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Miscellaneous Projects in Sports Drug Testing at the National Measurement Institute, Australia, 2005

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Background

This paper covers a collection of small projects that may be considered as topical and that has been undertaken in our laboratory over the past year or so. The topics presented cover a rapid method to distinguish between ephedrine and pseudoephedrine as well as some studies into supplements that appeared within Australia and on the US market in 2005 and then rapidly disappeared as authorities realised that many contravened the recently introduced steroid supplements laws.

Distinguishing ephedrine from pseudoephedrine

Within Australia ephedrine use by sports persons is very rare with pseudoephedrine making up a large proportion of suspicious cases within our laboratory. In 2004 we had 135, in 2005 228, and in the first 3 months of 2006 56, cases of pseudoephedrine detected in the stimulants screen. The issue for anti-doping laboratories is that pseudoephedrine is not banned but ephedrine is banned for use in sport. Similarly cathine is banned but phenylpropanolamine is not. Up to now we have used the traditional "screen 1" using the GC/NPD and then had to perform the equivalent of a confirmation using the pentafluorobenzoyl derivative (Kazlauskas *et a*l 1999) to distinguish whether the finding was due to pseudoephedrine or ephedrine. The time taken for this was considerable and a simpler method was required.

A paper on the use of cyclohexanone (El-Haj *et al* 2003) to perform this differentiation was published in 2003. This method was analogous to the use of formaldehyde in our laboratory to provide sharper peaks in the GCNPD quantification but with no separation of the enantiomers, but the larger cyclohexanone function provided a marked difference in retention time between the various enantiomers. Thus the simple procedure of taking 200 µL of the t-butylmethylether layer from the basic stimulants extraction and adding 50 µL of cyclohexanone provided the new derivatives. Our normal protocol was to run any suspicious sample from the stimulants screen

using GC/NPD on the GC/MS to determine the composition of the peaks. This included samples that contained ephedrines to ensure the peak was not due to some other substance. The derivatised samples were simply added to the end of the GCMS sequence and analysed in the same batch with no extra work.

The reaction of cyclohexanone with ephedrine at room temperature was found to be much slower than with pseudoephedrine. Cathine and phenylpropanolamine reacted almost immediately. Thus by putting the samples at the end of the sequence time was allowed for the reactions to proceed to an appreciable amount. The time course for the various ephedrines is shown in Figure 1. Heating the sample to 50ºC greatly reduced the time for the reaction to go to completion.

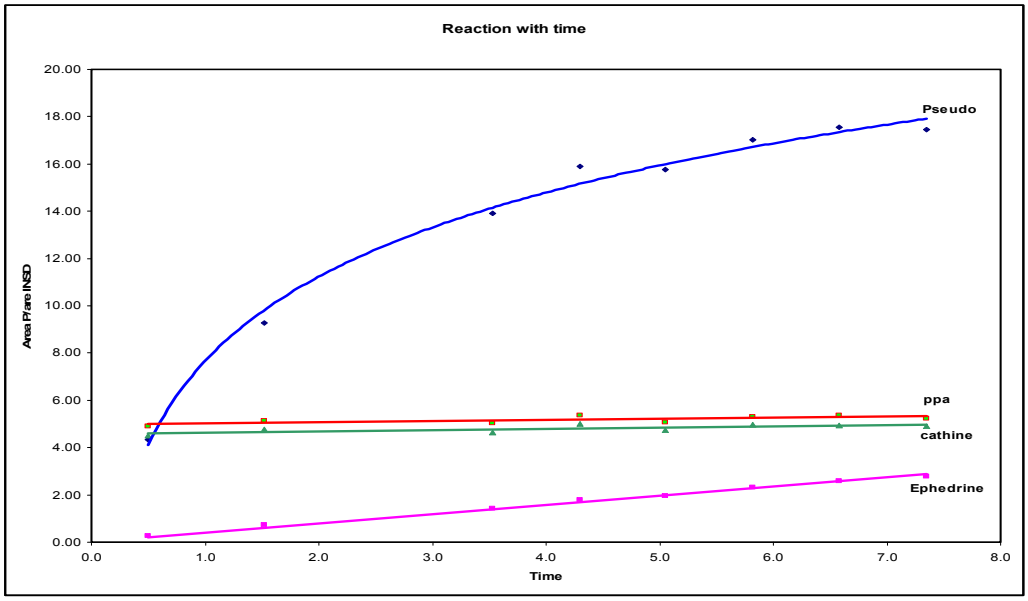

Fig 1. Rates of the reaction of cyclohexanone with ephedrines relative to the internal standard. The ephedrine was at 10 ng/mL, pseudoephedrine at 25 ng/mL and the cathine at 5ng/ml and phenylpropanolamine (ppa) at 25 ng/mL.

The separation of the various derivatives was sufficient to ensure unambiguous identification using relative retention times. A typical chromatogram is shown in Fig 2 and the mass spectral fragmentation is shown in Fig 3. Note that the fragmentation is different for the nor – ephedrines and this is reflected in the pseudoephedrine and ephedrine forming isoxazolidines and the cathine and phenylpropanolamine forming simple Schiff's bases.

The overall cost saving for us through the introduction of this method is substantial since we no longer had to do the equivalent of 228 (in 2005) confirmations for a substance that was not banned. The current procedure adds very little time to our analysis time.

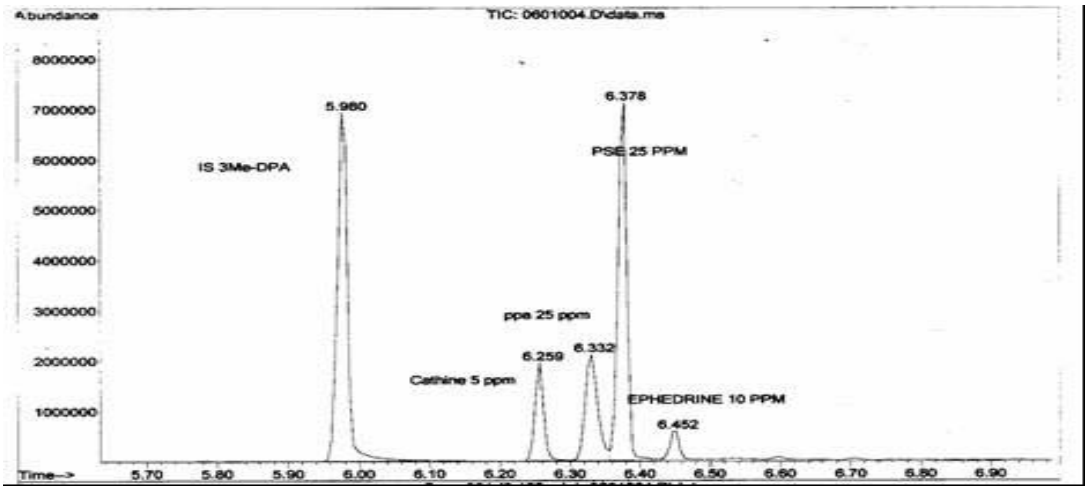

Fig 2. The TIC for the reaction showing the separation of the cyclohexanone derivatives of the four ephedrines.

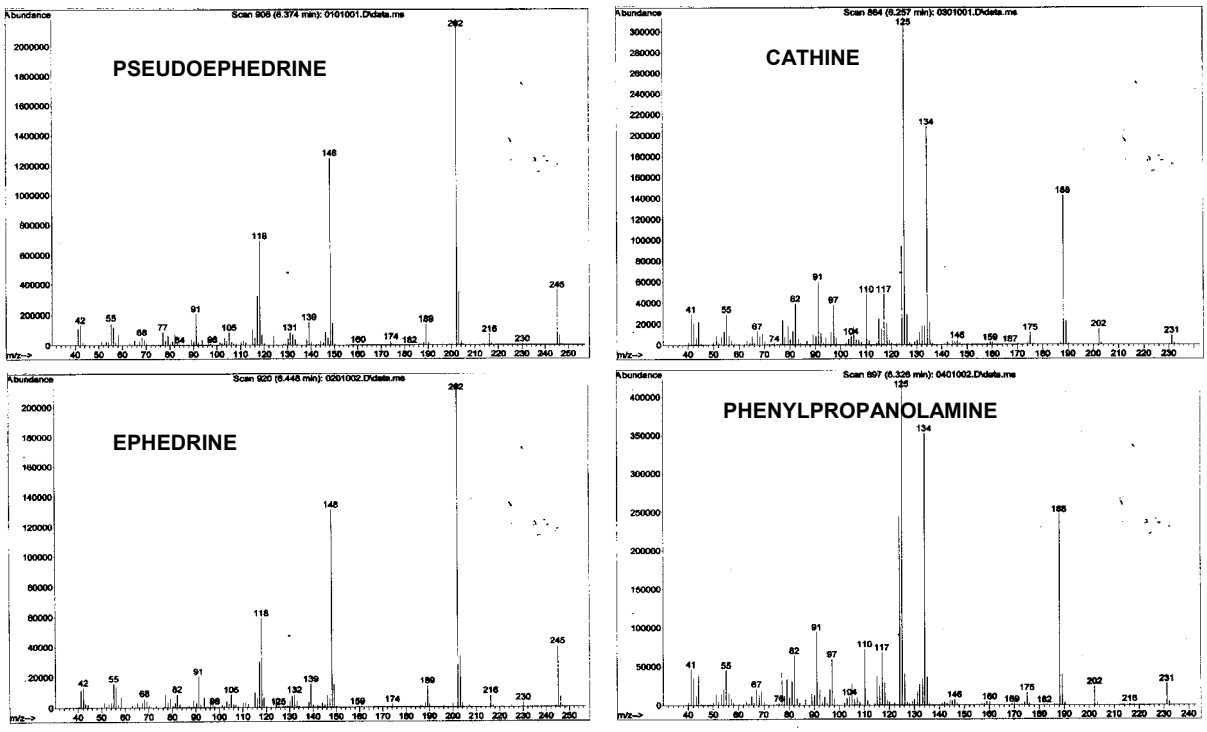

Fig 3. Mass spectrum of each of the cyclohexanone derivatives.

Benzylpiperazine

Benzylpiperazine is used as substitute for amphetamines and its pharmacology is similar to methamphetamine. It is restricted in most of Australia but is sold over the counter in New Zealand where even the local shops have it available as a party pill.

A description of the detection of benzylpiperazine was presented in Cologne previously (C.Manzoni *et al* 2001.) but it does not seem to have ever been reported as a banned substances nor as an adverse analytical finding within sport. Recently in Australia and New Zealand seven

weightlifters were found positive for benzylpiperazine and they were sanctioned for 2 years. An investigation into this was carried out. The athletes claimed it was from a supplement to which benzylpiperazine had been added. When the analytical finding was made we referred to WADA to determine if benzylpiperazine was classified as a banned substance. They confirmed that they considered it as a related substance but it did not appear on the 2007 draft as a named substance.

The detection of benzylpiperazine is very straight forward as it is volatile and is seen in the stimulants screen "screen 1" with a RRT to DPA of 0.85. The typical TIC trace for a positive sample is shown in Fig 4 and the full scan spectrum in Fig 5.

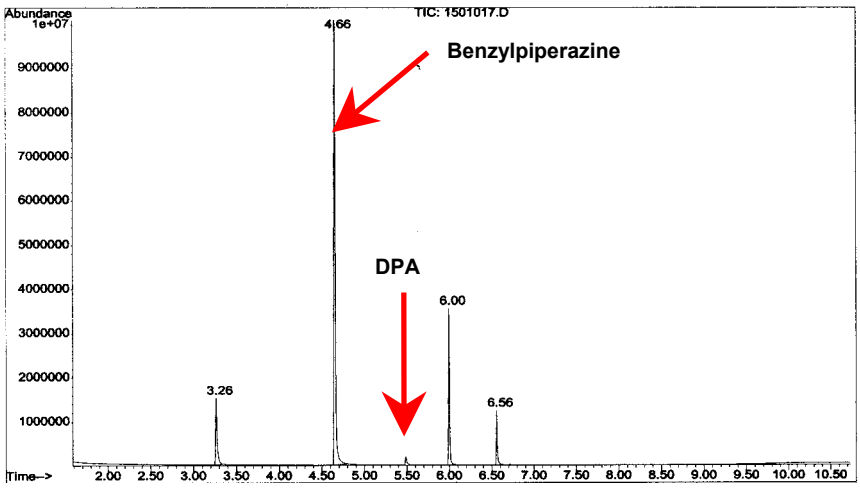

Fig 4. The TIC spectrum for the analysis of benzylpiperazine in a urine sample. The DPA is present at 200 ng/mL.

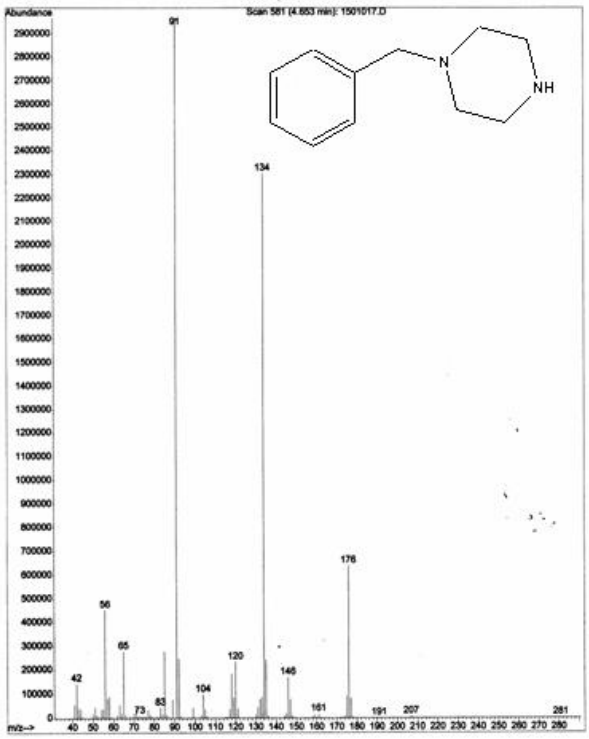

Fig 5. Mass spectrum of benzylpiperazine showing good molecular ion and three diagnostic peaks.

Supplements

During 2004/early 2005 a number of steroid supplements were available on the web as nutritional agents sold by bodybuilding sites. Several articles appeared in the Washington Post with information provided by Professor D. Catlin (UCLA) showing that these were in fact steroids that should not be available in this type of market. Many of these were rapidly withdrawn and are now unavailable. We were able to purchase many of these from the websites before they were withdrawn and have managed to look at the structures and metabolism.

Prostanazol

Prostanozol is sold by Anabolic Xtreme and Orastane-E by Gaspart Nutrition. It was labelled as [3,2-c]-pyrazole-5alpha-etioallocholane-17beta-tetrahydropyranol 25 mg – again presumably in an effort to hide its relationship to androstane.

The main component was isolated and recrystallised, and spectral data (MS, 1H-NMR) were obtained. By GCMS and derivatisation with MSTFA a mass spectrum of the 17-hydroxy compound was obtained, and no tetrahydropyranyl (THP) derivative was found. By LCMS we found both the hydroxy compound and the THP derivative. The THP derivative appears to readily hydrolyse even in methanol to give the hydroxy parent but hydrolyses much more readily in methanol/acetic acid. All data obtained were consistent with it being analogous to the 17-desmethyl-stanozolol structure.

An administration of prostanozol (100 mg) to a male volunteer was undertaken and urine samples were collected for 48 h. Extraction of the urine samples using the routine steroid GCMS (as derivatives with MSTFA/TMSI/Ethanethiol) screening process showed three main metabolites under the corticosteroid area of the chromatogram. This made it very hard to detect them at low levels.

Extraction of the metabolites using the routine stanozolol confirmation procedure (enzyme hydrolysis at pH 7 with E. coli, BondElute SPE washed with water, 1M acetic acid, methanol, drying the column and eluting the compounds with dichloromethane/isopropanol/ammonia) gave a very clean extract. This showed four compounds with the diTMS/enolTMS derivative having mass m/z 544 which changed to m/z 472 for the diTMS derivative on derivatisation with MSTFA/TMSImidazole reagent, indicating that the oxygen function at C17 was a carbonyl. The TMS/enolTMS derivatives are shown in Fig 9 and the corresponding spectra in Fig 10.

The GCMS data give m/z 254 for compounds I and II (see Fig 9) which indicates 3'-OH and 4-OH derivatives. Metabolites III and IV give no m/z 254 in GCMS. These may be hydroxylated in position 16 or other positions in rings C or D (Schänzer and Donike 1993)

For further structural identification, as well as analysis with much increased sensitivity, the LCMS and LCMSMS data were obtained for the extract from routine sample preparation for diuretics and corticosteroids (Trout 2005). The LCMS was run using a Prevail column 2mm x 50mm on the Quatro micro MSMS. This gives similar metabolites.

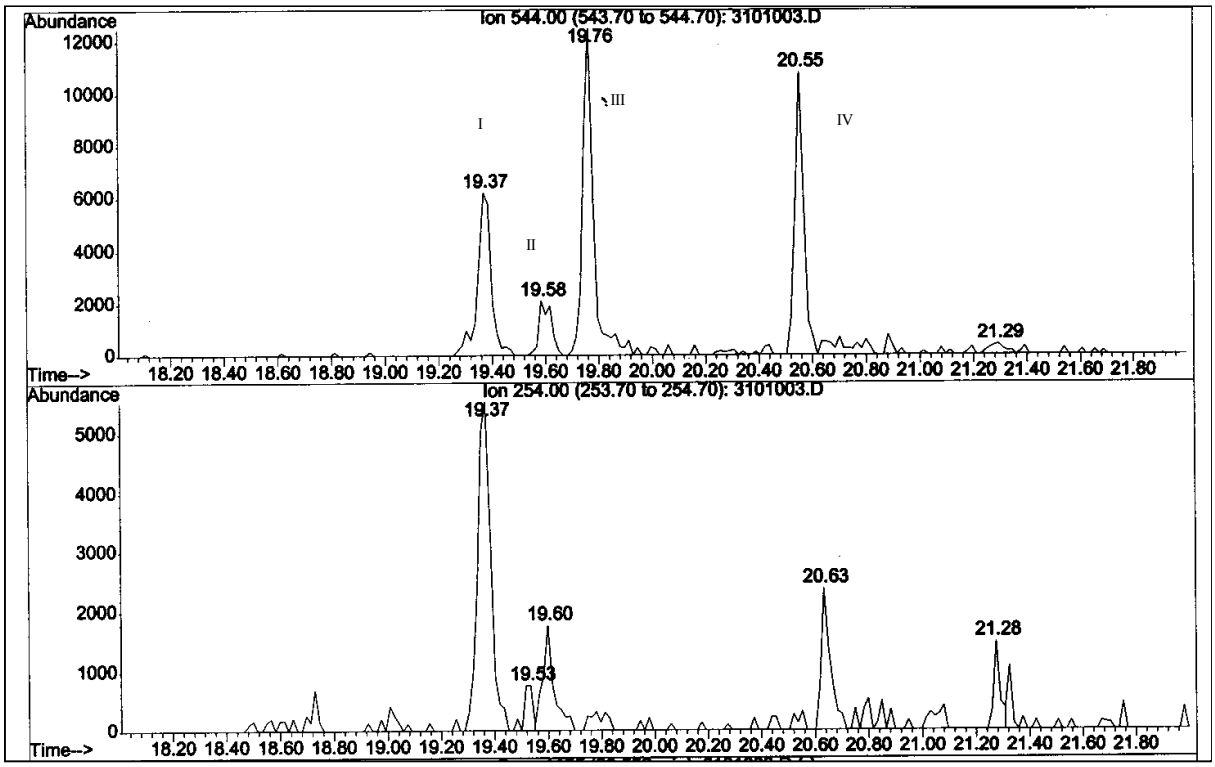

Fig 9. Extracted ions of the Prostanozol metabolites

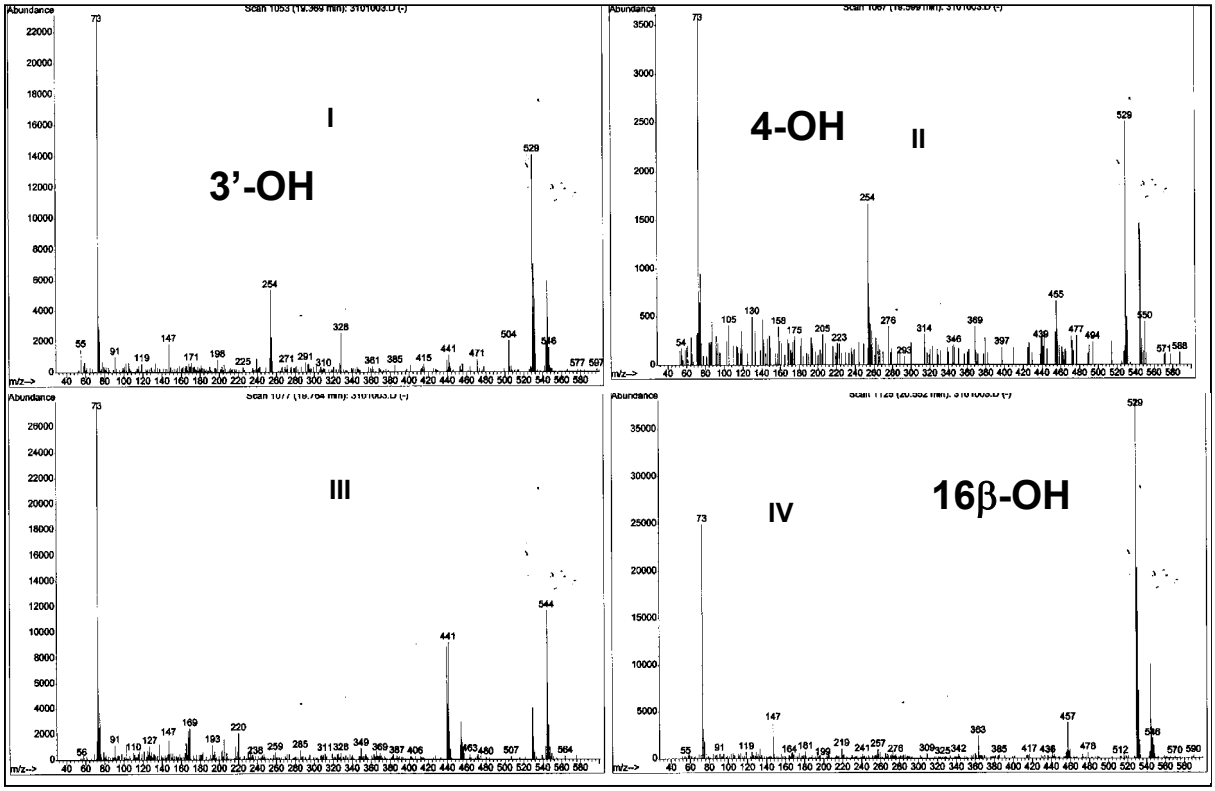

Fig 10. Mass spectra of the metabolites and proposed structures.

The LCMS chromatogram shows four metabolites with the same mass m/z 329 ($M+H^+$) corresponding to the TMS/enol-TMS derivatives seen in the GCMS analysis. This chromatogram is shown in Fig 11.

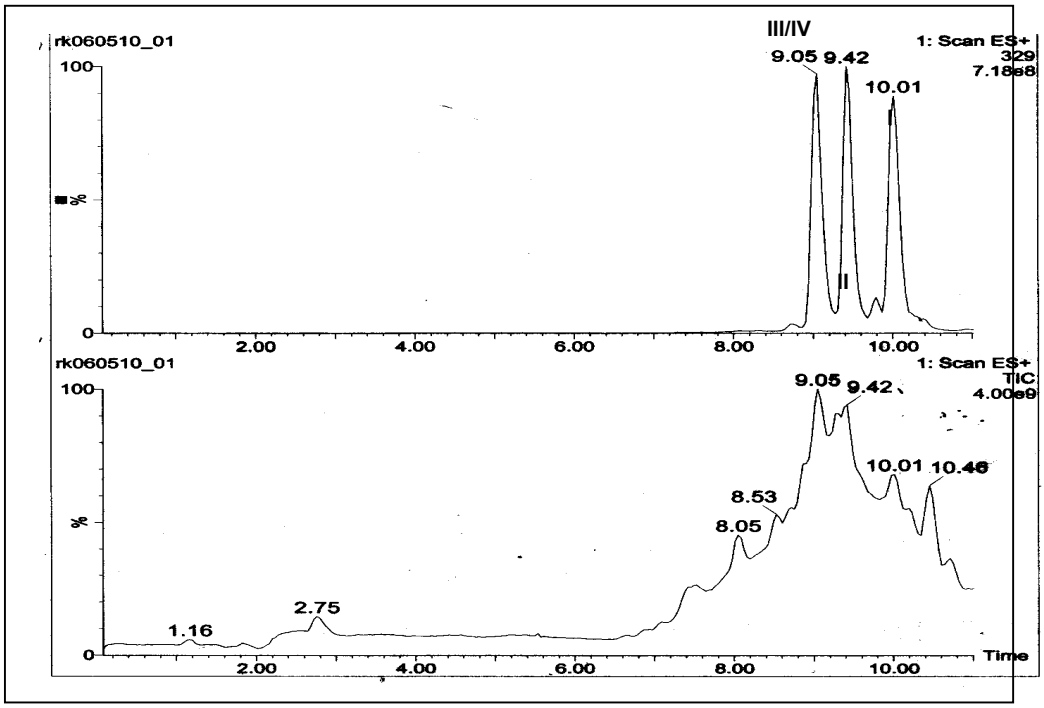

Fig 11. LCMS Chromatogram showing the TIC and the extracted $M+H^+$ ions at m/z 329.

The LCMSMS data from the fragmentation of the ion 329 for each of these compounds allow confirmation of the GCMS structural data to be obtained by comparison to the data seen for the hydroxylated stanozolol metabolites. The compound at 10.0 min gives fragmentation at m/z 85 and 97 similar to 3'-hydroxystanozolol and the compound at 9.77 min is small compared to the others, but gives a complex spectrum with fragments at m/z 81 and 95 in a similar fashion to 4-hydroxystanozolol. The two peaks at 9.42 and 9.05 min give similar simple spectra and may be the 16-hydroxylated compounds again by analogy to the 16β-hydroxystanozolol (Mück and Henion 1990). Thus tentative structures for these compounds are shown in Fig 12.

A sample of [3,2-c]-pyrazole-5a-androstan-16b-hydroxy-17-one O,N-diacetate was obtained from BDG Synthesis (New Zealand) as an intermediate from their synthesis of 16β-hydroxystanozolol. This diacetate was hydrolysed in methanol containing 5% hydrochloric acid at 60ºC for 2 days to give the deacetylated compound which corresponded to the metabolite IV (20.55 min) in the GCMS.

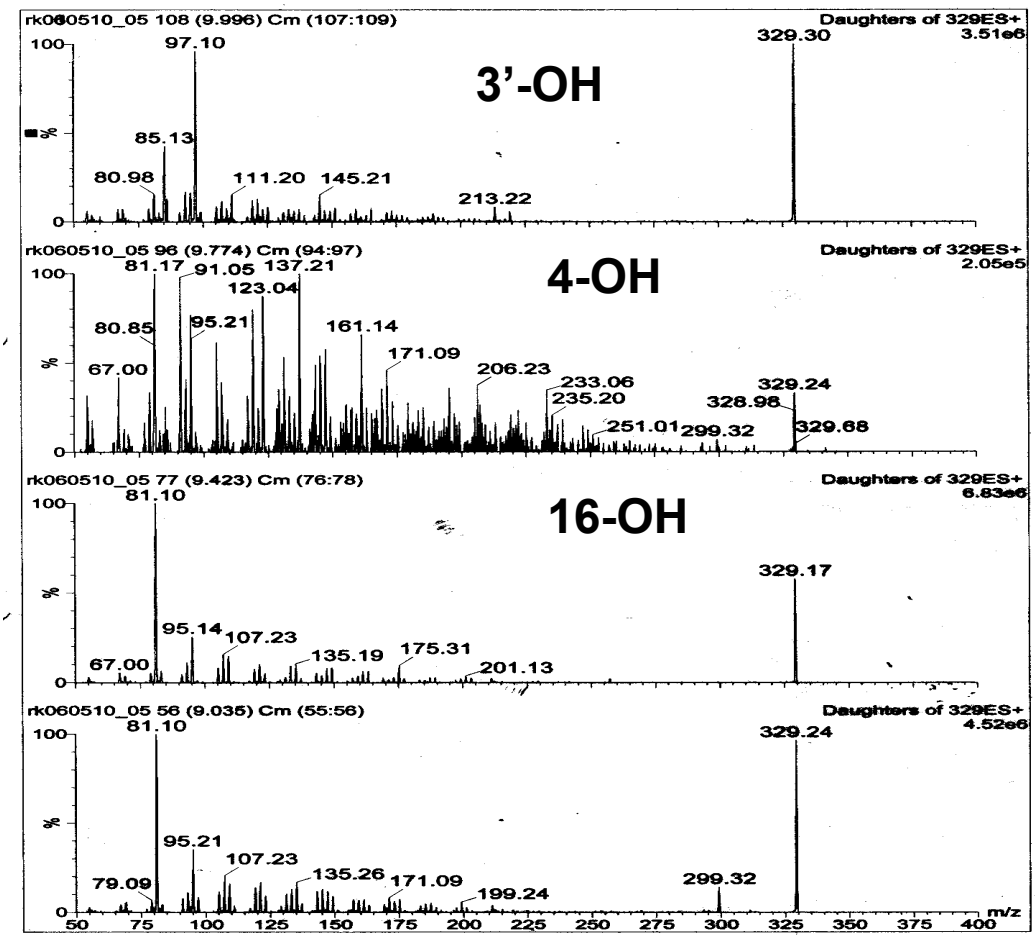

Fig 12 shows the LCMSMS spectra for each of the compounds arising from fragmentation of the $M+H^+$ ion m/z 329.

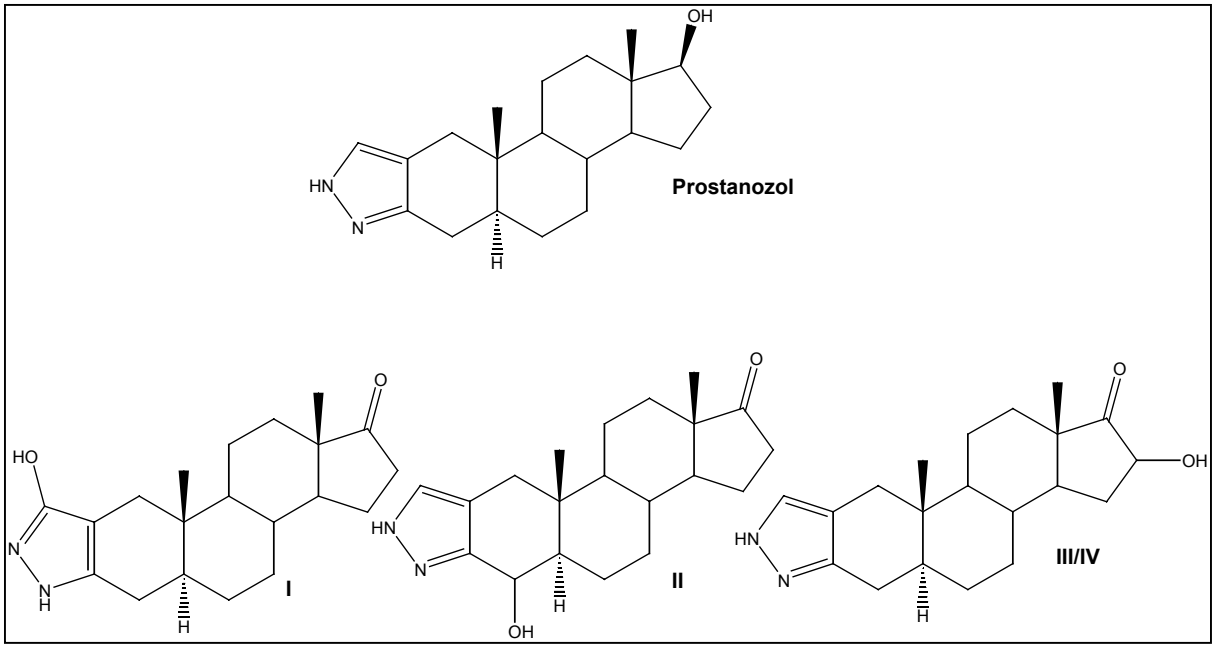

Fig 12. Structures for 3 of the metabolites from administration of prostanozol**.**

Formadrol

The bottle obtained as Formadrol was supposed to contain 6α-methyletiocholene-3,17-dione but was actually a mixture of androstenedione and methylandrostenedione, presumably methylated in the 6 position. The methylandrostenedione isolated by chromatography appears to be a mixture of isomers with one major component as shown by GCMS on the underivatised compounds (Fig 13). The 1H-NMR of the purified material indicates that it is methylated and consistent with it being methylated in position 6 as indicated on the label of the container. The mass spectrum has ions at m/z 258 and 138 whereas androstenedione has these at m/z 244 and 124. The fragment at m/z 258 in the 6-methyl and 244 in the androstenedione is due to fragmentation with loss of the portion from the 1,2 and 3,4 position cleavage showing retention of a methyl in the 6-methyl derivative and indicating it is not at C2. The fragments m/z 138 for the 6-methyl analogue and m/z 124 for androstenedione is due to the B ring cleavage across the 9,10 and 6,7 positions showing the retention of the methyl group in 6-methylandrostenedione thus adding to the conclusion the labelling is correct (Brown and Djerassi, 1980)

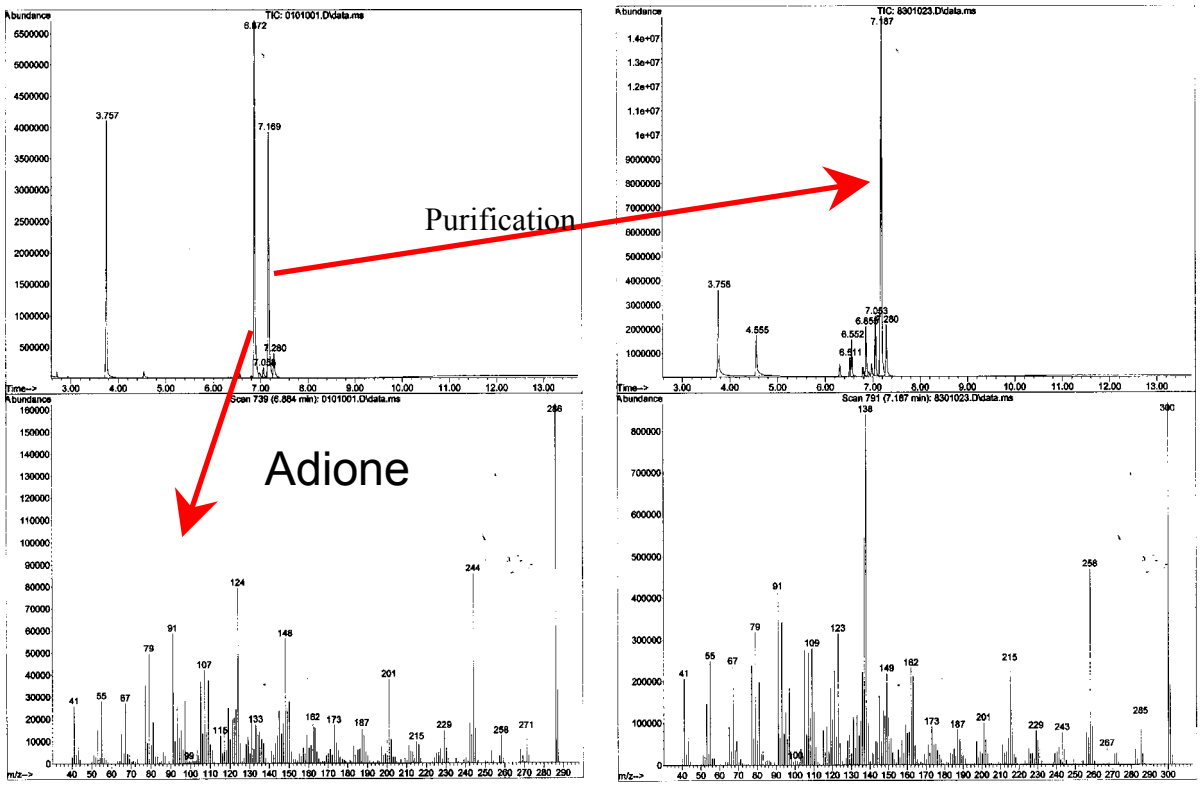

Fig 13. GCMS spectra of the mixture in the capsule and the purified crystalline compound, and the corresponding mass spectra of the underivatised compounds.

An excretion study using one capsule (20 mg) was performed in a male volunteer and urine collected for 24 h. The full scan specta obtained from the routine steroid screening sample

preparation and derivatisation (MSTFA/TMSI/Ethanthiol) showed two closely eluting metabolites for the 6-methyl derivative. It also showed large androsterone and etiocholanolone peaks from the androstenedione present in the formulation. These are shown in Fig 14 as the chromatogram TIC traces for the 0 and 6 h urine samples collected. The extracted ions and the corresponding spectra are shown in Fig 15. The metabolites are very similar to those formed for drostanolone (the 2-methyl isomer) and have almost the same retention times. Thus screening for drostanolone will detect the metabolites of 6-methylandrostenedione but much care will be needed to distinguish between the two positional isomers.

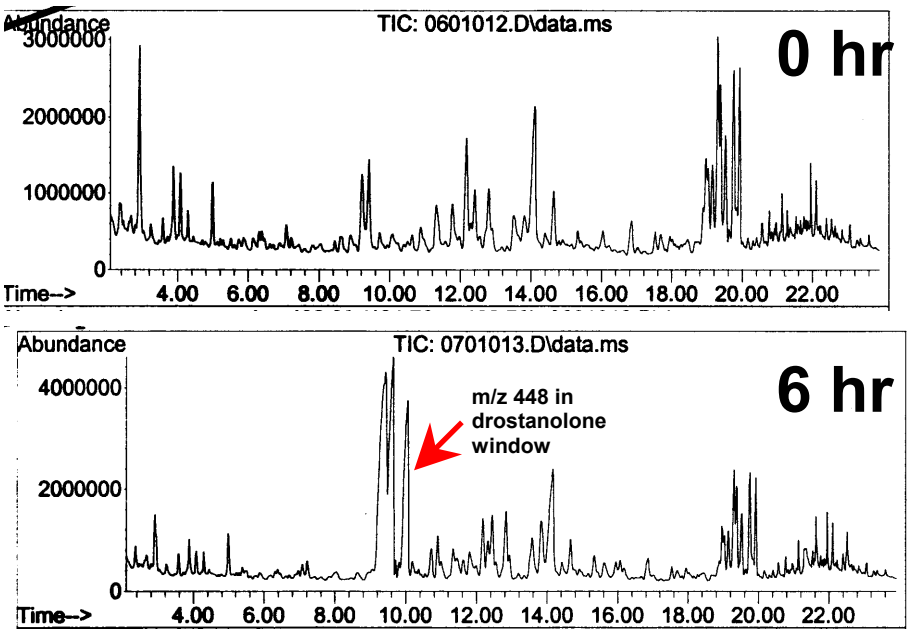

Fig 14. TIC spectra showing excretion of the metabolites of 6-methylandrostenedione.

By analogy with drostanolone the most likely metabolites are reduction at C3 and both reduction at C3 and reduction of the double bond. In both metabolites only one carbonyl function is present as derivatisation with MSTFA/TMS; Imidazole gives compounds with mass 72 less (m/z 374 and 376) indicating one carbonyl and one hydroxyl function in each. Thus the structures in Fig 16 are most likely.

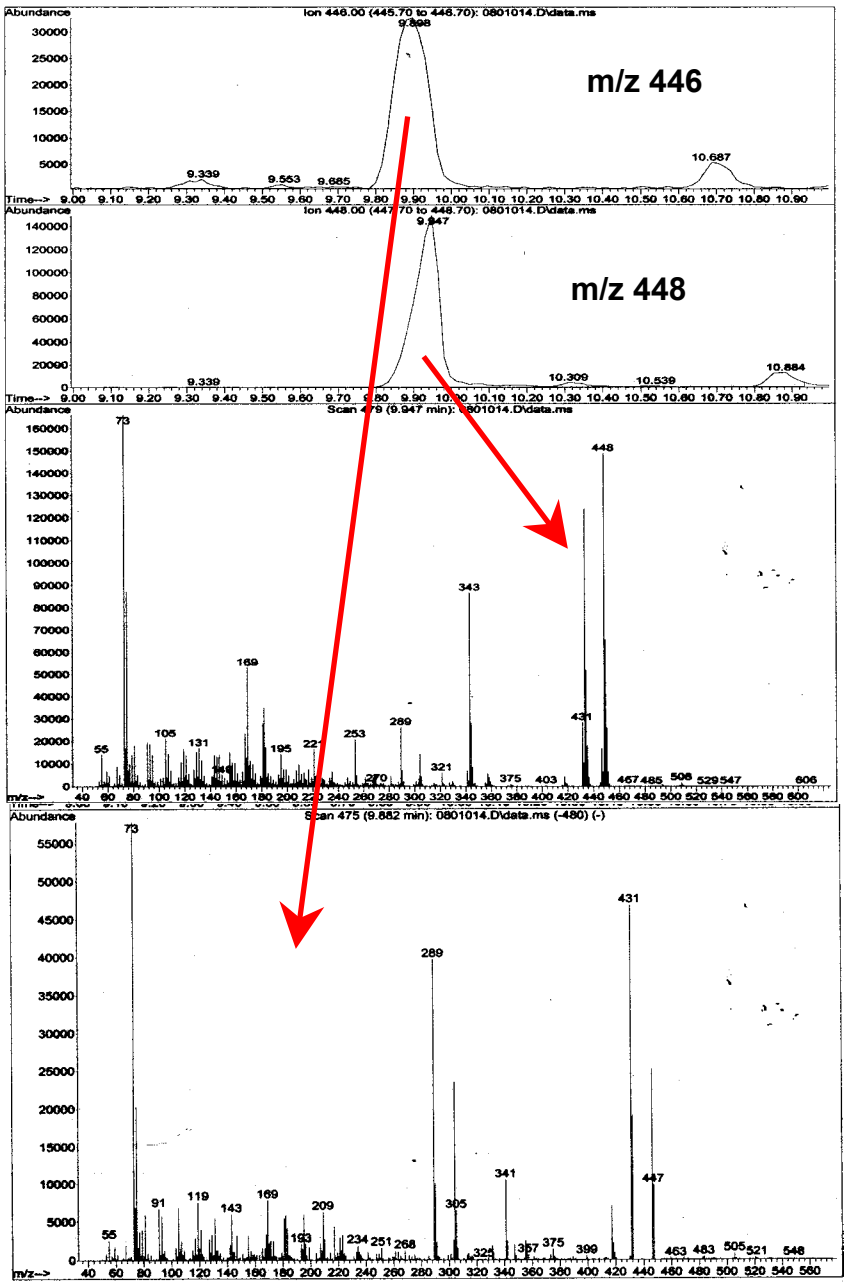

Fig 15. The extracted ions and corresponding spectra for the two metabolites as TMS/enolTMS derivatives.

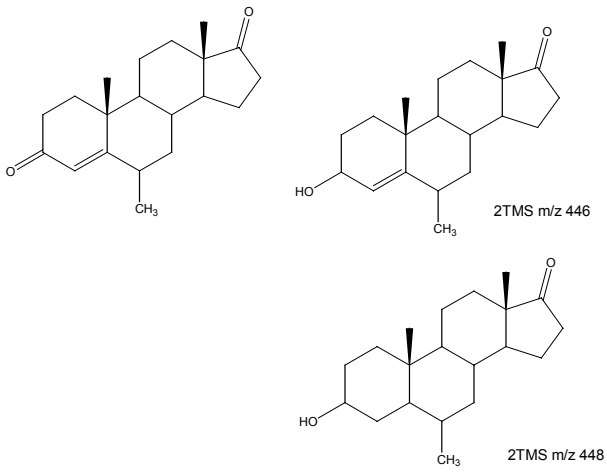

Fig 16. Proposed structures for metabolites of 6-methylandrostenedione.

Conclusions

There is still considerable work to be done to prove the structures of the supplements as it is not trivial to work backwards from an unknown structure or even to confirm the identity of a compound listed on the label. A process of isolation and spectral data gives some conclusions but synthesis in an unambiguous manner is needed for final confirmations.

Much more study needs to be done to determine the structures of the metabolites but screening for some of these products can be undertaken from the data obtained so far.

Acknowledgements

The staff at NMI needs to be acknowledged for sample preparation from the excretion studies and obtaining GCMS and LCMSMS data. A. Lisi and K. Gazi performed the study of the cyclohexanone derivatives of the ephedrines and benzylpiperazine, and Ben Parker and George Wang, both Year in Industry students from the University of Sydney, did much of the isolation work for the supplements. We acknowledge the Southern Cross University Ethics Committee for approval of single dose studies.

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