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Immunoaffinity chromatography combined with the ion trap technique in order to detect traces of 19-norandrosterone

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Abstract

The detection limits requested last year by the International Olympic Committee for 19-norandrosterone, 19-nortestosterone's main metabolite is 2 ng/ml. To confirm the presence or not of this substance, urine samples were analysed via basic (Procedure 4) or immunoaffinity extraction and then, either an ordinary MSD or an ion trap apparatus was used for the analytical part. In order to diminish the biological background, 19-norandrosterone was isolated via immunoaffinity chromatography (IAC) using specific antibodies directed towards 19-nortestosterone. Once the samples were purified, the MSD in SCAN mode or the ion-trap instrument set-up in SCAN or MS/MS mode was used. In some cases, a full daughter ion SCAN of a parent ion was performed, and in others, just two daughter ions in a SRM (Selective Reaction Monitoring) mode were recorded. This last mode has the advantage of reducing enormously the background in favour of an increased sensitivity. The aim of this work was to determine whether immunoaffinity chromatography was compulsory to confirm the presence of traces of 19-norandrosterone in urine with the use of an ordinary MSD, or whether a basic extraction combined with an instrument set up in MS/MS mode could give similar results. The best detection limit was obtained with an immunoaffinity extraction and the ion trap instrument in MS/MS mode and the ultimate concentration of 19-norandrosterone that can be detected in 5 millilitres of urine was 200 picograms per millilitre.

Experimental

After administration of 19-nortestosterone, most of the metabolites are excreted in urine in their conjugated form. For the experiments described below, a standard solution of the unconjugated metabolite was used, but a hydrolysis was performed in order to be closer to real conditions.

Steroids, immunoaffinity gel and reagents

The Institut für Biochemie, Deutsche Sportochschule, Köln, Germany synthesised and provided us the 19-norandrosterone. The immunoaffinity gel was obtained through the Laboratoire d'Hormonologie - Marloie (Belgium) - and the other solvents and reagents were of analytical grade.

Extraction of 19-norandrosterone from urine for GC/MS identification

Negative urine samples were spiked with several concentrations of 19-norandrosterone and 19-nortestosterone was used as an internal standard (IS, normally 250 ng per sample). Between 5 and 40 ml of urine were necessary to confirm the presence of traces of 19-norandrosterone. C18 (500 mg) solid phase extraction columns were prewashed with 5 ml methanol and 5 ml water. Then the samples were applied on the columns, and were rinsed with 5 ml of water. The steroids were eluted with four times 1 ml of methanol. A rotary vacuum evaporator got rid of the alcohol. After that, 1 ml of phosphate buffer (0.2 M, pH 7.0) and 30 µl of *E. coli* β-glucuronidase (5000 units glucur.) were added to the dry residue. The hydrolysis took 1 hour at 50°C or overnight at 37°C. The metabolite was then purified via a chemical extraction or via an immunoaffinity chromatography.

Isolation of 19-norandrosterone via a basic extraction

Once the hydrolysis was completed the basic extraction included the following steps: *A.* Adding 100 mg of solid buffer ($\text{Na}_2\text{CO}_3:\text{NaHCO}_3$, 1:10, w:w) to the hydrolysat in order to have a pH contained between 8.5 and 9, *B.* Adding 6 ml of n-pentane, *C.* Shaking the mixture for 20 min, *D.* Centrifugating the samples at 3000 rev/min for 10 min, *E.* Pipetting the organic phase into a new tube.

Isolation of 19-norandrosterone via immunoaffinity chromatography (IAC)

References: [Björkhem *et al.*, 1982], [Daen *et al.*, 1991], [Nakamura *et al.*, 1990], [Schänzer *et al.*, 1992], [Schänzer *et al.*, 1996], [Van Ginkel *et al.*, 1989],

In order to isolate 19-norandrosterone, an immunoaffinity gel directed towards 19-nortestosterone (for 1 ml of gel, maximum capacity around 300 ng) was used. The gel was put into a Econo column (1.5 cm ID, 14 cm length, Biorad, Glattbrugg, Switzerland) containing 10 ml of PBS (120 mM NaCl, 2.7 mM KCl, 10 mM $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 0.05 % NaN_3 , adjusted to pH 7.4 with concentrated HCl). The purification of our samples via immunoaffinity chromatography included the following steps: *A.* Washing the column with 5 ml of PBS buffer, *B.* Equilibrating the column with 3 times 3 ml of water, *C.* Applying the hydrolysed urine sample previously diluted with 5 ml PBS, *D.* Washing the gel with 3 times 3 ml of water, *E.* Washing the gel with 3 ml of methanol-water solution (10:90, v:v), *F.* Eluting the bound fraction with 2 times 3 ml of methanol-water solution (80:20, v:v), *G.* Rinsing the column with 2 times 5 ml of water, *H.* Equilibrating the column with 5 ml of PBS buffer, *I.* Storing the column full with PBS buffer at 4 °C.

Derivatization for GC/MS and GC-Q analysis

TMS enol-ether derivatives were formed for GC/MS and GC-Q analysis. The extracts were dried with a speedvac and left for half an hour in a vacuum chamber containing some di-phosphorus pentoxide. Then, they were dissolved in 50 μ l MSTFA/DTE/TMIS (1000:5:5, v:w:v) and heated at 60 °C for 30 minutes.

GC/MS analysis

Instrument: GC/MS Hewlett & Packard (GC 5890/MS 5970), electron impact ionisation with 70 eV; column: Ultra-2, fused silica capillary column crosslinked 5 % phenylmethylsiloxane, 25 m, ID 0.2, film thickness 0.33 μ m; carrier gas: helium 1 ml/min; splitless injection mode (1 μ l injected); temperature program: initial 100 °C; program rate 16 °C/min until 220°C, then 3.8 °C/min; final temperature 300 °C; final time 10 min; injector temperature 270 °C; interface temperature 310 °C.

GC-Q analysis

Instrument: ion trap Finnigan (GC /MS), electron impact ionisation with 70 eV; column: Ultra-2, fused silica capillary column crosslinked 5 % phenylmethylsiloxane, 25 m, ID 0.2 mm, film thickness 0.33 μ m; carrier gas: helium 1 ml/min; splitless injection mode (1 μ l injected); temperature program: initial 100 °C; program rate 16 °C/min until 220 °C, then 3.8 °C/min; final temperature 320 °C; injector temperature 270 °C; transfer line temperature 280 °C; ion source temperature 200 °C. SCAN mode 70-700 amu; SRM mode, isolation of parent ion 405 notch 1.0 for 19-norandrosterone, fragmentation energy 1.0 eV and mass production between 100 and 410 amu 1 scan/min or mass production from 223 to 227 and from 313 to 317, isolation of parent ion 418 notch 1.0 for 19-nortestosterone, fragmentation energy 0.9 eV and mass production between 140 and 420 amu 1 scan/min.

Results

Detection of small amounts of 19-norandrosterone (0.5 ng/ml) in a complex matrix such as urine was realised with a normal MSD (MS 5970) in a FULL SCAN mode. In the first case 10 ml of urine were purified via a basic purification (Figure 2). It was impossible to get a proper extracted ion chromatogram and a specific mass spectrum. The augmentation of urine volume up to 40 ml of urine did not give better results; in both cases, the signal was mistaken with the enormous biological background level. The experiment was also realised with the immunoaffinity purification. 10 ml of urine gave quite a good extracted ion chromatogram, but it was impossible to obtain a good mass spectrum. With the use of 40 ml of urine, there was hardly any increase of the biological background level. The extracted ion chromatogram and the mass spectrum were nice and clean. In order to get these results, it was necessary to purify large amounts of urine, but usually laboratories get samples containing only 30 to 50 ml of urine. So with the use of a MSD (GC 5890/MS 5970), most of the urine would be used in order to confirm the presence of this specific metabolite of nandrolone. Taking into consideration these results and the different constraints, this approach was more of a theoretical approach than a practical one.

The purification of "dirty" urine samples (old urine samples contaminated with bacteria or moisture) with a specific gravity above 1.020 was realised with both purification techniques. In that case, 5 ml of urine were spiked with a total of 10 ng of 19-norandrosterone (Figure 3). With the use of immunoaffinity purification, a huge decrease of the background level on the TIC was noticeable. The extracted ion chromatogram showed a significant signal to noise ratio, but the mass spectrum was not specific at all. Too many contaminating ions were present. With the basic purification, the signal was again mistaken with the background, so in this particular case, immunoaffinity and basic extraction purification did not give good results.

Fortunately, it was possible to inject the same samples on an ion trap instrument, the GC-Q. The apparatus was set in MS and MS/MS mode. In the first case (Figure 5), a very good

extracted ion and mass spectrum were obtained with the immunoaffinity purification. On the contrary, with the use of the Procedure 4, it was difficult to get a nice and clean chromatogram; 19-norandrosterone's mass spectrum was hardly recognisable, there were always too many non-specific ions. The possibility of getting rid of most of the biological background with the use of the GC-Q in a MS/MS mode was an interesting alternative after a basic purification in order to get good results (Figure 7). The chromatogram was lacking of contaminating peaks and the MS/MS mass spectrum had very few odd ions. All of them disappeared with the immunoaffinity purification. That meant that these odd daughter ions certainly came from the 405 parent ions produced by the biological matrix. Thus a better purification combined with the MS/MS mode could certainly improve the detection at very low concentration.

The use of both techniques, immunoaffinity purification and the MS/MS analytical method on the same "dirty" urine samples gave quite good results. It was possible to get a proper chromatogram and mass spectrum with only 20 pg/ μ l injected (Figure 8). At this concentration with a basic purification, a same ghost peak at the retention time of 19-norandrosterone was noticeable in the sample and the negative urine, but the mass spectrum had nothing to do with this compound (data not shown).

In order to see very low concentrations (0.2 ng/ml of urine) of nandrolone's main metabolite during a screening procedure via a basic purification, it was necessary to plot only two of the major ions (± 1 m/z). The trouble with this technique is, that there could be an increase of false positive results. Unfortunately it was difficult to increase the urine volume in order to get a better mass spectrum. Even with the selectivity of the apparatus, there was a limit with the amount of urine to purify. Indeed with an augmentation of the urine volume up to 20 ml in order to inject 1 μ l containing 80 pg of 19-norandrosterone, a bad extracted ion chromatogram and mass spectrum were obtained. That meant that there were too many 405 ions coming from the matrix. Doing a fragmentation of this particular ion produced then a lot of daughter ions which in terms increased enormously the background. In this case, the MS/MS mass spectrum was contaminated with full of non-specific daughter ions of 19-norandrosterone. The behaviour of the trap was noticeable on the chromatograms when different volumes of urine

were purified. The increase of the area between 5 ml and 20 ml of urine spiked with 2 ng/ml of 19-norandrosterone was not four times more. So even with the selectivity of the GC-Q in MS/MS mode, an augmentation of the biological background was a limiting factor. Thanks to the immunoaffinity chromatography it was possible to overcome such problems, and theoretically, it should be possible to purify urine samples containing pico traces as far as the urine volume is big enough.

Conclusion

Giving a full mass spectrum of 19-norandrosterone from 5 ml of a dirty urine containing 2 ng/ml of this particular metabolite was possible with the combination of immunoaffinity purification and the GC-Q set up in MS mode. The MSD used during the study seemed to be incapable in giving such results, because the signal was mistaken with the electronic and biological background. With very low concentrations and a basic extraction, the biological background level was so high that even a more sensitive instrument such as the GC-Q was not able to produce a proper mass spectrum. Thanks to the selectivity of the GC-Q set-up in MS/MS mode, it was possible to get rid of the biological noise left from the basic extraction and to obtain a nice MS/MS mass spectrum. Doing an immunoaffinity purification was no need for these concentrations. On the other hand, immunoaffinity was compulsory in order to detect 200 pg/ml of urine. If there was some need for detecting rapidly so low concentrations of 19-norandrosterone in urine, there was always the possibility of screening urine samples with the GC-Q set-up in MS/MS mode. In stead of plotting all the ions between 100 and 410, only the major ions such as 315 and 225 (± 1 m/z) were plotted. In that particular case, with a basic extraction, it was possible to detect so low concentrations of 19-norandrosterone. Of course this last technique could be only used for screening purposes, because an increase of false positive results could emerge. Then, after this screening procedure, a confirmation one involving immunoaffinty could be done.

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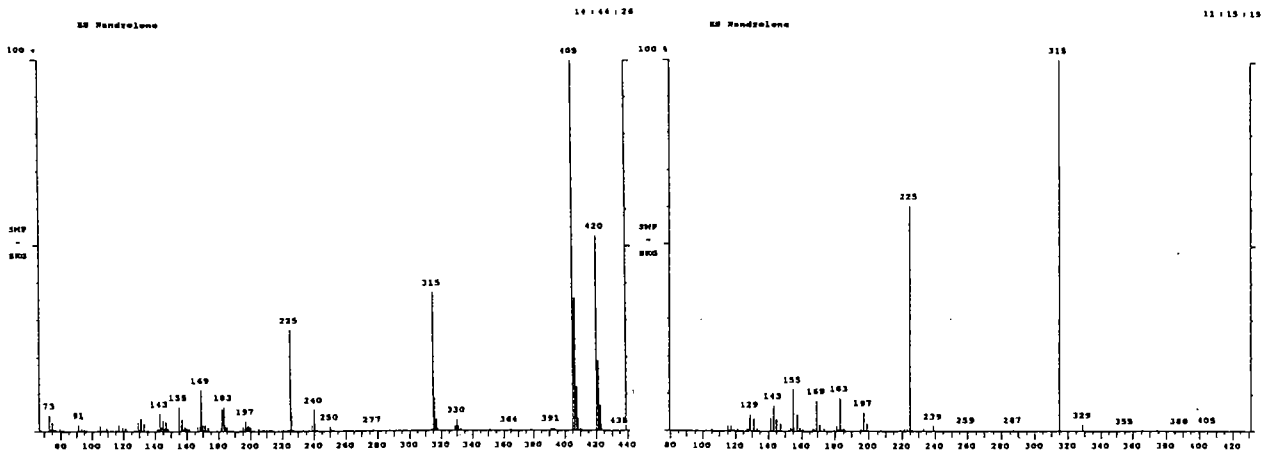


Figure 1: MS and MS/MS mass spectrum of 19-norandrosterone after derivatization (TMS).

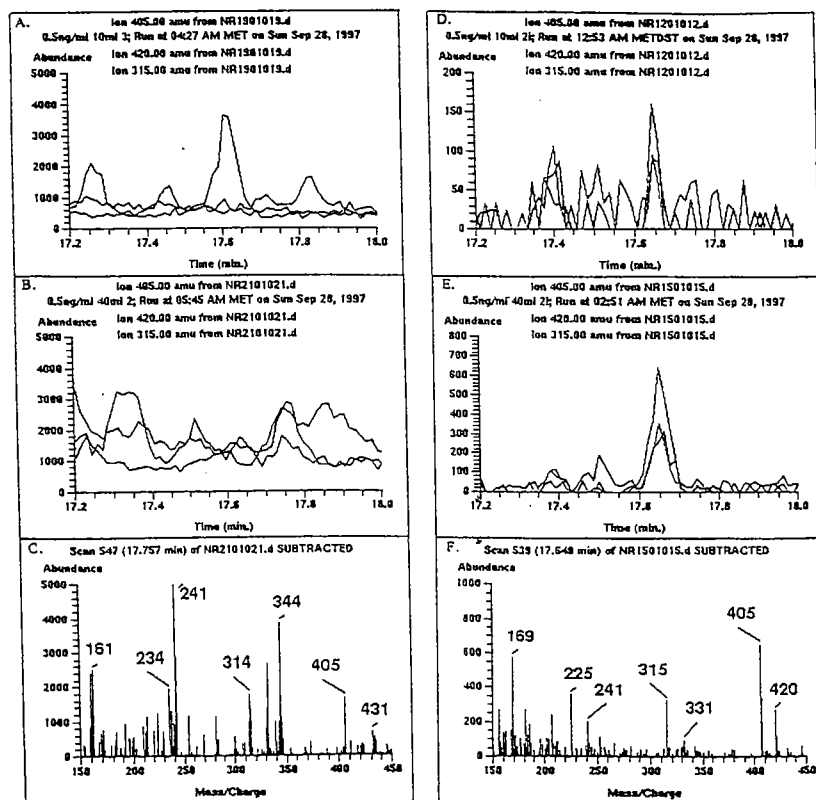


Figure 2: Extracted ion chromatograms and mass spectrums obtained after basic (left column) or immunoaffinity (right column) purification of different urine volumes containing 0.5 ng/ml of 19-norandrosterone. A and D: extracted ion, 10 ml of urine purified; B and E: extracted ion, 40 ml of urine purified; C and F: mass spectrum at the retention time of 19-norandrosterone, 40 ml of urine purified.

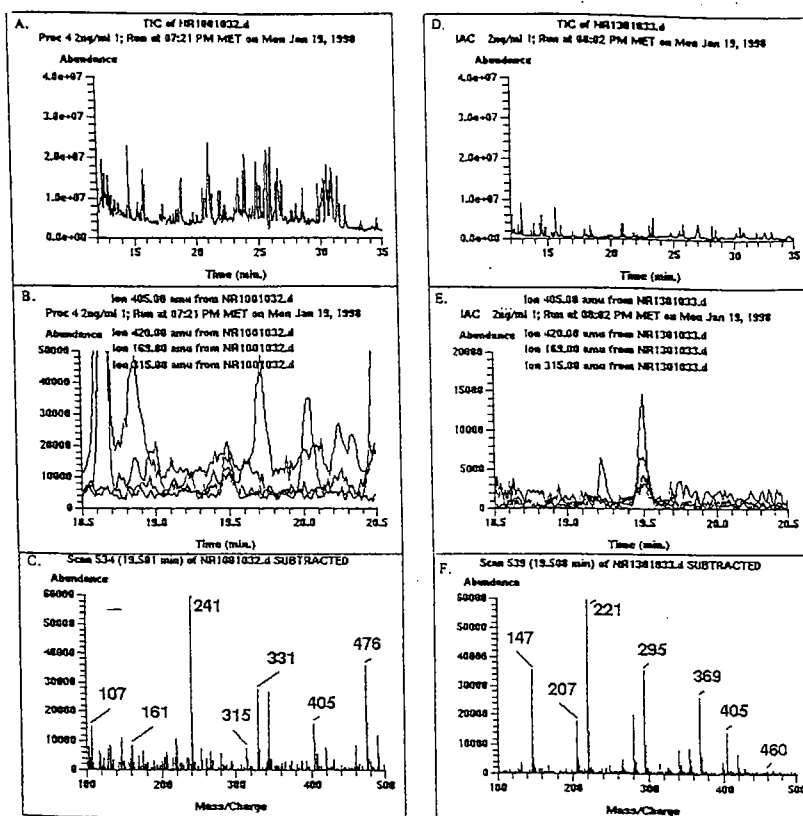


Figure 3: 5 ml of urine spiked with 2 ng/ml of 19-norandrosterone purified either with a basic (left column) or an immunoaffinity (right column) extraction. A and D: total ion current, B and E: extracted ion, C and F: mass spectrum at the retention time of 19-norandrosterone. These samples were injected on the MSD.

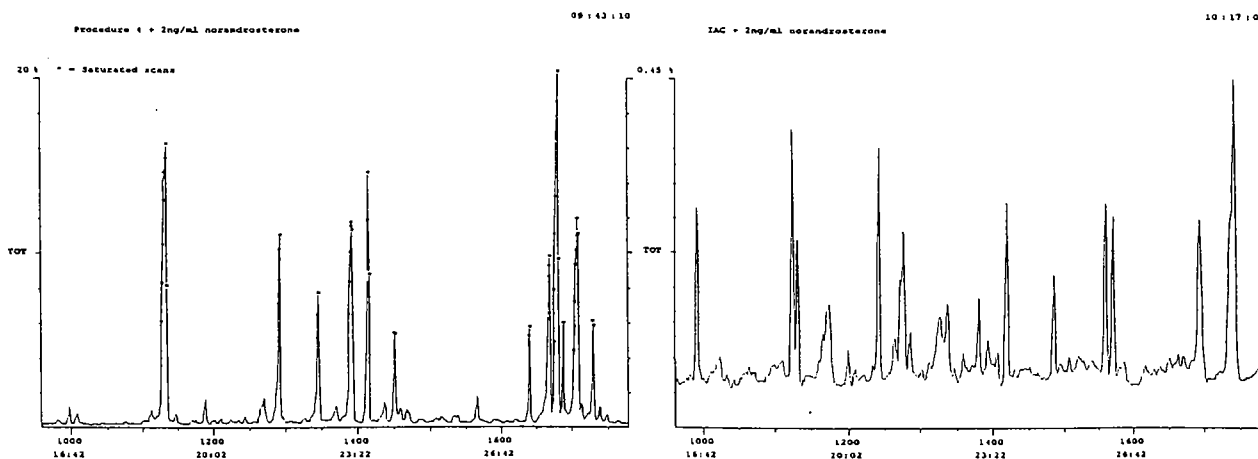


Figure 4: Total ion current after a basic (left) or an immunoaffinity (right) purification observed on a GC-Q in MS mode.

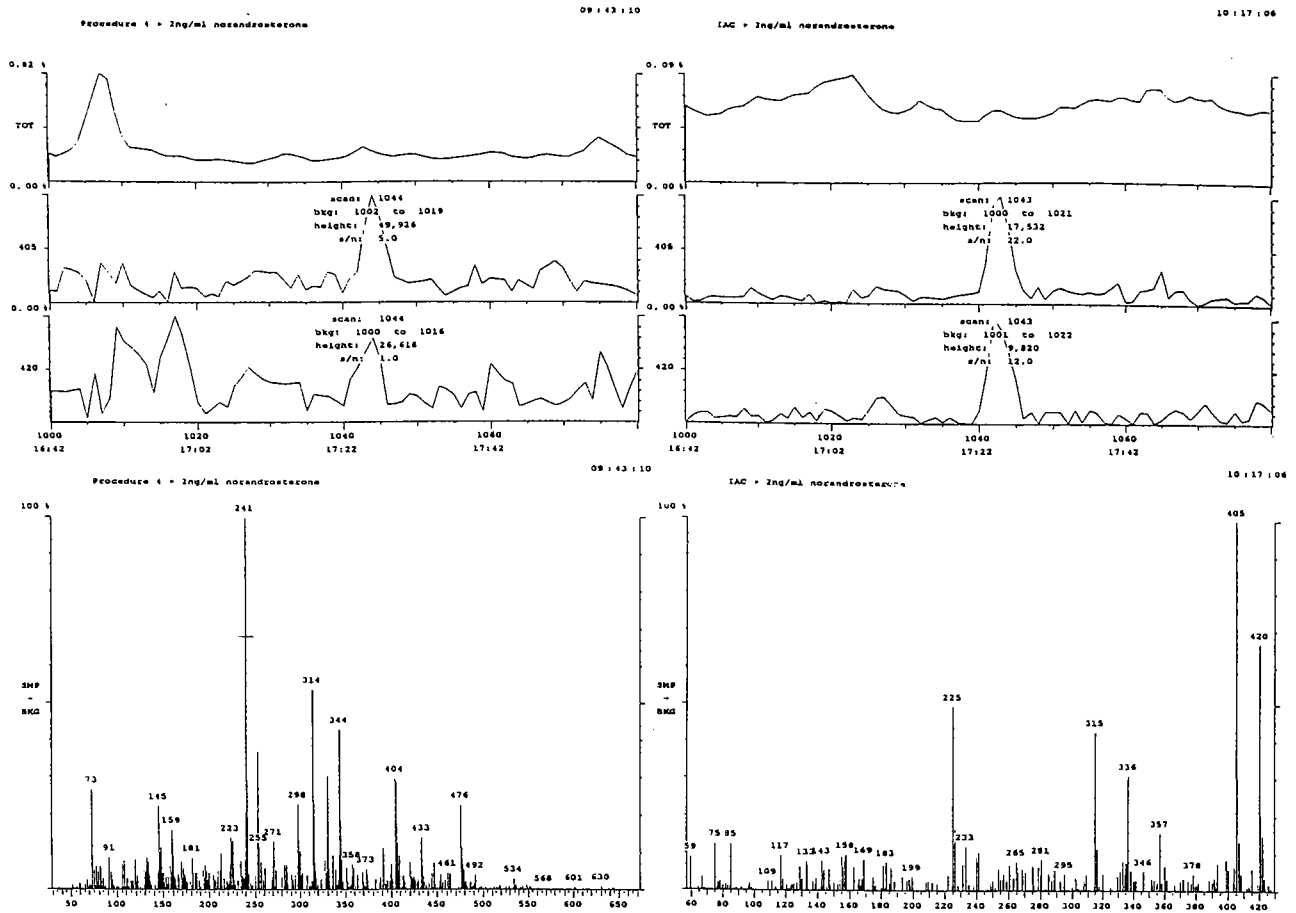


Figure 5: Same samples as in Figure 3 excepted that they were injected onto a GC-Q in MS mode.

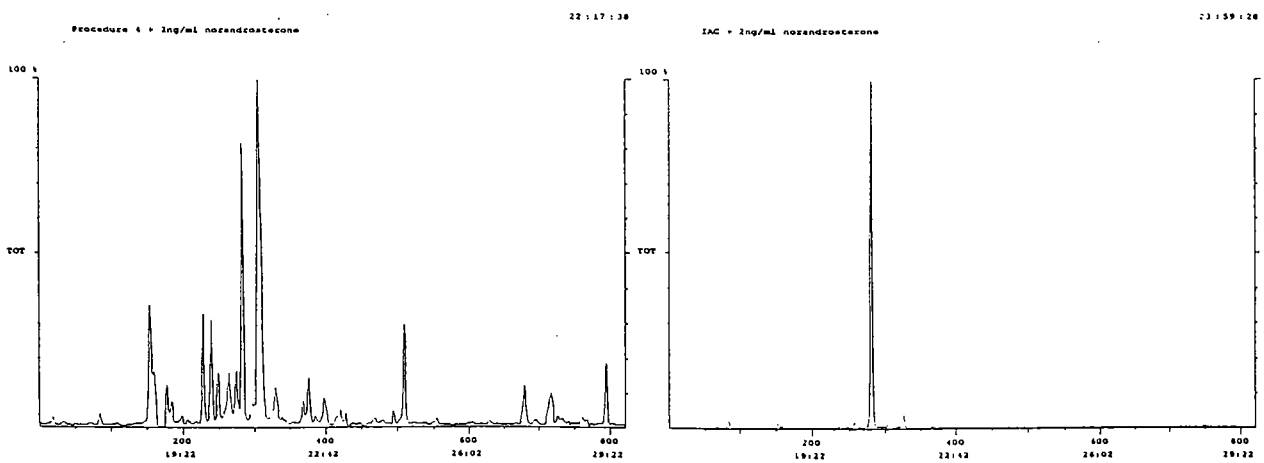


Figure 6: Total ion current after a basic (left) or an immunoaffinity (right) purification observed on a GC-Q in MS/MS mode.

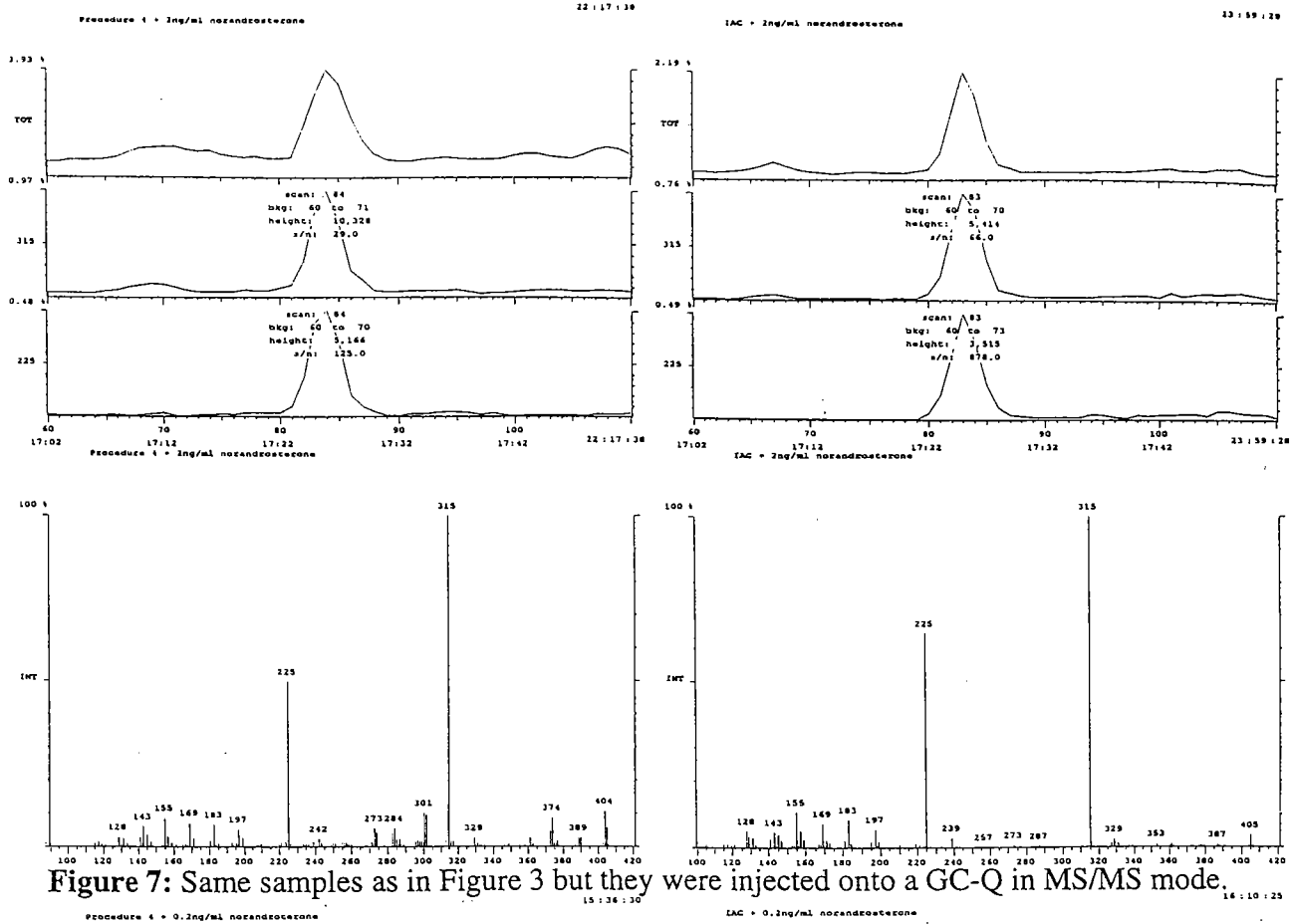


Figure 7: Same samples as in Figure 3 but they were injected onto a GC-Q in MS/MS mode.

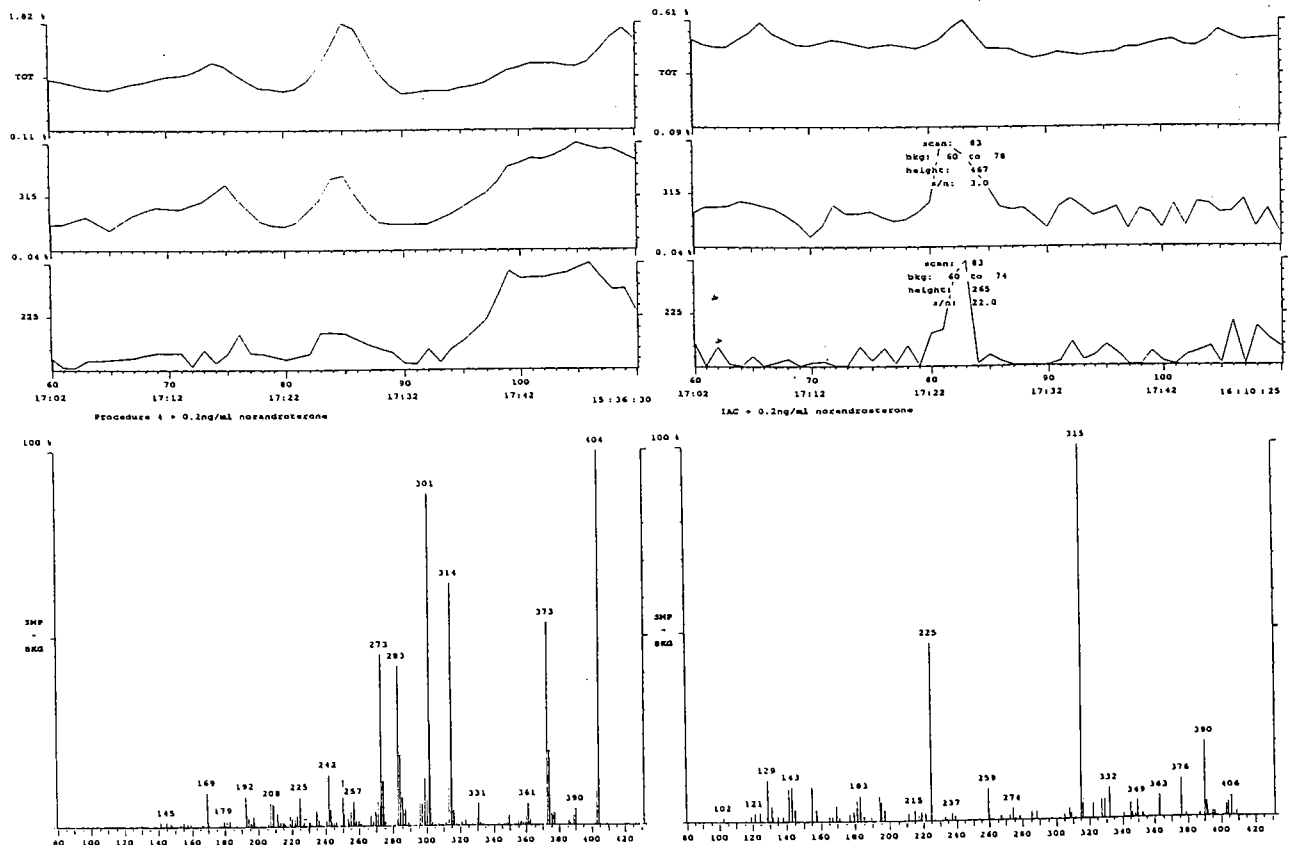


Figure 8: 5 ml of urine with 0.2 ng/ml of 19-norandrostosterone purified with a basic (left column) or an immunoaffinity (right column) purification. The samples were injected in MS/MS mode.

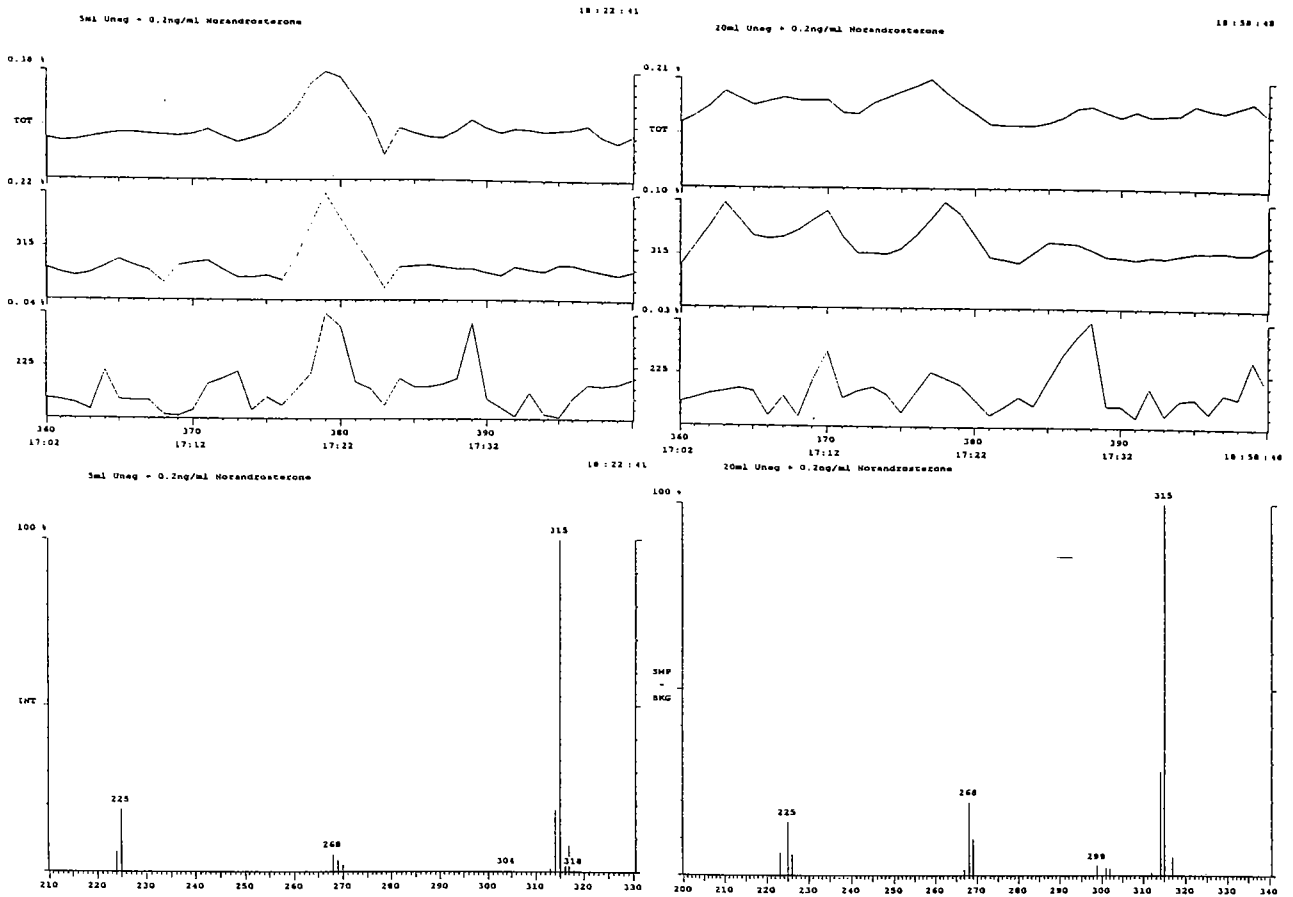


Figure 9: Detection of 0.2 ng/ml of 19-norandrosterone from 5 ml or 20 ml of urine after a basic purification and an injection onto a GC-Q in a SRM mode in order to decrease the biological background.

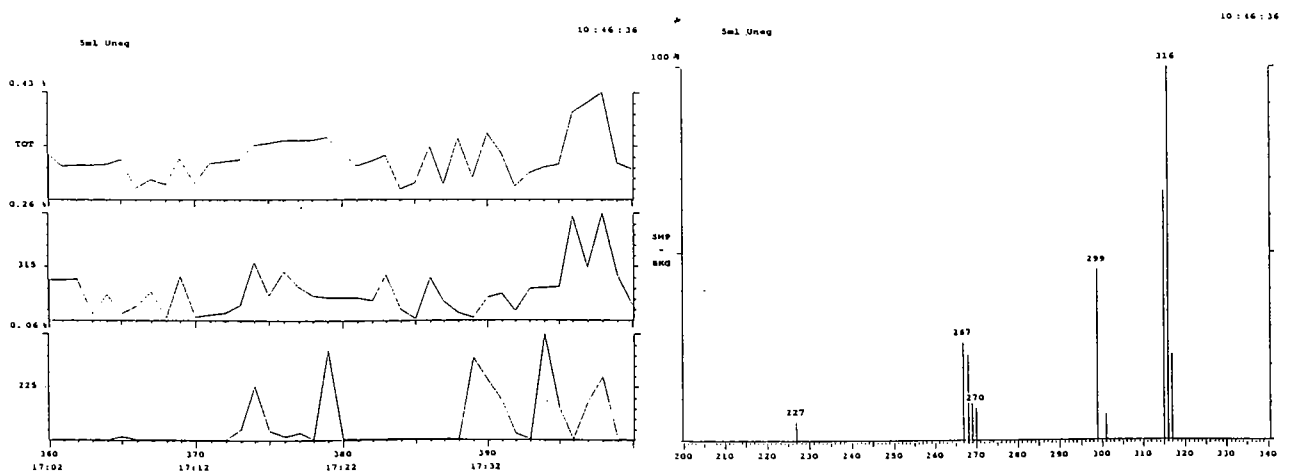


Figure 10: Extracted ion and mass spectrum at the retention time of 19-norandrosterone of 5 ml of negative urine.