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A radioimmunoassay for the metabolites of the anabolic steroid nandrolone

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Abstract: A new radioimmunoassay for the major metabolites of nandrolone was developed to overcome the interference of certain contraceptive steroids in the original assay of the parent steroid. The new assay gave greater screening capability by increasing retrospective detection and the signal-to-background ratio.

Keywords: *Nandrolone; 19-norandrosterone; 19-norepiandrosterone; lynoestrenol.*

Introduction

The radioimmunoassay for nandrolone (17β-hydroxyestr-4-en-3-one), which was introduced in 1976, has the disadvantage that false positives have been obtained from women taking contraceptive steroid preparations containing lynoestrenol [1]. Fotherby [2] considered that norethisterone which has an oxo group at C-3 was probably a metabolite of lynoestrenol (Fig. 1). Indeed, lynoestrenol may owe its biological activity to its conversion to norethisterone which is also an oral contraceptive. It is extremely likely that norethisterone would cross-react with the nandrolone antiserum owing to the presence of the 3-oxo group combined with the A/B ring linkage.

Only about 0.05% of injected nandrolone is excreted unchanged in the urine. The amount of free steroid is increased to 0.4% if the urine is first hydrolysed with β-glucuronidase, but there is an unwanted increase in non-specific background values. A much larger proportion (about 30%) is excreted in the urine in the form of the tetrahydro 17-oxosteroid derivatives, 19-norandrosterone, 19-noraetiocholanolone and the 3β-hydroxy epimer, 19-norepiandrosterone [3]. These metabolites are conjugated in the urine to glucuronides and sulphates.

It was thought that if an antiserum was developed to these metabolites there would be a better chance of avoiding false positives from lynoestrenol administration. The plan was to prepare an immunogen by linking the steroid haptens of the metabolites to the protein through the D-ring of the steroid. In this work a carboxymethyloxime link through the 17-oxo group was used thereby directing the specificity to the left-hand side of the molecule, in particular the 3-hydroxy group and the A/B ring linkage.

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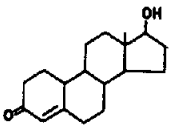
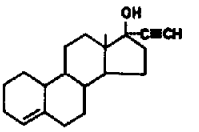
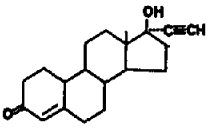


NANDROLONE



LYNOESTRENOL



NORETHISTERONE

Figure 1
Nandrolone and the oral contraceptives lynoestrenol and norethisterone.

To obtain these metabolites in high concentrations relative to those of endogenous steroids, a large dose of unesterified nandrolone was administered orally after suppressing gonadal and adrenal secretion. The paper describes the isolation of these metabolites and the subsequent development of a radioimmunoassay. A comparative study of urinary values of nandrolone and its metabolites was undertaken for two male subjects after a single intramuscular dose of Deca-Durabolin® (nandrolone decanoate) and one female subject after administration of lynoestrenol.

Experimental

The scheme for isolation of the metabolites of nandrolone is outlined in Fig. 2.

Administration and collection

Androgen production was suppressed from both the testes and the adrenal glands of a male volunteer before administration of a large oral dose of nandrolone. Informed consent was obtained from the volunteer.

A single dose of Deca-Durabolin (100 mg) was administered intramuscularly to subject A.K. at 1400 h on day 1. At 2400 h on day 3 the powerful synthetic corticosteroid dexamethasone (1 mg) was given orally to suppress the adrenal glands. On day 4 at 1400 h, 1 g of nandrolone was given orally and at 2400 h dexamethasone (1 mg) was again given to keep the adrenal glands suppressed. Twenty-four hour urine samples were collected from 1400 h on days 1 to 5.

Measurement of urinary oxosteroids

The 17-oxosteroid concentration in each 24-h urine sample was measured [4]. Urine collected on day 4 was selected for isolation of the metabolites.

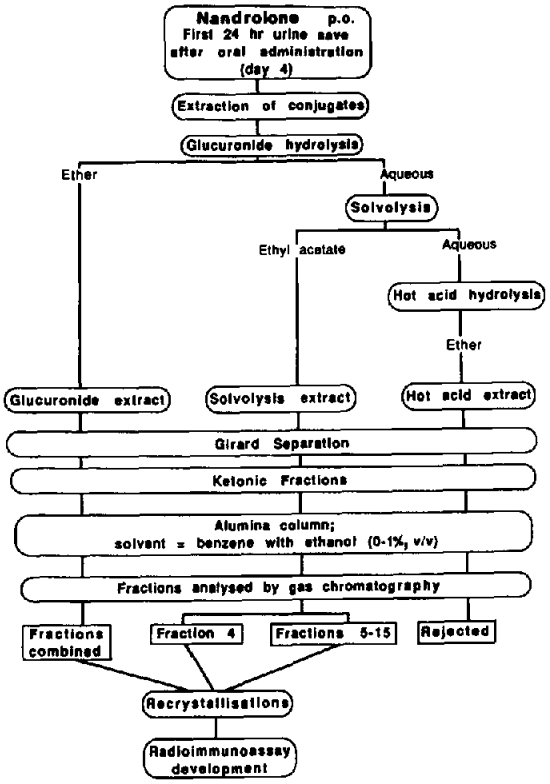


Figure 2
Development scheme for the isolation of nandrolone metabolites.

Hydrolysis and extraction

The conjugated 17-oxosteroids were extracted from the urine [5] and redissolved in 200 ml of distilled water. The concentration of the extract was adjusted to 0.07 M with respect to acetate buffer (pH = 4.3) and incubated overnight at 37°C with glucuronidase (*Patella vulgata*, 220,000 Fishman units). After incubation the solution was cooled, extracted with ether (2 × ½ vol), washed with water (1 × 1/10 vol), 1 M sodium hydroxide (1 × 1/10 vol) and water (2 × 1/10 vol).

Solvolysis using a method essentially that of Burstein and Lieberman [6] was performed on the aqueous fraction combined with the first water wash. The urinary oxosteroid sulphates were extracted with ethyl acetate (2 × ½ vol) and the combined extract allowed to stand for 30 min to allow complete separation of the aqueous layer. The organic phase was carefully decanted and incubated overnight at 37°C for solvolysis to occur. The extract was then cooled in ice and washed with iced water (1 × 1/10 vol), iced sodium hydroxide solution (9 × 1/10 vol) and then with iced water (3 × 1/10 vol).

The aqueous fraction together with the first water wash from the solvolysis were combined and subjected to hot acid hydrolysis. Hydrochloric acid (11 M, 1/10 vol) was added and the solution heated at 100°C for 20 min. After cooling, the solution was extracted with ether (2 × ½ vol) and washed with iced water (1 × 1/10 vol), 1 M sodium hydroxide (2 × 1/10 vol) and water (3 × 1/10 vol).

The glucuronide, solvolysis and acid hydrolysis extracts were evaporated to dryness. Duplicate 1/800 portions of each hydrolytic procedure were taken for 17-oxosteroid determination.

Separation into ketonic and non-ketonic fractions

The steroids in each hydrolysed extract were separated into ketonic and "non-ketonic" fractions by means of Girard reagent-T (trimethyl-acetoaminohydrazide hydrochloride). The method used was essentially that of Pincus and Pearlman [7]. However, Brooks [8] showed that some acetylation of the free hydroxide groups took place under those conditions. This was overcome by leaving the reaction mixture overnight at room temperature. Double the quantities of all the reagents were used for the solvolysis extract. Each ketonic extract was dried and the non-ketonic fraction rejected.

Chromatography of ketonic extracts

Each ketonic extract was fractionated on an alumina column (10 g, Brockman activity III) as described previously [8]. Each extract was dissolved in benzene (15 ml) and transferred by pipette on to the top of the column. Fractions were successively eluted using a column solvent of benzene containing increasing proportions of ethanol (0%, 0.2%, 0.5%, 1%, v/v). 15-ml portions of the eluent were collected, dried and each residual fraction weighed. A small part of each fraction was derivatised and subjected to gas-liquid chromatography (GLC).

The GLC used was a Packard 429 chromatograph with a DB1 fused silica capillary column (film thickness, 25 μm; length 30 m) and a solid injector. The temperature of the injection port and flame-ionisation detector heaters was 300°C. The temperature was programmed at 180°C for 3 min followed by a 3°C min^{-1} rise to 291°C.

A portion of each fraction was converted to its mono-trimethylsilyl ether derivative (mono-TMS) by the addition of trimethylchlorosilane in *N*-methyl-*N*-(TMS)trifluoro-acetamide (2%, v/v) and heating at 60°C for 25 min. The derivatives were dried under air and redissolved in ethyl acetate. An aliquot approximately equal to 50 ng of each fraction was analysed.

Mono-TMS derivatives of androsterone, aetiocholanolone, nandrolone and testo-sterone were also sampled to establish their retention times.

The retention times were calculated in methylene units using a basic computer program kindly supplied by the Drug Control and Teaching Centre of King's College, London. Peak areas were also recorded.

The hydrolysed glucuronide and solvolysis fractions were selected for further work and the acid fractions were rejected.

Preparation of immunogens of nandrolone metabolites

Fraction 4 (48 mg) of the solvolysis extract was chosen for preparation of the antigen because of its large yield. The crystalline residue was recrystallised in light petroleum (b.p. 60–80°C) (6 ml) with benzene (400 μl) and washed twice with cold light petroleum:benzene (24:1, v/v). The recrystallised product was checked for purity by GLC.

The product was converted to its carboxymethyloxime derivative and extracted by the method of Johnson *et al.* [9].

Eight milligrams of the carboxymethyloxime derivative was conjugated to bovine serum albumin [10], the remainder being used for radiolabel preparation.

Immunisation

Multiple intradermal injections of the immunogen were administered into 10–12 dorsal sites of a New Zealand White rabbit. The conjugate was dissolved in saline and initially emulsified with complete Bacto adjuvant H37Ra (*M. tuberculosis*) and thereafter with incomplete Freund's adjuvant for booster injections. Booster injections were administered after injection and the antiserum was harvested after 6 months.

Preparation of a radioiodine label

The method used was that of Nars and Hunter [11] as described by Brooks *et al.* [12].

Recrystallisation of combined glucuronide fractions and combined remaining solvolysis fractions of interest

Recrystallisation of the combined glucuronide fractions was in light petroleum (b.p. 60–80°C); recrystallisation of the combined solvolysis fractions 5–15 was in light petroleum (b.p. 60–80°C) (3.5 ml) with benzene (2.2 ml).

Separation of 3α- and 3β-hydroxy steroids

The solvolysis extract was a mixture of two steroids (see Results). Nine-hundred micrograms was taken for the analysis of α- and β-ketonic steroids using the digitonin method of Butt *et al.* [13]. The α- and β-fractions were derivatised to mono-TMS ethers and run on the GLC.

Characterisation of the antiserum

As the glucuronide extract consisted mainly of a 3α-hydroxy steroid and the solvolysis extract of a 3α/3β-hydroxy steroid mixture (see Results) it was important to characterise the antiserum. Working solutions of each recrystallised extract were prepared and run as calibration curves (5–500 pg/tube) in the metabolite assay. The calibration curve for the α/β mix was selected for further use.

Procedure for the assay of nandrolone metabolites in urine

As the major metabolites of nandrolone would be conjugated as glucuronides and sulphates in the urine, a rapid, hot acid hydrolytic procedure was established.

Urine (25 μl) was diluted with distilled water (500 μl) using an automatic diluter into 75 × 12 mm i.d. glass tubes. Three drops of concentrated hydrochloric acid (equivalent to approximately 120 μl) were added using a long glass Pasteur pipette. The tubes were immersed in a boiling water-bath for 20 min. After hydrolysis, distilled water (250 μl) was added to replace the amount lost by evaporation. The tubes were then cooled in an ice bath.

Extraction was performed by the addition of dichloromethane (4 ml) and shaking for 10 min on a multivortex mixer. The aqueous layers were removed by suction and the extracts were washed for 30 s with 0.5 M sodium hydroxide (1 × 500 μl) and with distilled water (2 × 500 μl). Between mixing procedures the extracts were kept cool.

1-ml aliquots of the extracted samples were dispensed into 100 × 10 mm i.d. glass tubes and dried under air at 37°C. The samples were redissolved in the assay buffer (pH = 7.4) (1 ml). The extracted metabolites were assayed by the same procedure as that described for urinary testosterone by Brooks *et al.* [1]. Normal ranges for 22 men and 19 women (excluding one pregnant female) were established.

Detection of urinary nandrolone and metabolites after administration of Deca-Durabolin®

A single dose of Deca-Durabolin (nandrolone decanoate, 100 mg) was administered intramuscularly to two male volunteers, A.K. and S.C. at 1000 h on day 1.

Spot urine samples were collected from days 0 to 18 and subsequently on days 24, 28, 35 and 43. Additional collections were made on days 53 and 64 for subject S.C. Urinary nandrolone was measured as described by Brooks *et al.* [12].

Comparison of the urinary values obtained from the assay of nandrolone and metabolites after lynoestrenol administration

A female volunteer, K.F., took the oral contraceptive Minilyn (lynoestrenol 2.5 mg and ethinyl oestradiol 50 μg) for 7 days. Spot urines were collected from days 0 to 7.

Results

Isolation of metabolites of nandrolone

By far the greatest quantities of 17-oxosteroids were excreted in the first 24 h after oral administration of nandrolone. The 17-oxosteroid concentration in each hydrolysed fraction is shown in Table 1, the highest concentration being given by solvolysis.

Table 1
The determined concentration of 17-oxosteroids in each hydrolysed fraction

Hydrolysed extract	Total 17-oxosteroids (mg)
β-glucuronidase	24
solvolysis	133
hot acid	42

After fractionation of these extracts on an alumina column the mono-TMS derivatives were analysed by GLC. The quantities of steroids were calculated from relative peak areas. Only one large peak having MU values of 23.81–23.86 was observed for the hydrolysed glucuronide fractions. This was defined as Peak 1. The estimated total weight calculated from areas of Peak 1 for these fractions was 14.8 mg. Recrystallisation of these combined fractions gave 11.8 mg, confirmed by GLC to consist of 98.4% Peak 1. One other small peak was seen in the glucuronide fractions which upon recrystallisation gave the remaining 1.6% with an MU value of 24.59 and this was called Peak 2.

Two main peaks were identified in the solvolysis fraction with MU values of 23.81–23.85 and 24.60–24.63. These corresponded with Peaks 1 and 2 seen in the glucuronide fractions but with a predominance of Peak 2. The estimated total amounts of each component for these fractions (not yet combined) were 65.5 and 157.1 mg, respectively. Fraction 4 was chosen for radioimmunoassay preparation, recrystallisation yielding 20.7 mg of the first crop and 1.8 mg of the second crop. GLC of both crops showed only Peaks 1 and 2. The two crops were combined and 27.3 mg of the carboxymethyloxime derivative was prepared. The remainder of the solvolysis fractions of interest (5–15) were combined and recrystallised giving 107.1 mg of the first crop and 24.2 mg of the second crop. The results of the recrystallisations are summarised in Table 2.

Table 2
Results of recrystallisation of fractions of hydrolysed extracts

Hydrolysed extract	First crop weight (mg)	First crop % present peak 1	First crop % present peak 2	Second crop weight (mg)	Second crop % present peak 1	Second crop % present peak 2
glucuronide	11.8	98.4	1.6	np*	np	np
solvolysis fraction 4	20.7	32.2	67.8	1.8	76.9	23.1
solvolysis fractions 5–15	107.1	32.4	67.6	24.2	77.1	22.9

*np = not performed.

Analysis of 3α/3β-ketonic steroids

The mono-TMS derivative of the α-fraction gave one peak which was identified as Peak 1. The β-fraction gave the other peak identified as Peak 2. The fractions were analysed by GLC–mass spectrometry at King's College, London. The electron impact mass spectra of androsterone (3α-hydroxy-5α-androstan-17-one) is different from its 5β isomer, aetiocholanolone. The steroids were confirmed to have spectral characteristics similar to those of androsterone but with a shift of 14 mass units, i.e. a replacement of a methyl group with a hydrogen atom on the A/B ring linkage. This information together with the knowledge of the presence of a 3β-hydroxy steroid points to the presence of 19-norandrosterone and the 3β-hydroxy epimer 19-norepiandrosterone as the 2 metabolites isolated.

Antiserum to the metabolites

The immunogen injected into the rabbit contained more of the 3β-hydroxy steroid than the 3α-hydroxy steroid. Thus it was essential to determine the specificity of the antiserum to the 3α-steroid alone. Prepared working solutions of the glucuronide extract consisting of 98% of the 3α-fraction and the solvolysis extract consisting of 33% of the 3α-fraction and 67% of the 3β-fraction were run as calibration curves in the metabolite assay (Fig. 3). The curves were very similar, the 3α-fraction cross-reacting 75% with the antiserum with respect to the 3α/3β calibration curve at 50% B/B_0.

Normal ranges of metabolites

Twenty-two men gave a range of 8.0–49.4 $\mu g\,l^{-1}$ (mean = 21.6 ± 9.6) and 19 women a range of 3.8–44.8 $\mu g\,l^{-1}$ (mean = 19.8 ± 9.9). An arbitrary cut-off point of 100 $\mu g\,l^{-1}$ was chosen for the presence of nandrolone metabolites. One female was excluded from

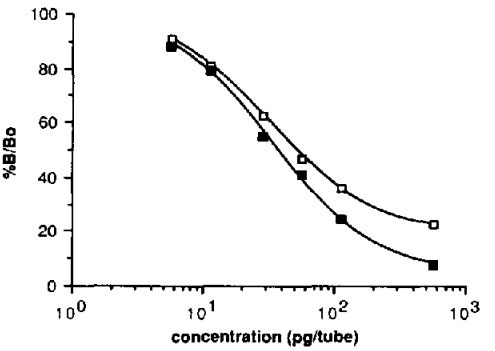


Figure 3
Comparison of standard curves of working solution α (□) and working solution α/β (■).

the original batch of 20 because she gave a value of 124 $\mu g\ l^{-1}$, which was above the cut-off limit, but she was known to be pregnant.

Administration of Deca-Durabolin®

Figures 4 and 5 display graphically the assay values of nandrolone and its metabolites after intramuscular administration. For subject A.K. the maximum concentration of nandrolone was only 3–4 times the assay cut-off point of 2 $\mu g\ l^{-1}$. However, the maximum 19-nor metabolite concentration was more than 13 times the chosen cut-off value of 100 $\mu g\ l^{-1}$. For subject S.C. the maximum concentration was 6 times above the positive threshold whereas his metabolite concentration was 10 times above the cut-off point.

In both cases the metabolite assay gave a greater degree of retrospectivity for nandrolone drug use.

Adminstration of lynoestrenol

The results of the administration of the oral contraceptive lynoestrenol on nandrolone and metabolite assay values are shown in Fig. 6. The nandrolone assay values increased beyond the cut-off point of 2 $\mu g\ l^{-1}$ for days 1–7. The metabolite values remained well below the cut-off point of 100 $\mu g\ l^{-1}$, the highest value of 51.4 $\mu g\ l^{-1}$ being just above the normal range on day 4.

Discussion

Two major metabolites of nandrolone were isolated, the antiserum raised recognising both steroids well. The larger quantity of the 3β-hydroxy metabolite 19-norepiandrosterone compared to the 3α-hydroxy metabolite 19-norandrosterone was unexpected in the light of other studies [3, 14, 15, 16]. The large amounts of 19-norepiandrosterone may be due to the large oral administration of nandrolone or alternatively may be unique to that individual. Almost all the 19-norepiandrosterone was present in the solvolysis fraction, which is in accordance with other conjugated 3β-hydroxy steroids in humans, e.g. epiandrosterone [17].

The new nandrolone metabolite assay gave greater screening capabilities than the present steroid assay by showing a larger signal-to-background ratio. The fluctuations in assayed values were due to changes in the volume of urine excreted in unit time. Continuous 24-h urine collections were made for 8 days and showed a smooth elimination of the metabolites (not published). The metabolite assay also gave a larger degree of retrospectivity for nandrolone doping, giving a positive result up to day 43 for subject S.C. This length of retrospectivity may appear unimportant compared to the 3 months or longer using the technique of gas chromatography–mass spectrometry (GC–MS). However, only a single dose was administered and the quantity of urine used in the method was very small. If a larger aliquot was taken with an intermediate purification step, the sensitivity of retrospective detection could be increased. This was not the main objective for two reasons. First, the method was developed as a fast and cost-effective screening technique for the major intramuscular anabolic steroid abused in sport. Second, and more important, there was a possibility that increasing the sensitivity for nandrolone metabolites might result in the detection of small amounts of these steroids that had been endogenously produced. Although the proposed mechanism for the major human biosynthetic pathway of oestrogens from androgens involves enzymatic

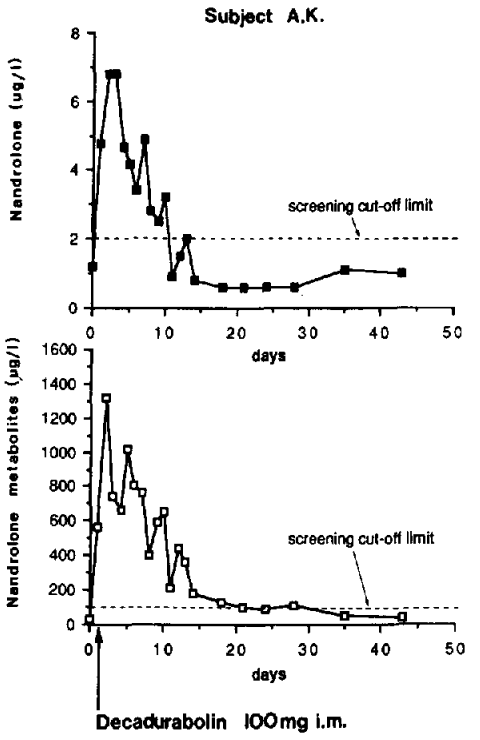


Figure 4
Urinary values of nandrolone and metabolites after Deca-Durabolin administration; subject A.K.

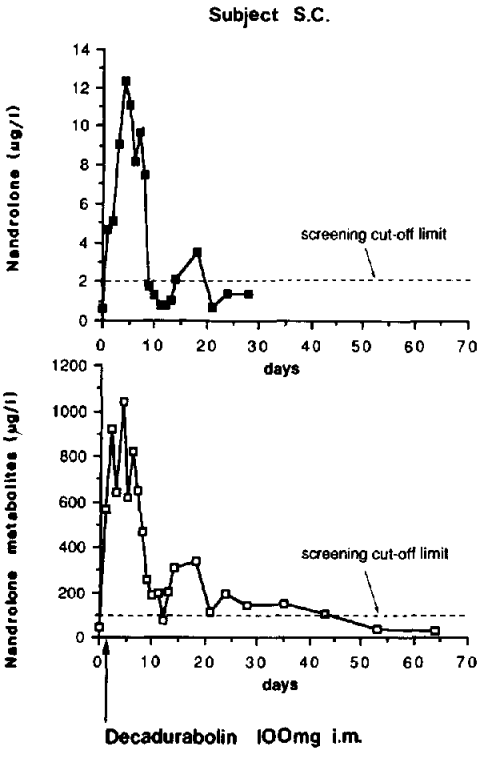


Figure 5
Urinary values of nandrolone and metabolites after Deca-Durabolin administration; subject S.C.

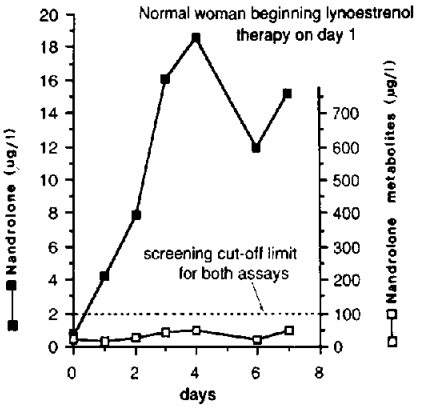


Figure 6
The effect of lynoestrenol on the nandrolone and metabolite assays.

hydroxylation at the C-19 methyl group [18] a minor pathway involving 19-nor steroids cannot be ruled out. 19-Norandrostenedione and nandrolone have been isolated in mare follicular fluid [19, 20] and nandrolone is present in stallion urine [21]. *In vitro* studies on human ovary [22] and prostate [23] have suggested that 19-nor steroids may play an intermediate role in the aromatisation process and nandrolone has been detected in human follicular fluid using GC–MS [24]. A small increase in urinary oestrogens after nandrolone injection has also been demonstrated [25]. The high value for the metabolite assay carried out in a control subject who was pregnant is explained by the very recent report of GC–MS evidence that nandrolone is present in plasma throughout gestation [26].

Study of large populations is necessary to ensure that increasingly sensitive methods do not detect endogenous nandrolone metabolites as athletic positives. Alternatively, a quantitative value or ratio may be used to establish whether doping has occurred. However, introducing out-of-competition testing on athletes would remove the need for concentrating on sensitivity and retrospectivity.

The effect of the contraceptive lynoestrenol on the metabolite assay was minimal, the value for day 4 being just above the normal range. It is conceivable that the values for the metabolites might be slightly elevated due to the metabolism of lynoestrenol; *in vivo* studies on human subjects have indicated that lynoestrenol is metabolised to norethisterone, which is also an oral contraceptive [2]. This possibility has been strengthened by the characterisation of lynoestrenol metabolites in rabbit liver homogenates, showing the presence of norethisterone and an isomer of tetrahydronorethisterone [27]. Norethisterone in turn is metabolised in very small quantities to nandrolone and subsequently to 19-norandrosterone. However, the suggested metabolite cut-off point for a positive result was far above the day 4 assay value. The interference of the metabolites of lynoestrenol in the nandrolone assay was thus overcome by the introduction of the nandrolone metabolite assay.

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