.11
16α-OH-17-epi-stanozolol	21.03
4α-OH-stanozolol	2.00
4β-OH-stanozolol	0.95
3'OH-stanozolol	0.07
17-epi-3'OH-stanozolol	0.01

Fig.1 Stanozolol Antibody: cross-reactivity of metabolites.

Figure 3 shows the immunoassay results for a batch of extracted samples, analysed on an individual microtitre plate.

The B/Bo values for all blank samples were taken to produce a mean. The mean +/- 2 SDs is plotted to encompass 95% of the blank sample scatter.

The results show that 2ng/ml 16β-OH stanozolol is readily detectable. There is good distinction between the negative scatter and positive samples on the plate.

There is some variation in results of the samples spiked at 1ng/ml. Extraction recovery and the amount of analyte actually 'applied' to the plate may well be factors at this level. Nevertheless, it is reasonable to expect that both samples spiked @1ng/ml 16β-OH stanozolol (shown in Fig.4) would be sent for further work. A post administration sample at this level may contain small amounts of other metabolites which would enhance the B/Bo value vs. a spiked sample. One other consideration is the efficiency of hydrolysis. The PA sample shown in Fig.4 is the result of 250µl of extracted urine! It would however, be advantageous to analyse some very low level PA samples as well.

The extraction recovery for 16β-OH stanozolol from the method shown in Fig.2 is 33%. Different elution solvents can improve this to more than 80%. However the negative scatter observed in these instances was poor due to the increase in matrix components eluted.

Further work will be carried out to optimise the extraction for use as a confirmatory method. This may also result in a further increase in sensitivity for the analysis.

This method allows the rapid analysis of large numbers of samples. It is fully automatable or may be performed manually. Due to the specificity of the assay, confirmatory analysis of suspected positives can be solely concentrated on the detection of stanozolol and its metabolites.

References

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2 – Schänzer ,W., Opfermann, G. and Donike, M., *J. Steroid Biochem., 1990, **36**, 153.*

3 – Schänzer, W., Delahaut, P., Geyer, H., Machnik, M. and Horning, S., *J. Chrom. B , 1996, **687**, 93.*

Sample preparation

To 5ml urine sample add: 1ml Ammonium acetate, 2.5M, pH6.8, 400µl β-glucuronidase and incubate at 37°C overnight.

Extraction of stanozolol and metabolites from hydrolysed urine

Condition Certify mixed mode SPE cartridge

(2ml methanol, 2ml water)

↓

Apply urine sample (6ml)

↓

Wash cartridge

(1ml, 10% methanol in 0.1M pH 5.5 phosphate buffer)

(2ml, 1M acetic acid)

(3ml, Hexane)

↓

Wash cartridge

(3ml, Ethyl acetate : Hexane , 3:1 v/v) → Neutral fraction

(3ml methanol)

↓

Elute cartridge

(3ml, 3% ammonia in ethyl acetate)

↓

Evaporate to dryness

(Centrifugal evaporator or under oxygen free nitrogen @ 40^0C)

↓

Reconstitute residue for Immunoassay

(400µl, phosphate buffered NaCl + 0.5% Bovine serum albumin)

Figure 2

Stanozolol immunoassay procedure

Reconstitute the plate before use with wash buffer

↓

Add 20µl sample, 80µl assay buffer and 100µl antiserum to each well. Incubate immediately for 1 hour at 37°C with shaking

↓

Wash the plate 3 times with buffer

↓

Add 200µl of protein G – alkaline phosphatase reagent to each well. Incubate immediately for 1 hour at 37°C with shaking

↓

Wash the plate 3 times with buffer

↓

Add 200µl p-nitrophenyl phosphate substrate to each well.

Allow to incubate for 30 minutes at room temperature, without shaking.

↓

Add 50µl stopper solution to each well.

Read the plate at 405nm with a reference of 630nm to give absorbance values for each well.

Figure 3

Fig. 4 STANOZOLOL ELISA