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Miscellaneous Projects 2004

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Background

This paper covers a collection of small projects which may be considered as topical for our laboratory over the past year or so. These are a continuation of the study of elimination studies using available substituted nandrolone analogues, the issue of obtaining reasonable results for morphine and especially codeine analysis taking into account the poor hydrolysis of codeine glucuronide and some important observations on the analysis of hCG in the presence of urines which contain sediment.

Designer drugs based on substituted nandrolones

In the Cologne Workshop in 2004 a talk was presented on some methyl-susbtituted nandrolone analogues, namely 18-methylnandrolone and 7a-methylnandrolone (MENT) (Kazlauskas, 2004). Other substituted nandrolone derivatives that were available commercially were 17α-methylnandrolone, 4-fluoronandrolone and 19-norclostebol.

All studies were carried out by our routine steroid screening procedure. To the urine (3 mL) were added internal standards, 0.2 M phosphate buffer pH 7, E. coli glucuronidase and the mix was incubated at 50 ^{o}C for 1.5 h. The mixture was loaded onto a 3M Empore C18 cartridge (using the automated Gilson ASPEC system) and eluted with water, 25% methanol/water, hexane and the steroids eluted with 5% ethyl acetate/methanol. The collected fraction was evaporated to dryness and derivatised with MSTFA/TMSI/ethanethiol. The sample (3 uL) was injected onto an Agilent 6890/5973 mass spectrometer using an Agilent HP1 column, 17 m length, 0.20 mm id, and 0.11 um film thickness. The temperature programme used was 184 ^{o}C then 3 ^{o}C /min to 235 ^{o}C then 10 ^{o}C /min to 265 ^{o}C then 30 ^{o}C /min to 310 ^{o}C and then hold for 2 min.

17a-methylnandrolone

The study of the methyl substituted nandrolones was felt to be important because of the possibility that they may be masked by endogenous steroids which in the screen have the same molecular weight as the proposed metabolites of the "designer drugs". Thus 17α-methylnandrolone itself has the molecular weight of testosterone and its possible reduced metabolites will correspond to molecular ions and (D_2M^+) isotope of androsterone and etiocholanolone as shown in Figure 1. If they have similar retention times then it may be impossible to identify their presence due to the comparatively large amount of the natural metabolites.

The 17α-methylnandrolone was obtained from Steraloids (E3900-000, 4-estren-17α-methyl-17β-ol-3-one) and on receipt the identity and purity was checked. For the study, 15 mg in a gel capsule was administered to a volunteer. Urine samples were collected at 0, 4, 8, 12, 17.5 and 28 h. The extracted ions for the Enol-TMS derivatives of the parent substance and the postulated metabolites are shown in Figure 2. The mass spectra corresponding to the two metabolites are shown in Figure 3.

Both metabolites have molecular ions 4 mass units higher than the parent and suggest reduction of both the keto and double bond. By analogy to the metabolites of methyltestosterone and of nandrolone it may be postulated that the most likely stereochemistry would be 3α,5α and 3α,5β for the M1 and M2 metabolites. Both of the metabolites are well separated from androsterone and etiocholanolone and it appears there is no interference with detection of these substances.

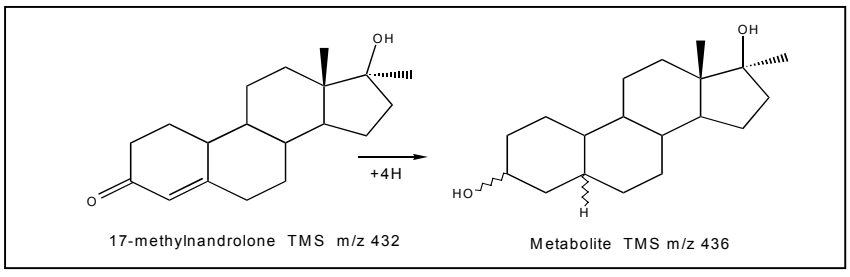

Figure 1

The elimination profile of the two metabolites is shown **in Figure 4** and they remain detectable for about 24 h after an oral dose. The data is presented as the peak height ratio of the signal relative to the peak height of the internal standard (methyltestosterone) added at 100 ng/mL.

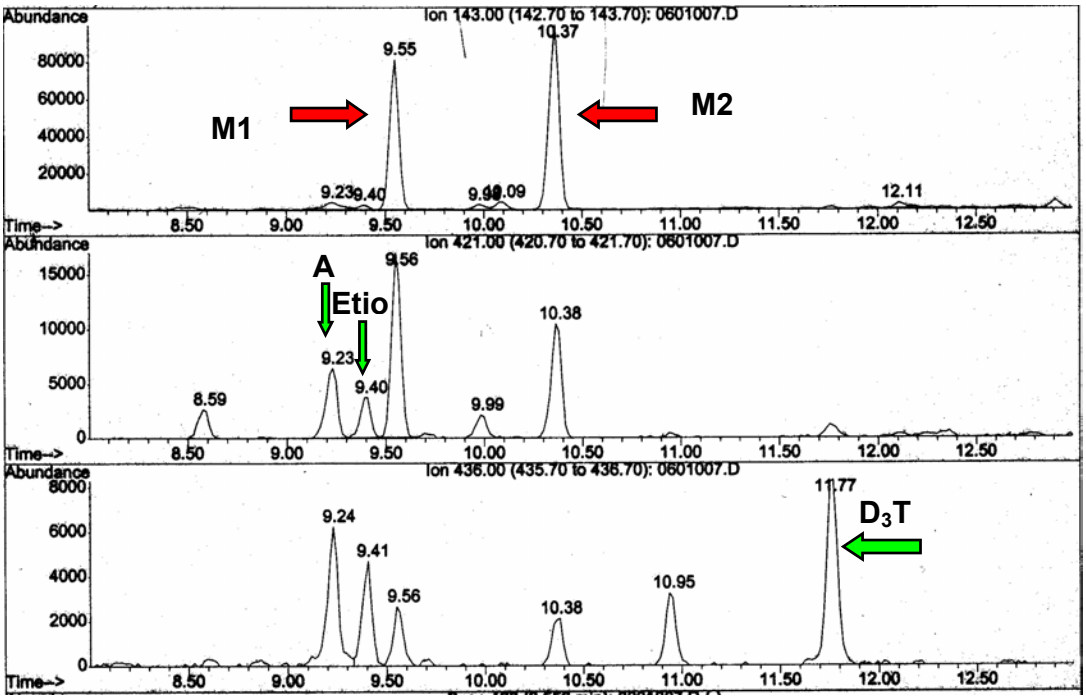

Figure 2 Extracted ions from TMS derivatives of the excretion study for 17-methylnandrolone showing the two metabolites.

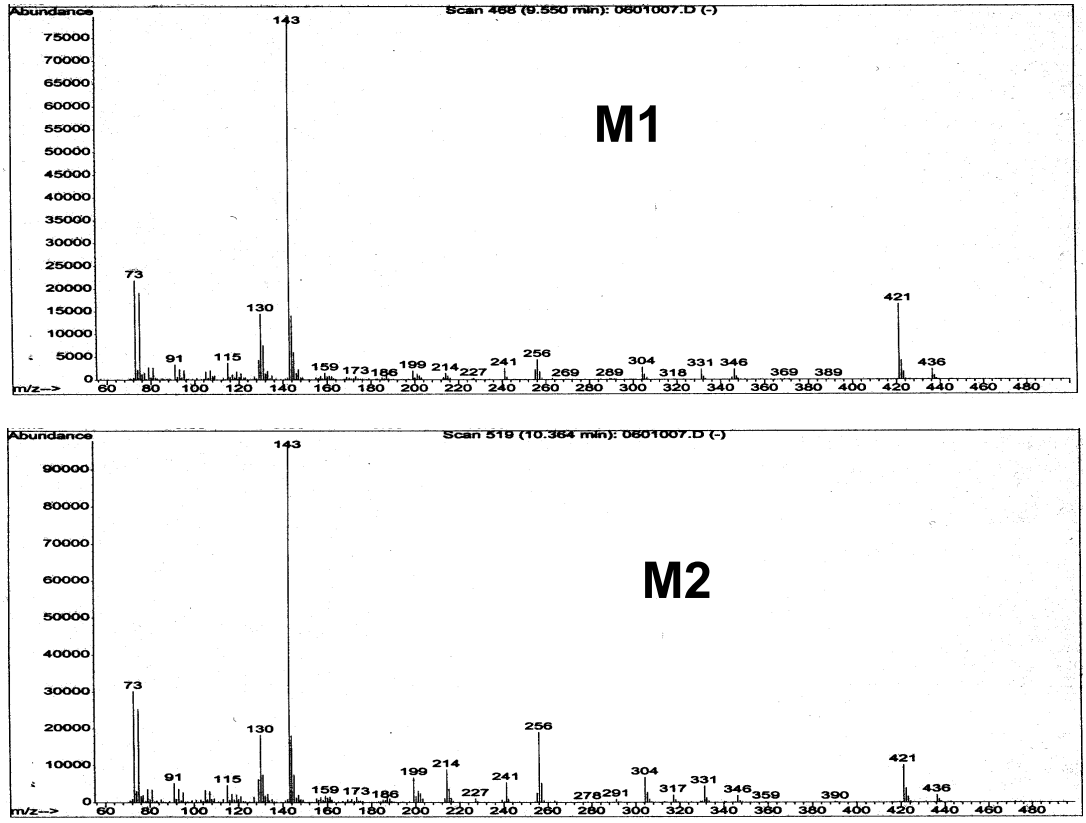

Figure 3 Mass spectra of the two metabolites from 17-methylnandrolone

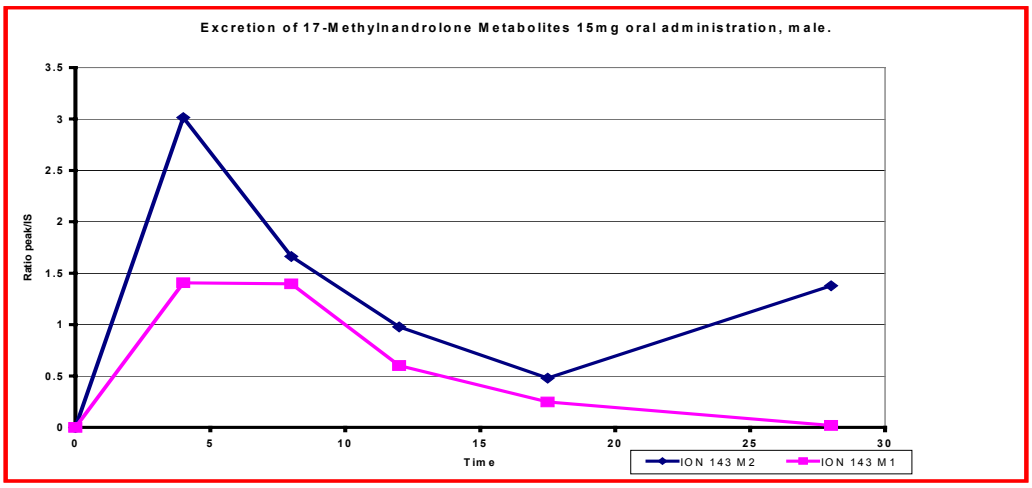

Figure 4 The elimination profile of the two metabolites after ingestion of 15mg 17-methylnandrolone

4-fluoronandrolone

4-Fluoronandrolone is of interest because it has a molecular weight of 436 and reduction of keto and/or double bonds may give metabolites hidden under deuterated internal standards d4-androsterone/d5-etiocholanolone.

For the excretion study a volunteer ingested 5 mg of 4-fluoronandrolone (Steraloids, E3675-000, 4-estren-4-fluoro-17β-ol-3-one). Urine samples were collected at 0, 3.5, 7, 14, 20, 26 and 30 h. Analysis of the urine samples indicated one major metabolite eluting before androsterone and different to the starting 4-fluoronandrolone (11.82 min, m/z 436(100%), 421(5), 305(5), 212(5)) which was found in very small amount just before testosterone. In keeping with typical 17-keto steroids it mainly gave the molecular ion. The main metabolite had a retention time of 8.79 min (see **Figure 5** for the extracted ions at 7 h after ingestion) and a molecular ion at m/z 436 (see **Figure 6**). The proposed structures for the metabolites is shown in **Figure 7 (X=F)**. The stereochemistry at the 3 position is not known but may be expected to be 3α by analogy to other steroids in this class.

One further metabolite at low level was also seen at 9.56 min. This may be the compound further reduced at C4,5 as it gives a molecular ion at m/z 438 and retains the 169 ion characteristic of enol TMS at the 17 position. The stereochemistry is not evident as only one metabolite can be seen. The profile for the excretion of the two metabolites is shown in **Figure 8**. Note the delayed excretion of the metabolite M2.

Norclostebol

Norclostebol was of interest as it should have a metabolism similar to the 4-fluoronandrolone and it has been available as a steroid for some time. Norclostebol (4-chloronandrolone) was available as its acetate. For the excretion study a volunteer took 11 mg of norclostebol

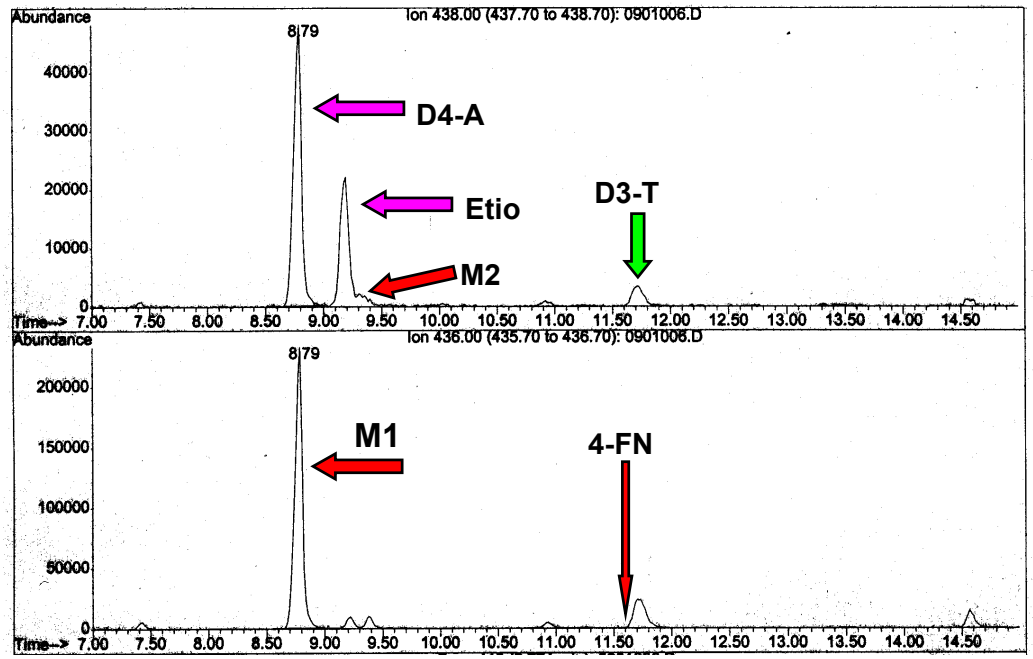

Figure 5 The extracted ions for the TMS derivative of the excretion study for 4-fluoronandrolone

acetate in a gel capsule and collected urine samples at 0, 5, 13, 16, 28 and 37 h. Three metabolites were found. The major metabolite M1 was at 11.78 min with a m/z 452, the same molecular weight as that expected for norclostebol (see **Figure 9**, for the extracted ions and **Figure 10** for the full scan spectrum). This metabolite was different from the norclostebol prepared from the norclostebol acetate by acid hydrolysis which had retention time of 15.66 min m/z 452(100%), 454(50), 437(6), 417(5), 321(6), 216(5) and as expected gives mainly the parent ion. Base hydrolysis only gives a mixture of dechlorinated compounds. Its proposed structure is given in **Figure 7** (X=Cl, m/z 452 TMS derivative).

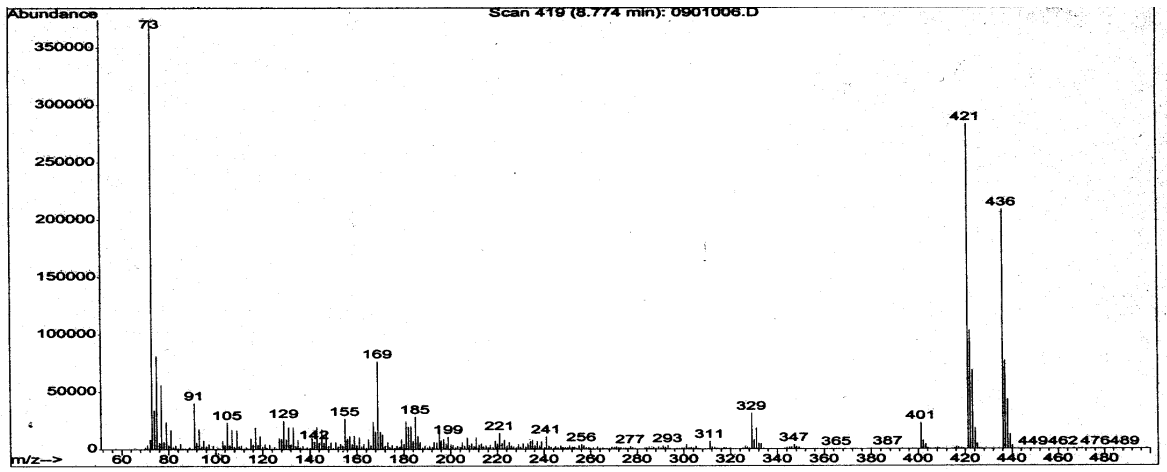

Figure 6 Mass spectrum of metabolite 1,TMS derivative from 4-fluoronandrolone.

Two further metabolites were also seen (see **Figure 9**). These gave similar spectra with ions at m/z 454(56%), 456(30), 439(100), 441(48) and may arise through reduction of the double bond in a similar fashion to the 4-fluoronandrolone

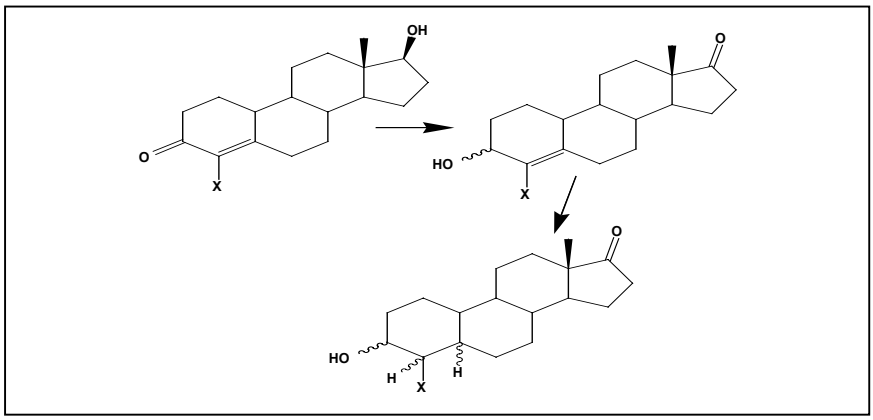

Figure 7

MORPHINE/CODEINE MEASUREMENTS

After administration of codeine the major components in urine from the elimination are free codeine, codeine glucuronide, morphine 3-o-glucuronide, and free morphine. Codeine and morphine quantification is required for drug in the workplace testing and in Australia this is set out in the Australian standard AS4308. For doping analysis codeine is not banned but WADA has put the morphine/codeine ratio on the monitoring list. Presumably this is linked to reports that this ratio may allow distinction between heroin, morphine and codeine ingestion (Posey 1984).

In general it appears that codeine quantification in proficiency studies is very poor. Morphine is better but needs improvement. This appears to result from incomplete enzymatic hydrolysis or incomplete acid hydrolysis, which in the absence of the glucuronide standards, has prevented measurement of reasonable recovery information. If the quantification of either one or both of these parameters is poor then the morphine/codeine ratio results will be meaningless.

In general the 6-O glucuronides of these narcotics are difficult to hydrolyse while the 3-O-glucuronide of morphine is able to be hydrolysed more efficiently. Due to the lack of appropriate glucuronide standards the recovery is particularly difficult to estimate.

For the hydrolysis and quantification procedure to be under control one needs to use the glucuronides for calibration and their deuterated forms as internal standards in order to make the analysis independent of the recovery. There are several possibilities depending on which standards are available. For example:

- If calibration is performed using codeine and d3-codeine then the recovery of glucuronides can not be taken into account at all.
- if calibration is performed using codeine glucuronide and d3-codeine the slope of the calibration curve will be low due to poor recovery of unlabelled codeine and high recovery of deuterated codeine
- If calibration is performed using codeine and d3-codeine glucuronide the slope will be too high due to high recovery of codeine and low recovery of the d3-codeine.
- Some correction may be made for the latter two cases as it is possible to estimate recovery by comparing the data from the slope of the calibration curve to the expected ratio measured from the free forms of standards. Thus by measuring as a separate mixture, a codeine/D3-codeine ratio using a mixture of codeine and d3-codeine standards and comparing it to the equivalent amount of codeine and d3-codeine that would theoretically be released from codeine glucuronide and D3-codeine glucuronide. This would provide a useful correction factor.

Both codeine glucuronide and d3-codeine glucuronide are recently available from the Chemical Reference Materials Section of the NMI, Pymble Australia (http:\\www.measurement.gov.au) and this allowed the quantification of codeine to be revisited. We analyse both codeine and morphine using an extractive alkylation procedure with ethyl iodide to allow differentiation between codeine and morphine.

Since codeine is excreted as the glucuronide and as the conjugated form it is necessary to remove the free codeine and analyse it separately. If this is not done the recovery of codeine will be too high. An example of this was seen in our lab for the AUSTOX Proficiency study performed in September 2004 (pooled patient sample). The sample provided was a spiked sample containing 250 ng/mL codeine. We analysed it as though it was a real excretion using the glucuronide materials and obtained a figure of 500 ng/mL. This overestimation was due to high recovery when a low recovery was expected as it was **not a realistic sample**.

Figure 12 shows results for the August 2004 AUSTOX study where the ASDTL result was one of the higher values. In this study the other laboratories with high codeine values perform mainly acid hydrolysis, but without a deuterated internal standard there is no control of the hydrolysis on a sample to sample basis giving rise to large uncertainty.

hCG Issue

We have routinely performed the analysis for hCG in the following way:

- Screening assay - DPC Immulite hCG CLIA on unaltered urine

- Confirmation - DPC Immulite hCG CLIA after buffer washing over 10,000 MWCO ultrafilter
- Second immunoassay - Bioclone Elegance hCG ELISA on unaltered urine. Bioclone assay usually returns lower apparent hCG concentrations than the Immulite method

Recently we obtained a result after repeated analysis which indicated to us that hCG is strongly absorbed onto sediment that is often seen in urine samples. Initial analysis gave good data for hCG. After freezing and thawing the precipitate was removed by centrifugation at 1500g and the supernatant transferred to a new tube. No HCG was found in the supernatant! The pellet was redissolved in water and values similar to that previously measured were found. **Figure 13** shows the results, where S2A is the original sample, S2B is the sample after freezing and thawing and S7 is a sample from an athlete who was shown to have a midline chest tumour. The best recovery was from a the samples diluted 50:50 with water, thoroughly mixed to dissolve all residue and the measurements obtained corrected for the new volume.

Since the practice of removing the precipitate has been part of our procedure for screening hCG the questions arise: Have we been throwing away the HCG in samples for years? Are there other analytes that absorb onto the sediment? Since most of the sediment usually dissolves on heating at 50C in a water bath for about 10minutes it is easier to ensure dissolution than to remove it and have the possibility of variation in measurement.

Acknowledgements

The staff at ASDTL need to be acknowledged for sample preparation, K. Gazi for the study of the codeine analysis, NARL for preparation of glucuronides of codeine, C.Howe for work on the hCG study and John Lewis and Peter Bowron for the AUSTOX data

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Posey, BL., Kimble, SN., High-Performance Liquid Chromatographic Study of Codeine, Norcodeine and Morphine as Indicators of Codeine Ingestion, J. Anal. Tox., 1984, 8, 68-74.

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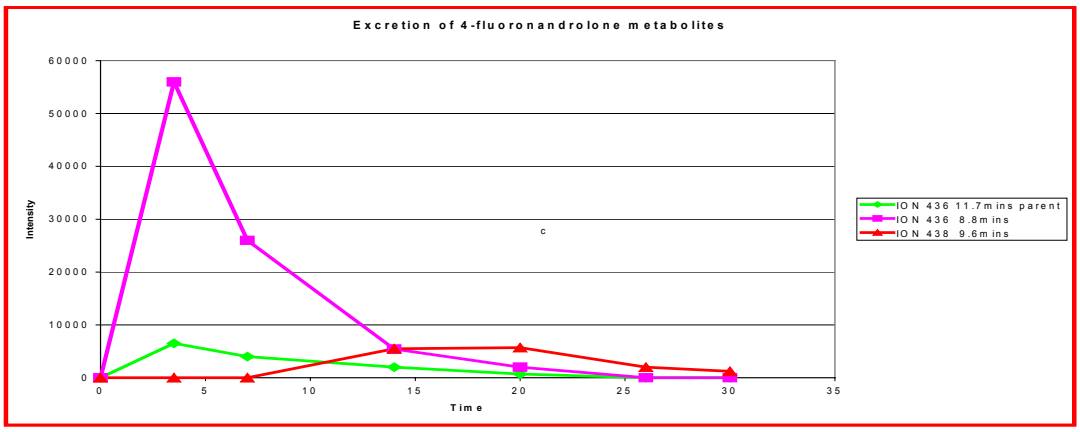

Figure 8 The elimination profile of the two metabolites of 4-fluoronandrolone.

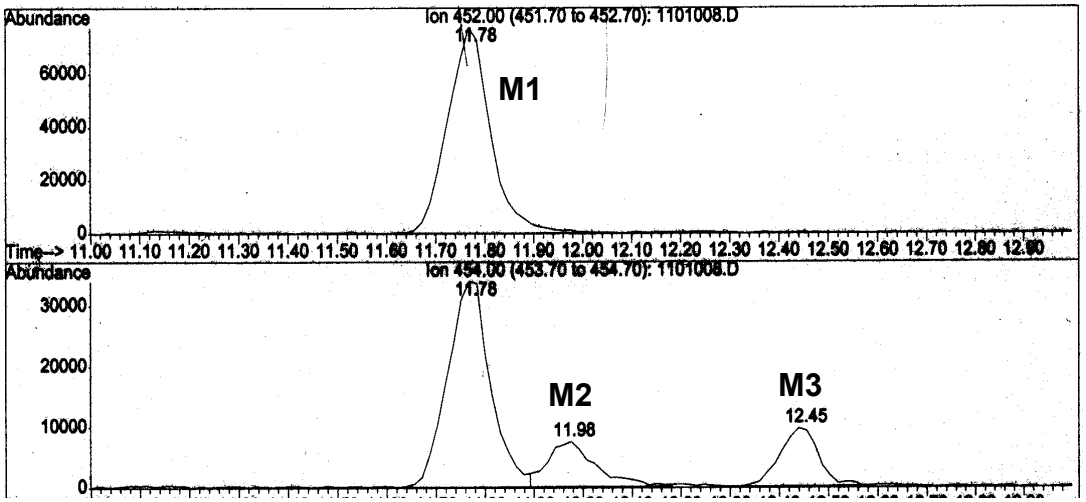

Figure 9 The extracted ions for the TMS derivatives for the three metabolites of norclostebol.

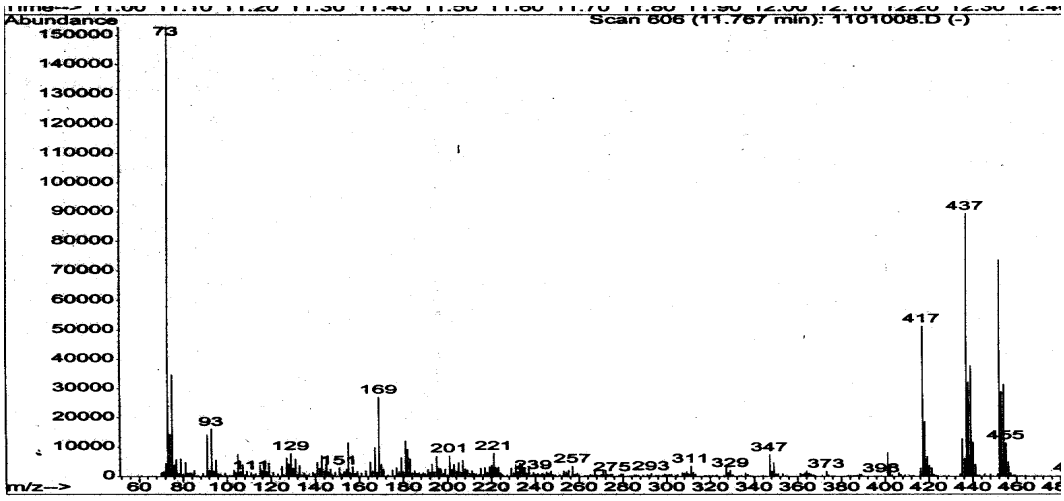

Figure 10 The mass spectrum of the TMS derivative of the main metabolite of norclostebol.

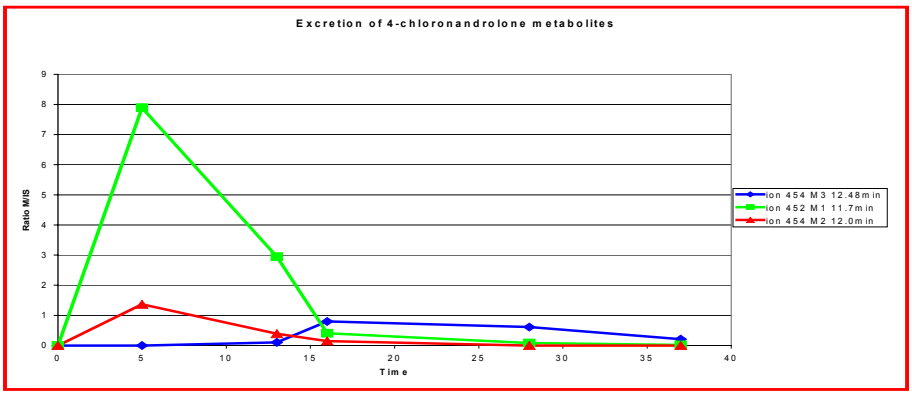

Figure 11 Elimination profile of the 3 metabolites of norclostebol.

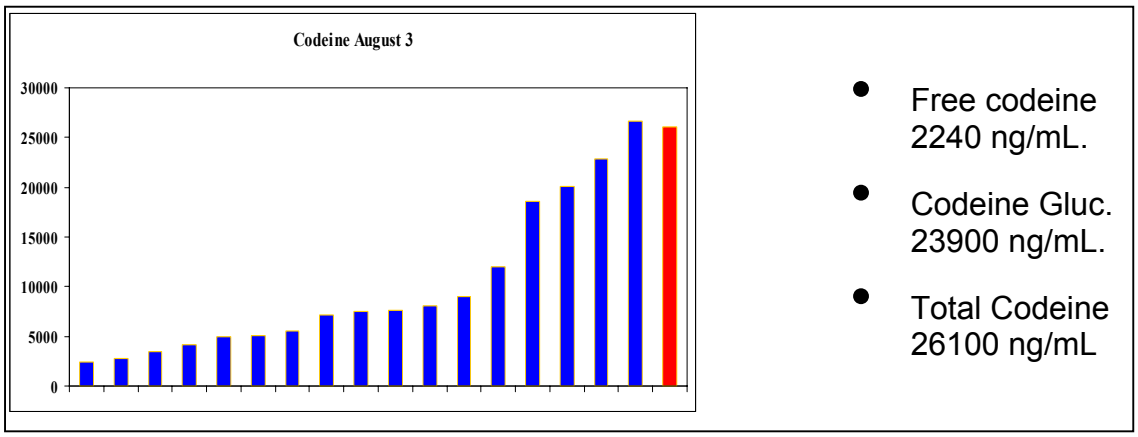

Figure 12 Comparison of the 18 laboratories for quantification of codeine in a pooled urine sample. The result using glucuronide standards is at the right hand end.

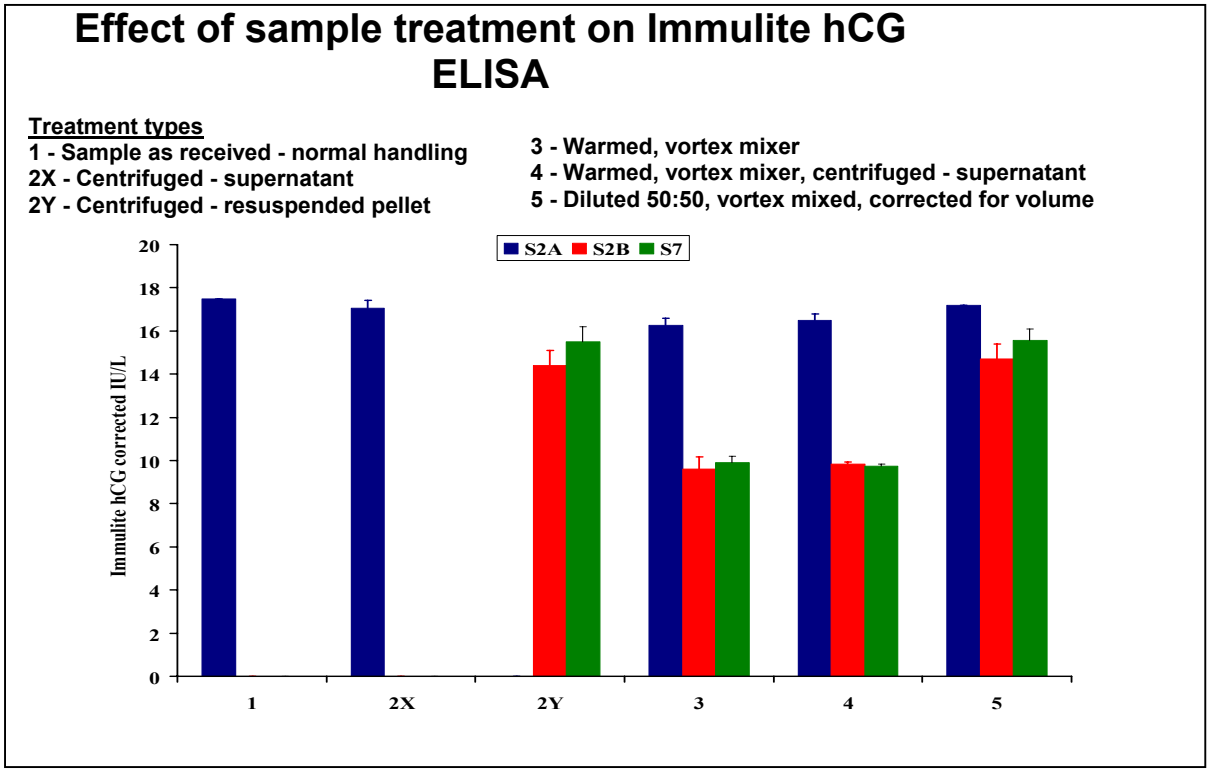

Figure 13 Comparison of the results for the measurement of hCG in the sample (S2A), the same frozen thawed sample (S2B) and a sample from another athlete (S7)