Stability of 19-norandrosterone and 19-noretiocholanolone glucuronide in urine under various storage conditions

P J van der Merwe (PhD)
E Grobbelaar (MSc Chemistry)
South African Doping Control Laboratory, Department of Pharmacology, University of the Free State, Bloemfontein

Abstract

Introduction. The International Olympic Committee (IOC) has ruled that 19-norandrosterone should be quantified if present in low concentrations in urine of sport competitors. The question, however, is how stable is 19-norandrosterone in urine. In the light of this IOC ruling it is therefore important to know if the concentration of 19-norandrosterone can change during the transportation process of urine samples to the laboratory or during the handling and storage of the samples in the laboratory.

Objective. To study the stability of 19-norandrosterone and 19-noretiocholanolone glucuronide in urine under various storage conditions.

Method. Four subjects volunteered for the study. Two subjects received 100 mg of oral 19-norandrostenediol and two subjects 100 mg of oral 19-norandrosterone as a single dose. Fractional urine samples were collected over a period of 5 days. The urine samples collected on day 5 were analysed immediately for 19-norandrosterone and 19-noretiocholanolone concentrations and then stored under four different storage conditions. The samples were stored in direct sunlight, at room temperature (± 22°C) and refrigerated (± 5°C) were analysed on day 1 and day 3. The samples stored frozen (-20°C) were analysed on day 30 and day 60. 19-Norandrosterone and 19-noretiocholanolone were quantified using gas chromatography-mass spectrometry.

Results. There were no changes in the concentration of 19-norandrosterone or 19-noretiocholanolone when urine samples containing the glucuronide of these two products were stored under the various storage conditions.

Conclusion. Although it is recommended that urine samples should be stored frozen, this study showed that the concentration of 19-norandrosterone and 19-noretiocholanolone were not influenced by different storage conditions.

Introduction

Nandrolone and its precursors, 19-norandrostenediol and 19-norandrostenedione are metabolised in the human body to 19-norandrosterone (19-NA) and 19-noretiocholanolone (19-NE) (Fig. 1). These two metabolites are excreted in the urine in the glucuronide form, with 19-NA predominant.

It is well established in the literature\(^*\) that the presence of 19-NA in human urine may be the consequence of normal excretion but only in trace amounts. For this reason the International Olympic Committee (IOC) has established cut-off values in urine for 19-NA. These concentrations are 2 ng/ml for

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CORRESPONDENCE:

P J van der Merwe
Department of Pharmacology
University of the Free State
PO Box 339
Bloemfontein
9300
Tel: 051-401 3182
Fax: 051-444 1523
E-mail: gnmpvdm@med.uovs.ac.za

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Fig.1. Metabolism of nandrolone in humans.
male and 5 ng/ml for female athletes. A variety of evidence supports this IOC cut-off value* for male athletes. An endoge-
nous concentration exceeding this cut-off value was not proved
in the cited literature.

Urinary samples are not analysed immediately after collection
but are first transported to the laboratory. Since it has been
reported that bacterial activity may influence the concentra-
tion of certain steroids it is important to know if the concentra-
tion of 19-NA glucuronide can change during the transpor-
tation process of urinary samples to the laboratory or during the
handling and storage of the samples in the laboratory. If so, this
may lead to a false-negative or false-positive result.

No data are available on the stability of 19-NA and 19-NE
glucuronide in urine and therefore we undertook this study on
the stability of these two products in urine under various stor-
age conditions.

Objective
The aim of this study was the determination of the stability
of 19-NA and 19-NE glucuronide in human urine under various
storage conditions.

Method
Subjects
Four subjects received either a single oral 100 mg dose of 19-
norandrosteroneolol or 19-norandrostenedione (2 subjects for
each product). Fractional urinary samples were collected over a
period of 5 days to obtain information on the excretion profile of
these products. The project was approved by the Ethics
Committee of the Faculty of Health Sciences of the University
of the Free State and the subjects gave written informed con-
sent prior to entering the study.

Sample storage
Urinary samples obtained from the excretion on day 5 of each
product (one sample from each subject) were analysed direct-
ly after collection to obtain baseline concentrations and then
stored under various storage conditions. Samples were cho-
sen so as to obtain one low and one higher concentration for
both 19-NA and 19-NE.

Each urine sample was divided into 6 x 20 ml aliquots in
clear stoppered glass bottles and then stored as follows:

Short term stability: in direct sunlight, at room tempera-
ture (± 22°C) and in a refrigerator (± 5°C). Samples were
analysed after 1 and 3 days, respectively. The pH of all these
samples was measured prior to analysis.

Long-term stability: in a freezer at ± 20°C and then
analysed after 1 and 2 months, respectively.

Freeze and thaw stability: in a freezer at −20°C for 24
hours (duplicate samples from each subject). Half of the sam-
ples were thawed unassisted at room temperature while the
other half were thawed in warm water. When the samples were
completely thawed they were transferred back to the freezer
and kept frozen for another 24 hours. The cycle of thawing and
freezing was repeated two more times and then analysed on
the third cycle (3 days).

Quantification
The analytes 19-NA and 19-NE were quantified in duplicate
as follows:

Preparation of standards. Five standards were prepared
for each analyte by spiking blank urine samples with a fixed
amount of 17α-methyltestosterone (as internal standard) and
various amounts of the analytes to bracket the concentrations
of the test samples.

Solid phase extraction. A 2.5 ml aliquot of sample/stand-
ard was applied to a Sep Pak C18 cartridge which was pre-
treated with 5 ml methanol followed by 5 ml water. The
cartridge was washed with 5 ml water and the eluates eluted
from the cartridge with 2 ml methanol. The eluate was evapo-
rated to dryness under a stream of high-purity nitrogen.

Hydrolysis. The glucurono-conjugates of the analytes were
hydrolysed enzymatically by dissolution of the dry residue
in 1 ml phosphate buffer (pH 7) and incubation at 50°C with 25 μl
of β-glucuronidase (from Escherichia coli).

Liquid-liquid extraction. After the addition of 200 μl 20%
K2CO3/NaHCO3, the analytes were extracted with 4 ml n-pen-
tane. The organic phase was transferred to an ampoule and
evaporated to dryness under a stream of high-purity nitrogen.

Derivatisation. After the residue was left in a desiccator for
at least 1 hour, the trimethylsilyl derivatives were prepared by
adding 80 μl of MSTFA/NH4I/ethanolol (1000:2.3:1/v/v/v) and
heating at 60°C for 30 minutes.

Chromatography. Toluene (140 μl) was added and 1 μl
analysed by using GC-MSD operated in selected ion moni-
toring (SIM) mode. Each standard and test sample was analysed
by recording the responses (A) at the masses m/z 405 for 19-
NA and 19-NE and m/z 301 for the internal standard. Data pro-
cessing involved integration and the area ratios A19-NA/A19-NE
were used to construct calibration curves. The concentrations
of 19-NA and 19-NE were calculated from the calibration curves.

Results
According to the literature the accuracy of an analytical
method should be within 15%, i.e. repetitive measurements
on different days should not vary by more than 15%. This is indi-
cated in each graph by drawing the ± 10% lines. The concen-
trations of 19-NA and 19-NE as measured on the different days
were all, except for two, within the 10% boundaries. The two
concentrations outside these boundaries were well within 15%
and thus still acceptable.

The results of two of the urine samples are illustrated in Figs
2 and 3. The results of the other two samples were similar but
are not presented.

The pH values of the urine samples stored at the different
temperatures did not change over the 3-day period.

Short-term stability. Fig. 2a and Fig. 3a clearly illustrate that
both 19-NA and 19-NE glucuronide are stable when stored for
at least 3 days in direct sunlight, at room temperature and
in a refrigerator.

Long-term stability. From Figs 2b and 3b it is clear that
both 19-NA and 19-NE glucuronide are stable for at least 2
months when stored frozen at −20°C.

Freeze and thaw stability. Freezing and thawing had no influ-
ence on the stability (Figs 2c and 3c), even if the thawing
Fig. 2. Stability of 19-norandrostenediol or 19-norandrostenedione after the intake of 19-norandrostenediol or 19-norandrostenedione: (a) Short-term stability, (b) Long-term stability and (c) Freeze-thaw stability.

Fig. 3. Stability of 19-noretioccholanolone glucuronide after the intake of 19-norandrostenediol or 19-norandrostenedione: (a) Short-term stability, (b) Long-term stability and (c) Freeze-thaw stability.
was done by placing the samples in warm water.

**Discussion**

Transport and storage of urine samples at high temperatures may lead to bacterial degradation. It is documented⁳ that androsterone- and etiocholanolone glucuronide can be converted to 5α-androstan-3β-ol and 5β-androstan-3β-ol, respectively, by bacterial activity. Likewise it should be possible that bacterial activity can convert 19-NA and 19-NE glucuronide to 19-nor-5α-androstan-3β-ol and 19-nor-5β-androstan-3β-ol, which will cause a decrease in the concentration of 19-NA and 19-NE.

The results of this study clearly showed that there was no significant decrease in the concentration of 19-NA and 19-NE when urine containing glucuronides of these two products were stored under various conditions. It is therefore unlikely that the abovementioned conversion did occur.

Furthermore, elevated pH values are usually the first indication of bacterial activity but the pH values of the urine samples stored at different temperatures, even in direct sunlight, were stable indicating that no bacterial activity was present. This means that if 19-NA or 19-NE glucuronide is present in a urine sample from a competitor at a concentration just above the cut-off value set by the IOC, it should still be present in the sample at the same concentration when it arrives at the laboratory. Transport of the samples from the place of collection to the laboratory is normally by courier and usually does not take more than 3 days.

Also, the handling and storage of the samples in the laboratory will not influence the concentration. Although the stability was only tested for 3 days, no decreasing trend in the concentrations of both metabolites was observed for this period and it is therefore expected that no decrease will occur if the samples are stored for a longer period in a refrigerator.

Sometimes samples removed from the freezer are thawed in warm water to save time. It is advantageous to know that this process does not influence stability. It is therefore unlikely that storage and transport conditions will be responsible for a false-negative result for 19-NA.

One question that remains to be answered is if it is possible that the concentration of 19-NA can increase to give a false-positive result. This may occur if the volume is reduced by evaporation during the exposure of urine samples to high temperatures. Although the specific gravity or creatinine were not measured in this study it is highly unlikely that evaporation may have occurred, as the urine samples were stored in tightly sealed containers. Urine samples collected from competitors are also transported in tightly sealed containers and in the real life situation an increase in concentration due to evaporation is also highly unlikely. No significant increases in concentration of 19-NA or 19-NE were observed in this study.

During collection of a urine sample from a competitor the sample is divided into A and B sample containers and transported as such to the laboratory. The A samples are analysed while the B samples are stored in a freezer at −20°C. In the case of a positive test the competitor involved may request the analysis of the B sample and the analysis must take place within 30 days. The results of this study are of particular interest to this situation. The results prove that if 19-NA or 19-NE glucuronide is present in a urine sample stored in a freezer, these compounds are stable for at least 60 days. Analysis of the B sample containing 19-NA and 19-NE glucuronide within this time period should, within analytical variability, confirm the result of the A sample.

**Conclusion**

The results show clearly that there is no significant decrease in the concentration of 19-NA or 19-NE when urine containing the glucuronides of these two products are stored under various storage conditions. Although it is recommended that urine samples should be stored frozen, it is beneficial to know that the concentration of 19-NA and 19-NE in urine samples will not be influenced by other storage conditions.

**References**