Journal of Steroid Biochemistry, Vol. 11, pp. 933 to 936
Pergamon Press Ltd 1979. Printed in Great Britain

PRACTICAL ASPECTS OF SCREENING OF ANABOLIC STEROIDS IN DOPING CONTROL WITH PARTICULAR ACCENT TO NORTESTOSTERONE RADIOIMMUNOASSAY USING MIXED ANTISERA

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SUMMARY

A radioimmunoassay of two main classes of anabolic steroids, suitable for screening of these drugs in doping control is described and evaluated. Antisera against 17α-methyltestosterone-3-carboxymethyl-oxime-BSA were raised in both rabbits and goats. To enhance the specificity of nortestosterone assay, two goat antisera against nortestosterone-17β-hemisuccinate-BSA (Ab. 1) and nor-testosterone-3-carboxymethyloxime-BSA (Ab. 2) were raised, which recognized selectively 19-nor-3-oxosteroids and 19-nor-17β-hydroxysteroids, respectively. Using their mixture, the cross-reaction of naturally occurring steroids except for testosterone could be minimized. Tritiated steroids were used as tracers. Iodinated methyltestosterone and nortestosterone, prepared by simple melting of these steroids with $Na^{125}I$, could be used as alternative radioligands. Some questions concerning the expression of results and practical limitations of doping control of anabolics are discussed.

The mass abuse of anabolic steroids in sports has dated roughly since the Olympic Games in Tokyo in 1964 and has led in following years to a ban on these drugs by most of international sport organizations. Consequently, the problem arose of the detection of this large group of steroids and their metabolites.

In 1974, Brooks group [1, 2] introduced a radioimmunoassay method based on rabbit antisera raised against 3-carboxymethyloxime-bovine serum albumin conjugates of methyltestosterone and nortestosterone and ^{125}I-labelled derivatives of these steroids with aromatic amines as tracers. The method has become a method of choice for screening of anabolics in urine at a number of international sport competitions including Olympic Games. The positive results have been corroborated by gas chromatographic–mass spectrometric analysis. Doping with a great number of known anabolic steroids can be detected by this radioimmunoassay with an exception of naturally occurring androgens (i.e. testosterone and dihydrotestosterone) and several synthetic C_{19}-17β-hydroxysteroids with alkyl group(s) in position different from 17α (*e.g.* methenolone and adrostanolone).

In practical performance of doping control there was no difficulty in detection of 17α-alkylated steroids since the cross-reaction effect of testosterone and its 5α-reduced analogues could be completely overcome by acetylation of secondary 17β-hydroxygroup; the resulting acetates hardly cross-react with antiserum. The problem that we feel remains not completely solved is the detection of 19-nortestosterone and its analogues. The situation is complicated by the fact that this steroid is rapidly metabolized in the similar way to testosterone. When only nortestosterone-3-carboxymethyloxime conjugate as an immunogen is used, considerable cross-reaction of various 17β-hydroxysteroids occurs, whereas detection of 19-nortestosterone derivatives without free 17β-hydroxygroup (nortestosterone esters, 17-oxo-19-norsteroids) is limited. Table 1 shows the cross-reactivities of several typical steroids using our antisera and that of Brooks *et al.* against nortestosterone-3-CMO-BSA, as measured in our laboratory. With our antiserum, [^{3}H]-labelled tracer was used.

In order to increase the selectivity of steroid radioimmunoassay the use of mixed antisera obtained with two different immunogens was suggested by Sekihara *et al.*[3] and the concept was developed by Rodbard[4]. This approach has also been applied in this work.

The formulas of immunogens prepared as well as respective steroid/BSA molar ratios are shown in Fig. 1. The same conjugate as used by Brooks[2] group was prepared from methyltestosterone, both 17β-hemisuccinate- and 3-carboxymethyloxime-conjugates were synthetized to obtain different antisera

Table 1. Cross-reactivities of several steroids with antisera against nortestosterone-3-carboxymethyloxime-BSA

Steroid	Cross-reactivity in %	
	Brooks *et al.* (1975)	Hampl *et al.* (1978)
Nortestosterone	100	100
Nortestosterone phenylpropionate	0.92	1.73
Nordihydrotestosterone	54.4	80.7
Norethandrolone	49.8	6.40
Testosterone	4.33	6.50
Estradiol	3.45	7.50
Androstenedione	0.81	0.93
5α-Androstane-3α,17β-diol	3.08	6.00
Methenolone	1.56	0.30
Drostanolone	1.96	1.98

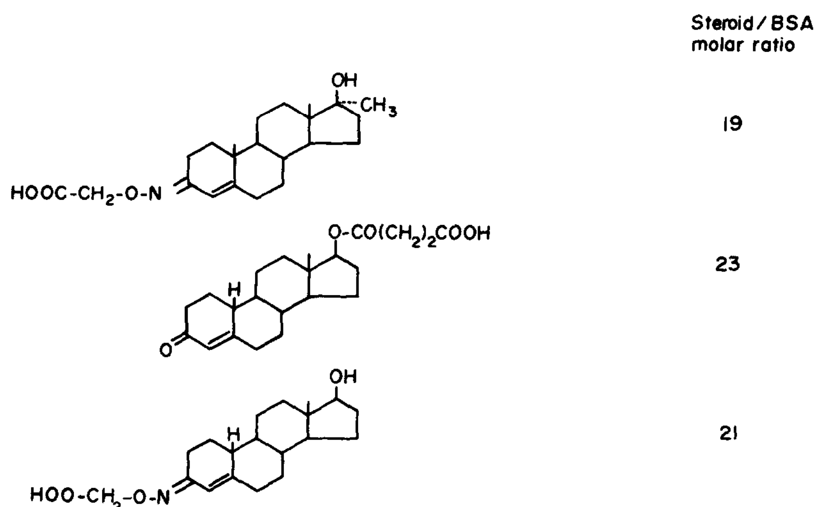

Fig. 1. Formulae for immunogens used for radioimmunoassay of anabolic steroids.

for nortestosterone. Rabbits and goats were immunized in the usual way with methyltestosterone conjugate. Since a higher titre was achieved in goats, these animals only were immunized with nortestosterone immunogens.

Twenty-two steroids including orally as well as parenterally active anabolics, principal androgens and their metabolites and several other hormonal steroids were examined for their ability to compete with [^{3}H]-methandienone for binding sites of rabbit and goat methyltestosterone antisera. The results, summarized in Table 2 do not differ substantially from those obtained with Brooks[2] antisera. The only exception is a lower cross-reaction of stanazolol and the higher one of methylandrostenediol.

Cross-reactions of 24 steroids with both nortestosterone antisera are shown in the first two columns of Table 3. As can be seen, both antisera bind 19-norsteroids but not to the same extent. The antibody raised against 17β-conjugate recognized preferably those steroids possessing Δ^4-3-oxo-grouping (nortestosterone 17β-hydroxyesters, testosterone, androstenedione and even progesterone). A structural determinant for the second antibody (i.e. against 3-oxime) appeared to be 17β-hydroxygroup with less regard to a stereochemistry of the A-ring. Besides 17β-hydroxy-19-norsteroids, oestradiol. testosterone and 17β-androstanediols were the most effective competitors.

Thus, in order to minimize cross-reactions of naturally occurring steroids and at the same time to retain the ability to bind 19-norsteroids, we were trying to establish the optimal composition of the mixture of both antisera. The method suggested by Rodbard[4] has been applied. Androstenedione and 5α-androstane-3α,17β-diol were chosen as model compounds: the former cross-reacted considerably with antibody against 17β-conjugate (antibody 1, Ab. 1) whereas the latter showed high cross-reaction with antibody raised against 3-conjugate (antibody 2, Ab. 2) only. Various volumes of both antisera, each in dilution at which given amount of nortestosterone (1 ng) caused 50% displacement of [^{3}H]-nortestosterone (1:12000 for Ab. 1 and 1:8000 for Ab. 2, respectively), were combined to obtain mixtures of various compositions of antisera (from 100% of Ab. 1 to 100% of Ab. 2). For each mixture, the amounts of nortestosterone and both competitors required for 50% displacement of tracer (designated according to Rodbard ID_{50} and ED_{50}, respectively) were determined (Ab. 2). Two straight lines were obtained, their intercept giving on the abscissa the optimal composition of the antisera, i.e. 60% Ab. 1 +40% Ab. 2. The cross-reactivities of 24 steroids examined are shown in the third column of Table 3. As can be seen, the undesired

Table 2. Cross-reactivities of various steroids with rabbit and goat methyltestosterone antisera

Steroid	Cross-reaction in %	
	Rabbit	Goat
Methandienone	100	100
Methyltestosterone	132	163
Dimethylandrostanolone	16.6	39.5
Methylandrostenediol	20.2	39.0
Stanazolol	5.9	7.2
Fluoxymestrone	4.7	6.5
Oxymetholone	3.2	9.6
Norethandrolone	2.3	5.5
Nortestosterone	2.9	1.9
Nortestosterone phenylpropionate	0	0
Methenolone	0.05	0.09
Drostanolone	0.08	0.35
Testosterone	47.6	19.8
Testosterone acetate	0.01	0.02
Dihydrotestosterone	6.9	9.7
Dihydrotestosterone acetate	0.01	0.01
Androstenedione	1.00	0.09
Dehydroepiandrosterone	0	0
Androsterone	0	0
Oestradiol	0.06	0.03
Cortisol	0.01	0.02
Progesterone	0.06	0.13

Table 3. Cross-reactivities of various steroids with goat nortestosterone antisera and their mixture. Antibody 1, Ab. 1.—anti-nortestosterone-17β-hemisuccinate-BSA. Antibody 2, Ab. 2.—anti-nortestosterone-3-carboxymethyloxime-BSA

Steroid	Cross-reactivity in %		
	Ab. 1	Ab. 2	Ab. 1 + Ab. 2
Nortestosterone	100	100	100
Nortestosterone phenylpropionate	10.1	1.73	5.02
Nortestosterone acetate	30.2	2.81	19.2
Nortestosterone hemisuccinate	18.9	2.50	10.5
Nordihydrotestosterone	21.4	80.7	27.4
5α-Androstane-19-nor-3ξ,17β-diol	0.46	20.4	8.26
Norethandrolone	37.6	6.40	29.9
Drostanolone	0.25	1.98	0.67
Methandienone	1.09	0.60	0.97
Methenolone	5.30	0.30	1.74
Stanazolol	0	0.12	0
Testosterone	7.70	6.50	6.90
Dihydrotestosterone	2.06	3.12	2.59
Androstenedione	3.86	0.93	1.83
Androsterone	0.30	0	0.11
Dehydroepiandrosterone	0.40	0	0.15
Aetiocholanolone	0.20	0	0
5α-Androstane-3α,17β-diol	0.90	6.00	1.01
5β-Androstane-3α,17β-diol	0.45	4.01	0.76
Epitestosterone	1.98	1.92	1.94
Cortisol	0	0	0
Oestradiol	0.38	7.50	0.79
Ethinylestradiol	0	0	0
Progesterone	2.13	0.56	1.32

cross-reactions of various naturally occurring steroids are reduced without remarkable loss of ability to detect nortestosterone analogues. The only hormones, cross-reaction of which cannot be overcome are testosterone and dihydrotestosterone.

The method itself (for both 17α-alkylsteroids and 19-norsteroids) involves the following steps. Extraction of urine (5 ml) with diethyl ether followed by washing and evaporation of the solvent. Acetylation of dry residues with acetic anhydride and pyridine (only when 17α-alkylsteroids are assayed), addition of tracer, diluted antiserum and buffer, incubation at 37°C for 30 min and then at 4°C for 1 h. Addition of stirred dextran-coated charcoal and a brief incubation, centrifugation and removal of an aliquot of the supernatant for liquid scintillation counting.

The method can be also employed for detection of conjugated steroids and their metabolites. Urine (0.5 ml) is hydrolyzed with β-glucuronidase from *Helix pomatia* digestive juice (5000 I.U. per sample) in sodium acetate buffer pH 4.5 at 37°C overnight and extracted with ether. An equivalent of 100 μl of urine is then taken into the assay.

The reliability criteria of the method are briefly summarized in Table 4. Accuracy was expressed as average recovery of increasing amounts of methandienone or nortestosterone (usually 50–200 pg) added to analyzed material. The precision as respective coefficients of variation. The sensitivity was determined according to Ekins[5] as least amounts of steroids which can be distinguished from zero sample with 95% probability.

The method described has been applied in doping

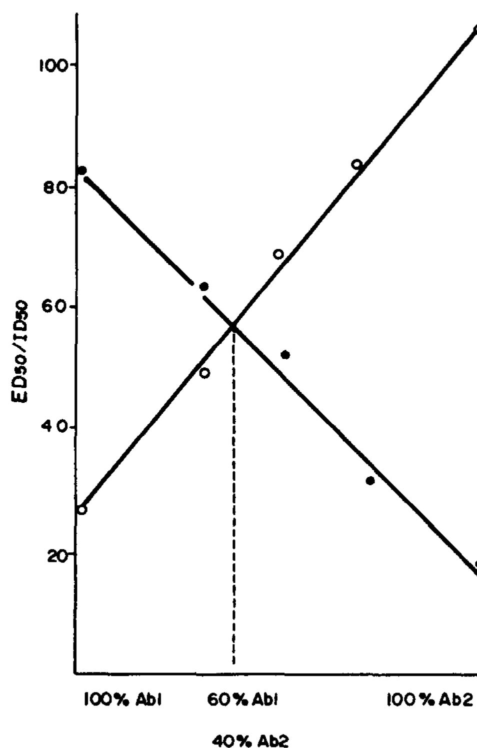

Fig. 2. Estimation of optimal composition of antisera mixture. Ratios ED_{50}/ID_{50}, where ED_{50} represents the amount of the competitor and ID_{50} the amount of nortestosterone, respectively, required for 50% displacement of [^{3}H]-nortestosterone were plotted against the percentual composition of the antisera-mixture. Full circles: ED_{50} of 5α-androstane-3α,17β-diol/ID_{50}. Open circles: ED_{50} of 4-androstene-3,17-dione/ID_{50}.

Table 4. Reliability criteria of radioimmunoassay of 17α-alkylated- and 19-norsteroids

Material analyzed	17α-Alkylated anabolic steroids			19-Norsteroids		
	Average recovery (%)	Coefficient of variation (%)	Sensitivity (pg)	Average recovery (%)	Coefficient of variation (%)	Sensitivity (pg)
Untreated urine	87.3	4.60	128	100.1	6.19	287
Hydrolyzed urine	—	—	—	101.1	6.92	329
Plasma	91.8	4.95	86	95.0	9.30	22

control at the European championship in light athletics in Prague, this year. Both unprocessed and hydrolyzed urine were taken for radioimmunoassay of nortestosterone. Ten control urine samples were usually processed within each series of analyses. Those results (in terms of radioactivity) which differed by more than two S.D. from the mean of controls were considered as positive. With manual processing, 30 duplicate analyses of both types of anabolics could be performed by two technicians in one day.

From the point of view of radioimmunoassay of other anabolics, one of the pertinent problems is the availability of a suitable tracer. The use of tritiated steroids or [^{125}I]-iodo-tyraminyl- or histaminyl-derivatives need not be the only way of labelling. Recently a method has been reported by Thakur and Waters[6] of direct (i.e. in steroid nucleus) iodination of several steroids, based on 1–2 min melting of an excess (100–1000 fold) of steroid with carrier-free [^{125}I]-Na. We have modified the procedure for testosterone. Following two-step chromatographic separation on t.l.c., the radioactive product obtained appeared to be immunoreactive and thus a suitable tracer for testosterone radioimmunoassay [7]. Similarly, we have prepared both iodinated methyltestosterone and nortestosterone. Following chromatographic separation both radioactive products appeared to be immunoreactive and could be displaced from their binding to respective antisera by non-radioactive steroids. Association constants with methyltestosterone- and both nortestosterone-antisera for ^{3}H- and ^{125}I-labelled radioligands could then be calculated (Table 5).

In conclusion, we would like to emphasize that many problems concerning the detection of anabolic steroids in doping control remain still opened.

Table 5. Association constants of three antisera raised against 17α-alkylated- and 19-norsteroids with ^{3}H- and ^{125}I-labelled radioligands at 25°C

Immunogen	Association constant $1.mol^{-1} \times 10^{-9}$	
	^{3}H	^{125}I
Methyltestosterone-3-carboxymethyloxime-BSA	2.95	2.26
Nortestosterone-3-carboxymethyloxime-BSA	1.78	1.14
Nortestosterone-17β-hemisuccinate-BSA	1.31	0.88

Using radioimmunoassay it is practically impossible to distinguish endogenous and administered androgens (namely testosterone and dihydrotestosterone). These compounds and particularly their metabolites can be detected by gas chromatography–mass spectrometry, but the problem of their origin remains unsolved. It has been known that secretion of certain metabolites (*e.g.* etiocholanolone and epiandrosterone) is increased considerably when exogenous testosterone is administered. It is, however, difficult to establish some limit from which "doping" takes place. Perhaps the solution may be the simultaneous estimation of free testosterone and LH, radioimmunoassay could be used for both hormones. When both hormones are elevated, the endogenous origin of testosterone could be expected whereas in the case of increased testosterone and low LH the exogenous origin of the steroid is very probable.

The definition of "banned anabolics" itself is also broad and vague. There are for instance contraceptives (including several 17α-alkylated-C_{19}-steroids) many of which are known as potent anabolics. Should they be included into the list of doping compounds or not? They have been usually detected by radioimmunoassay but they could be distinguished by mass spectrometry.

Hence, radioimmunoassay is the best tool for screening of these steroids and the definitive evidence for the drug abuse remains with gas chromatography–mass spectrometry.

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