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Introduction

Human Chorionic Gonadotrophin (hCG) is used therapeutically to stimulate gonadal steroid production in both men and women usually connected with an infertility issue. In sports it is abused by some athletes, particularly in males, to stimulate testosterone functions after administration of testosterone or other anabolic steroids since it can counter-act the negative feedback loop by restoring the natural steroid production.

It is also a natural hormone produced in pregnancy that supports the corpus luteal cells to maintain progesterone production and it is the analyte measured clinically in over-the-counter pregnancy tests (at about 50 IU/L). The detection of hCG can be also used as a tumour marker in certain cancers, such as choriocarcinomas and testicular germ cell malignancies. These have been detected by further medical examinations after hCG has been reported in doping control in some male athletes on two occasions within our laboratory.

In both males and females the normal level of hCG is <5 IU/L but for doping control purposes, the World-Anti Doping Agency (WADA) requires hCG levels above 5 IU/L to be reported as an adverse analytical finding in men.

Methods

The 2-Dimensional gel electrophoresis was carried out using commercial Immobilised pH Gradient (IPG) gel strips with a range of pH 3 to 10 and pH 3 to 6 for the first dimension and a Criterion pre-cast (10 % Bis-Tris) SDS-PAGE gel for the second dimension. The standards or purified urine extracts were first reduced and alkylated using tributyl phosphine (TBP), acrylamide and dithiothreitol (DTT). The treated samples were quenched and then desalted and washed prior to their application on the IPG strips. The isoelectric focussing (IEF) took place for approximately 24 hours using a programmed voltage. The developed strips were incubated in equilibration buffer and then transferred to the prepared Criterion sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. The SDS-Page gel was run for approximately 90 minutes at a constant voltage (160V). The gel was blotted onto a PVDF

membrane and visualised for hCG using the polyclonal hCG antibody and chemiluminescent substrate from the DPC Immulite hCG assay kit.

Results

hCG is an intact heterodimer, consisting of 2 subunits, α-and β-, each stabilised by 3 disulfide bonds forming a cystine knot. The α-subunit is common to other glycoprotein hormones such as luteinizing (LH), follicle-stimulating (FSH), and thyroid-stimulating (TSH) hormones. The β-hCG subunit is unique to the hCG hormone and functionally important variations in structure occur in post-translational modifications which only attach carbohydrates at certain amino acid residues, Serine (O-linked) and Asparagine (N-linked) and they are complicated by differences in sugar branching (mono-, bi- and tri-antennary structures). These isoforms provide differences in the carbohydrate structures and thus make the separation possible by 2-D gel electrophoresis. The First dimension consists of an IEF step which separates the isoforms of a protein based on their isoelectric point (pI) using an immobilised pH gradient (IPG) strip in the presence of an electric field. The second dimension consists of an SDS-PAGE process which separates the proteins by their size; smaller proteins migrates faster than larger proteins. The last Western Blot (Immunoblot) step allows the proteins to be transferred to a membrane by electroblotting. The membrane is the incubated with an antibody specific to the protein of interest that is conjugated to an enzyme (e.g. alkaline phosphatase) and finally the membrane is incubated with a substrate that forms an intermediate and generates chemiluminescence only where the antibody is bound.

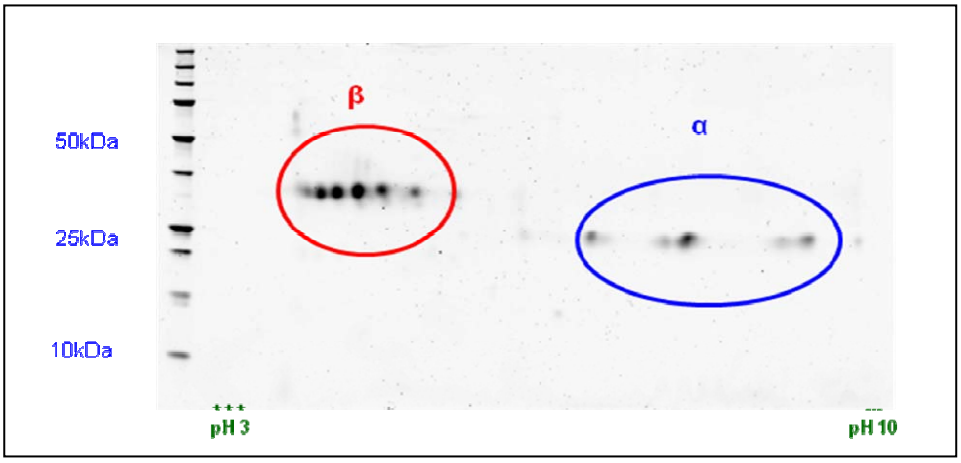


FIG 1. 5ug Pregnyl Standard Separated by 2D Electrophoresis. Reduced (DTT) Alkylated (TBP). 1st dimension IEF: Bio-Rad pH3-10 11cm ready strip. 2nd dimension Gel separation: Criterion 4-12% Bis/Tris XT gel, 150V, 45 min. The gel is visualised using Flamingo Red staining solution viewed with a wavelength of 600nm.

The results from a number of gels are shown in the following figures. FIG 1 shows the separation of the α and β subunits for natural human hCG in the preparation Pregnyl. These

subunits are clearly separated by molecular weight as well as a number of isoforms within each subunit. The gel shown in FIG 2 is that of recombinant hCG in the preparation Ovidrel. Here it can be seen that the β subunit consists of a number of isoforms that vary both in molecular weight as well as in glycosylation.

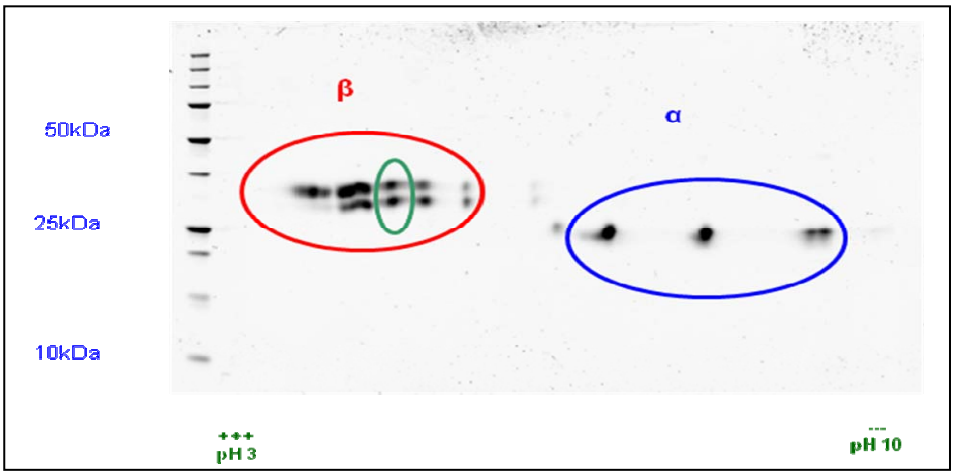


FIG 2. 5ug Ovidrel (rhCG) Standard Separated by 2D Electrophoresis..

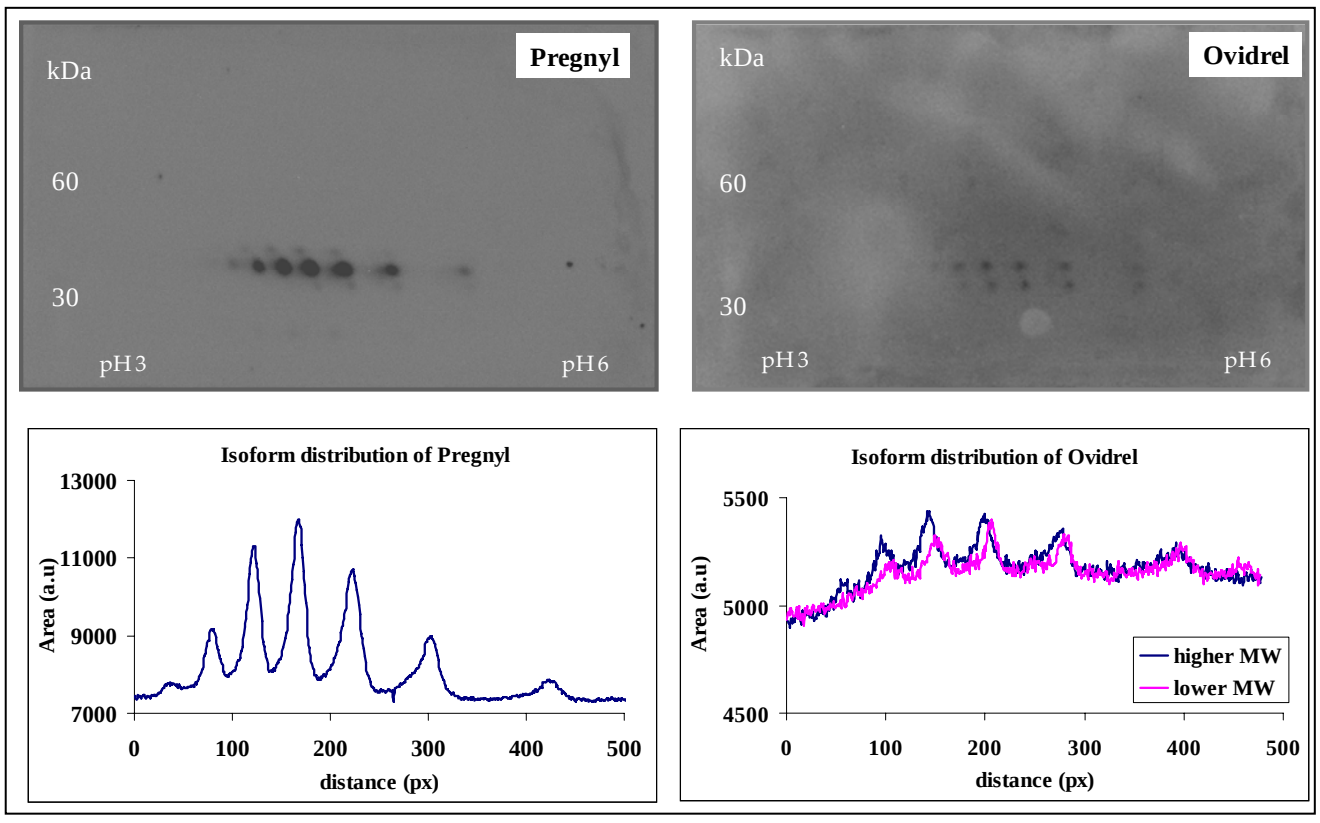


FIG 3. 2D-gels obtained using a pH gradient from 3 to 6 in the electrofocusing step.

The α subunit remains the same as in the natural material. This difference in the β subunit is a means of allowing detection of the use of recombinant hCG. While both gels shown in FIG 1

and FIG 2 represent gels from the pharmaceutical products and are produced from application of a larger quantity of material, these same differences can be clearly seen in gels run using material obtained from spikes into urine. FIG 3 shows the β subunit visualised using antibody specifically for the β-subunit portion of the molecule, in urine spikes of 50 IU/L of both Pregnyl and Ovidrel as well as using a tighter pH gradient and densitometry results.

Quantification of hCG using LCMSMS

The β-hCG subunit is unique to this protein and contains glycosylation at several quite specific amino acid positions. Since the glycosylation varies considerably fragments from tryptic digests containing these amino acids are not suitable for hCG quantification.

The digest fragment described as bT5 (VLQGVLPALPQVVCNYR, peptide mass 1869.255 Da) obtained from cleavage at position 43 and 60 with 17 amino acid residues, is of a molecular weight that is amenable to LC/MS measurement as well as being specific to hCG. This fragment was synthesised by Cambridge Peptides (UK) as well as the isotopically labelled compound using 13C,15N valine giving a mass 24Da higher. This labelled compound was used as the internal standard and gave highly reproducible data. Further ion ratios for the measured fragment met WADA criteria using the precursor ion as well as 2 product ions.

The sample preparation described in FIG. 4 includes an initial purification using immunoaffinity columns. These were prepared in house from a monoclonal antibody of hCG (HCG1, Cat#120-40000 obtained from Abcam distributed by Sapphire Bioscience,NSW, Australia) which was coupled to sepharose activated with cyanogen bromide via amide groups. Labelled internal standard was added and the, protein was denatured using heat followed by an alkylation step. Tryptic digest was used to cleave the molecule and analysis by HPLC (Phenomenex Jupiter 4u Proteo 90A, 1 mm ID x 150 cm) and mass spectrometry using appropriate MRM transitions (638.7/1023, 638.7/698.8, and for IS 646.6/1035, 646.6/705) detected the bT5 fragment. The calibration curve across a wide concentration range 50 to 2700 IU/L had an acceptable linearity (R^2 = 0.9995). The reproducibilty of measuring bT5 at a spiked level of hCG of 5 IU/L (equivalent to 54 ng/mL) is shown in FIG 5. The results at this low concentration using the labelled bT5 fragment are acceptable.

Designer steroids

In the course of our work at NMI, samples are received from Australian Federal Police and Customs whithin the NMI Australian Forensic Drugs Laboratory (AFDL) section. When

unusual samples are submitted which appear to contain steroids and hormones these are often discussed with the anti-doping area within NMI at ASDTL.

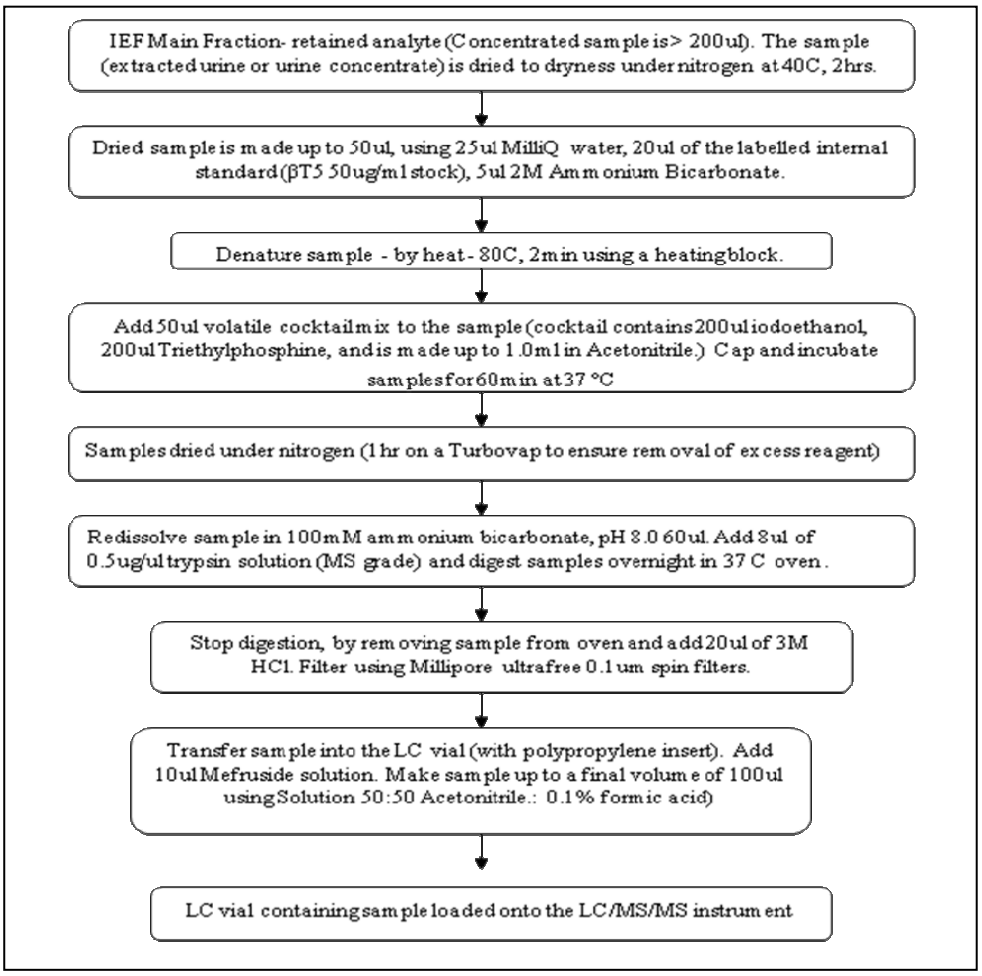


FIG 4. Sample preparation steps.

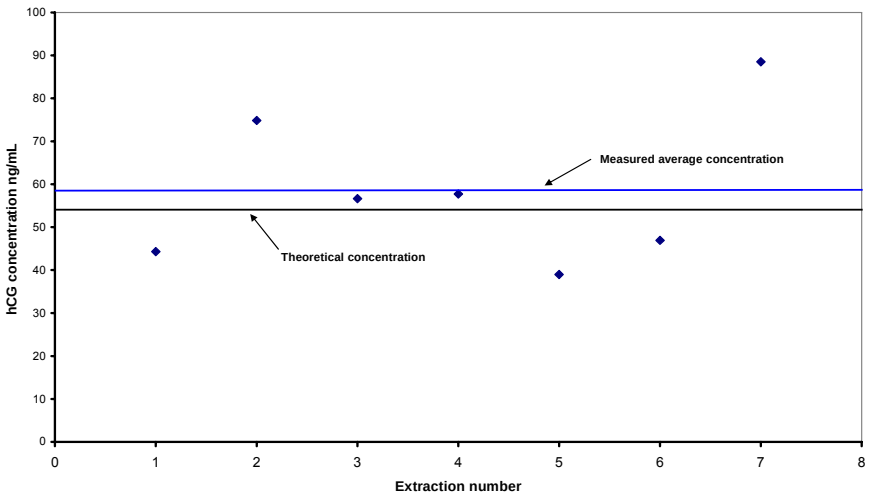


FIG 5. Reproducibility for seven different urines spiked with Pregnyl at 5 IU/L.

This interaction complements our studies into supplements containing steroids which can be purchased through the internet. As part of a seizure, AFDL obtained an oily injection material labelled methenolone enanthate. When this was checked it was found to be incorrectly labelled and had an unusual mass spectrum. Purification of the oil (0.5 g) using column chromatography allowed isolation of the main steroid component (about 20 mg). The highest ion was at m/z 430 in the GCMS and m/z 431.3143 measured using high resolution on the Orbitrap in positive mode using ESI which gave a formula of $C_{27}H_{43}O_4$ [CALC 431.3156]. Use of LCMSMS (HCD mode) gives ions at m/z 135, 145 and 187. Derivatisation with MSTFA/TMSI/Ethane-thiol gave no TMS derivative. Inspection of the mass spectral data and the ^{1}H NMR data indicated that this compound had an enathate ester group. The enanthate ester group was removed by mild treatment of a methanolic solution with sodium hydroxide. The product was purified by column chromatography to give an alcohol (A). The GCMS gave a molecular ion at m/z 318 (see FIG. 6) as well as characteristic fragments at m/z 152, 139 and 98. Treatment with MSTFA/TMSI/Ethanthiol gave a monoTMS derivative with M^+ at m/z 390 plus the same fragments as the underivatised compound. The LC/ESI/ HRMS gave the $M+H^+$ ion at 319.2300 [C_{20} H_{31} O_3 319.2268]. Treatment with dilute hydrochloric acid in methanol gave a compound which formed a bisTMS derivative with GC/MS ions at m/z 448 with masses found in the underivatised compound at m/z 98, 139, 152 shifted to m/z 156, 197, 210. Traces of this compound had been seen during derivatisation of the parent compound. A. This equates to a loss of a methyl group.

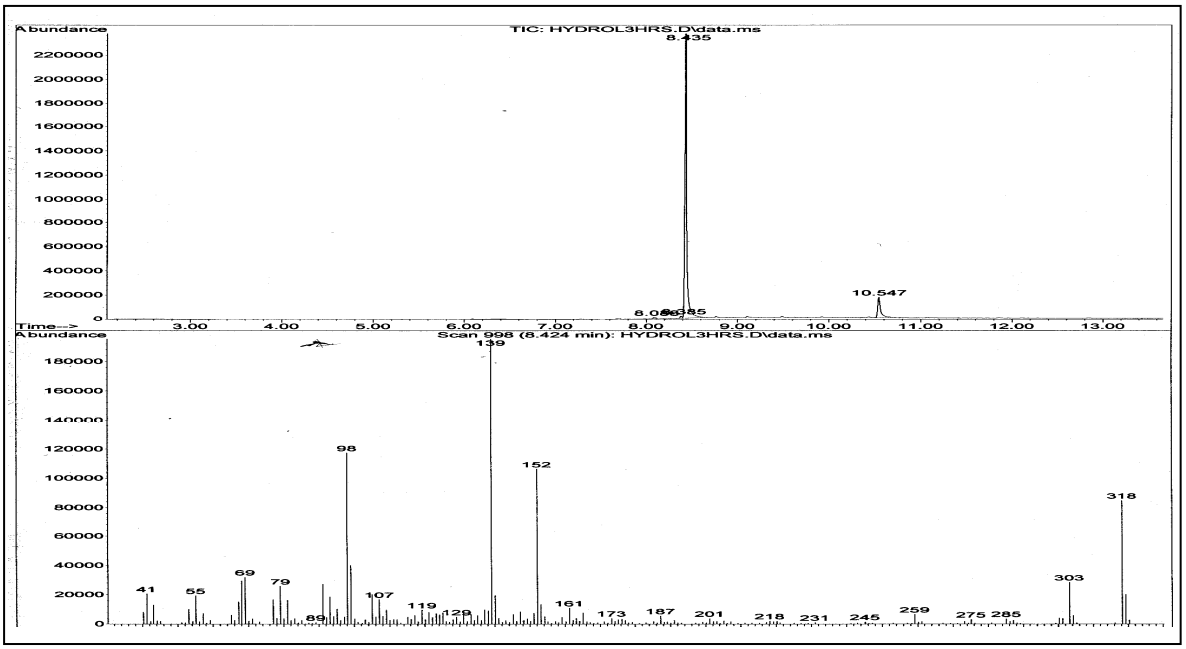


FIG. 6 Mass spectrum of underivatised alcohol (A) at 8.44 min.

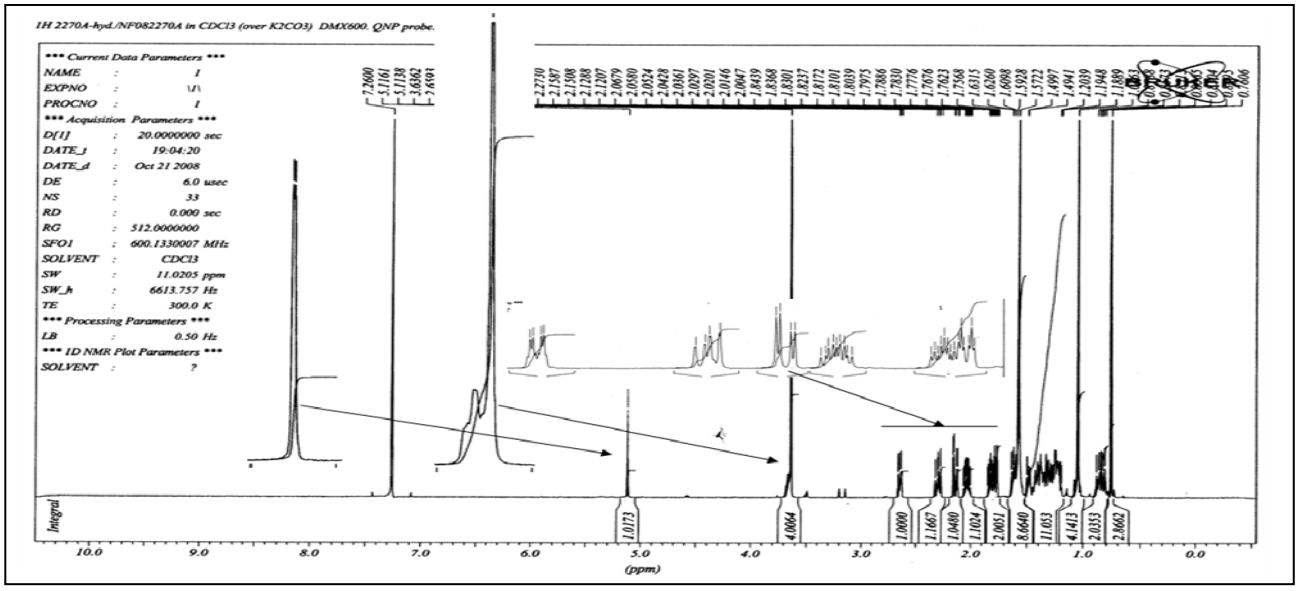


FIG 7: 1H NMR spectrum of the alcohol after base hydrolysis of the enanthate ester.

The structure of this unknown compound was assigned by extensive NMR (^{1}H, ^{13}C, COSY, HMQC and HMBC) experiments. The ^{1}H NMR spectrum of alcohol (A) is shown in FIG. 7 and a summary of the combined results from all the experiments is shown in FIG 8. The two three proton singlets at 0.77 and 1.06 ppm supports the presence of two methyl groups at position 18 and 19 in a steroid skeleton. The fine coupled doublet at 5.11 ppm in the ^{1}H NMR spectrum and the resonance at 100.9 ppm in the ^{13}C NMR spectrum is consistent with a proton that is part of an enolised β-diketone. The three proton singlet at 3.64 ppm is consistent with the presence of a methoxy group. This finding supports a methylated form of a β-diketone. The COSY experiment showed the proton within the methylated β-diketone, coupled to an allylic proton which is itself part of a methylene group.

The HMBC experiment show the carbonyl group at 206.1 ppm correlates to single protons at 5.12 and 1.82 ppm, plus a correlation to methyl group at 1.05 ppm. The alkene carbon at 173.6 ppm shows correlations to single protons at 5.12, 2.30 and 2.14 ppm, plus a correlation to a methoxy group at 3.64 ppm. The alkene carbon at 101 ppm correlates to the two protons at 2.30 and 2.14 ppm and the carbon attached to these two protons at 33.3 ppm correlates to single protons at 5.12, 2.30 and 1.82 ppm.

Hence from these extensive NMR experiments the only possible combination to place these four carbons within the A-ring of a normal steroid skeleton is shown in FIG. 9. This structure also explains the unusual mass spectral fragmentations. Therefore the unknown steroid (A) is androst-2-en-3-methoxy-17-ol-1-one.

Position	Proton	Carbon	COSY	HMBC
C1		206.1		5.12, 2.14, 1.82, 1.05
C2	5.12	101	2.30	3.64, 2.30, 2.14
C3		173.6		5.12, 3.64, 2.30, 2.14
C4	2.30, 2.14	33.3	2.30, 2.14, 5.12, 1.82	5.12, 1.82
C5	1.82	41.8	2.30, 2.14	2.30, 2.14
C9	1.25			
C10		46.2		
C11	2.64, 1.77	23.2		
C13		43		
C16	2.03, 1.46	30.2		
C17	3.66	82		0.76, 2.03
C18	0.76	11.5		
C19	1.05	11.4		
CH3-O	3.64	55.4		

FIG 8. Summary of the combined assignments from all the NMR experiments

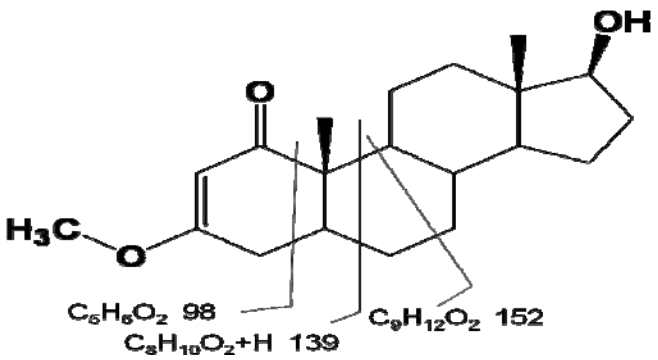


FIG 9: proposed structure of the unknown substance.

An interesting feature was the proton signals at C11 one of which had a very substantial downfield shift to 2.64. This signal had a coupling to a proton which had an interaction to the carbonyl group at C1. This can be explained by the close proximity of the carbonyl function to the 11 position of the steroid having a strong deshielding effect.

2,3 epithio-17α-methylandrostane-17β-ol

This compound was listed in the contents of a capsule labelled Epistane from a seizure by Customs and also the supplement Hemapolin bought from Bodybuilding.com. GCMS from both supplements give mainly MADOL m/z 288 plus another peak m/z 320 (MADOL + H_2S) corresponding to 2,3 epithio-17α-methylandrostane-17β-ol as specified on the label of the supplements.

In the two samples the epithio compound had different retention times. LCMS was similar for both supplements giving loss of H_2O [m/z 303] then H_2S [m/z 269]. The two compounds were isolated by column chromatography and the ^1H NMR of each is shown in FIG 11. These are two related epithio compounds which appear to differ in the stereochemistry at C2,C3. These should have the αα or the ββ stereochemistry but further work is needed to assign the structures to a specific material.

The facile loss of sulphur from these molecules shows Madol as a single peak and this is characteristic for the thio compounds. Madol which occurs in supplements always has a small later peak associated with it as it is always accompanied by the 3,4-ene isomer.

The two epithio isomers can be synthesised by similar pathways but the starting materials determine which is produced as shown in **Scheme 1**. Thus the stereochemistry of the starting epoxide determines which epithio compound is synthesised as the epoxides open in opposite ways. Further differences in the mass spectra are shown in FIG. 12 and appear to be mainly due to variations between the two isomers of loss of either a sulphur atom or hydrogen sulphide.

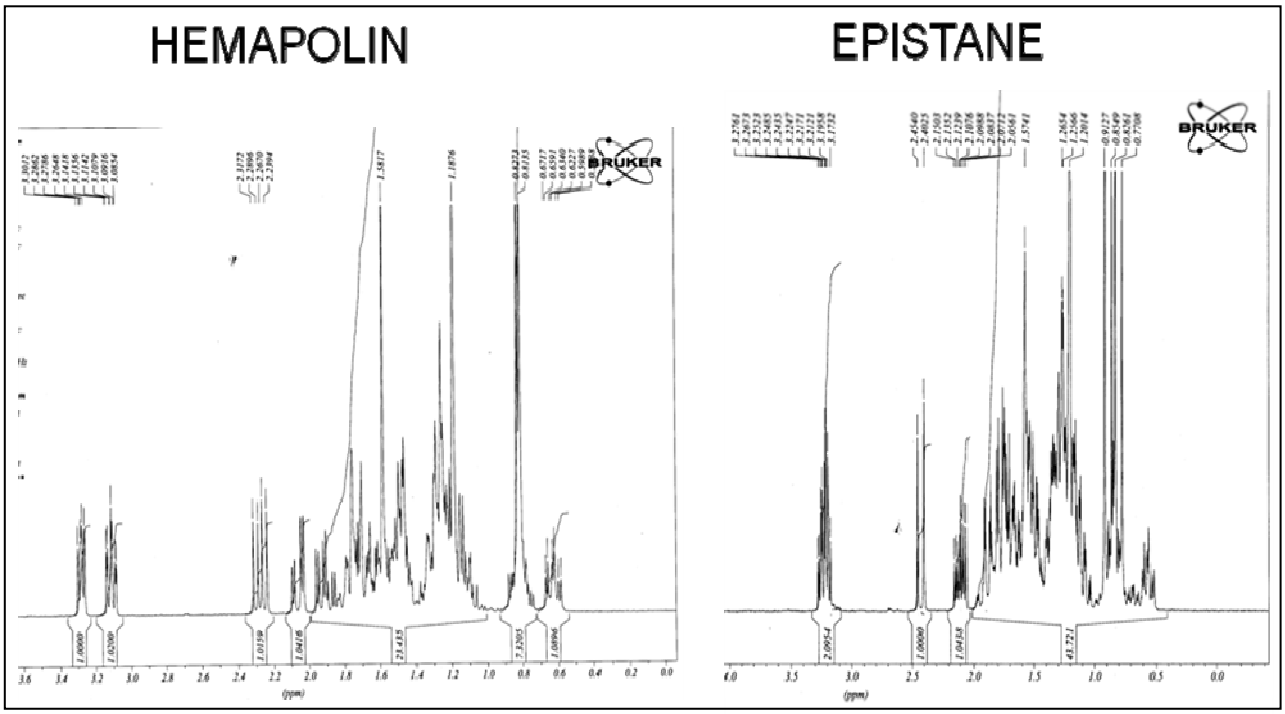
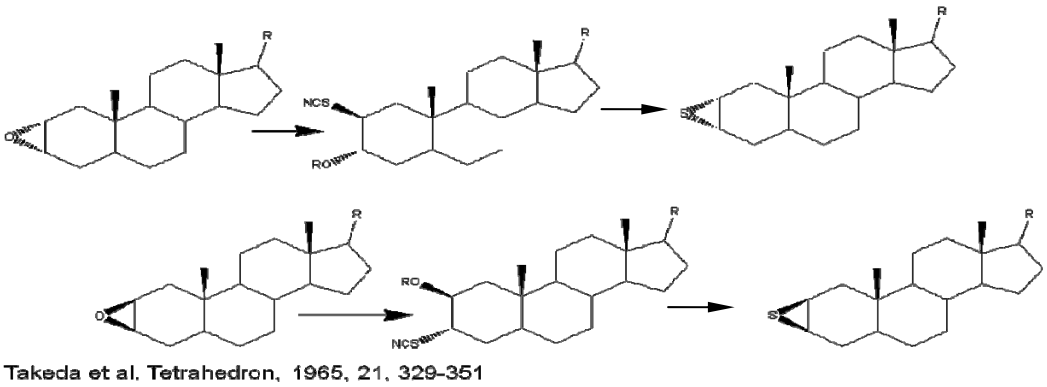


FIG 11: 1H NMR spectra of the two isomers of 2,3 epithio-17α-methylandrostane-17β-ol

Acknowledgements

We would like to thank both WADA and the Australian Government through the Anti-doping Research panel for funding of projects. We would like to thank Shane Cameron for continued

collaboration with AFDL and Greg Tarrant for his skilful assistance with review of the NMR data and general advice.



Scheme 1: Literature synthesis of the epithio compounds from the epoxides.

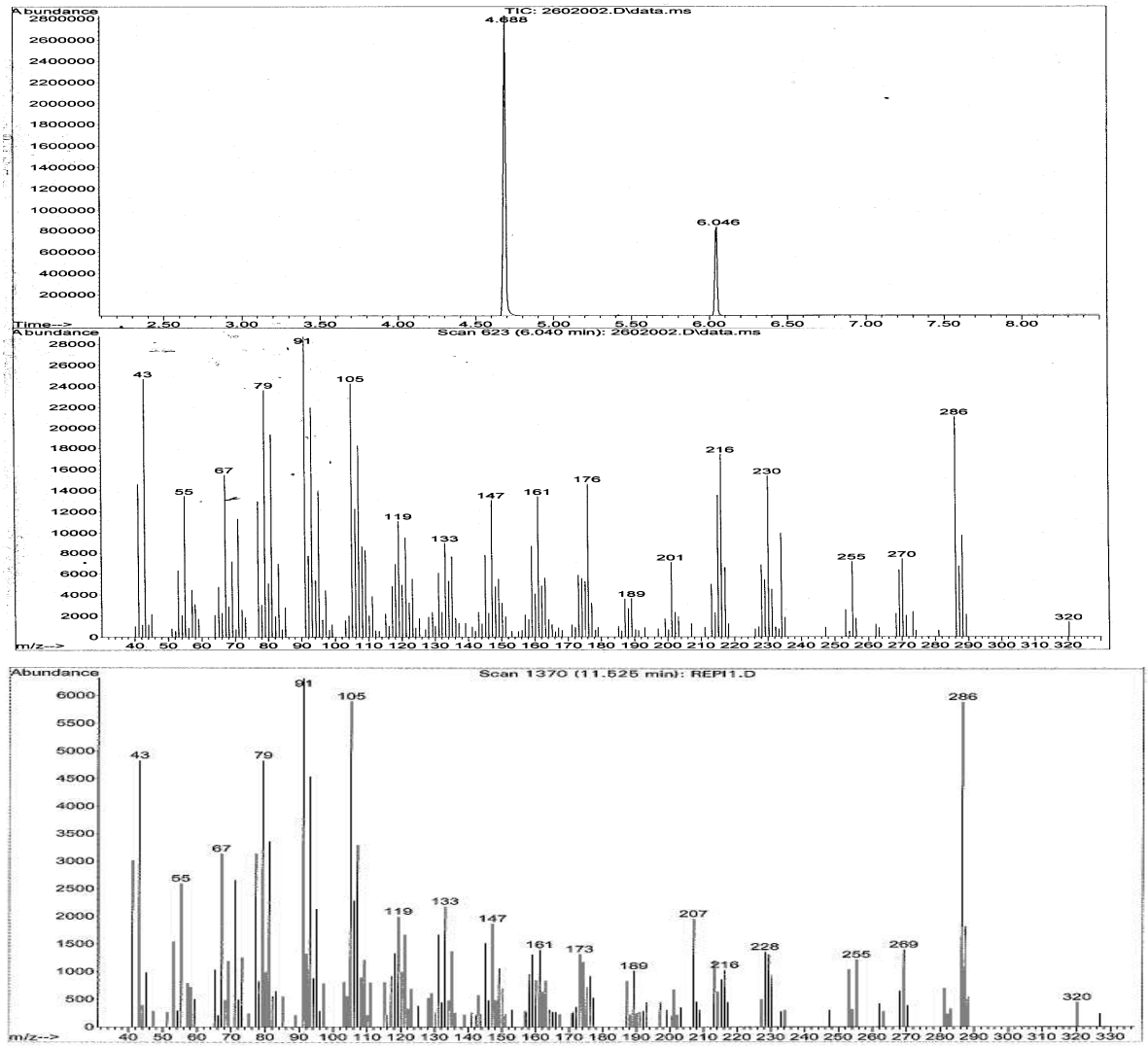


FIG 12: Chromatogram and Mass spectrum of compound from Hemapolin (upper traces) and the mass spectrum from Epistane (lower trace).