

## REVIEW

## Urine nandrolone metabolites: false positive doping test?

R M N Kohler, M I Lambert

*Br J Sports Med* 2002;**36**:325–329

The aim of this review is to analyse the studies on nandrolone metabolism to determine if it is possible for an athlete to test positive for nandrolone without having ingested or injected nandrolone.

The anabolic androgenic steroid 19-nortestosterone, also called nandrolone, was first synthesised by Birch in 1950.<sup>1</sup> Nandrolone has an anabolic effect, and is used in the treatment of certain chronic diseases.<sup>1–3</sup> The use of nandrolone by athletes became popular in the late 1950s.<sup>4</sup> Athletes use nandrolone in an oral or injectable form to increase muscle strength and improve performance.<sup>5</sup> As a result of the potential performance enhancing benefits<sup>6</sup> and potential health risks associated with anabolic steroid use,<sup>7</sup> the International Olympic Committee (IOC) prohibited the use of nandrolone in sport in 1976.

“A doping offence for nandrolone was defined as a concentration of NA in human urine exceeding 2 ng/ml in men and 5 ng/ml in women”

When nandrolone is ingested or injected by humans subjects, three metabolites of nandrolone can be isolated and measured in the urine by gas chromatography-mass spectrometry. These metabolites have been identified as 19-norandrosterone (NA; 3 $\alpha$ -hydroxy-5 $\alpha$ -oestrane-17-one), 19-noretiocholanolone (3 $\alpha$ -hydroxy-5 $\beta$ -oestrane-17-one), and 19-norepiandrosterone (3 $\beta$ -hydroxy-5 $\alpha$ -oestrane-17-one).<sup>8–13</sup> These metabolites are isomeric compounds, having the same chemical composition and molecular mass but different chemical structure. NA is usually the most abundant urine metabolite of nandrolone.<sup>9–13</sup> The presence of these metabolites in the urine forms the basis of doping analysis for the illegal use of nandrolone by athletes.<sup>8, 12, 14–16</sup> This was based on the premise that these urine metabolites could only have been derived from exogenous nandrolone. A study in 1982 appeared to have found NA, or a similar compound, in the urine of athletes who had not used nandrolone.<sup>8</sup> In 1996, the IOC stated that a critical concentration for nandrolone metabolites in the urine had been established. A doping offence for nandrolone was defined as a concentration of NA in human urine exceeding 2 ng/ml in men and 5 ng/ml in women.<sup>17</sup>

Recently, the possibility of false positive tests for nandrolone has been raised. Explanations for false positive tests have included supplement contamination<sup>18</sup> and endogenous production of nandrolone and regulation of metabolic pathways of nandrolone metabolism by various physiological factors and supplement interventions. The aim of this review is to analyse the studies on nandrolone metabolism with the overall goal of determining whether it is indeed possible for an athlete to test positive for nandrolone without having either ingested or injected nandrolone. The question of a positive test resulting from nutritional supplements<sup>14, 18</sup> and food contamination<sup>19, 20</sup> is beyond the scope of this review.

**EVIDENCE FOR ENDOGENOUS NA**

The origin of endogenous NA in the urine of athletes who have not knowingly ingested or injected nandrolone is central to resolving the question of whether it is possible to have a false positive test. The first study to suggest that NA could be found in the urine of people free of exogenous nandrolone was a study on laboratory staff (n = 14).<sup>8</sup> Their urine was analysed using isotope dilution mass spectrometry, and NA or a similar compound was suspected. This suspicion was based on the detection of a small peak for the ion at m/z 256.<sup>8</sup> In retrospect, this signal may have been caused by interference of other endogenous compounds (noise) and perhaps represents a false positive finding. The authors acknowledged the limitations of the study, the analytical technique lacking specificity and sensitivity.

Studies in 1988 and 1990 again raised the possibility of endogenous NA in the urine of humans. Kicman and Brooks<sup>21</sup> used radioimmunoassay and measured NA in the urine of men and women, who were supposedly free of exogenous nandrolone, ranging from 3.8 to 49.4 ng/ml. However, these data should be interpreted with caution as it could be argued that the analytical technique again lacks both specificity and sensitivity. Debruyckere *et al*<sup>11</sup> measured NA in the urine of three subjects at concentrations of 9, 14, and 37 ng/ml.<sup>11</sup> These results were later attributed to nandrolone contaminated meat which the subjects may have eaten.<sup>19, 22</sup>

In 1996, the IOC declared that the presence of a small amount of NA in the urine was not considered to constitute a doping offence.<sup>17</sup> This suggests that they acknowledged the possibility

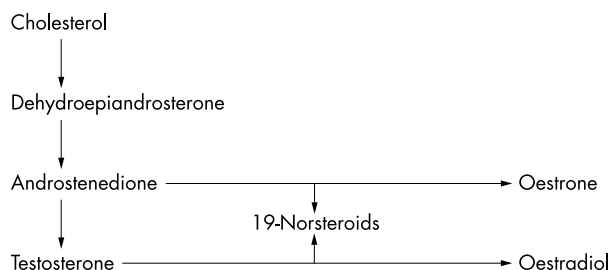
See end of article for authors' affiliations

Correspondence to:  
Dr Lambert, MRC/UCT  
Research Unit for Exercise  
Science and Sports  
Medicine, PO Box 115,  
Newlands 7925, South  
Africa; mlambert@  
sports.uct.ac.za

Accepted  
26 February 2002

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**Abbreviations:** IOC, International Olympic Committee; NA, 19-norandrosterone; GC-C-IRMS, gas chromatography-combustion-isotope ratio mass spectrometry



**Figure 1** The androgen biosynthetic pathway and the proposed aromatisation of androgen to oestrogen with the formation of 19-norsteroid intermediates.

of endogenous NA production. It can only be assumed that this decision was reached on the basis of data collected by IOC laboratories during routine drug testing, as the scientific evidence at the time was equivocal. In the late 1990s, analytical procedures for the detection and quantification of steroid metabolites in urine had become increasingly sensitive.<sup>23</sup> This may have accounted for an appreciable number of positive urine samples for NA being analysed in certain anti-doping laboratories. Many of the positive samples were from participants of sports that had previously not been associated with anabolic steroid use.<sup>1</sup> Further research with more sensitive equipment was undertaken to determine if NA could be produced naturally by the human body. This research showed convincing evidence that NA was found in the urine of subjects free of exogenous nandrolone.<sup>1 15 18 24-28</sup> The urine NA concentrations in these studies ranged from 0.01 to 1.79 ng/ml. In a study by Galan Martin *et al.*,<sup>29</sup> high NA concentrations in five sportspeople (4, 5, 6, 8, and 14 ng/ml) were measured. One woman in the study, who was postmenopausal, had a NA concentration of 22 ng/ml. It could be argued that these athletes had administered nandrolone.<sup>29</sup> These results are difficult to explain and perhaps further investigation of these subjects is necessary before a definite opinion can be formed.

## METABOLISM OF NA

### Aromatisation

Metabolic pathways for the endogenous production of NA in the human body need to be considered. Under normal circumstances, testosterone is aromatised to oestrogen by the aromatase enzyme complex.<sup>30</sup> Androstenedione, the direct precursor of testosterone, is also aromatised to oestrogen by the aromatase enzyme.<sup>31 32</sup> The important step in this metabolic process is the removal of the methyl group from the 19th carbon of either testosterone or androstenedione. Nandrolone differs structurally from testosterone and androstenedione in lacking the methyl group at the 19th carbon, and it is additionally different from androstenedione in substitution of a ketone group for a hydroxy group at the 17th carbon. Is it feasible that 19-norsteroids (nandrolone and metabolites) are intermediates in the aromatisation process? (fig 1).

Animal studies, *in vitro* experiments, and observations in humans, particularly pregnant women, add support to the proposal that 19-norsteroids are intermediates in the aromatisation of androgens to oestrogen.<sup>8 15 18 21 25 26 33-37</sup> Oestrogen concentrations in women increase significantly both at the time of ovulation and during pregnancy.<sup>26 38</sup> Raised urine NA concentrations have recently been identified in women at the time of ovulation<sup>26 39</sup> and during pregnancy.<sup>26</sup> Mareck-Engelke *et al.*<sup>40</sup> reported that during pregnancy the concentration of NA in human urine may reach 20 ng/ml. In these cases, pregnancy is confirmed with a blood test for human chorionic gonadotrophin.

A recent study by Reznik *et al.*<sup>25</sup> examined the sequelae of giving human chorionic gonadotrophin to 10 men. Human chorionic gonadotrophin increases serum testosterone in healthy men<sup>41-43</sup> and stimulates the aromatase enzyme, causing a gradual increase in serum oestrogen.<sup>41 44 45</sup> The serum testosterone and oestrogen increased in the 10 subjects after human chorionic gonadotrophin administration, and NA excretion in the urine increased by 250%. It may be concluded from this study that the increase in nandrolone biosynthesis was possibly associated with the increased aromatisation of testosterone to oestrogen.

"Factors that could increase the flux of androgen precursors through the testosterone biosynthetic pathway could theoretically increase the amount of nandrolone produced."

Although the pathways proposed are theoretical, the available evidence suggests that it is possible for the flux of androgen precursors through the testosterone biosynthetic pathway to result in the production of endogenous nandrolone. Therefore it can be assumed that factors that could increase the flux of androgen precursors through the testosterone biosynthetic pathway could theoretically increase the amount of nandrolone produced.

## FACTORS WITH THE POTENTIAL TO AFFECT NA METABOLISM

### Genetics

There is a wide range of serum testosterone concentrations in men,<sup>32</sup> suggesting large genetic interindividual and intraindividual variability in sex steroid production and excretion over a 24 hour period.<sup>46</sup> The possibility therefore exists that there is a variable rate of NA excretion.<sup>1</sup> Indeed, endogenous NA urine excretion in a male athlete varied by 680% over a three month period<sup>1</sup> and in another subject by 72% over a 24 hour period.<sup>15</sup> The enzyme complex 17 $\beta$ -hydroxysteroid dehydrogenase, which is responsible for converting androstenedione into testosterone, and the aromatase enzyme complex, which converts testosterone into oestrogen, occur in muscle and fat.<sup>31 47</sup> Therefore, it is conceivable that people with higher muscle and fat content may be more proficient in the production of 19-norsteroid intermediates. The aromatase enzyme complex *per se* can also show considerable genetic variability in expression and activity in certain people,<sup>48</sup> with increased activity of the aromatase enzyme producing larger amounts of oestrogen. This prompts the question of whether genetic upregulation of the aromatisation process in these people increases the production of 19-norsteroids.

### Exercise

Intense exercise has been associated with raised levels of NA in the urine.<sup>1 27 28</sup> Le Bizec *et al.*<sup>28</sup> studied professional soccer players over 19 months and collected 385 urine samples. Urine NA concentrations after soccer games were significantly higher than before games. For NA concentrations after games, 70% of the urine samples were below 0.1 ng/ml, and 20% were between 0.1 and 0.2 ng/ml. NA in four urine samples were above 1.0 ng/ml, the maximum value being 1.79 ng/ml.<sup>28</sup> When urine is tested for banned substances and the specific gravity of the urine sample is measured above 1.020, urine metabolite concentrations are adjusted by a correction factor.<sup>23</sup> This analysis is based on the premise that urine flow rate and urine metabolite excretion remain constant during and directly after exercise. However, this is an erroneous assumption as it has been shown that excretion of pseudophedrine after exercise was increased in subjects in whom urine volume remained constant.<sup>49</sup> Thus urine metabolite excretion may not remain constant during and directly after exercise, and

random urine sample collection after exercise may be unreliable.<sup>15, 42</sup> A more accurate measure would be to collect a urine sample over a 24 hour period, allowing the calculation of excretion rates of urine metabolites.<sup>15</sup> However, this is not practical, particularly when testing for drug use in sport.

The serum androgen response to exercise in athletes can vary according to the type, duration, and intensity of the exercise task.<sup>50-56</sup> Serum concentrations of testosterone, androstenedione, and dehydroepiandrosterone increase with short term, intense exercise as the result of increased testicular production by an unknown mechanism.<sup>57</sup> An increase in serum testosterone after exercise may also be caused by a decrease in the plasma volume<sup>58</sup> or a decrease in hepatic clearance.<sup>54</sup> The effect of exercise on serum oestrogen is also extremely variable.<sup>53</sup>

“A urine specimen collected after high intensity exercise could have a higher concentration of NA”

It is conceivable that the increase in circulating androgens in people participating in short duration, high intensity exercise could result in the stimulation of the aromatase enzyme complex, resulting in an absolute increase in the amount of NA in the urine. Therefore, there are sufficient data<sup>1, 27, 28</sup> to suggest that a urine specimen collected after high intensity exercise could have a higher concentration of NA for reasons other than dehydration.

#### Trauma and hypoglycaemic stress

As yet, no study has investigated the possible effect that traumatic stress (musculoskeletal injury) may have on 19-norsteroid metabolism. Interestingly, two international male athletes, one an international rugby player<sup>59</sup> and the other a paraOlympian (B Frasure, personal communication), recently tested positive for NA above 2 ng/ml, after both having suffered significant injuries just before passing a urine sample for drug testing. Both athletes claimed to be innocent of a doping offence. The concentration of NA in the urine samples of both athletes was about 6 ng/ml, which is slightly above the IOC cut off concentration for men (2 ng/ml).

Reznik *et al*<sup>23</sup> have provided some insight into the effect of a stress response on nandrolone metabolism. Hypoglycaemia was induced in 10 subjects by intravenous injection of 0.1 IU/kg insulin. Urine samples were collected at 0–2, 2–4, and 4–10 hours after the insulin injection. They concluded that hypoglycaemic stress did not significantly alter NA excretion. However, inspection of their data shows that, in certain subjects, NA excretion increased in the first two hours after induction of the hypoglycaemic stress. Had the study included more than 10 subjects, it is likely that there would have been sufficient statistical power to show that the increase in NA in the first two hours after hypoglycaemic stress would have produced a significant finding. Hypoglycaemic stress is associated with the production of glucose counter-regulatory hormones: cortisol, glucagon, growth hormone, and adrenaline.<sup>32</sup> Cortisol is produced in the adrenal cortex when stimulated by adrenocorticotropic hormone. The latter also stimulates the production of androgens and mineralocorticosteroids from the adrenal cortex.<sup>32</sup> It is tempting to speculate that the increased production of adrenal androgens results in increased NA excretion as described above. Further studies need to evaluate whether the increase in adrenal androgens and their aromatisation could produce any changes in NA excretion after traumatic musculoskeletal stress.

#### Mineral cofactors and herbal products

There is also a theoretical argument that certain substances not prohibited in sport may alter nandrolone metabolism. For example, the trace element zinc is a cofactor in many enzymic processes in the body. An increase in serum testosterone in

men who are marginally zinc deficient has been shown after zinc supplementation.<sup>60</sup> Also, diets deficient in zinc resulted in a significant decrease in serum testosterone concentration. Therefore it can be concluded that zinc supports testosterone production.<sup>60</sup> Although there is a linear relationship between serum zinc and serum testosterone concentrations,<sup>61</sup> it is not known whether supraphysiological doses of zinc are associated with higher levels of testosterone production. Certain athletes are marginally zinc deficient<sup>62</sup> because of inadequate intake<sup>63</sup> and considerable sweat loss.<sup>64, 65</sup> As zinc status may not be optimal in these athletes, can zinc supplementation enhance testosterone production and could this increase in testosterone production increase the production of aromatisation intermediates? This question was partially addressed when a zinc/magnesium supplement (30 mg zinc) was given to football players nightly for eight weeks. This treatment increased free and bound serum testosterone by about 33%.<sup>66</sup> These findings were not attributed to haemoconcentration because the blood samples were taken 24 hours after exercise. On the basis of the possibility that 19-norsteroid metabolism may be associated with testosterone metabolism and the aromatisation process, it is feasible that zinc supplementation, combined with exercise, may increase nandrolone metabolites in the urine.

The herbal product *tribulus terrestris* (tribestan), which has been used in Eastern cultures since ancient times to treat impotence and improve libido, is another substance that has been associated with an increased serum testosterone concentration (S Milanov, unpublished work). Could tribestan in combination with exercise increase NA in the urine? Further research is necessary.

#### CHALLENGING THE IOC CUT OFF CONCENTRATION FOR URINE NA

Until recently, studies involving large numbers of subjects to determine the physiological range for the concentration of NA in the urine of men and women free of exogenous nandrolone were lacking. Data on the range of NA could only be drawn from the analysis of urine from sedentary and recreational people at rest.<sup>1, 15, 18, 24, 25</sup> The total number of subjects from all these studies is about 150. No specific mention is made in any of the studies of the age of the male subjects. This is relevant because testosterone production decreases with advancing age.<sup>67</sup> Therefore one might expect 19-norsteroid production to decrease also with advancing age, making the age of study populations an important consideration. The amount of NA in the urine from the subjects did not exceed 1 ng/ml, except in the study of Galan Martin *et al*,<sup>29</sup> the concerns in which have already been raised.

“There is no explanation by the IOC of why the threshold concentration for NA is higher in women.”

Two recent studies involving larger numbers of sportsmen have provided further evidence. Urine samples collected after exercise in these studies showed that the concentration of NA in the urine increased, and in certain men the concentration of NA was close to the cut off concentration of 2 ng/ml.<sup>27, 28</sup> Should this be combined with other stressors and possible supplement interventions (mentioned above), the concentration of NA in the urine is most unpredictable.

The IOC have also apparently collected data and measured NA urine excretion in elite male and female athletes at the 1996 Nagano Olympic games, but these data have not been released into the public domain.<sup>68</sup> It would be beneficial for the IOC data to be made public to support reasoning behind the calculation of cut off concentrations for NA in the urine of men and women. There is also no explanation by the IOC of why the threshold concentration for NA is higher in women. If

the reason for it is the higher circulating levels of oestrogen, particularly at the time of ovulation, is this not indirect support for the presence of 19-norsteroids as intermediates in the aromatisation of androgens to oestrogen?<sup>26</sup> Bradford-Hill has stated: "It is the essence of science to disclose both the data upon which a conclusion is based and the methods by which the conclusion is obtained".<sup>69</sup>

The IOC has defended the status quo on nandrolone and confirmed these threshold values of 2 ng/ml in men and 5 ng/ml in women in Monaco in October 1999.<sup>70</sup> The conditions of strict liability are currently applied in the case of any athlete contravening the above thresholds.

## METHODS TO TEST FOR NA

A solution to the controversy surrounding nandrolone in sport is to develop a testing procedure that can accurately differentiate endogenous nandrolone metabolites from nandrolone that is ingested or injected. The technique of gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) to calculate the <sup>13</sup>C/<sup>12</sup>C ratio is currently being developed as a method to fulfil this purpose.<sup>1 71-73</sup> This is based on the principle that natural steroids have a different carbon isotopic signature from synthetic steroids. The <sup>13</sup>C/<sup>12</sup>C ratio for synthetic nandrolone metabolites is lower than that for endogenous metabolites, therefore administering exogenous nandrolone will lower this ratio. This ratio has also been proposed as a method of detecting the use of synthetic testosterone as an alternative to the testosterone/epitestosterone ratio.<sup>71-73</sup> However, a potential problem with GC-C-IRMS is the lack of reproducibility and sensitivity because of the low levels of endogenous nandrolone metabolites present in the body. At present, this method can only be applied to "high" concentrations of NA (60 ng/ml) in the urine.<sup>73</sup>

Le Bizec *et al*<sup>74</sup> has proposed examining the steroid conjugates as an additional criterion to distinguish between the endogenous or exogenous origin of nandrolone metabolites. Endogenous NA was found to be 30% sulpho-conjugated in contrast with administered nandrolone, which was found to be 100% conjugated to glucuronic acid when excreted in the urine.<sup>74</sup>

Kintz *et al*<sup>75 76</sup> proposed that analysis of hair samples from athletes is another option to consider for detecting the presence of exogenous nandrolone. The analysis of hair samples could be used to accurately verify positive results obtained by gas chromatography-mass spectrometry.

Until the hair sample and GC-C-IRMS techniques have been validated on a large scale, a prudent approach after the detection of NA in urine samples above the cut off concentration is for the athlete to have further blood tests before the sample is declared positive, as is done for athletes with a high testosterone/epitestosterone ratio.

## CONCLUSION

The abuse of the steroid testosterone presented a new problem for drug control in sport.<sup>42</sup> Perhaps the same can now be said for nandrolone. According to the Olympic movement anti-doping code, NA is not a prohibited substance.<sup>77</sup> However, should NA in the urine exceed a certain threshold concentration, the interpretation is that nandrolone has been ingested or injected. There is strong scientific evidence to show that NA can appear in the urine of people free of exogenous nandrolone. Evidence suggests that NA may occur as an intermediate in the aromatisation of testosterone to oestrogen. Recent evidence has shown that the amount of NA in the urine can be regulated by the administration of human chorionic gonadotrophin. Therefore, threshold concentrations for men (2 ng/ml) and women (5 ng/ml) as defined by the IOC are still open to debate because conclusive scientific evidence showing how these values may be altered by various physiological stimuli is lacking. In accordance with this,

## Take home message

19-Norandrosterone (NA) is produced endogenously as an intermediate in the aromatisation of androgen to oestrogen. Intense exercise may increase its concentration in the urine. Future laboratory testing methods need to distinguish endogenous from exogenous nandrolone metabolites accurately, as this has important implications for doping control in sport.

multicentre studies need to answer further specific questions on the current urine threshold concentrations for nandrolone metabolites and whether physiological stressors and permitted supplement interventions can alter NA excretion.

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## Authors' affiliations

R M N Kohler, M I Lambert, MRC/UCT Research Unit for Exercise Science and Sports Medicine, Department of Human Biology, University of Cape Town, Sports Science Institute of South Africa, Newlands, South Africa

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