J. steroid Biochem. Vol. 17, pp. 447 to 451, 1982
Printed in Great Britain. All rights reserved

0022-4731/82/100447-05$03.00/0
Copyright © 1982 Pergamon Press Ltd

DETECTION AND QUANTITATION OF 19-NORANDROSTERONE IN URINE BY ISOTOPE DILUTION–MASS SPECTROMETRY

INGEMAR BJÖRKHEM and HÅKAN EK
Department of Clinical Chemistry and the Research Centre at Huddinge University Hospital, Karolinska Institutet, Stockholm, Sweden

(*Received 5 February 1982*)

SUMMARY

A highly accurate method has been developed for detection and quantitation of 19-norandrosterone, the major urinary metabolite of 19-nortestosterone in man. A suitable ^{14}C labelled standard was obtained by i.m. injection of [4-^{14}C]-19-nortestosterone into a human volunteer. A fixed amount of this internal standard was added to a fixed amount of urine and the mixture was treated with *Helix pomatia* for 24 h. After extraction and purification by t.l.c., the mixture was converted into methoxime-trimethylsilyl derivative and analyzed by combined GC–MS. Unlabelled 19-norandrosterone could be quantitated from the ratio between the tracings of the ions at m/z 256 and m/z 258, corresponding to M-90–31 ions. In alternative procedures the ions at m/z 346 and 348 (corresponding to the M-31 ions) could be used.

Under the conditions employed, urinary 19-norandrosterone could be identified and quantitated in concentrations exceeding 20 ng/ml. The steroid could be traced in urine up to 6 weeks after i.m. administration of 25 mg of the decanoate of 19-nortestosterone (Deca-Durabol®). When using radioimmunoassay with antibodies towards unmetabolized 19-nortestosterone, it was possible to trace urinary 19-nortestosterone only for 1–2 weeks after the administration. The present method has been successfully used for analysis of 19-norandrosterone in urine samples obtained from athletes involved in competition.

INTRODUCTION

It is well known that 19-nortestosterone and different esters of it are used as doping agents in man and horses in connection with competitions. Sensitive and accurate tests are required to detect illegal use of this steroid. Antibodies have been raised against 19-nortestosterone [1–5] and radioimmunoassay can thus be used for detection of this compound in urine. 19-Nortestosterone as well as its esters are however extensively metabolized into 19-norandrosterone and 19-noretiocholanolone in human subjects [6–8]. In horses, estrane-3,17-diols and various 16-hydroxylated metabolites are formed in addition to 19-norandrosterone and noretiocholanolone [9–11]. A sensitive assay should therefore preferably be directed towards the metabolites rather than towards unmetabolized 19-nortestosterone. In doping-detection laboratories, radioimmunoassay is often used as a screening test and positive samples are then analyzed for presence of the major metabolites of 19-nortestosterone by combined gas chromatography–mass spectrometry (GC–MS). From a legal point of view, only the latter method gives sufficient evidence that this specific anabolic steroid has been administered.

In previous publications, we demonstrated the usefulness of isotope dilution–mass spectrometry for doping analyses of anabolic steroids. Such specific and accurate methods have hitherto been developed for methandienone [12] and stanozolol [13].

In the present work we describe such a method also for assay of 19-norandrosterone, the major urinary metabolite of 19-nortestosterone. This method has proved to be of great value in doping analyses of urine samples obtained from different competitions to provide evidence that doping has been carried out.

Nomenclature: 19-Nortestosterone, 17β-hydroxy-4-estren-3-one; 19-norandrosterone, 3α-hydroxy-5α-estran-17-one; 19-noretiocholanolone, 3α-hydroxy-5β-estran-17-one.

MATERIALS AND METHODS

Materials

[4–^{14}C]-19-nortestosterone (SA 60 mCi/mmol) was obtained from Radiochemical Centre, Amersham, England. Unlabelled 19-norandrosterone and 19-noreticholanolone were kind gifts from Dr Stárka and Dr Hampl at the Research Institute of Endocrinology, Prague, Czechslovakia. Blank urine samples were collected from the laboratory staff.

Preparation of [4-^{14}C]-19-norandrosterone

A healthy male volunteer (IB) was injected intra-

muscularly with 15 μCi of the above 19-nortestosterone dissolved in a mixture of 0.5 ml ethanol and 1.5 ml of sterile saline solution (0.9%, w/v). The collection of urine started immediately and the urine samples were stored frozen at −20°C until used. See Results for details with respect to recovery and identification of 19-norandrosterone.

Radioimmunoassay

The radioimmunoassay kit for analysis of anabolic steroids was obtained from Professor R. V. Brooks at St. Thomas Hospital, London, U.K. [1]. The antibodies had a small cross reactivity towards 19-norandrosterone and 19-noreticholanolone (<3%). The analysis was performed according to the instructions, with one exception. After treatment with dextran-coated charcoal and centrifugation, an aliquot of the supernatant was used for gamma counting.

Preparation of samples for isotope dilution—selected ion monitoring

Urine from the first hours after injection of [4-^{14}C]-nortestosterone was used directly as source of internal standard. Thus 0.5 ml of this urine, containing about 100 ng of [4-^{14}C]-19-norandrosterone, was added to 1 or 2 ml of the urine samples to be analyzed. Standard mixtures were obtained by using 2 ml of urine from an untreated subject together with 0, 20, 50, 100, 200, 400, 800 and 1600 ng of unlabelled 19-norandrosterone. *Helix pomatia* digestive juice, 50 μl, was added together with 1 ml of 0.15 M sodium acetate buffer, pH 4.6, and the mixture was incubated at 37°C for 24 h. The steroids were then extracted twice with 5 ml of ethyl acetate and the combined organic phases were washed twice with 0.1 M NaOH (2 ml), and once with distilled water (2 ml). The organic phase was dried with anhydrous sodium sulfate and the solvent was evaporated under a stream of nitrogen. The residue was subjected to preparative t.l.c., using chloroform–ethyl acetate (1:1, v/v) as solvent. The zone containing 19-norandrosterone (R_f = 0.58) was detected by radioscanning, using a Berthold Dunnschicht-Scanner II (Wildbad, GFR). The appropriate zone was scraped off and eluted with methanol. After evaporation of the solvent, the material was converted into the methoxime—trimethylsilyl derivative [14]. Before analysis by GC–MS, the mixture was extracted with 50 μl of hexane.

Combined gas chromatography–mass spectrometry

About 5 μl of the above hexane solution was analyzed by GC–MS using an LKB 9000 or 2091 instrument equipped with a 1.5% SE-30 column (on Chromosorb W, 80–100 mesh, 2 mm × 1.5 M). The carrier gas was helium and the flow rate was 30 ml/min. The temperature of the column was about 260°C. The electron energy was set to 20 eV. The first channel of the multiple ion detector (MID) was focused on the ion at m/z 256 and the other on the ion at m/z 258. In general the amplification of the tracing of the ion at m/z 258 was three times higher than that of the tracing of the ion at m/z 256. In an alternative procedure, the ions at m/z 346 and m/z 348 were used in the analysis.

RESULTS

Preparation of [4-^{14}C]-19-norandrosterone

[4-^{14}C]-19-Nortesterone, 15 μCi, with about 85% excess with respect to ^{14}C, was administered intramuscularly to a volunteer (IB) as described in detail in Methods. Of the total administered dose, about 46% was recovered in urine after the first 4 h and additional 28% during the subsequent 20 h After hydrolysis with *Helix pomatia* digestion juice, extraction and t.l.c., it was shown that about 75% of the radioactivity had chromatographic properties as 19-norandrosterone and about 21% as 19-noretiocholanolone (Fig. 1). Less than 4% of the radioactivity had chromatographic properties of unmetabolized 19-nortestosterone. Combined GC–MS confirmed the identity as [4-^{14}C]-19-norandrosterone and 19-noretiocholanolone, respectively. The 19-norandrosterone contained about 75% of ^{14}C. Apparently there had been dilution with small amounts of endogenous 19-norandrosterone or some compound(s) similar to this steroid during the conversion.

Assay of unlabelled 19-norandrosterone

In Fig. 2, the mass spectrum of methoxime-trimethylsilyl ether of unlabelled 19-norandrosterone is shown. The ions at m/z 256 (M-90-31), and m/z 346 (M-31) were found to be suitable for a mass fragmentographic assay.

In Fig. 3, the ions at m/z 256 and m/z 258 were followed through a gas-chromatographic analysis of a hydrolyzed and purified extract of a combination of 0.5 ml of urine containing about 100 ng of [4-^{14}C]-19-norandrosterone and 2 ml of urine from an untreated subject. Only a small peak was obtained in the tracing of the ion at m/z 256, corresponding to unlabelled norandrosterone, whereas a prominent peak was obtained in the tracing of the ion at m/z 258, corresponding to 4-^{14}C-labelled internal standard. In Fig.

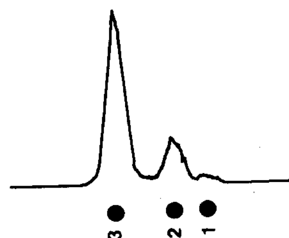

Fig. 1. Thin layer radiochromatograms of hydrolyzed urinary products isolated after injection of [4-^{14}C]-19-nortestosterone (*cf.* Results). Chloroform–ethyl acetate (1:1, v/v) was used as solvent. 1, 19-nortestosterone; 2, 19-noretiocholanolone, 3, 19-norandrosterone.

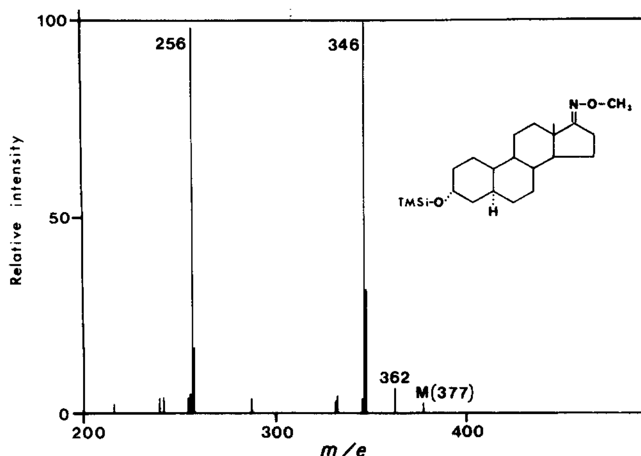

Fig. 2. Mass spectrum of methoxime–trimethylsilyl ether derivative of unlabelled 19-norandrosterone.

3B, 200 ng of unlabelled 19-norandrosterone had been added to the urine sample. A prominent peak was now obtained also in the tracing of the ion at *m/z* 256. In Fig. 2C, a corresponding mass fragmentographic tracing is shown obtained in the analysis of a urine sample from a subject who had been treated with 25 mg of Deca-Durabol® 2 days prior to the collection of the urine sample.

Identical results were obtained when using the ions at *m/z* 346 and *m/z* 348 as those obtained with *m/z* 256 and *m/z* 258.

A standard curve was constructed by addition of increasing amounts of unlabelled 19-norandrosterone to urine samples from untreated subjects. Figure 4 shows the result. Due to the presence of small amounts of unlabelled 19-norandrosterone (or a similar compound) in the internal standard (see above) and in the urine from the untreated subject (maximally about 5 ng/ml), there was an intercept. Due to the contribution at *m/z* 258 from unlabelled 19-norandrosterone from the natural isotopic abundance, there was no linearity in the higher concentration range. After correction for the intercept, and the overlapping at *m/z* 258 from the unlabelled derivative, the standard curve was linear with increasing concentrations of unlabelled 19-norandrosterone.

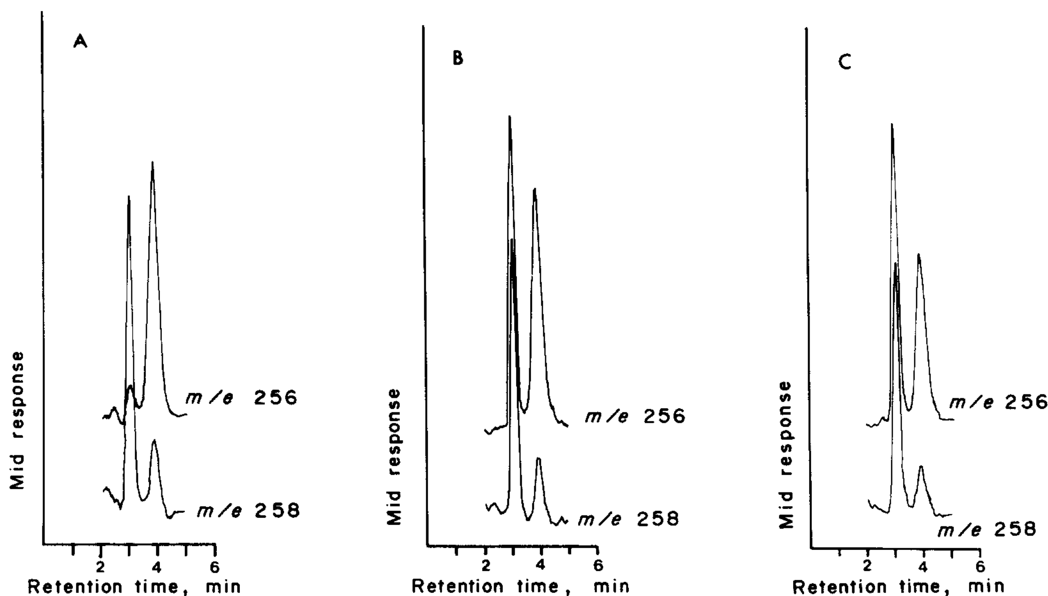

Fig. 3.(a) Multiple ion detector recording of derivative of a purified extract of 0.5 ml of urine containing 100 ng of [4-^{14}C]-19-norandrosterone and 2 ml of urine from an untreated subject. (B) Multiple ion detector recording of the same extract as above with the exception that 200 ng of unlabelled 19-norandrosterone had been added to the urine. (C) Multiple ion detector recording of derivative of a purified extract of 0.5 ml of urine containing 100 ng of [4-^{14}C]-19-norandrosterone together with 2 ml of urine from a subject to which 25 mg of Deca-Durabol® had been administered. The amplification of the recording of the ion at *m/z* 256 and *m/z* 258 was 300 × and 900 ×, respectively.

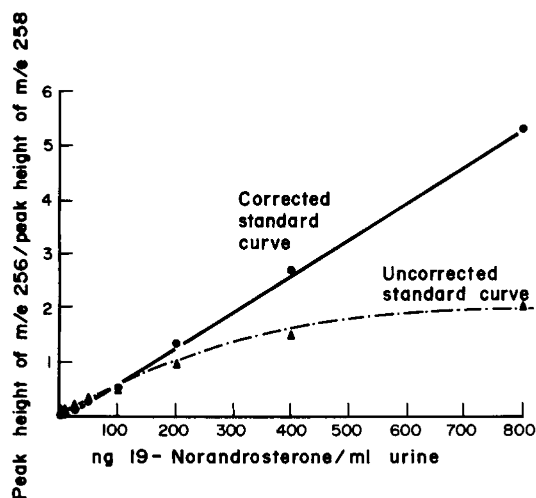

Fig. 4. Standard curve for determination of 19-norandrosterone in the range 0–800 ng/ml (for details, see Materials and Methods).

As pointed out above [4-^{14}C]-19-norandrosterone had apparently been diluted with small amounts of endogenous 19-norandrosterone or some similar interfering compound during its formation from [4-^{14}C]-nortestosterone. Thus it was important to exclude the possibility that some subjects may have such a high excretion of endogenous 19-norandrosterone that it could influence the present assay. When using the above standard curve, however, urine samples obtained from 14 untreated subjects (7 males and 7 females) were found to contain less than 5 ng/ml of 19-norandrosterone.

The precision of the assay was tested by five replicate measurements of urine samples containing about 100 and 300 ng of 19-norandrosterone. The coefficients of variation were 6 and 2%, respectively.

Comparison between isotope dilution–mass spectrometry and radioimmunoassay

After intramuscular administration of 25 mg of Deca-Durabol® to a male volunteer, significant amounts (more than 20 ng/ml) of 19-nortestosterone could be detected in urine for about 6 weeks (Fig. 5A). The total excretion of the metabolite during this period of time was calculated to be about 7 mg. The same urine samples were also tested by radioimmunoassay, using antibodies directed towards 19-nortestosterone [1]. This radioimmunoassay is generally used in our laboratory for screening analyses of 19-norsteroids in urine. Under the conditions employed, this assay gives a response corresponding to an apparent concentration of 0.5–1.5 ng/ml of 19-noretestosterone in urine samples from untreated subjects. From the results obtained it is evident that significant excretion of 19-nortestosterone could be detected only for about 2 weeks with this method. This conclusion was confirmed also in a second experiment in which the urinary excretion of 19-norsteroids from another male volunteer was followed for 18 days after administration of the same dose as above of Deca-Durabol® (results not shown).

DISCUSSION

The present method for assay of 19-norandrosterone seems to have merits with respect to accuracy and sensitivity as compared to previous methods [1–7]. In an isotope dilution assay, the internal standard should preferably be labelled with a stable isotope. A very small amount of radioactivity was used in each analysis, however, and could hardly be harmful for those involved in the analysis. In addition, the radioactivity was an advantage in the detection of the appropriate zone in the t.l.c. [*cf.* ref. 15]. It may be

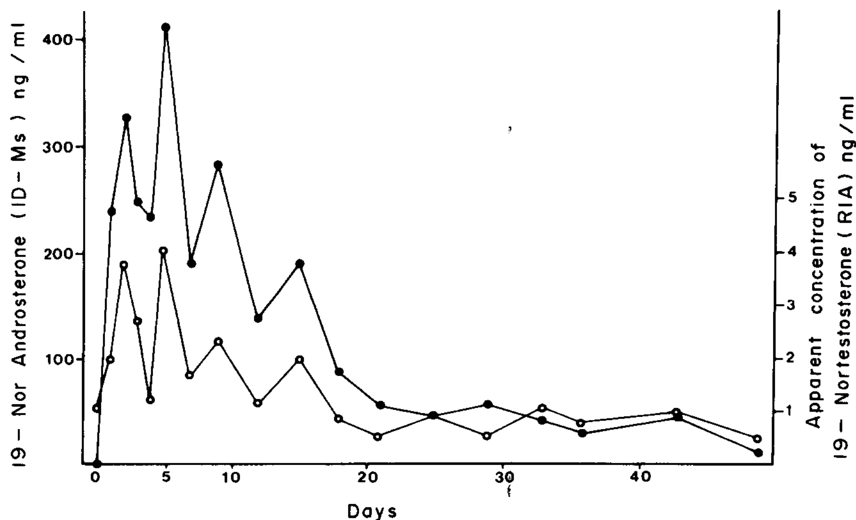

Fig. 5. Excretion of 19-norandrosterone (●——●) and 19-nortestosterone (○——○) in urine as determined by isotope dilution–mass spectrometry and radioimmunoassay respectively. Both subjects received 25 mg of Deca-Durabol® intramuscularly (for details, see Materials and Methods).

pointed out that the biosynthetic preparation of [4-^{14}C]-19-norandrosterone may be performed with experimental animals instead of a human subject.

A complicating factor in the assay of 19-nortestosterone and its metabolites in connection with doping analysis is the fact that 19-norsteroids are intermediates in the formation of estrogens. Thus, at least in theory, such endogenous compounds may appear in urine. It is clear from the present work, however, that if such endogenous compounds occur, their concentrations in urine are negligible as compared to those obtained after administration of an ester of 19-nortestosterone in a therapeutic dose. It is well known that much higher doses than those used here may be used in connection with doping. When using the present isotope dilution assay, and the present mode of calibration, the interference due to endogenous compounds never exceeded 5 ng/ml of urine in any of the untreated subjects tested. The concentration of 19-norandrosterone in urine during the first week after administration of Deca-Durabol® varied between 200 and 500 ng/ml. Under the conditions employed, we have chosen 20 ng/ml of urine as a practical detection limit. Prior to more experience with the present method, we do not consider an athlete as guilty of doping from a legal point of view if the concentration of 19-norandrosterone in urine is below 100 ng/ml.

During the last year we have tested about 300 urine samples obtained from different competitions for the presence of 19-norsteroids, using the same radioimmunoassay as here. Of these 300 samples, 15 gave a significant response in the radioimmunoassay and these samples were therefore subjected to analysis by isotope dilution–mass spectrometry. Three of the samples contained high amounts of 19-norandrosterone, 350, 480 and 670 ng/ml, respectively. All the other samples contained less than 20 ng/ml of 19-norandrosterone. The presence of 19-norandrosterone in the three urine samples was confirmed also by using the alternative ions in the selected ion monitoring and by monitoring a full mass spectrum of the methoxime trimethysilyl ether of 19-norandrosterone. There is no doubt, that these subjects were guilty of having administered 19-nortestosterone or an ester of this steroid.

Acknowledgements—The authors are grateful to Professor Brooks for supplying antibodies and to Professors Hampl and Stárka for the samples of unlabeled 19-norandrosterone and 19-noretiocholanolone. This work was supported by a grant from Sveriges Riksidrotts-Förbund.

REFERENCES

1. Brooks R. V., Jeremiah G., Webb W. A. and Wheeler M.: Detection of anabolic steroid administration to athletes. *J. steroid Biochem.* **11** (1979) 913–917.
2. Dugal R., Dupuis C. and Bertrand M. J.: Radioimmunoassay of anabolic steroids: An evaluation of three antisera for the detection of anabolic steroids in biological fluids. *Br. J. Sport Med.* **11** (1977) 162–169.
3. Rogozkin V. A., Morozow V. I. and Tchaikovsky V. S.: Rapid radioimmunoassay for anabolic steroids in urine. *Schweiz. Z. Sportmedizine* **4** (1979) 169–173.
4. Hampl R., Picha J., Chundela B. and Stárka L.: Radioimmunoassay of nortestosterone and related steroids. *J. clin. Chem. clin. Biochem.* **17** (1979) 529–532.
5. Jondorf W. R. and MacDougall D. F.: Application of RIA method for detecting nandrolone in equine and canine plasma. *Vet. Res.* **100** (1977) 560–562.
6. Ward R. J., Lawson A. M. and Shackleton C. H. L.: Gas chromatographic–mass spectrometric methods for the detection and identification of anabolic steroid drugs. *Br. J. Sport Med.* **9** (1975) 93–97.
7. Ward R. J., Lawson A. M. and Shackleton C. H. L.: Screening by gas chromatography–mass spectrometry for metabolites of five commonly used anabolic drugs. In *Mass Spectrometry in Drug Metabolism*, Plenum New York (1977), pp. 465–474.
8. Dimick D. F., Heron M., Baulieu E. E. and Jayle M. F.: A comparative study of the metabolic fate of testosterone, 17α-methyltestosterone, 19-nortestosterone, 17α-methyl-19-nortestosterone and 17α-methylestr-5(10)-ene-17β-ol-3-one in normal males. *Clin. chim. Acta* **6** (1961) 63–71.
9. Houghton E., Oxley G. A. and Moss M. S.: Studies related to the metabolism of anabolic steroids in the horse: a gas chromatographic–mass spectrometric method to confirm the administration of 19-nortestosterone or its esters to horses. *Biomed. Mass Spectrum.* **5** (1978) 170–173.
10. Houghton E. and Teale P. Capillary column gas chromatographic–mass spectrometric analysis of anabolic steroid residues using splitless injections made at elevated temperatures. *Biomed. Mass Spectrom.* **8**, (1981) 358–361.
11. Houghton E. and Dumasia M. C.: Studies related to the metabolism of anabolic steroids in the horse: The identification of some 16-oxygenated metabolites of 19-nortestosterone. *Xenobiotica* **10** (1980) 381–390.
12. Björkhem I., Lantto O. and Löf A.: Detection and quantitation of methandienone (Dianabol®) in urine by isotope dilution–mass fragmentography. *J. steroid. Biochem.* **13** (1980) 169–175.
13. Lantto O., Björkhem I., Ek H. and Johnston D.: Detection and quantitation of stanozol (Stromba®) in urine by isotope dilution–mass fragmentography. *J. steroid. Biochem.* 14 (1981) 721–727.
14. Björkhem I., Blomstrand R., Lantto O., Löf A. and Svensson L.: Plasma cortisol determination by mass fragmentography. *Clin. chim. Acta* **56**, (1974) 241–248.
15. Björkhem I., Lantto O. and Svensson L.: Serum testosterone determination by mass fragmentography. *Clin. chim. Acta* **60** (1975) 59–66.