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## **Miscellaneous Projects in Sports Drug Testing at the National Measurement Institute, Australia, 2006 or, “an ASDTL Assortment”.**

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### **Introduction**

Two minor projects carried out at ASDTL will be discussed at this meeting. The first relates to some further work which has been carried out on improving the development of the test for recombinant EPO in urine and the second relates to the detection of low level steroids and their metabolites using ESI and LC/MS/MS.

The EPO method development involved further studies using the immunoaffinity columns prepared from sheep serum, the preparation and use of which were described last year (Marinelli et al. 2006). The aim of the project was to determine if the extracts from the columns were pure enough to permit the detection of recombinant EPO in urine without the need for double blotting. The second project was an investigation into whether a new LC/MS/MS system was able to detect methandriol which is part of a screen for steroids in animal urine. We had previously reported (Huynh et al. 2003) that methandriol was unable to be detected using ESI on our existing Waters Quattro Micro system. Similar findings have been reported for androstenediols by a group in Ghent (Van Poucke et al. 2005).

### **Progress with EPO Detection**

#### **Experimental**

The immunoaffinity columns were prepared as described previously (Marinelli et al. 2006) and were capable of recovering more than 50% of spiked recombinant EPO from urine. A series of spiked and unspiked urines were prepared for analysis. For immunoaffinity purification a 20 mL

aliquot of urine was mixed with the gel for two hours and then filtered. The eluate after pH adjustment was then treated as a urine for the EPO gel method.

The methods used for detection of EPO were the standard gel electrophoresis method using double Western blotting developed by Lasne (Lasne et al. 2002) and a variation in which only a single Western blot was used. The second acid blot which normally follows incubation with the primary antibody was not performed. For comparison some urine samples were run using both methods.

## **Results and Discussion**

The rationale for attempting to use a single Western blot for EPO detection was based on two factors. The first was our observation that the transfer in the second blot was relatively inefficient resulting in a loss of more than 50% of the EPO signal compared to a single blot, and the second was that the double blotting procedure is a technically difficult procedure to perform. It was hoped that the use of a single blot after immunoaffinity purification would result in a more sensitive and simpler procedure. A series of spiked urines, using urine from a single collection, were prepared and analysed using a single blotting method. Four of these urines which were spiked at the relatively high level of 30 IU/L were purified by passage through immunoaffinity columns prior to analysis. The results are shown in Figure 1. It is surprising to note that in this particular urine sample both recombinant and natural EPO can be readily detected without double blotting and without immunoaffinity purification. The recombinant EPO can be detected with only single blotting at 5, 10 and 30 IU/L in urines which have not undergone immunoaffinity purification. Thus, for this particular urine sample, EPO can be detected without the need for double blotting and without immunoaffinity purification. The experiment was repeated using the same urine with spikes at 5 and 10 IU/L. Similar results were obtained and are shown in Figure 2. Whilst this particular urine sample did not need double blotting to detect EPO this was not expected to apply for most urines. Samples of three different urines, labelled 722, 723 and 724 were spiked with rhEPO at 2 IU/L and analysed using the single blot procedure. The same spiked samples were analysed using the single blot procedure after immunoaffinity purification. The results are shown in Figure 3. The results demonstrate that single blotting is not useful for these urines as a virtual continuum of bands is observed for the unpurified urines. Sample 722 is particularly bad. There is no possibility of detecting the spiked EPO in any of these samples using a single blot. However, when the samples are purified by immunoaffinity chromatography prior to gel electrophoresis the spiked EPO can be detected in all samples. The difference

observed for sample 722 is particularly striking. The chemiluminescent profiles of the bands obtained are shown in Figure 4 where the recombinant EPO bands can be seen more clearly.

## Conclusions

- The immunoaffinity columns can be used as a cleanup for less selective detection methods.
- The columns can be reused several times provided additional elution buffer is used.
- The potential exists to use the columns for extracting EPO from urine for other identification methods such as MS.

## ESI and Steroids

### Experimental

The samples were analysed using an Applied Biosystems API 4000 QTrap using a Turbo V ESI ion source and an Agilent 1100 CapLC system. The source conditions were voltage 4600 V, temperature 350°C with ion source gas 1 at 60°C and ion source gas 2 at 35°C.

The LC conditions were :- Column Waters XTerra C18 3.5µm, 1 x 100 mm run at 50 µL/min on gradient of 70% A for 1 min, then to 30% A in 7 mins, then to 0% A in 1 min and hold for 1 min, then to 70% A in 2 mins. A is 10/90 Acn/H<sub>2</sub>O and B is 90/10 Acn/H<sub>2</sub>O both with 0.2% formic acid. 5µL sample injection.

The MS was run in MRM in positive ion mode with 10 transitions each of 100 ms. 275 > 109 and 145, 329 > 81 and 95, 345 > 81 and 95, and 287 > 121, 135, 133 and 147.

## Results and Discussion

We had previously attempted to analyse a range of anabolic agents extracted from bovine urine by ESI LC/MS/MS using our Waters Quattro Micro system. The compounds investigated were nandrolone and epinandrolone, boldenone and epiboldenone, stanozolol and 16OH-stanozolol, and methandriol. All but methandriol were able to be detected. Figure 5 gives the structure of methandriol and Figure 6 shows the results obtained when a methandriol solution was infused

into the Quattro ESI source. The response was very low being about  $10^5$  rather than  $10^7$  which was observed for similar concentrations of other steroids. It has been reported that 3OH steroids (actually 3,17 diols) give poor ESI response on a similar instrument (Van Poucke et al 2005). We recently purchased an API 4000 QTrap and decided to investigate its ESI response to methandriol and the other anabolic agents. A solution of approximately  $1\mu\text{g/mL}$  of methandriol in 50/50 methanol water was infused and the resulting spectrum is shown in Figure 7. The molecular weight of methandriol is approximately 304 Da and hence one would expect to see a protonated ion at  $m/z$  305  $[\text{M}+\text{H}^+]$ . It can be seen from Figure 7 that there is virtually no 305 ion but rather major ions at 301 and 269. The mass loss of 4 Da from 305 to 301 by dehydrogenation is not possible. The 269 ion corresponds to a loss of 36 from 305 which corresponds to the loss of two water molecules. This loss is likely for a diol. Examination of the spectrum showed a small peak at 287 corresponding to a single water loss. The ion 301 is 32 higher than 269 which could be due to a methanol adduct. Examination of the spectrum shows a small peak at 337 corresponding to  $\text{M}+\text{CH}_3\text{OH}^+$ . The major ion of 301 would then be due to a double water loss from this species. If the above assignments are correct then one would expect a different spectrum to be observed when methandriol was infused in a water acetonitrile mix. The spectrum obtained is shown in Figure 8 and is clearly different. The expected  $\text{M}+\text{H}^+$  of 305 is now visible and 337 is not present. The base peak is 287 corresponding to a single water loss and the next largest peak is 269 being a double water loss. The ion 287 was chosen as the precursor for MS/MS optimisation and the product ion spectrum obtained is shown in Figure 9. A standard solution of the seven analytes was analysed using LC/MS/MS, and all including methandriol could be readily detected at the required LOD (5 ng/mL for methandriol and 1 ng/mL for the rest). The resulting TIC is shown in Figure 10.

## Conclusions

- ESI ion sources from different manufacturers can give very different responses depending on the compound.
- One should be wary of using automated software that takes the empirical formula of the compound and calculates the  $\text{M}+\text{H}^+$  value for ion source optimisation.
- One should also be aware of possible solvent effects.

## **Acknowledgements**

The EPO project could not have proceeded without the generous support of the World Anti-Doping Agency.

## **References**

Marinelli, D., Agon, V., Howe, C., Stenhouse, A., Sillence, M., Trout, G.J., Kazlauskas, R. (2006) EPO immunoaffinity columns for purification. In: Schanzer, W., Geyer, H., Gotzmann, A., Mareck, U. (eds.) *Recent Advances in Doping Analysis (14)*, Koln, pp 345-352.

Huynh, T., Trout, G.J., Kazlauskas, R. (2003) The detection of low level anabolic agents in bovine and human urine using LC-ESI-MS-MS. In: Schanzer, W., Geyer, H., Gotzmann, A., Mareck, U. (eds.) *Recent Advances in Doping Analysis (11)*, Koln, pp 271-276.

Van Poucke C, Van De Velde M, Van Peteghem C. (2005) Combination of liquid chromatography/tandem mass spectrometry and gas chromatography/mass spectrometry for the detection of 21 anabolic steroid residues in bovine urine. *J Mass Spectrom.* 40(6):731-8.

Lasne, F., Martin, L., Crepin, N., de Ceaurriz, J. (2002) Detection of isoelectric profiles of erythropoietin in urine: differentiation of natural and administered recombinant hormones. *Anal. Biochem.*, 311(2), 119-126.

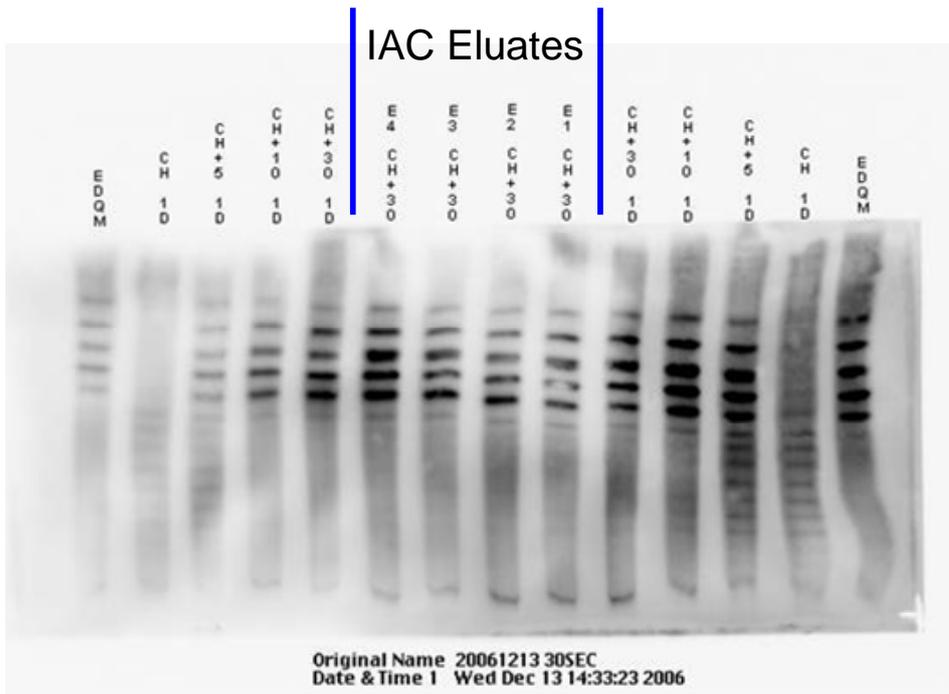


Figure 1. Isoform distributions obtained from single blotting for urine extracts spiked with 0, 5, 10 and 30 IU/L of rhEPO and urines spiked at 30 IU/L after immunoaffinity column purification.

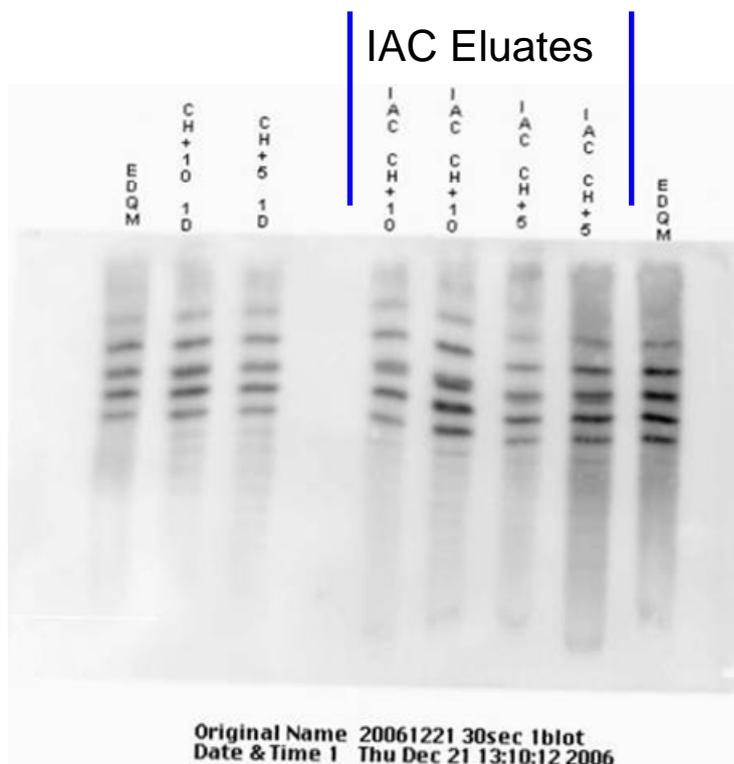


Figure 2. Isoform distributions obtained from single blotting for urine extracts spiked with 5 and 10 IU/L of rhEPO before and after immunoaffinity column purification.

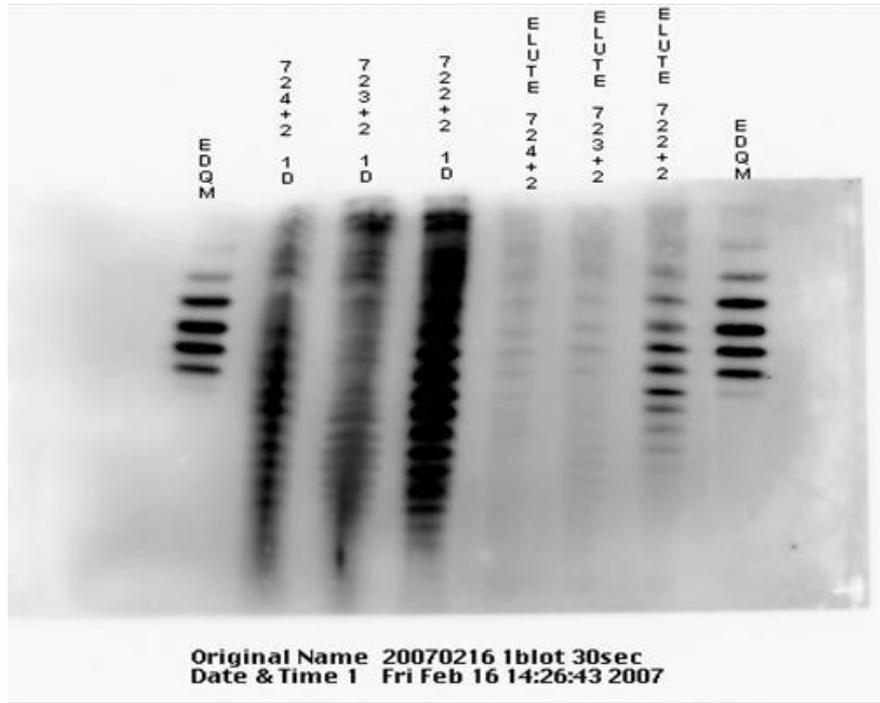


Figure 3. Isoform distributions obtained from single blotting of three different urine samples spiked with 2 IU/L of rhEPO. The results from the same samples after passage through the immunoaffinity column are shown on the right hand side of the gel.

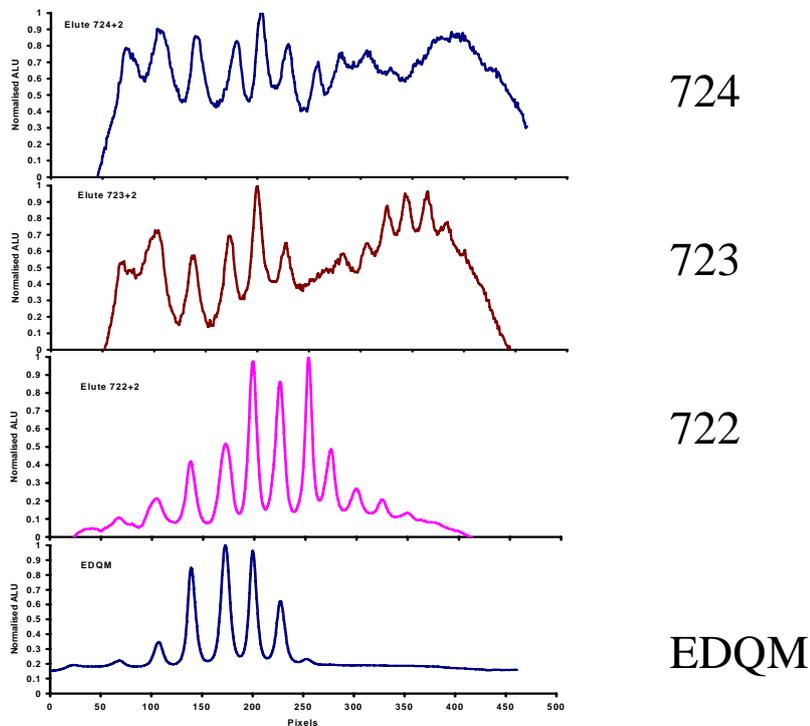
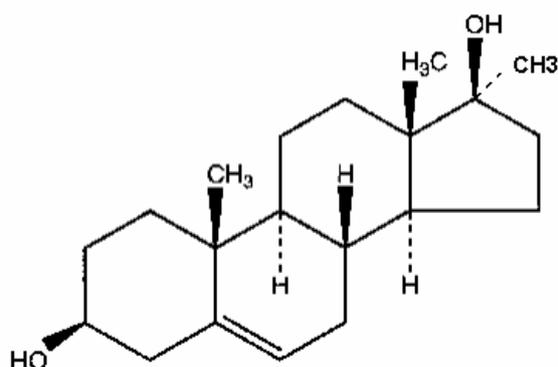


Figure 4. Profiles obtained from the immunoaffinity purified urine samples from Figure 3.



17 $\alpha$ -methyl-5-androstene-3 $\beta$ ,17 $\beta$ -diol

Molecular Weight 304.4

Figure 5. Methandriol

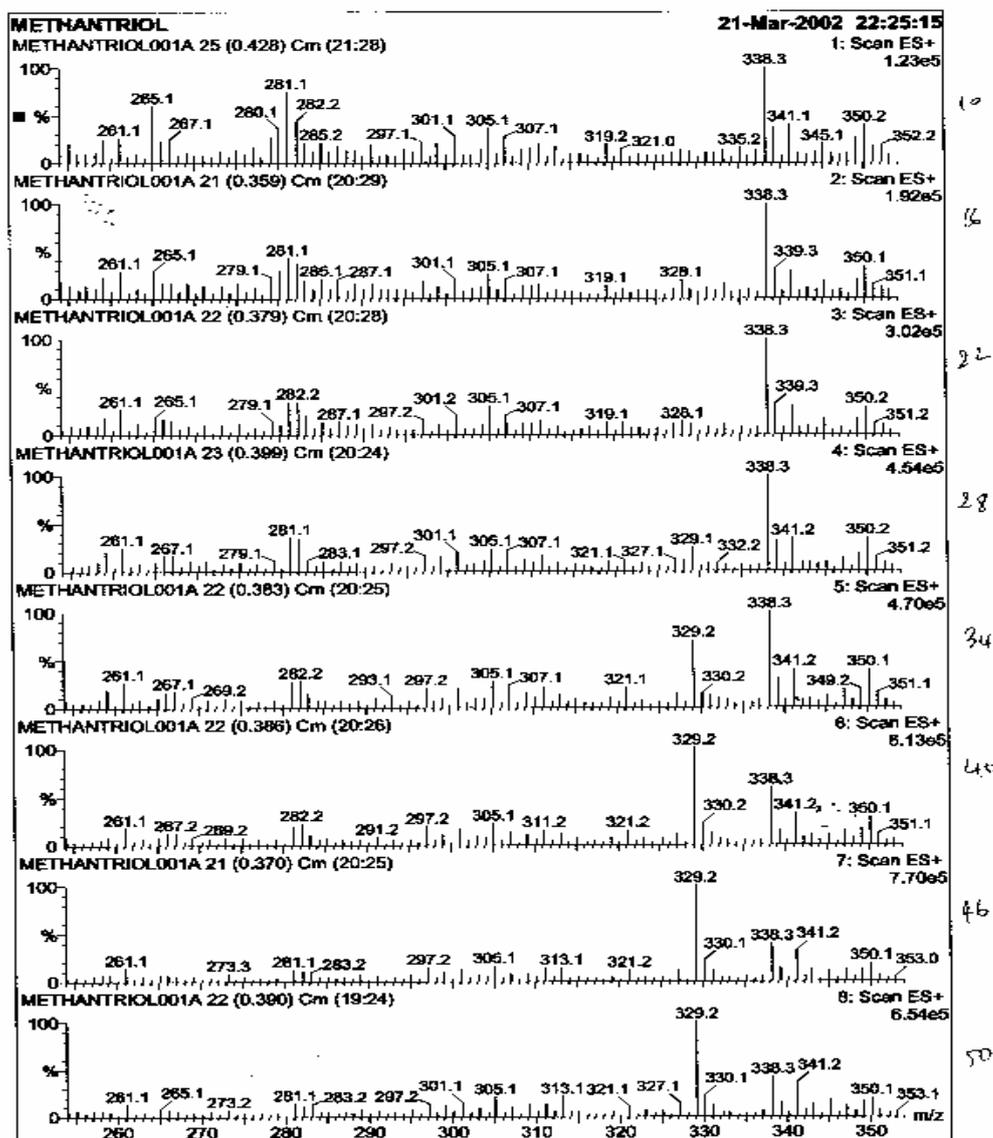


Figure 6. ESI spectra obtained from infusion of a solution of methandriol into a Waters Quattro Micro MS at cone voltages from 10 to 50 V.

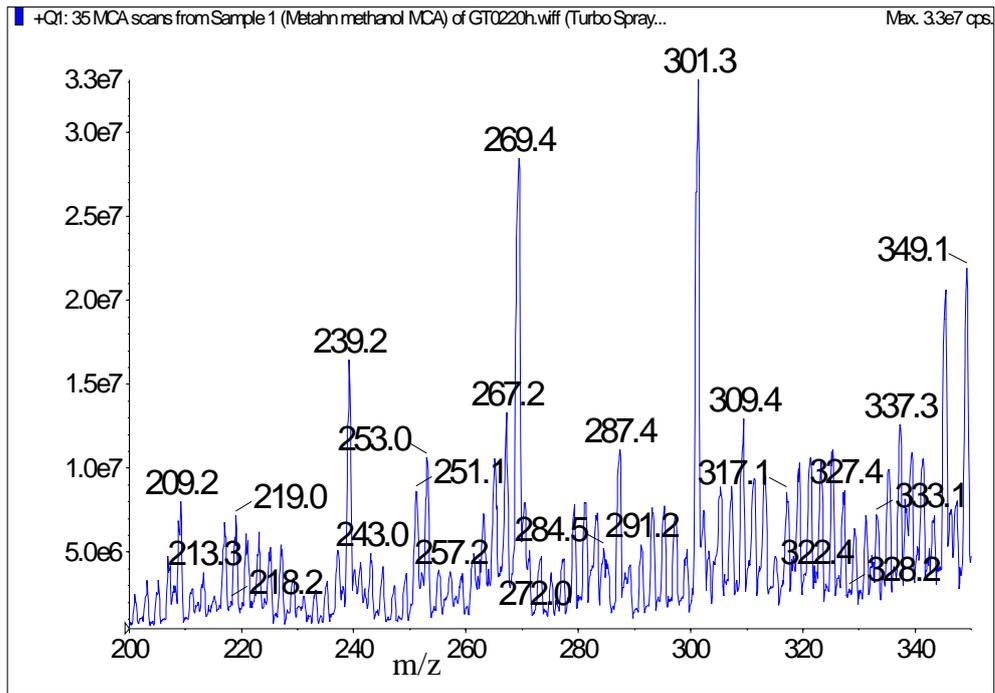


Figure 7. ESI spectrum obtained from a water/methanol solution of methandriol infused into an API 4000 Qtrap.

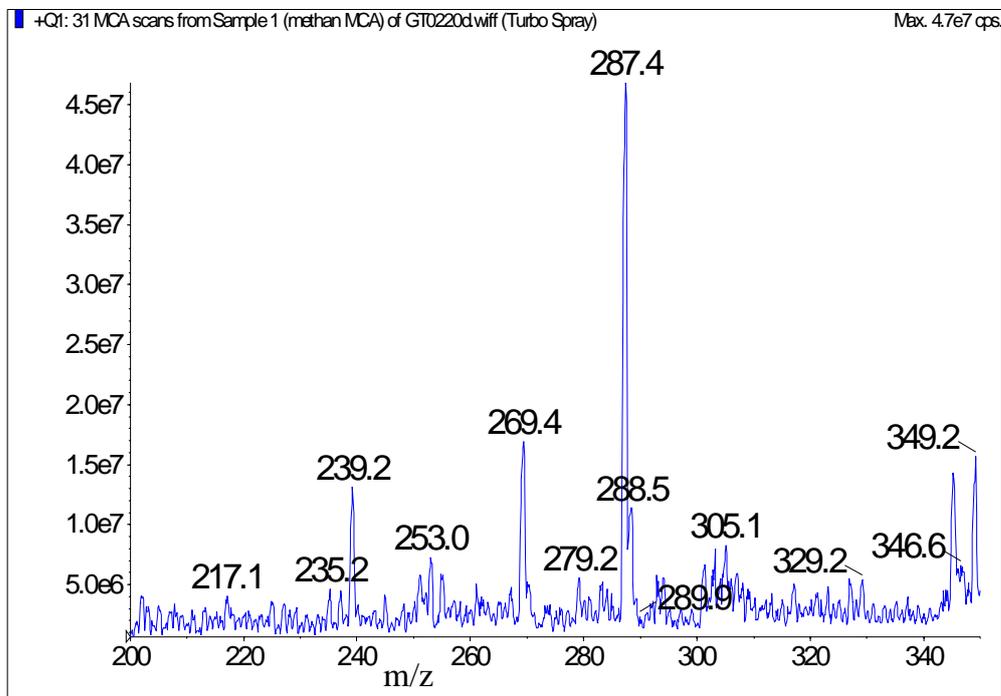


Figure 8. ESI spectrum obtained from a water/acetonitrile solution of methandriol infused into an API 4000 Qtrap.

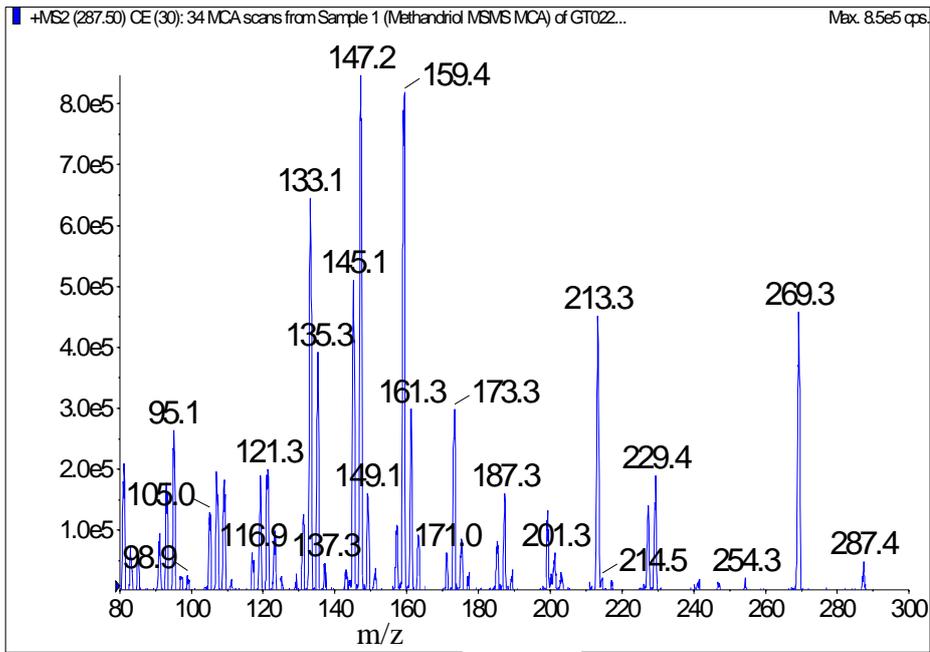


Figure 9. Product ion spectrum obtained from the precursor ion 287 from methandriol.

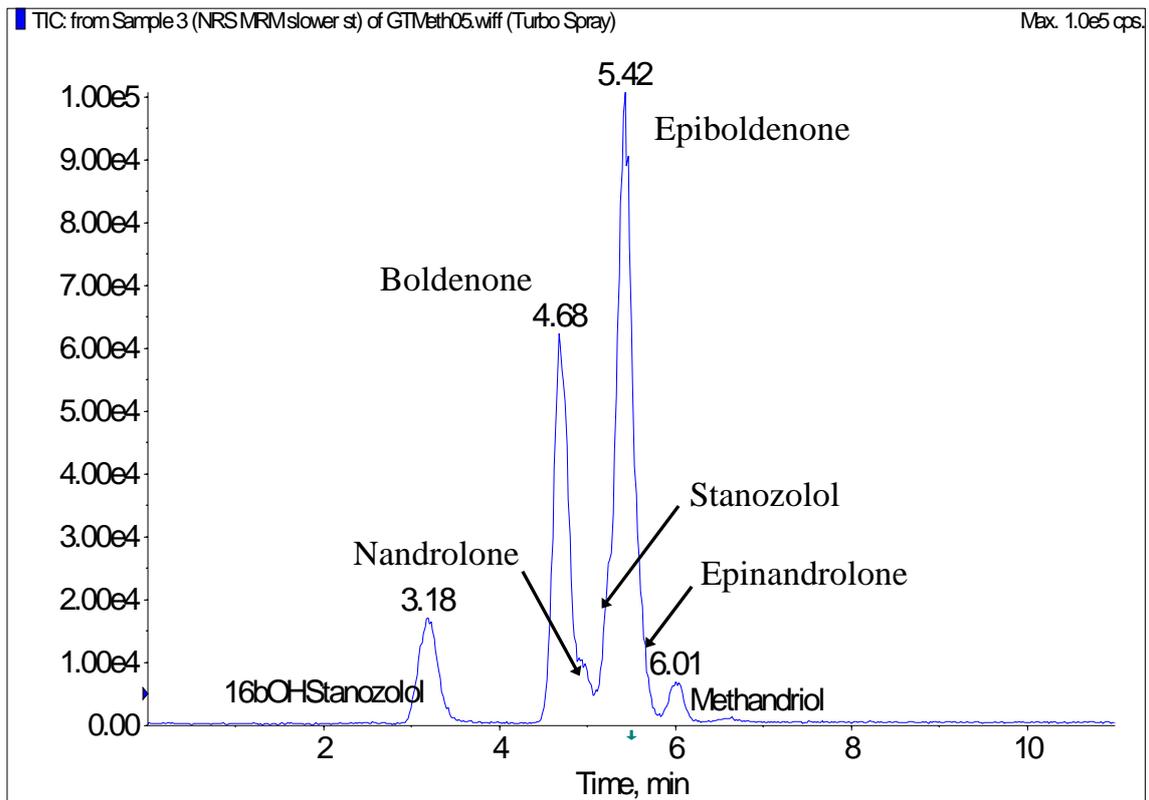


Figure 10. TIC from the 10 MRM transitions obtained using the API 4000 QTrap from a standard mix at a concentration equivalent to 3 ng/mL in urine.