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The Detection of low level anabolic agents in bovine and human urine using LC-ESI-MS-MS

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Introduction

As well as analysing human urine for sports drug testing, ASDTL also analyses bovine urine samples for the presence of stanozolol, nandrolone, boldenone and their metabolites for the export of meat to the European Union (EU). The method we have developed uses SPE extraction with LC/MS detection and is capable of detecting these analytes at the required detection limit of 1 ng/ml in bovine urine. Investigations have been made as to whether similar detection levels can be achieved for the human metabolites of stanozolol, nandrolone, clenbuterol and metandienone.

Method Summary

The analytes were extracted from the urine with 3M Empore C18 SPE columns using a Gilson ASPEC XL4. The extracts were separated on an Alltech Alltima C18 (150 x 2.1 mm) column using a Waters 2795 HPLC. The eluates were analysed using ESI MS-MS in positive ion modes with a Micromass Quattro Micro and the detection of each analyte was optimised..

The extraction protocol was:

- To each 2 mL of urine was added 1.5 mL of pH 7 phosphate buffer, 50ul of E.Coli glucuronidase and 50 uL of d3-nandrolone,d3-boldenone and d3-stanozolol internal standards (200 ng/mL).
- The tubes were hydrolysed at 50°C for 1 hour and the analytes extracted by passage of the sample through a 3M Empore C18 column, followed by a 1 mL water wash, a

0.5 mL wash with 25% methanol in water, 1 mL of hexane and elution with 2 mL ethyl acetate.

- The ethyl acetate extract was evaporated to dryness under nitrogen and reconstituted in 250 uL of methanol.
- The HPLC used methanol/water containing 0.2% formic acid with a gradient from 30% methanol to 90% methanol in 9 minutes. After 9.5 minutes the methanol was increased to 99% until 12.5 minutes.

Results and Discussion

Figure 1 shows the output obtained from the analysis of a bovine urine spiked at 1 ng/mL with the analytes that are required to be analysed for the EU. All can be clearly detected at this concentration. A human urine sample was spiked at 2 ng/mL with clenbuterol, epimetendiol, stanozolol, 3'OH stanozolol, 4βOH stanozolol and 16βOH stanozolol, and analysed using the same procedure. The results are shown in Figure 2. All the compounds are detected although the signal to noise ratio for epimetendiol is relatively low. As a comparison the results obtained from the analysis of a 2 ng/mL urine spike using our routine HRMS steroid screen are shown in Figure 3. For the stanozolol metabolites the signal to noise ratio is clearly superior in the LC-MS-MS method, whilst for epimetendiol the GC-HRMS result is better. For clenbuterol the results are comparable. The human metabolites of nandrolone could not be detected at 2 ng/mL using this LC-MS-MS method.

The method was used as an additional confirmation procedure for a sports sample which contained low concentrations of stanozolol metabolites. The chromatograms obtained are shown in Figure 4 and showed better signal to noise ratios than those obtained for this sample using GC-HRMS.

Conclusions

LC-ESI-MS-MS gives equal or superior sensitivity for the detection of clenbuterol and the metabolites of stanozolol compared to GC-HRMS. Epimetendiol can be detected but with an inferior signal to noise ratio. The human metabolites of nandrolone can not be detected at 2 ng/mL

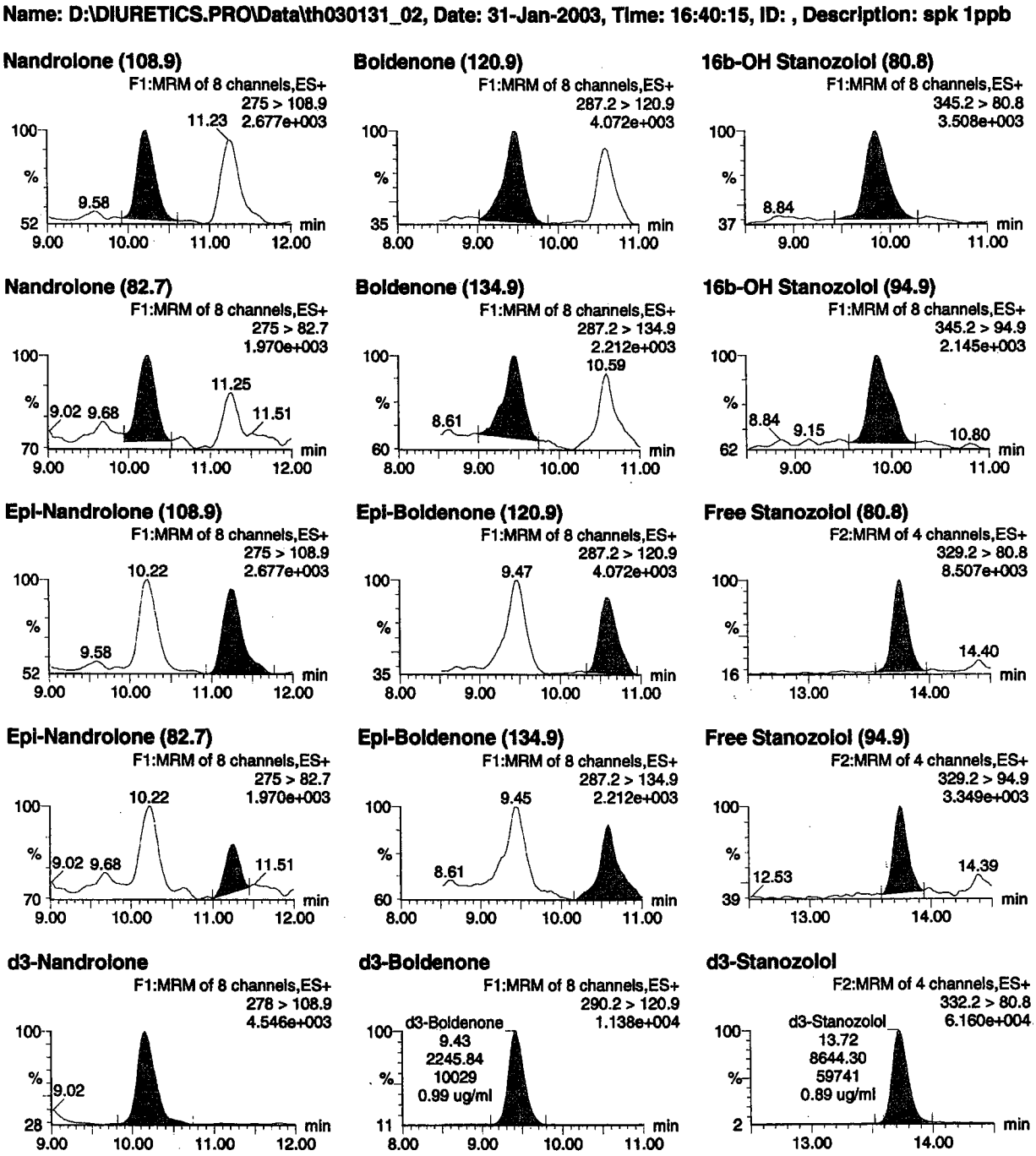

Figure 1. Chromatograms from a bovine urine sample spiked with 1 ng/mL of nandrolone, boldenone, stanozolol and their metabolites.

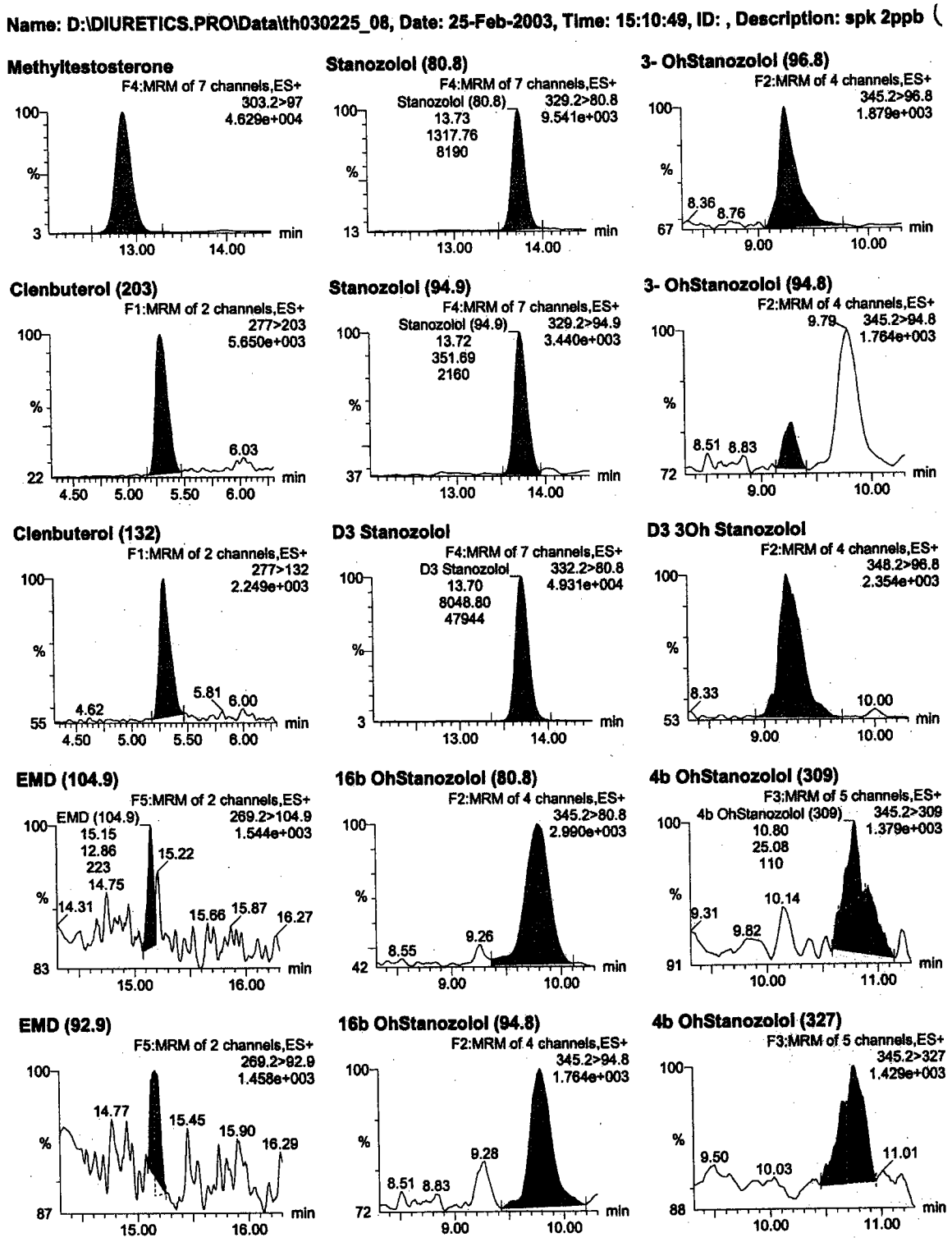

Figure 2. Human urine spiked at 2ng/mL with clenbuterol, epimetendiol, stanozolol, 3'OH stanozolol, 4βOH stanozolol and 16βOH stanozolol.

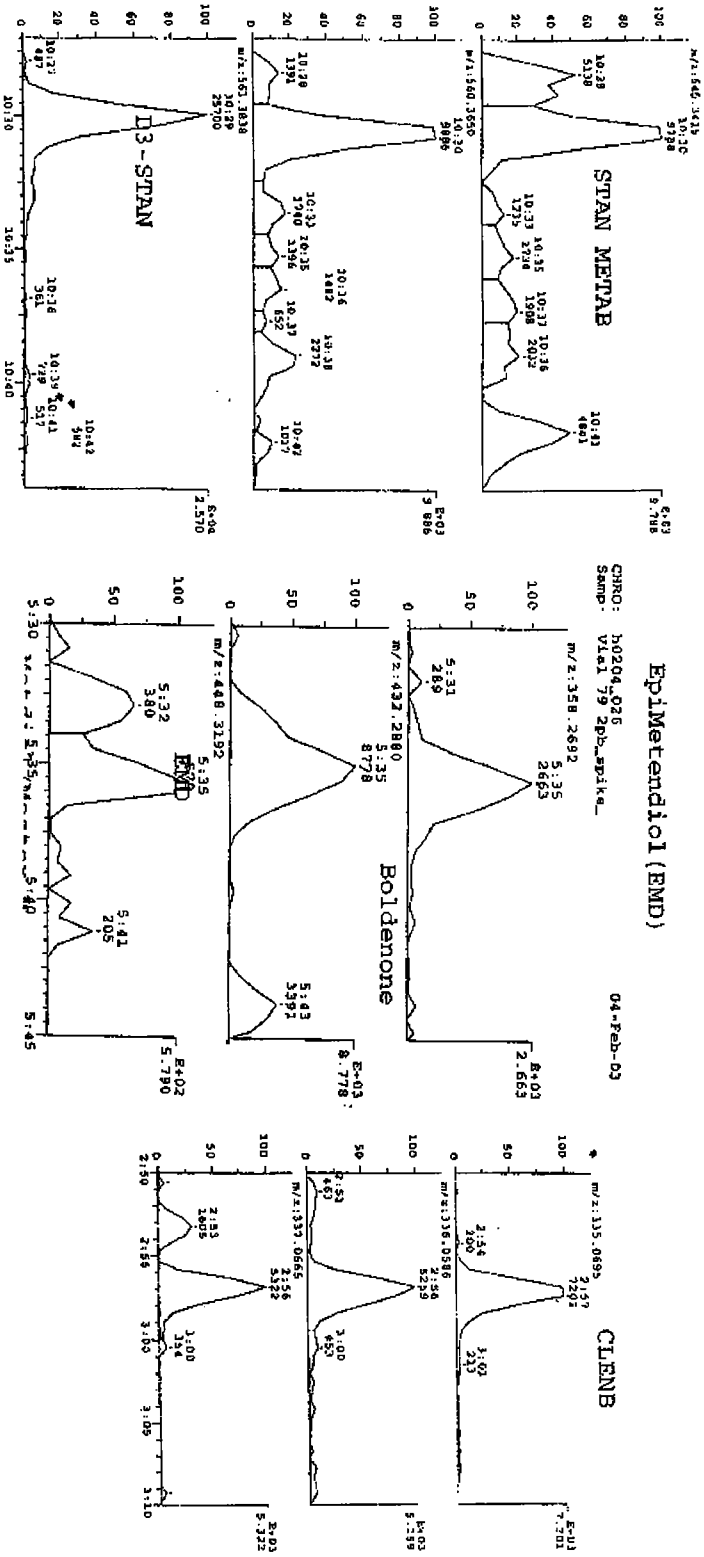

Figure 3. Chromatograms from the GC-HRMS analysis of a human urine sample spiked at 2 ng/mL with 3'OH stanozolol, epimetendiol and clenbuterol.

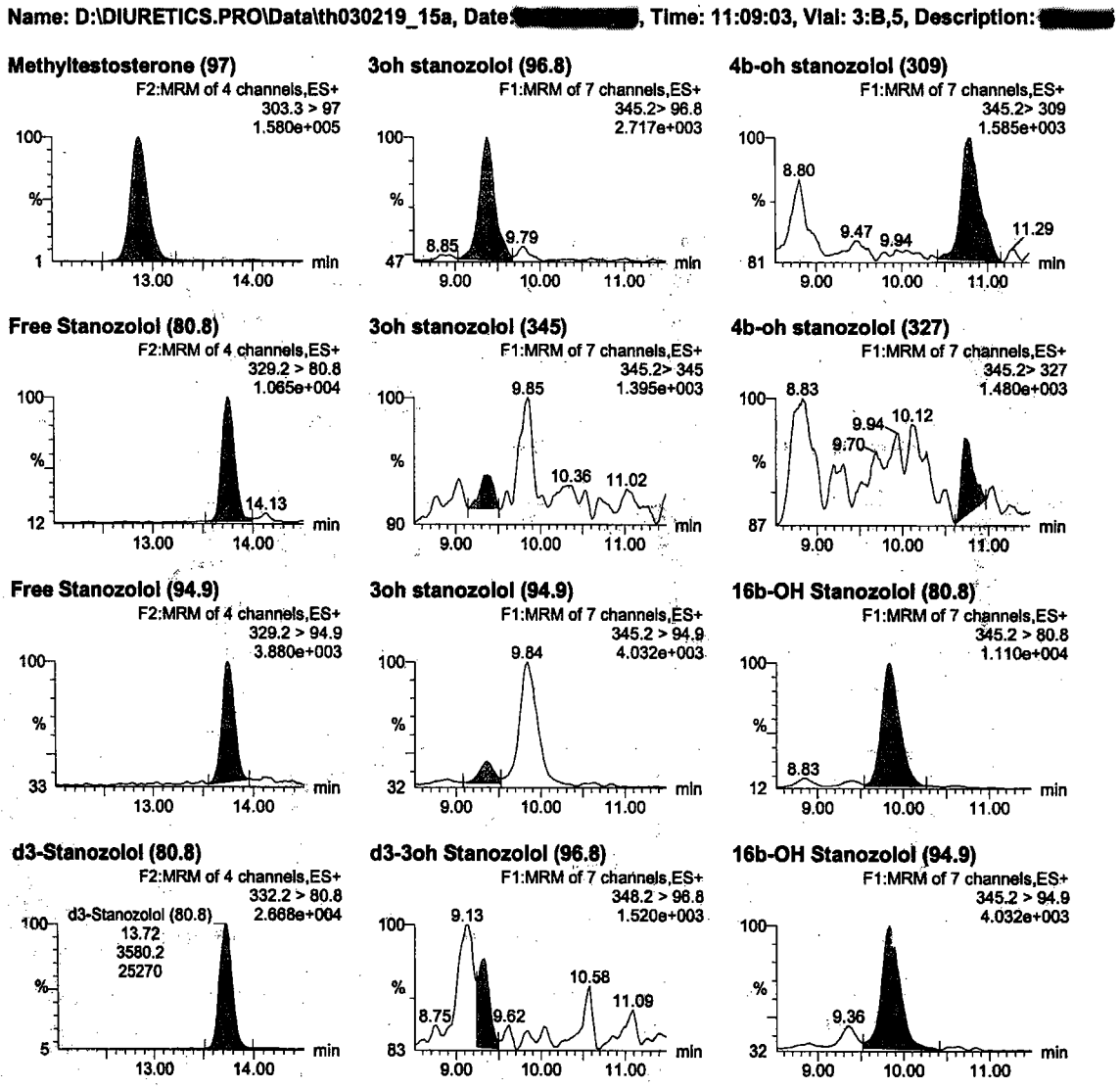

Figure 4. Positive human urine sample containing approximately 2 ng/mL of 3'OH stanozolol and 4 ng/mL of 16βOH stanozolol.