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19-Norandrosterone - Criteria for the Decision Making Process

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Abstract

Endogenous production of norandrosterone and meat consumption of nandrolone treated animals lead to urinary excretion of small amounts of norandrosterone.

Former misuse of nandrolone preparations show similar results.

At this time, it is not possible to discern clearly between endogenous production, consumption of contaminated meat or misuse.

In fact, it is difficult to assess urines containing small amounts of norandrosterone.

Pregnancy and application of norethisterone containing preparations (for female urine) excluded, analytical confirmation of norandrosterone is necessary.

Appropriate tools of judgment include endocrinological studies and isotope ratio mass spectrometry.

Introduction

Endogenous nortestosterone and metabolites are detected in urines of stallions, boars and specific pregnant female animals (cow, sheep, goat, deer).

Application of oral contraceptives containing norethisterone and pregnancy lead also to the urinary excretion of norandrosterone.

Nortestosterone containing drugs represents one of the most popular steroids on the market even leading to the prohibited cattle feeding.

Small amounts of nandrolone metabolites are detectable in the urine after consumption of meat generated from nandrolone treated animals (1).

The misuse of nortestosterone prepares by athletes to enhance their physical performance is well known. Varieties of nandrolone preparations are available on the legal and black market.

Moreover, nandrolone is often used for counterfeits (2).

The majority of the applied nandrolone products are injectable long-term preparations. The metabolite norandrosterone is detectable in the urine over a long period.

A. Procedure to detect nandrolone suspicious urine samples in doping analysis

I. Female urines

The following criteria for female urines should be taken into consideration (3). The sample should not be described positive if one of these criteria is correct.

Norethisterone

Norethisterone is frequently used as gestogen-compound in oral contraceptives. Two metabolites are detectable: norandrosterone and H4-norethisterone (Fig 1).

In the case, that both metabolites are detected norandrosterone indicates to be a metabolite of norethisterone.

Pregnancy

Norandrosterone is detected sometimes in urines of pregnant women. Some studies detected norandrosterone in the later phase of pregnancy (after week 14). The calculated concentrations were situated between 1 and 23 ng/ml.

The presence of norandrosterone caused by pregnancy influences the steroid profile parameters as follows: The concentration of pregnandiol and estrogens increases strong due to a missing feed back mechanism. Coelution with endogenous steroids and internal standards lead to shifts in retention times and bad chromatography (Fig. 2).

Determination of HCG is the best method to confirm pregnancy.

II. Confirmation of norandrosterone (4,5,6,7)

Fig. 3 shows the flow scheme of the standard operating procedure for confirmation of nandrolone metabolites.

The separation of the free fraction is added as additional cleaning step to the routine sample preparation. Also an extraction with n-pentane (instead of ether) is suggested to separate coeluting vitamine E (Fig.4).

Low resolution SIM-confirmation is performed for two different norandrosterone-derivatives:

- a) mono-TMS-norandrosterone and
- b) bis-TMS-norandrosterone

(GC/MS-parameters and ion masses are shown in tables 1-3.)

Criteria for evaluation of the received SIM-spectra (according to IOC-rules):

Three diagnostic ions are monitored.

The signal to noise ratio of the diagnostic ions has to be greater than three to one.

The relative abundance of any ion shall not differ by more than five per cent (absolute) or twenty per cent (relative), whichever is greater (positive control urine or repeated urine sample).

| | |
|----------------------------|---|
| GC/MS | HP 5890 II / HP 5971A |
| column | 17m Ultra 1 (OV1), 0.2 mm id, 0.11 μ m film thickness |
| carrier gas | 1ml helium at 180°C, split 1:10 |
| GC temperature program | 181°C, 3°C per min, 230°C, 40°C per min, 320°C |
| injection port temperature | 300°C |
| transferline temperature | 320°C |

Tab 1: Standard Operating Procedure (SOP) - Confirmation of nandrolone metabolite GC/MS-parameters

| m/z | fragment ions |
|--------|---|
| 348.20 | molecular ion (M^+) |
| 333.20 | M^+ - 15 (CH_3) |
| 258.20 | M^+ - 90 ($SiOC_3H_{10}$) |
| 276.20 | D4-Etiocholanolone-Mono-TMS - 90 (ISTD) |

Tab 2: Standard Operating Procedure (SOP) - Confirmation of nandrolone metabolite Table of ion masses used for selected ion analysis of 5 α -estran-3 α -ol-17-one-TMS (Norandrosterone-Mono-TMS)

| m/z | fragment ions |
|--------|--|
| 169.20 | d-ring-fragment |
| 225.20 | M^+ - 15 - 90 - 90 ($CH_3 - 2SiOC_3H_{10}$) |
| 315.20 | M^+ - 15 - 90 ($CH_3 - SiOC_3H_{10}$) |
| 405.20 | M^+ - 15 (CH_3) |
| 420.20 | molecular ion (M^+) |
| | |
| 434.20 | Androsterone / Etiocholanolone-Bis-TMS (M^+) |
| 438.20 | D4-Etiocholanolone-Bis-TMS / M^+ (ISTD) |
| 446.20 | Methyltestosterone-Bis-TMS / M^+ (ISTD) |
| 333.20 | Norandrosterone-Mono-TMS (M^+ - 15) |

Tab 3: Standard Operating Procedure (SOP) - Confirmation of nandrolone metabolite Table of ion masses used for selected ion analysis of 5 α -estran-3 α -ol-17-one-TMS (Norandrosterone-Bis-TMS)

III. Quantification of norandrosterone / statistical test

For quantification the suspicious urine and a positive control urine are prepared three times each and injected twice. The concentration of norandrosterone in the positive control urine should be similar to the expected concentration in the suspicious urine sample.

Evaluation of the data should be performed by calculation of a confidence interval for the observed difference of concentrations.

This method is based on t-statistics. It allows evaluation respective to the critical concentration for a given probability.

According to IOC-rules the cut off limits are 5 ng/ml for female and 2 ng/ml for male.

IV. Further aspects: noretiocholanolone and nortestosterone

Noretiocholanolone

The second metabolite of nandrolone usually is excreted in lower concentrations than norandrosterone.

For urines containing low concentration of norandrosterone the concentration of noretiocholanolone may be below the detection limit.

Additional confirmation of noretiocholanolone is not necessary.

Nortestosterone

Excretion of the parent compound is detectable short time after nandrolone application.

Later the excretion decreases below the detection limit.

B. Differentiation between endogenous production, consumption of contaminated meat and misuse

Experimental

Two studies were performed to obtain more information to determine the difference between consumption of contaminated meat and misuse.

- I. Fig 5 represents misuse: a single injection of nandrolone decanoate (50 mg) is shown. Over a time period of 13 months 8 urine samples were taken.
- II. Fig 6 shows the results of a single oral application of 20 µg nandrolone. This study may represent consumption of contaminated meat. (Similar results will be expected by misuse of oral nandrolone preparations.) 10 urine samples were taken within 24 hours.

Results and Discussion

Both studies were evaluated according to following criteria:

1. Cut off limits

According to IOC-rules the cut off limits are 5 ng/ml for female and 2 ng/ml for male.

2. Ratios

The area ratio between androsterone (A) and norandrosterone (N1) was used previously as additional tool. This ratio is based on long time history bearing no scientific basis.

A/N1 (m/z: 434/405) with the cut off limit 500 was also the confirmation limit for norandrosterone with low resolution mass spectrometry.

For inclusion not symmetric peakshapes and different excretion due to enzym activity androsterone (A) as well as etiocholanolone (E) should be used..

Proposal may be the area ratio (A+E)/N1 (m/z: 434/405) with cut off limits of 1000 for male and 500 for female. These limits correspond frequently with norandrosterone concentrations of 2 ng/ml for male and 5 ng/ml for female.

In study I. norandrosterone was confirmed in each urine sample collected after injection of nandrolone decanoate. The urine taken last still shows a concentration of 3.6 ng/ml which has to be judged for male as positive, for female as negative case. Each corresponding area ratio (A+E/N1) shows a positive result.

In study II. after oral application the concentration of norandrosterone decreases very fast. For female the concentration cut off limit is reached after 8 hours, the male one after 15 hours. Each corresponding area ratio (A+E/N1) shows a negative result.

Comparison of area ratios (A+E/N1) of both studies: Due to the fact, that the calculated area ratios show different results depending on the application form of nandrolone further investigations are necessary to determine whether the area ratio (A+E/N1) is an appropriate and more secure source for decisive information.

| sample | date | C(Norand.) [ng/ml] | Q(area) (A+E)/Norand. |
|--------|----------|-----------------------|--------------------------|
| 1 | 28.03.97 | 88.2 | 1.7 |
| 2 | 30.04.97 | 35.5 | 3.2 |
| 3 | 22.05.97 | 98.3 | 4.2 |
| 4 | 25.06.97 | 59.9 | 11.3 |
| 5 | 31.07.97 | 42.4 | 14.2 |
| 6 | 01.09.97 | 19.1 | 29.7 |
| 7 | 29.09.97 | 36.7 | 48.5 |
| 8 | 21.01.98 | 3.6 | 191.2 |

Fig 5: Injection of Nandolone-Decanoate
last injection: 31.12.96 (50 mg)

| sample | h | C(Norand.) [ng/ml] | Q(area) (A+E)/Norand. |
|--------|------|-----------------------|--------------------------|
| 1 | 1 | 3.6 | 467 |
| 2 | 2 | 30.0 | 65 |
| 3 | 3 | 22.1 | 100 |
| 4 | 4 | 9.5 | 324 |
| 5 | 5 | 9.1 | 601 |
| 6 | 8 | 4.0 | 1424 |
| 7 | 11.5 | 2.7 | 1854 |
| 8 | 15 | 2.1 | 2004 |
| 9 | 21.5 | 1.3 | 4173 |
| 10 | 24 | 1.6 | 3099 |

Fig 6: Oral application of 20 µg Nandrolone
volunteer: male, 34 years, 171 cm, 65 kg

Conclusion

At this time no differentiation between consumption of meat from nandrolone treated animals and misuse of nandrolone preparations is possible.

The main intention for judging criteria should be the prevention of false positive results and protection of the athlete.

Norandrosterone generated from endogenous production or consumption of contaminated meat should be excluded even with the ambiguity of positive samples regarded probably negative.

Proposed solution may be:

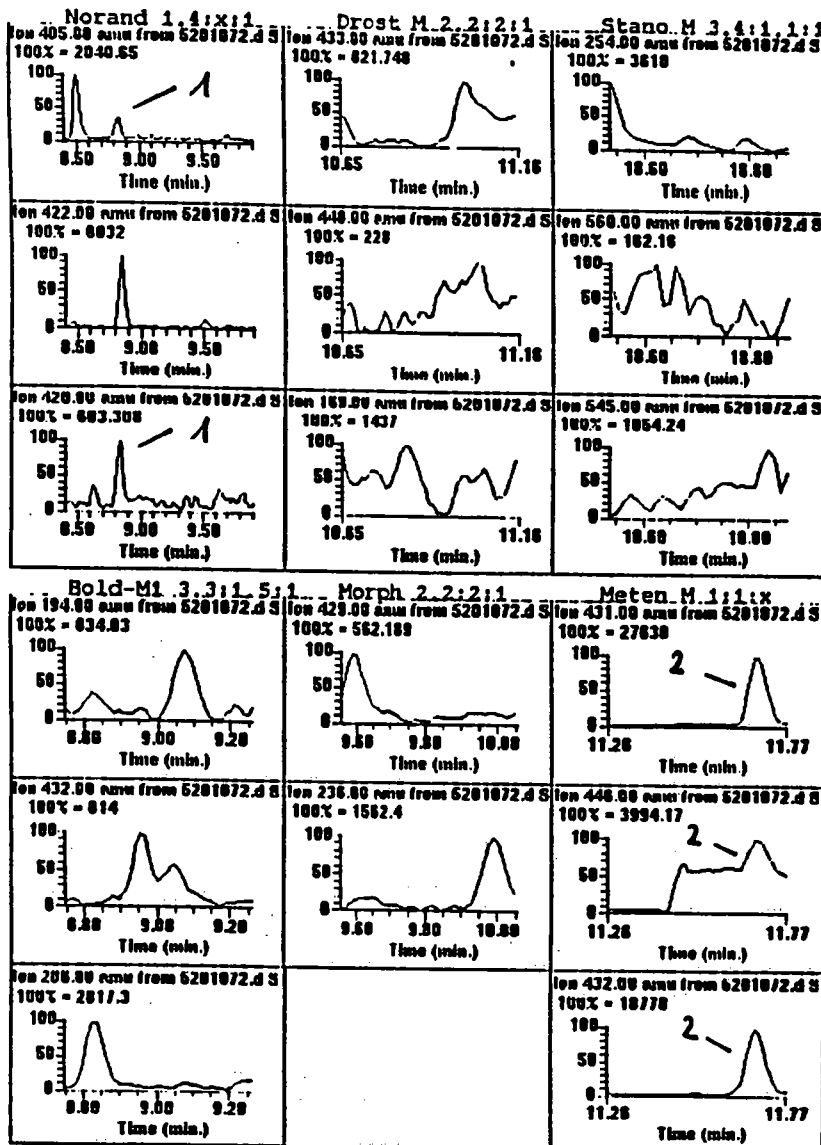
a) Investigations of norandrosterone with isotope ratio mass spectrometry is expected to lead to a differentiation between endogenous excretion and exogenous application.

b) Endocrinological studies should be performed similar to testosterone suspicious urine samples (8). Several urine samples will be collected over a period of days. For urines containing norandrosterone depending on oral application or consumption of contaminated meat the concentration will decrease significantly within this collection period.

Urines with norandrosterone from injection of nandrolone-preparations will show norandrosterone concentrations with little variation over a long time period.

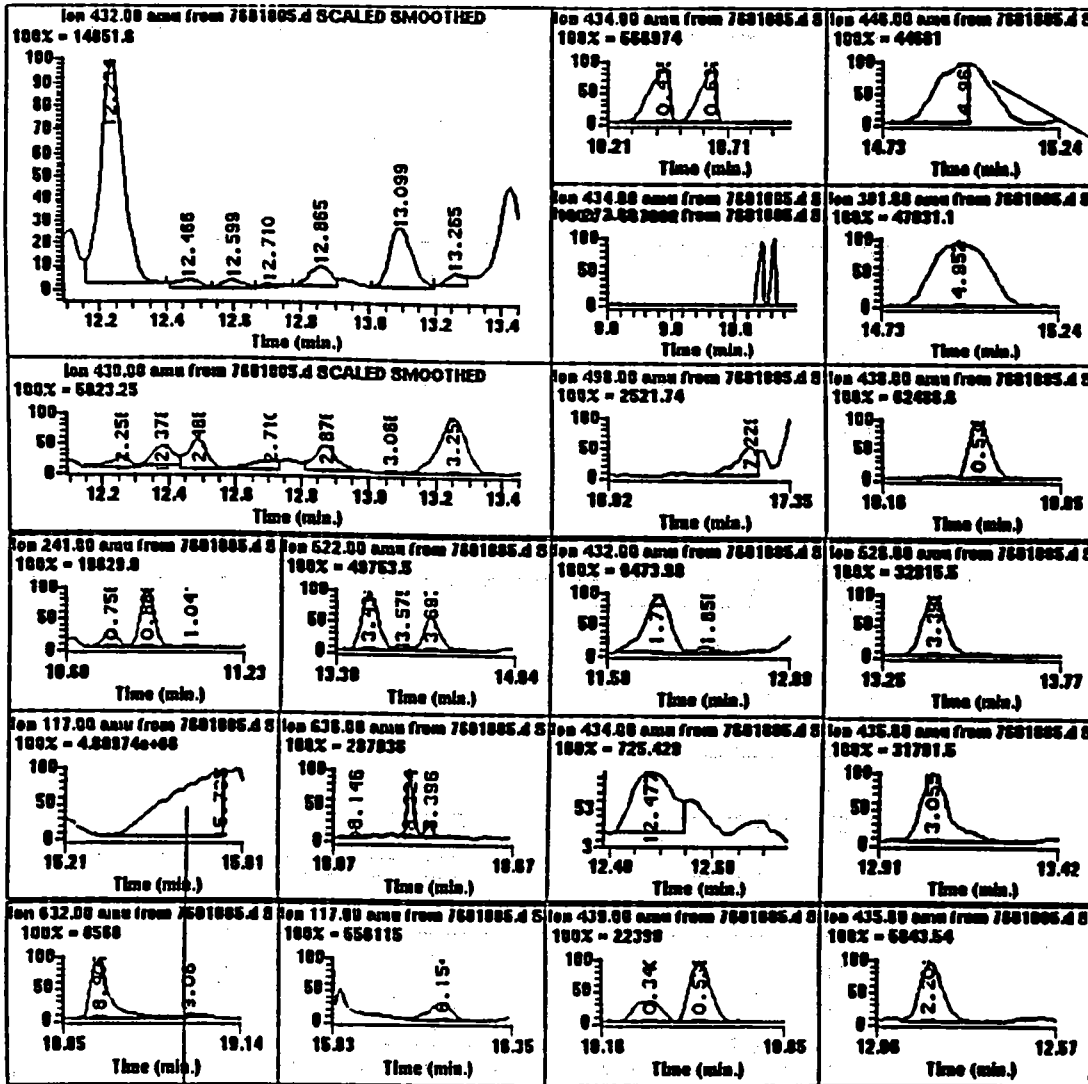
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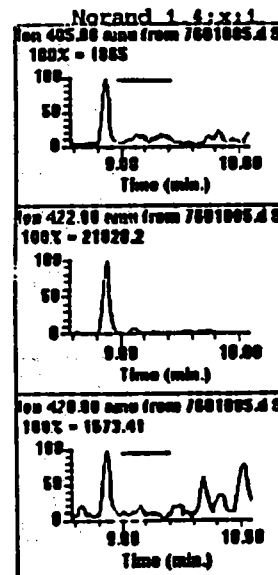
1. norandrosterone-bis-TMS
2. H4-norethisterone-bis-TMS

Fig 1: Screening printout of anabolic steroids (combined fraction) after application of norethisterone containing oral contraceptive



17 α -
methyl-
testosterone

Pregnandiol (20.000 ng/ml)



Norandrosterone
Q405 = 294
conc. = 23 ng/ml

Fig 2: Screening printout - steroid profile of a pregnant women

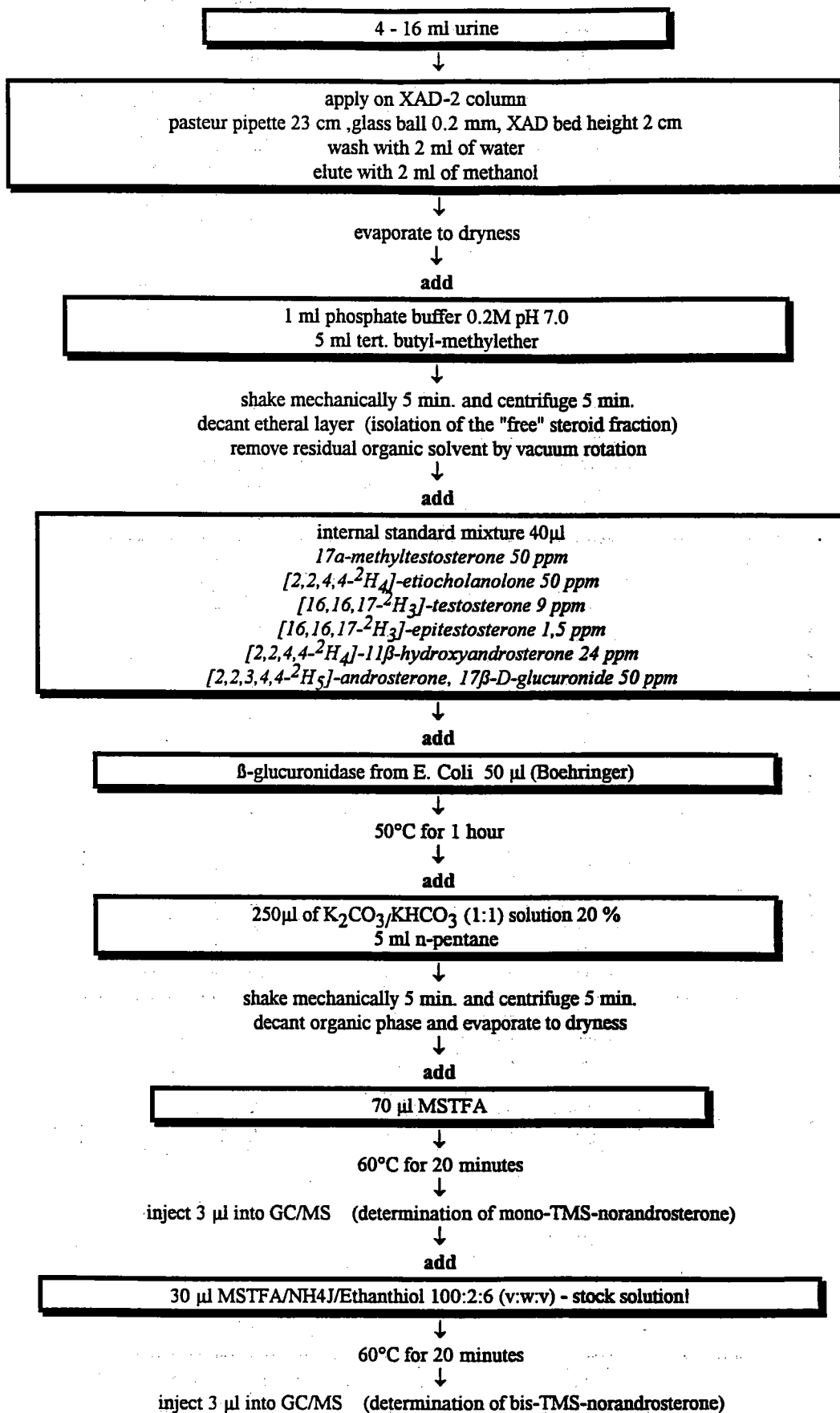
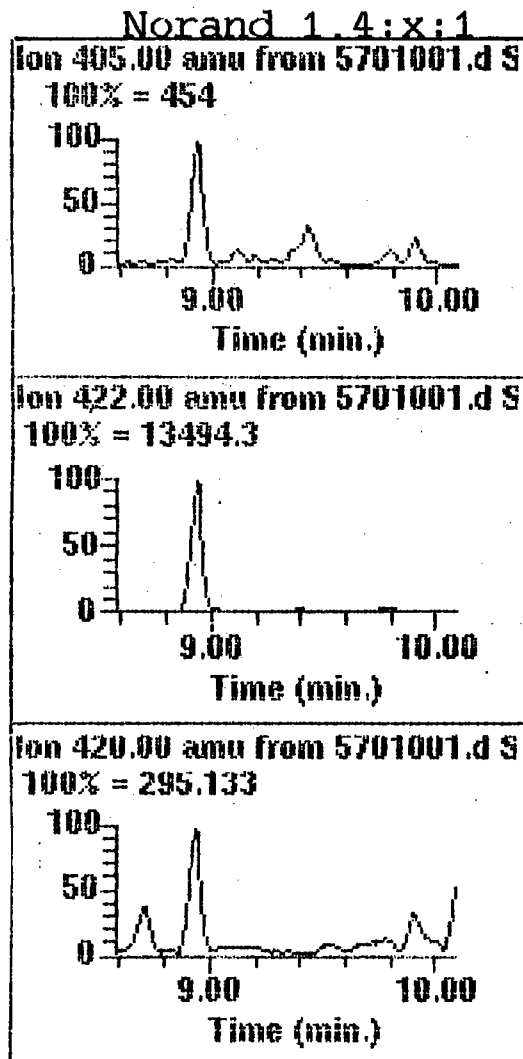
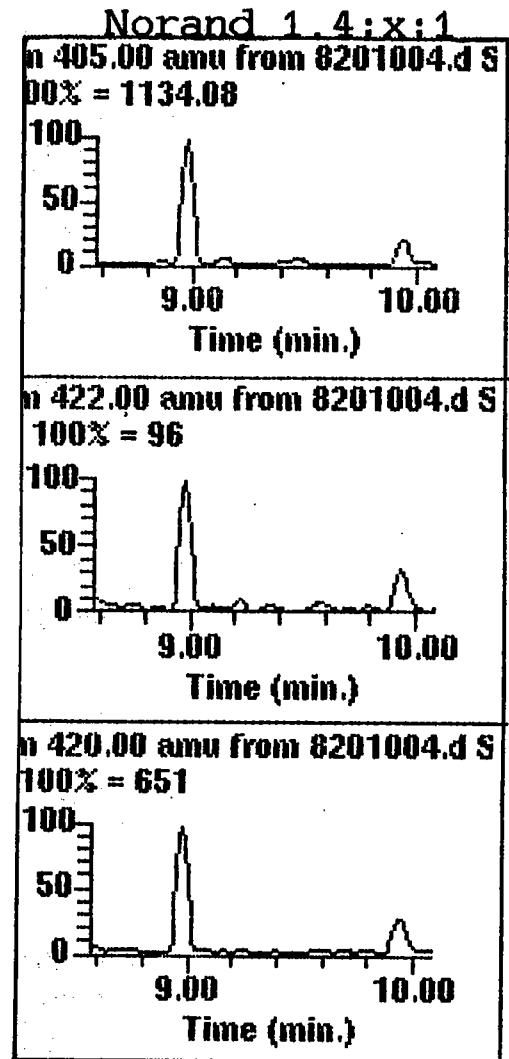


Fig 3: Standard Operating Procedure - Confirmation of nandrolone metabolites



a)



b)

Fig 4: Screening printout of norandrosterone

a) Routine sample preparation (combined fraction; 2 ml urine)

b) Extraction with n-pentane (confirmation; 4 ml urine)