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RECENT ADVANCES IN DOPING ANALYSIS (12)

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Miscellaneous Projects for 2003
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Miscellaneous projects for 2003

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Introduction

Over the past year a number of issues have emerged which required some research to be undertaken. These in themselves are small projects but yield results that may be of interest to the antidoping community. New designer drugs and analogues caused considerable upheaval to the work performed in 2002-3 with substances such as norbolethone and tetrahydrogestrinone (THG) (1,2) being found to be part of apparent major doping conspiracies. Bearing this in mind, a search of available androgen like substances available commercially was undertaken and a few of these were studied as possible doping agents and the results included in this paper.
From routine IRMS analysis of endogenous steroids, signals are occasionally noticed during studies of excretion urines which raise an interest especially when the delta value is similar to the ingested substance. These should be further studied as they may give rise to interesting new markers of clandestine use.

Designer drugs based on methylated nandrolones

In the draft list prepared by WADA and sent to stakeholders for review in early 2003 for the forthcoming year, 13-ethyl-17β-hydroxy-gon-4-ene-3-one was listed. This equates to 18-methylnandrolone. By the time the final list was released this substance had in fact for some reason, never specified, disappeared from the WADA 2004 list of prohibited substances [but is now on the 2005 list]. Our interest was raised from consideration of the possible metabolism of this substance. If it had similar metabolism to that of nandrolone then one could expect 18-methylnorandrosterone and 18-methylnoretiocholanolone to be produced. From an analyst's perspective this could be alarming since these two metabolites have the same molecular weight as androsterone and etiocholanolone and if the spectra were similar and if, in the chromatograms produced, the retention times were unfavorable then the extremely high levels of the two could be envisaged to swamp metabolites of methylated nandrolone analogues thus masking them. Of the few methylnandrolones available two were readily obtained and these were:

- 18-methylnandrolone (synthesised by NARL from hydrolysis of 18-methylnandrolone tetrapyranyl ether derivative obtained from China and used as a starting material in the synthesis of norbolethone)

- 7α-methylnandrolone (MENT) obtained from Steraloids. This has been used in studies by WHO to find an effective male contraceptive and has been around for some time as an androgen.

Experimental

18-Methylnandrolone and 7α-methylnandrolone were obtained from NARL as pure reference materials. A very small amount (10 mg and 5 mg respectively) was given to volunteers and the urine collected. The urines obtained were subjected to the standard combined steroid screening extraction procedure used in our laboratory (E.coli enzyme hydrolysis at pH7, C18 solid phase extraction and derivatisaton using MSTFA/TMSI/ethanethiol). The full scan mass spectrometry of the resulting extracts were performed with an Agilent 6890/5973 GCMS system using an Agilent HP-1 column (17 m, 0.11 μm film, 0.2 mm diameter) using the same temperature program as used for routine screening.

18-Methylnandrolone

The metabolism observed was as expected with formation of both 18-methylnorandrosterone and 18-methyl-noretiocholanolone (Figure 1). The metabolites gave spectral data similar to that observed for norandrosterone and noretiocholanolone see Figure 2. By analogy to the non-methylated metabolites and to androsterone and etiocholanolone, it is presumed that the compound eluting just before the androsterone is 18-methylnorandrosterone (9.14 min) (metabolite 1) and that eluting later at 10.02 min is the 18-methylnoretiocholanolone (metabolite 2).

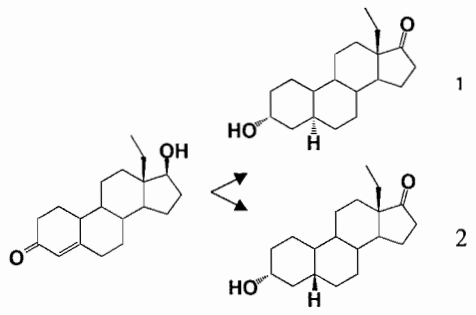


Figure 1.

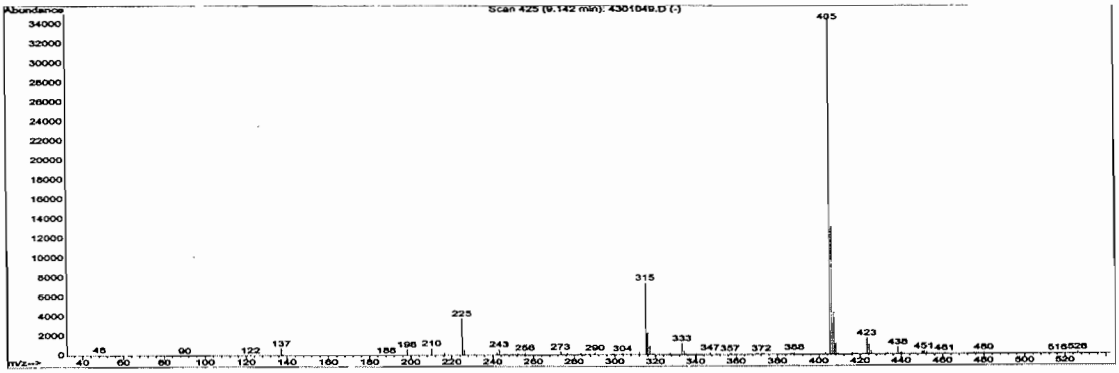


Figure 2a – mass spectrum of metabolite 1

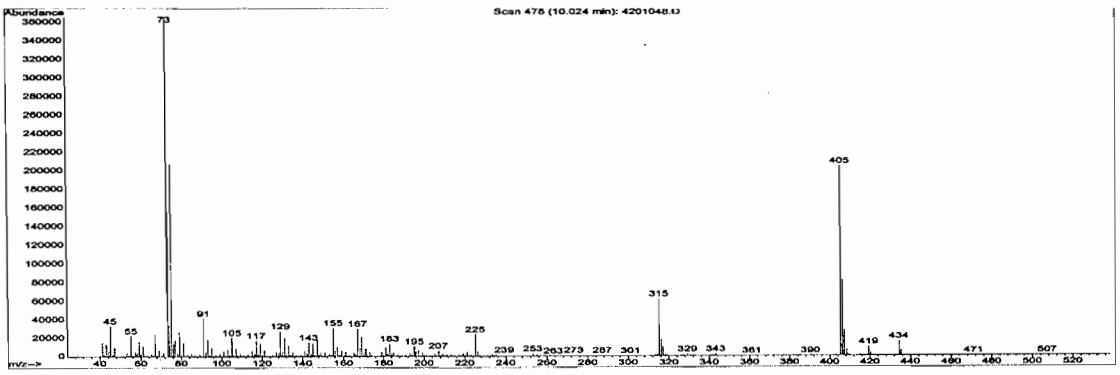


Figure 2b – mass spectrum of metabolite 2.

The main loss in the EI mass spectrum is that of the 13-ethyl group (analogous to loss of the 13-methyl group in norandrosterone) to give the m/z 405 ion. Thus the same ions used for screening norandrosterone (405, 315, 225) can be used. The Figure 3 shows the excretion of the metabolites over a 56 hr period.

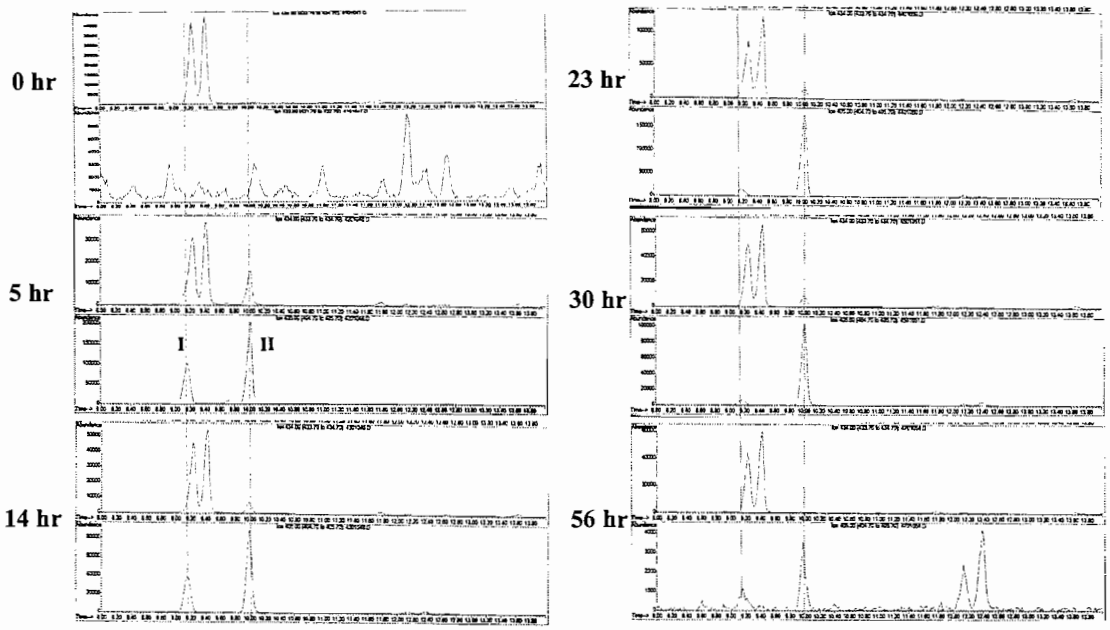


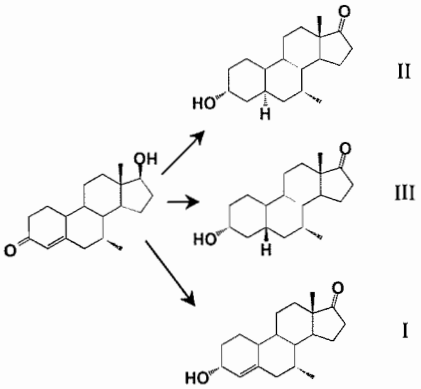
Figure 3 – excretion of the two metabolites over a 56 hr period. For each time point the top trace is for m/z 434 and the lower trace is m/z 405.

7α-Methylnandrolone (MENT)

This substance is a potent androgen studied by WHO as a possible male contraceptive. It is difficult to obtain, which is why it may have not been often used as a doping substance. It appears that bodybuilders would love to get it

"MENT has always been my favourite steroid, and that's just from reading the studies and looking at the structure of it. Thinking of what MENT can do should make every steroid user drool. This stuff is nearly as strong as its 17-alpha-alkylated counterpart mibolerone (cheque drops) but without the mad liver toxicity."
http://www.bodybuilding.com/fun/catment.htm

The analysis of the excretion urines collected over a 14hour period showed three major metabolites and another much smaller one (see Figure 4 for the chromatograms and Figure 5 for the mass spectra). Metabolite 1 (10.0 min, m/z 434, 419) appears to be 3-hydroxy-19-nor-7-methyl-androst-5(10)-ene-17-one as it has a molecular ion at m/z 432 (same as the parent drug) and may be the DHEA equivalent for testosterone. The double bond may be expected to be in the 5(10) position as this is the thermodynamically most stable one. Presumably the 7-methyl group hinders reduction of the double bond at the 4-5 position allowing this metabolite to accumulate.



The other main metabolite II (9.1 min) is the expected 7α-methylnorandrosterone and the peak at 10.2 may be the 7α-methylnoretiocholanolone (again by analogy to testosterone and nandrolone metabolite retention times).

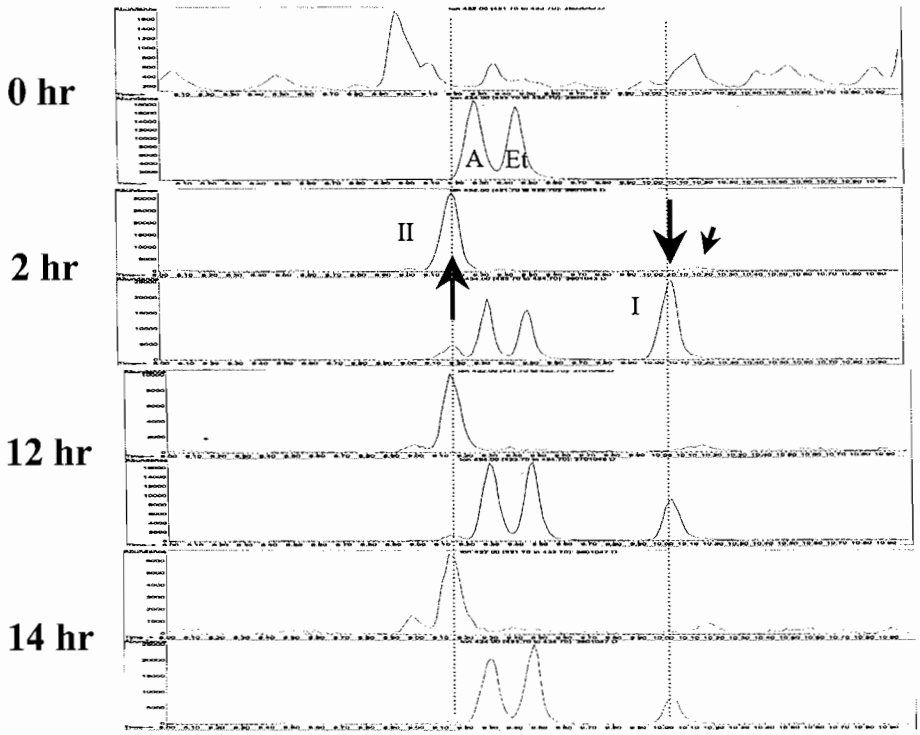


Figure 4 – chromatograms for collections up to 14hrs. For each time course the upper graph is the extracted ion at m/z 432 and the lower trace is the ion m/z 434.

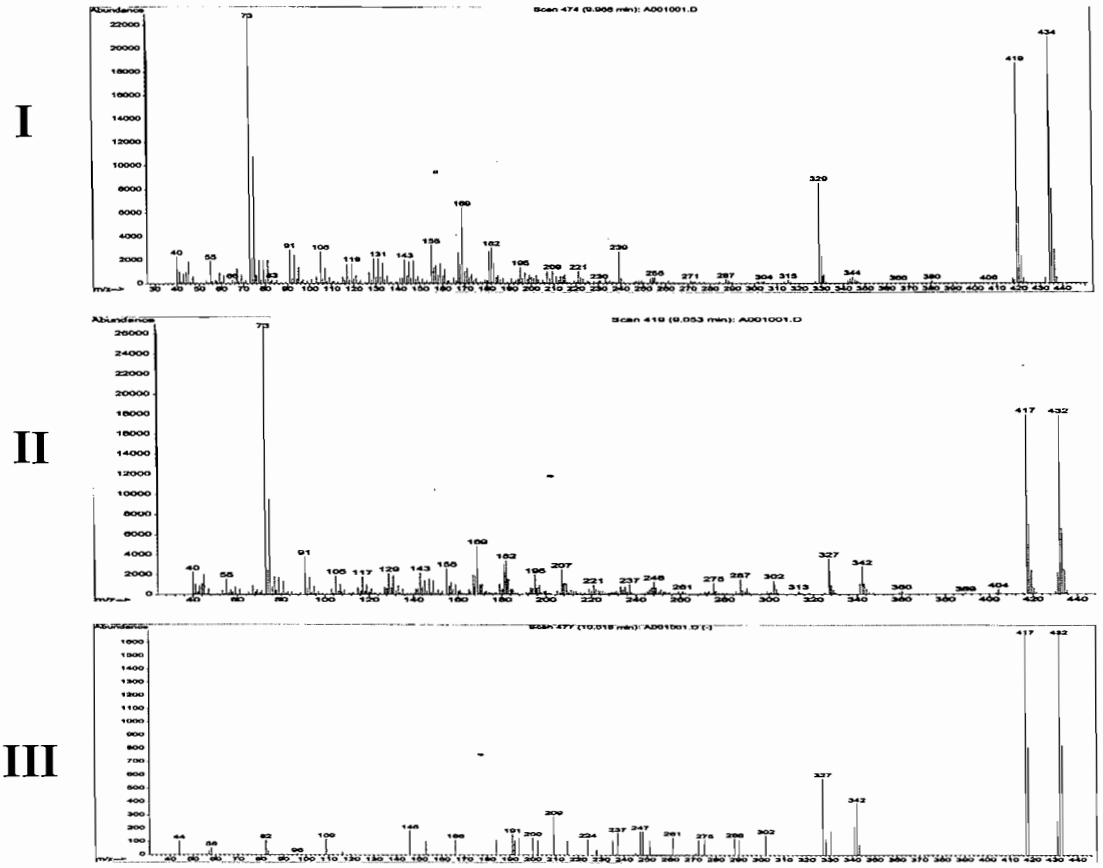


Figure 5 – mass spectra of the 3 metabolites.

Androgen receptor studies

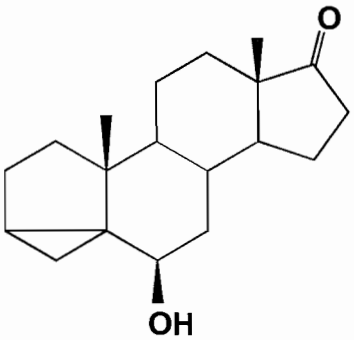
The substances were studied using a yeast based androgen receptor assay. This assay can be used to study the androgen activity of these steroids in order to get an idea of their relative potencies. This work has been published as a preliminary study (3). Table 1 shows the results for a series of agents and it can be seen that 18-methylnandrolone was inactive in this model.

Nandrolone	Active
Gestrinone	Active
THG	Active
Norbolethone	Active
5α-Norbolethonemetab.	Weak activity
5β-Norbolethonemetab.	Inactive
Norethandrolone	Active
18-Methylnandrolone	Inactive
Trenbolone	Active

Table 1 – Steroid hormone receptor activity for a series of compounds.

Searching for new markers of endogenous steroid abuse

The IRMS study of DHEA excretion urine samples provided traces which showed a consistent intense peak in the chromatogram with a similar depleted ^{13}C delta value to that of the ingested DHEA. The mass spectrum of derivatised and underivatised material indicated an unusual fragmentation pattern that had the same molecular ion as DHEA but unusual loss of m/z 55. A reference spectrum (4) was provided by Dr W. Schänzer of the Cologne laboratory, which allowed identification as 3α,5-cyclo-5α-androstan-6β-ol-17-one. This could be purchased from Sigma.



The single dose administration of DHEA gave elevated levels of this compound and its ^{13}C content decreased rapidly relative to the endogenous marker (11-ketoetiocholanolone) after ingestion of the DHEA as shown in Figure 6. When repeated doses of DHEA were administered the delta value of this substance (parallel to that of androsterone and etiocholanolone) decreased rapidly and fell considerably below the value of the DHEA administered (see Figure 7). This is most interesting as it suggests enrichment of the ^{12}C metabolites by enzymatic processes and is an issue for further study.

A survey of the normal population of this novel cyclo steroid using athlete urine samples is shown in Figure 8 and indicates that levels are normally very low. Using a log transformation, the mean value can be shown to be 22 ng/mL and the 95% confidence level is 140 ng/mL. Thus if values are found above this level, as may be shown by routine screening of the steroid profile, the sample can be considered for IRMS study as a suspect DHEA administration. This would also correspond to elevated androsterone and etiocholanolone levels also indicative of DHEA or androstenedione administration.

This work has been published fully (5)

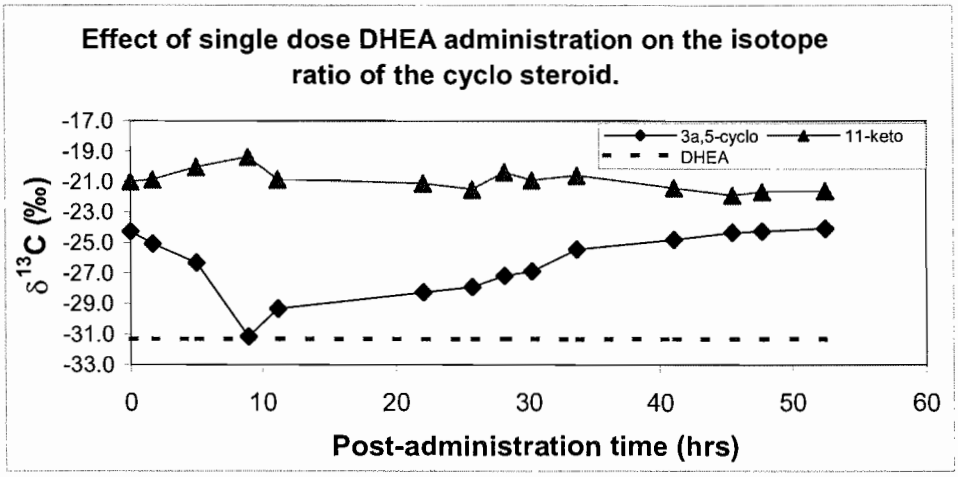


Figure 6 – single dose time course for DHEA (100 mg) administration.

References

1. Catlin DH, Ahrens BD, Kucherova Y., 2002 Detection of norbolethone, an anabolic steroid never marketed, in athletes' urine. Rapid Commun Mass Spectrom 16:1273–1275
2. Catlin, DH., Sekera, MH., Ahrens, BD., Starcevic, B, Chang, Y., Hatton, CK., Tetrahydrogestrinone: discovery, synthesis, and detection in urine, Rapid Communications in Mass Spectrometry, 2004, 18:1245-1249
3. Death, AK., Mcgrath, K.C.Y., Kazlauskas, R., and Handelsman, D.J., Tetrahydrogestrinone Is a Potent Androgen and Progestin, J. Clin. Endocrinol. Metab., 2004, 89: 2498-2500
4. H.L.J. Makin, D.J.H. Trafford, J. Nolan. *Mass Spectra and GC data of steroids.* Wiley-VCH, 1998, 49
5. Cawley, AT., Hine, ER., Trout, GJ., George, AV., Kazlauskas, R., Searching for new markers of endogenous steroid administration in athletes: "looking outside the metabolic box", Forensic Science International. 2004, 143:103-114.

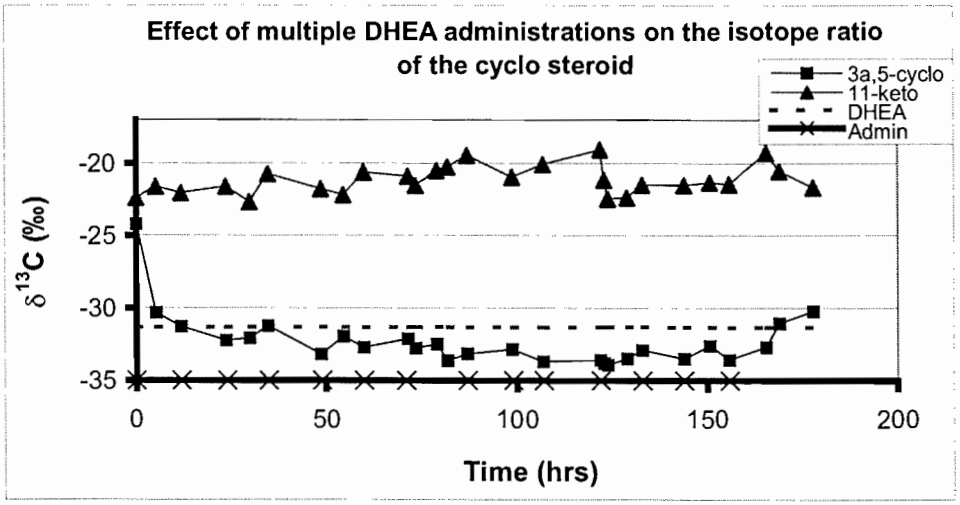


Figure 7 – Time course of the change in delta values for multiple 100 mg DHEA administrations. The x on the axis indicates administration points. The dotted line shows the delta value of the DHEA used for the study.

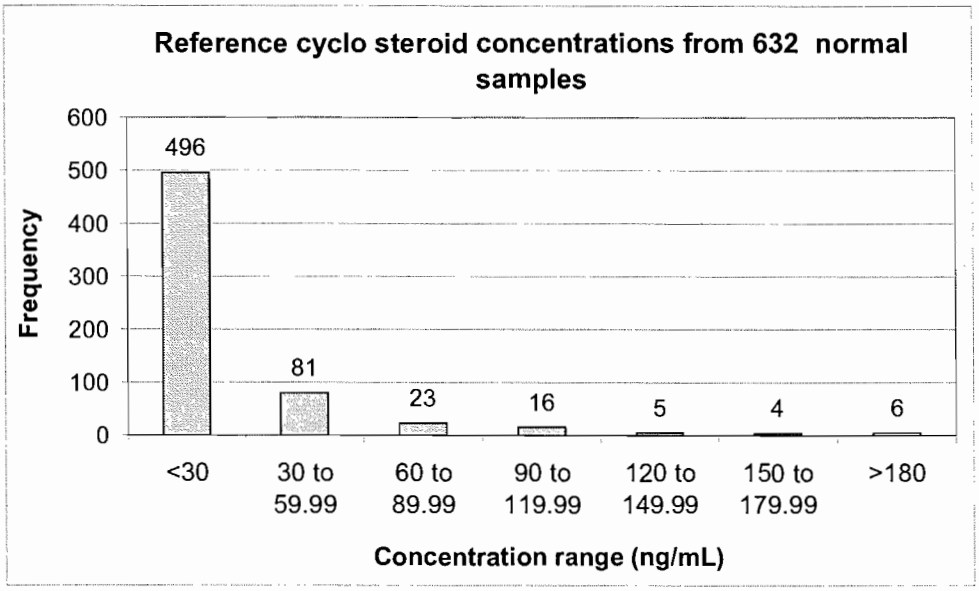


Figure 8 – Distribution of the cyclo steroid in 632 normal samples.

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