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SUPPLEMENTS & WADA LIST

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Background

Each year The World Anti-Doping Agency (WADA) releases a new Prohibited List of substances and this list can contain new drugs that laboratories have to test as part of the overall anti-doping programme. Since the list in 2005 the number of such added drugs is quite large as can be seen in Table 1. This Table shows several classes with new compounds including steroids, anti-estrogenic substances and stimulants.

1-androstendiol (5 α -androst-1-ene-3 β ,17 β -diol)	methylnortestosterone (17 β -hydroxy-17 α -methylestr-4-en-3-one)	fulvestrant	phenpromethamine
1-androstendione (5 α -androst-1-ene-3,17-dione)	methyltrienolone	finasteride	tuaminoheptane
bolandiol (19-norandrostenediol)	prostanazol	sibutramine	Norfenefrine
boldione (androsta-1,4-diene-3,17-dione)	tibolone	Fencamine	octopamine
desoxymethyltestosterone (17 α -methyl-5 α -androst-2-en-17 β -ol)	zilpaterol	Cyclazodone	Ortetamine
4-hydroxytestosterone (4,17 β -dihydroxyandrost-4-en-3-one)	testolactone	Fenbutrazate	Oxilofrine
methasterone (2 α , 17 α -dimethyl-5 α -androstane-3-one-17 β -ol)	dutasteride	isometheptene	phenpromethamine
methyldienolone (17 β -hydroxy-17 α -methylestra-4,9-dien-3-one)	raloxifene	p-methylamphetamine	tuaminoheptane
methyl-1-testosterone (17 β -hydroxy-17 α -methyl-5 α -androst-1-en-3-one)	toremifene	Oxilofrine	

Table 1: list of new additions to the WADA Prohibited List since 2005.

It has been the aim of our laboratory to obtain a standard for each of the compounds listed and to make either the standard and/or the excretion study available to all laboratories to enable the implementation of detection worldwide. All these compounds in Table 1 have been obtained within NMI/ASDTL or synthesised and many have been certified and are available

from the NMI. Some have been distributed through WAADS either as the parent compound and/or as an excretion study.

Raloxifene

Raloxifene (Evista 60mg) is a benzothiophene related to the nonsteroidal anti-oestrogens clomifene and tamoxifen and is used as a selective oestrogen receptor modulator for the prevention and treatment of postmenopausal osteoporosis. It is also under investigation for the prophylaxis of breast cancer.

The eMIMS Medicine Information entry gives limited data about its metabolism that includes:

The majority of a dose of raloxifene and its glucuronide metabolites are excreted within five days, primarily in the faeces, with less than 6% excreted in the urine and it appears to give all combinations of mono- and di- glucuronides. Thus the expectation is that it would be found as the parent drug after enzymatic hydrolysis and so we used our general procedure for diuretics for sample preparation and analysis ie hydrolysis with *E. coli* then SPE followed by LCMSMS using the Quatro Micro (Goebel et al, 2004). The optimised LCMSMS spectrum of the ion produced from the molecular ion at m/z 474 is shown in Figure 1. The detection limit is below 10ng/mL and the drug can be detected, after administration of one tablet (60 mg) to a volunteer, for more than 42 hrs after administration. The screen is performed using the transitions from m/z 474 to ions at m/z 269, 112 and 85.

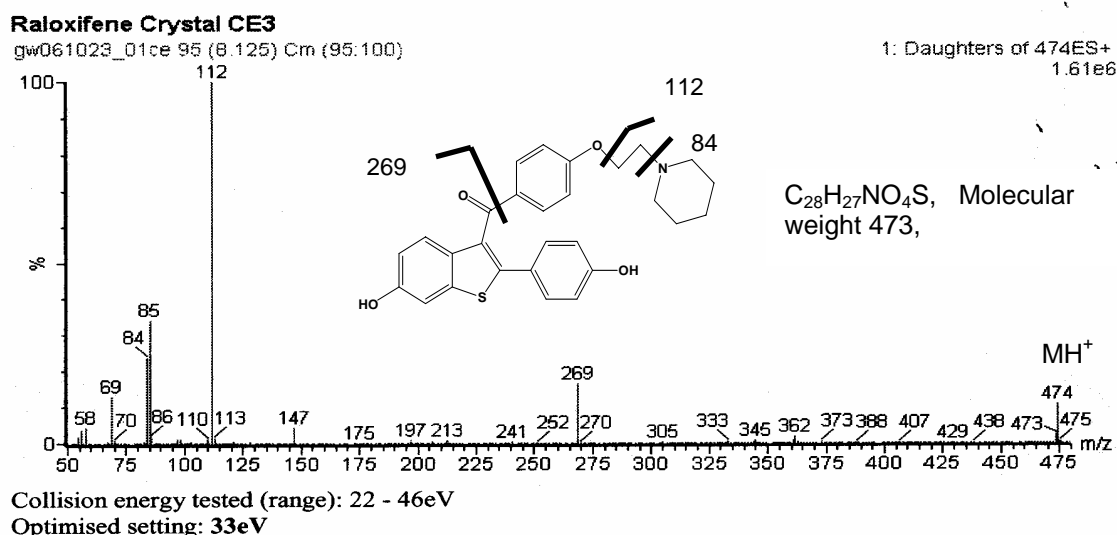


Figure 1: Optimised LCMSMS spectrum of raloxifene.

Toremifene

Toremifene (Fareston 60 mg) is an anti-oestrogen with properties similar to those of tamoxifen, and is used similarly in the treatment of advanced breast cancer in postmenopausal women. It is also being investigated as an adjuvant for the treatment of lung tumours.

The Martindale (The Extra Pharmacopoeia) has some information that states: Toremifene citrate is well absorbed from the gastrointestinal tract, reaching peak plasma concentrations of toremifene within 3 hours. It is extensively bound to plasma proteins, mainly albumin.

Toremifene is metabolised principally by the cytochrome P450 isoenzyme CYP3A4; some metabolites are reported to be active. It undergoes enterohepatic circulation and is eliminated mainly in the faeces as metabolites, with an elimination half-life of about 5 days. About 10% is excreted in the urine.

In the publication by Watanabe et al, using LC-API-MS the authors identified 4-hydroxytoremifene [M+H] 422 and they tentatively reported (structures not confirmed): dihydroxytoremifene [M+H] 438; N-demethylmonohydroxytoremifene [M+H] 408.

We undertook an excretion study using one tablet of Fareston and urine samples were collected for 60 hrs and another sample collected after 2 months. To analyse the excretion samples a general routine procedure for analysis of diuretics and some anabolic agents was used for sample preparation and analysis – SPE sample preparation after enzyme hydrolysis using *E. coli* then LCMSMS analysis using the Quatro Micro instrument (Goebel et al 2004). The optimised LCMSMS spectrum of toremifene is shown in Figure 2 for the transitions from the parent ion m/z 406.

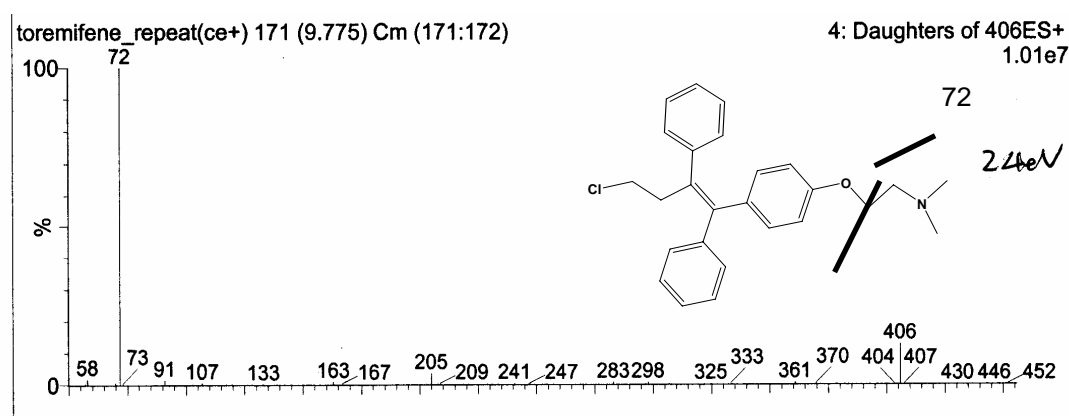


Figure 2: LCMSMS spectrum of toremifene.

Using 20 mL of the excretion urine collected at 14 hours it was possible to get sufficient sensitivity to optimise the detection of 3 metabolites as well as the unchanged toremifene. The chromatogram as well as the mass spectra of the metabolites from a 5 mL aliquot of the urines are shown in Figure 3. All the three metabolites had similar spectra and indicate the hydroxylation on one of the aromatic rings. The position of the hydroxyl groups is not known. The very long half life of the toremifene allows detection after a single dose of 60 mg for more than 2 months. Under the conditions of our screen we did not detect the other metabolites shown in the literature.

Fencamine

Very little information was available for this substance and it was not possible to purchase the material. Information on its metabolism can be found in Mallol et al, (1974) and this reference indicates that fencamine is mainly excreted as parent (used TLC to detect the compound).

The compound was prepared by Greg Tarrant in NMI and some data obtained for the parent. Analysis by spiking into urine and extraction using the routine screen 1 procedure gave the trace seen in Figure 4 with the fencamine being eluted very late with Relative Retention Time (RRT) of 1.977 to diphenylamine. At this point an excretion study has not been undertaken because of the lack of information on the compound's properties *in vivo*.

Cyclazadone

As with many of the new stimulants no literature other than original synthesis and activity as a CNS stimulant and anorexigenic agent was found (see Giudicelli et al, 1967).

The synthesis was therefore undertaken by NMI by Frank Corduroy and the compound is available as a certified standard. The structure is that of a substituted pemoline which would suggest that the parent would be mainly excreted and possibly as the des-isopropyl which is pemoline. The parent and pemoline can easily be detected in the steroid screen and at high concentrations in the stimulant screen. The GCMS trace for the stimulant screen (Screen 1) is shown in Figure 5.

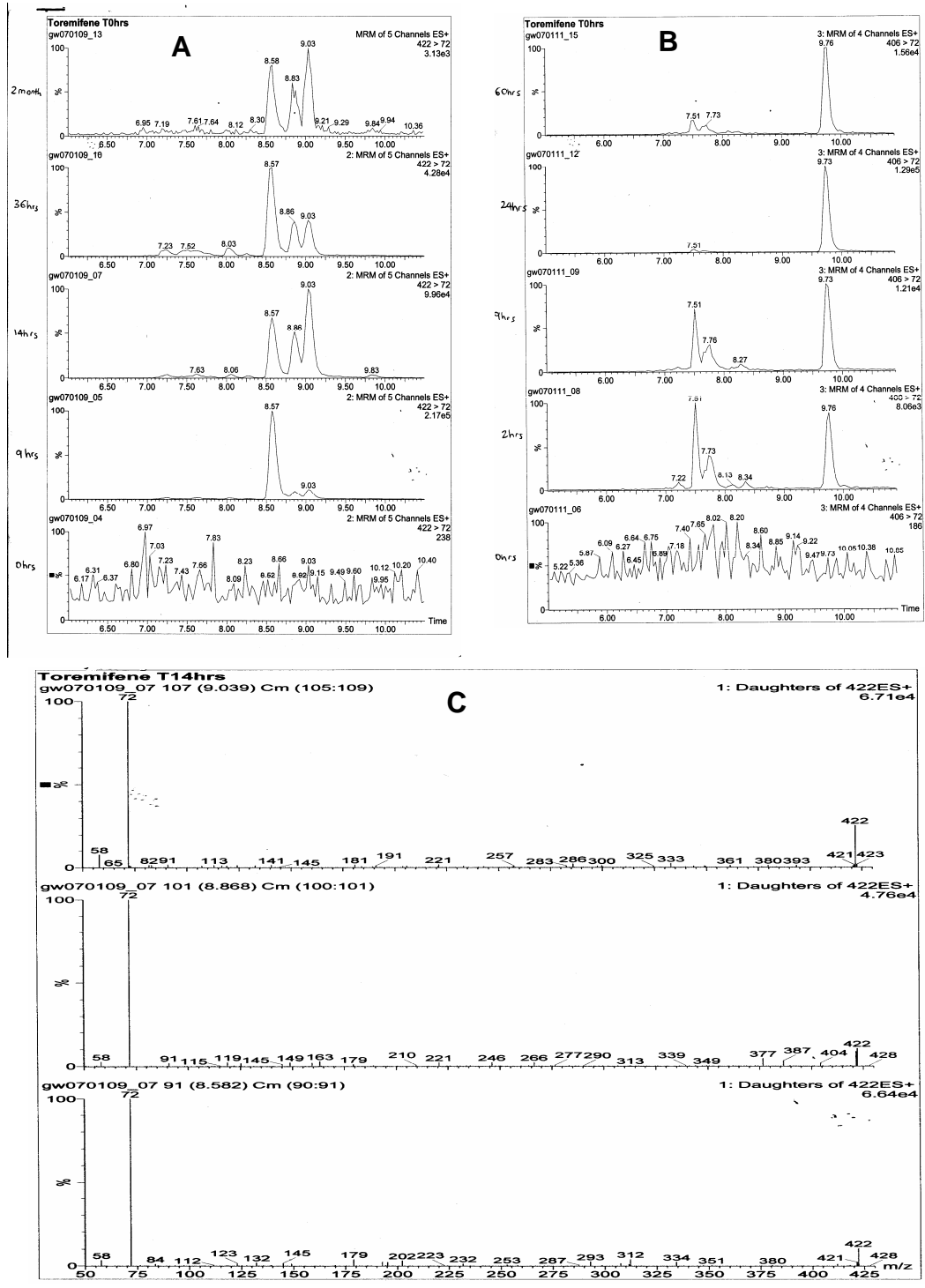


Figure 3: A) is the chromatogram for MRM transitions m/z 422 to 72 chromatogram of the extracted samples (5mL) showing the three metabolites of toremifene at the various time points (0, 9, 14, 60 and 2months) up to 2months after administration; B) is the MRM for the transition m/z 406 to 72 for toremifene up to 60hrs; C) is the mass spectrum for the three metabolites shown in A).

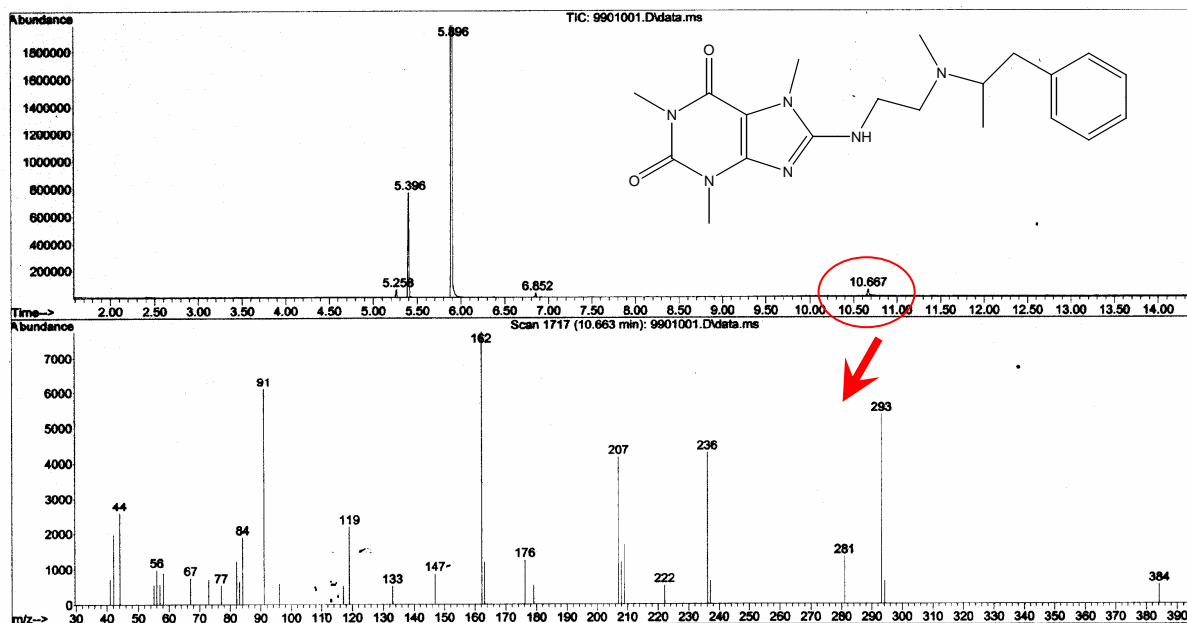


Figure 4. GCMS, chromatogram and spectrum for fencamine spiked into urine.

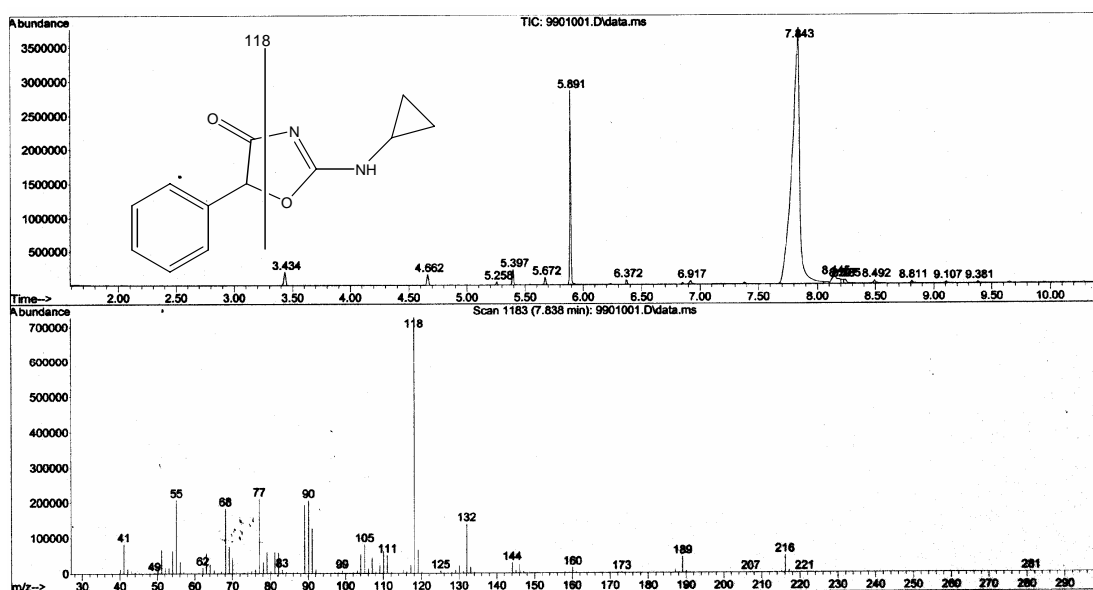


Figure 5: GCMS chromatogram and mass spectrum of cyclazadone using routine Screen 1 conditions. The internal standard diphenylamine is at 5.39 mins

p-Methylamphetamine

p-Methylamphetamine has not been used as a drug and no literature to that effect could be located. It has been used as an internal standard for amphetamine analysis. It was synthesised at NMI and is available as a certified reference substance. The chromatogram using the Screen 1 procedure is shown in Figure 6. The RRT is 0.4887 relative to

diphenylamine. It can be differentiated from its isomeric analogues as the pentafluorobenzoyl derivative. Its properties are not known so an excretion study has not been undertaken.

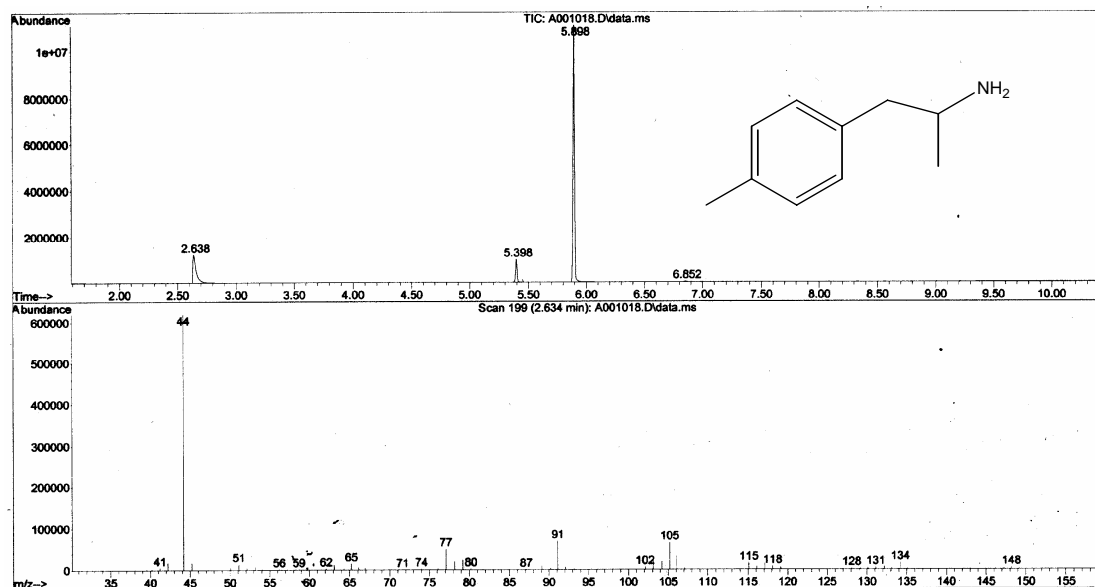


Figure 6: GCMS Chromatogram and spectrum of p-methylamphetamine

2-Aminoheptane (Tuaminoheptane)

2-Aminoheptane is sold as the sulphate in a nasal decongestant, Rhinofluimucil. It is very volatile as the free base. It was purchased from Sigma as racemate and also as the optical isomers. In the routine Screen 1 procedure 2-aminoheptane is not detected but can be seen if the GC temperature program starts at 50C (35C below our normal screen 1!) and elutes soon after the solvent at 1.5 mins (see Figure 7).

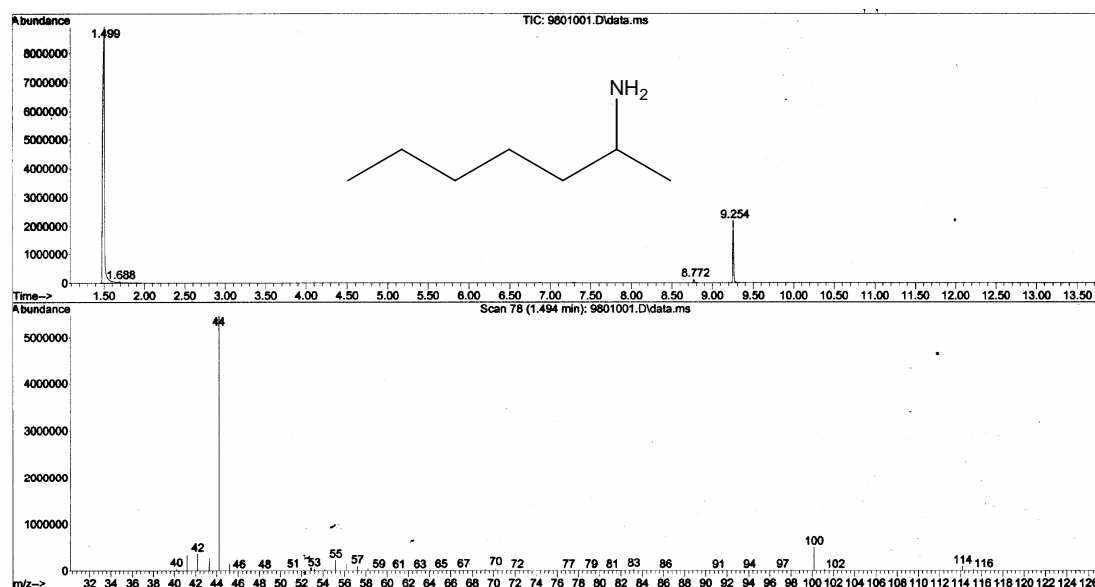


Figure 7: Chromatogram and mass spectrum of 2-aminoheptane in the modified GC temperature program from Screen 1 extraction. Diphenylamine is at 8.77 mins

Reaction of 2-aminoheptane with cyclohexanone as described for ephedrine (Cologne workshop) gives the Schiff's base which is far less volatile. and can be used for confirmation (see Figure 8).

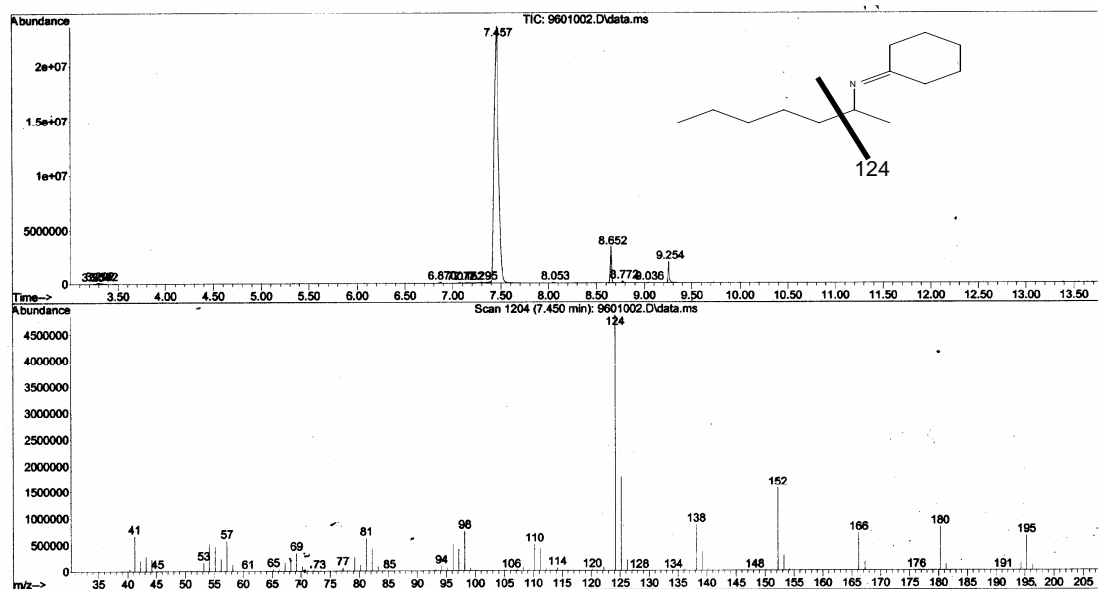


Figure 8: GCMS chromatogram and mass spectrum of the Schiff's base formed between 2-aminoheptane and cyclohexanone.

Supplements

For some time anabolic steroids have been sold via the internet, but many of them can be classified as designer drugs and to avoid issues with authorities the contents on the containers are often misrepresented by use of non IUPAC nomenclature. We analysed a number of supplements and some have been reported in the last Cologne Workshops.

Androst-1,4,6-triene-3,17-dione

GN Novedex and DS Rebound XT reportedly contain androst-1,4,6-triene-3,17-dione. GN Novadex also contains "6,17-Keto-etiochole-3-ol" if the label is to be believed. The capsules of each (10) were extracted with methanol and the residue on evaporation of the solvent was partitioned between t-butylmethyl ether and dilute sodium hydroxide solution. The organic layer was removed and the solvent evaporated. The residue was chromatographed on silica gel (TLC grade, 5 g) and the column eluted using a gradient from toluene-hexane to dichloromethane and then to t-butylmethyl ether in 10 ml aliquots. Novedex gave two fractions containing the separated steroids. The late eluting fraction was recrystallised from toluene/hexane and both the mass spectrum and ¹H NMR supported a structure of 6-hydroxyandrost-4-ene-3,17-dione (6-hydroxyandrostenedione).

The faster eluting compound from both supplements was recrystallised from toluene hexane and agreed with the structure androst-1,4,6-triene-3,17-dione by mass spectrometry and ¹H-NMR (CDCl₃, [CHCl₃ 7.26] 7.06 (d,1H, *J* 10.1), 6.315 (dd, 1H, *J* 10.1, 1.9), 6.26 (dd,1H, *J* 9.8, 2.9), 6.1 (dd, 1H, *J* 9.8, 1.8), 6.035 (bs, 1H), 1.23 (s, 3H), 1.0 (s, 3H)). GCMS for the underivatised compound gives m/z 282 as the parent ion, TMSI/MSTFA gives mono enol-TMS derivative (with traces of the bis derivative) at m/z 354 (see Figure 9) for the parent ion and TMSIm/MSTFA gives the underivatised compound.

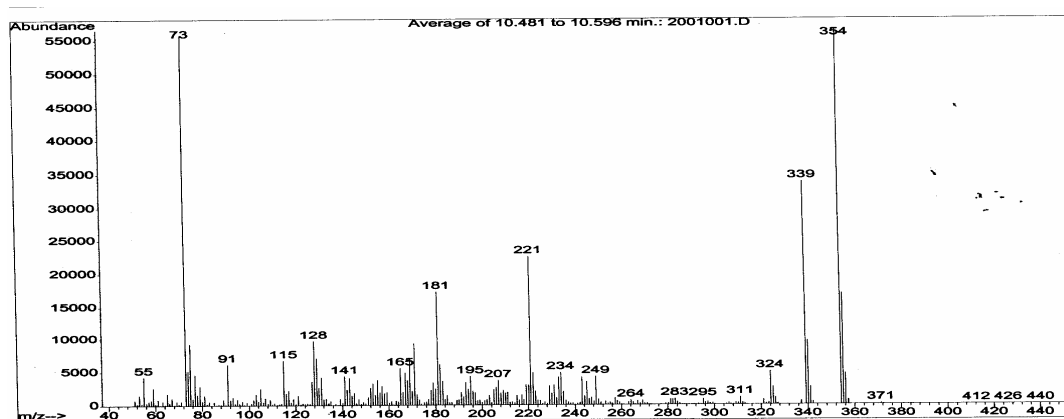


Figure 9. Mass spectrum of the enol-TMS derivative of androst-1,4,6-triene-3,17-dione.

An excretion study was performed using one tablet of Novedex XT and also with the Rebound XT. Sample preparation and analysis using the routine steroid GCMS screen showed that the 6-hydroxyandrostenedione in the Novedex XT gave a strong set of ions corresponding to the window for the main metabolite of androstenedione showing it is mainly excreted unchanged. The androst-1,4,6-triene-3,17-dione was also excreted unchanged and could be detected for at least 30 hrs. Its RRT to d3-testosterone was 0.934.

Acknowledgements

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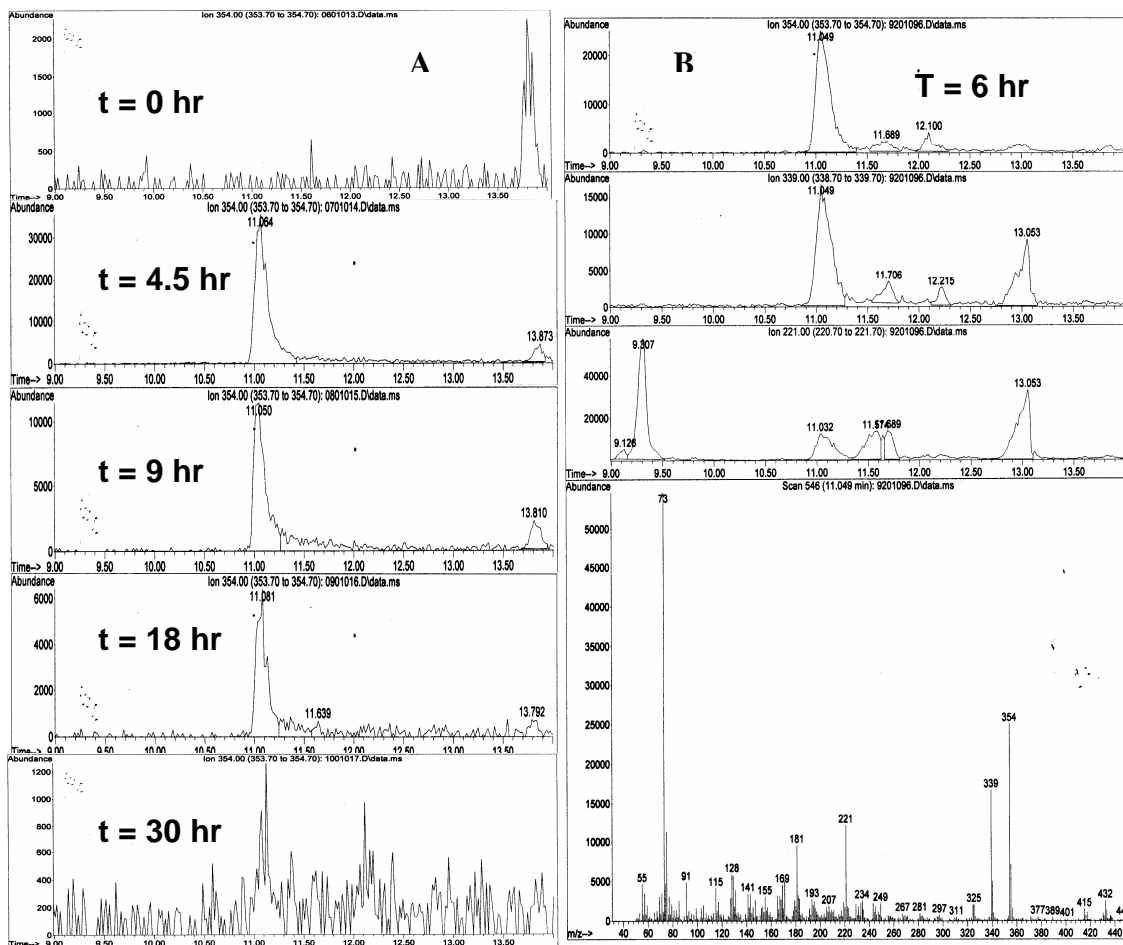


Figure 10. A) is the extracted ion m/z 354 for the urinary excretion of DS Rebound XT showing the triene at 11.1 min; **B)** is the 6hr excretion for Novedex XT showing the same compound and the spectrum identical to that extracted from the tablet.