Journal of Steroid Biochemistry, 1977, Vol. 8, pp. 1057–1063. Pergamon Press. Printed in Great Britain

METABOLISM OF ANABOLIC STEROID DRUGS IN MAN AND THE MARMOSET MONKEY (*CALLITHRIX JACCHUS*)—I. NILEVAR AND ORABOLIN

ROBERTA J. WARD, A. M. LAWSON and C. H. L. SHACKLETON
Division of Clinical Chemistry, Clinical Research Centre,
Watford Road, Harrow, Middlesex HA1 3UJ, U.K.

(*Received* 17 *March* 1977)

SUMMARY

The major urinary metabolites of the anabolic steroid drugs Nilevar (17α-ethyl-17β-hydroxyestra-4-ene-3-one) and Orabolin (17α-ethyl-17β-hydroxyestra-4-ene) have been detected and partially characterised in man and the marmoset monkey (*Callithrix jacchus*) by use of gas chromatography–mass spectrometry.

Nilevar was metabolised in man by "A" ring reduction (3ξ,17β-dihydroxy-19-nor-5ξ,(17α)pregnane epimers) and side-chain hydroxylation to form a 19-nor-pregnanetriol (19-nor-5ξ,(17α)pregnane-3ξ,17β,21-triol). Orabolin was oxidised at C-3 to form Nilevar which in turn was metabolised as described above. The marmoset monkey excreted Nilevar and Orabolin as the hydroxy and tetrahydro derivatives of Nilevar. However, this species did not metabolise these steroids by side-chain or D-ring hydroxylation.

Identification of the 19-nor-pregnanetriol metabolite forms the basis of a specific method devised for the detection of Nilevar and Orabolin metabolites in urine of athletes suspected of drug misuse.

INTRODUCTION

The misuse of anabolic steroid drugs (e.g. Methandienone [Dianabol], Stanazolol and Durabolin) has become so widespread that the governing bodies of international sport, including the International Olympic Committee, have imposed a ban upon their use. For control to be successful, sensitive tests are required for the detection of these anabolic drugs and their metabolites in body fluids.

Brooks and co-workers[1] have raised antisera against the 17α-hydroxy-17β-methyl substituents in the steroid molecule, e.g. Dianabol and Stanazolol, and 17α-hydroxy-17β-ethyl grouping, e.g. Nilevar and Orabolin, and have established a screening method for detection of these drugs in urine.

However, enforcement of the ban on the use of anabolic steroids by athletes requires more specific proof of drug identity than that afforded by radioimmunoassay. Gas chromatography–mass spectrometry has been chosen as the technique for this purpose. As is the case for naturally occurring steroids, anabolic drugs undergo extensive metabolism prior to excretion, and their metabolism must be known before sensitive methods can be established for their analysis. In a recent communication from this laboratory, preliminary studies on the metabolism of a series of anabolic steroid drugs was reported [2] and the present study describes the metabolism of two 19-nor steroids, Nilevar and Orabolin, in man and the marmoset monkey. The low levels of endogenous steroids in marmoset urine simplifies the identification of metabolites of administered drugs.

MATERIALS AND METHODS

Source of anabolic steroids

Nilevar: G. D. Searle, U.S.A. *Systematic name*:—17α-ethyl-17β-hydroxyestra-4-ene-3-one. *Other trivial or trade names*:—Norethandrolone, ethylnortestosterone.

Orabolin: Organon Laboratories Ltd., Morden, Surrey. *Systematic name*:—17α-ethyl-17β-hydroxyestra-4-ene. *Other trivial or trade names*:—Ethylestrenol, Maxibolin.

Instrumentation and materials

Gas chromatograph–mass spectrometer, Varian MAT-731, Varian GmbH, Bremen, Germany; *Gas chromatograph*, Model 104, Pye Unicam, Cambridge, England; Becker Model 409, Packard Becker, Delft, Netherlands; *Sephadex LH-20*, Pharmacia AB, Uppsala, Sweden; *Amberlite XAD-2*, Rohm & Haas, Philadelphia, U.S.A.; *Helix pomatia*, L'Industrie Biologique, Francaise, Gennevilliers, Seine, France; *Ketodase*, Warner–Chilcott Laboratories, Morris Plains, NJ, U.S.A.; *Hexamethyldisilazane* (*HMDS*), *Trimethylchlorosilane* (*TMCS*), Applied Science Laboratories, State College, PA, U.S.A.; *Methoxyamine hydrochloride*, Eastman Organic Chemicals, Rochester, NY, U.S.A.; *Pyridine* (*Analar*), BDH, Poole, England, distilled and stored over sodium hydroxide; *Cyclohexane* (*Analar*), BDH., Poole, England, charcoal washed and double distilled.

Urine collection, extraction and hydrolysis

Following collection of a 24 h control urine speci-

men the anabolic steroid under investigation was administered orally to a male volunteer (20 mg)* and to a marmoset monkey (5 mg). Urine was then collected for 48 h (two 24 h samples). Each marmoset urine specimen was diluted to 25 ml and 2.5 ml 5M acetate buffer was added (pH 4.8). The steroid conjugates were hydrolysed with *Helix pomatia* digestive juice and Ketodase for 48 h and the freed steroids extracted on columns of XAD-2 resin [3]. The human urine specimens were initially extracted on Amberlite XAD-2 columns in order to concentrate the steroids prior to hydrolysis. The dried extract was dissolved in 10 ml 0.5 M acetate buffer and hydrolysed with *Helix pomatia* digestive juice and Ketodase. After 48 h incubation at 37° the freed steroids were extracted on XAD-2.

Fractionation

The crude extracts were fractionated on Sephadex LH-20 columns according to the method described previously [3]. 6 g Columns were used with the solvent system cyclohexane–ethanol, (4:1, v/v). Two fractions were collected and dried, **A**, 12–55 ml, and **B**, 55–160 ml.

Derivatisation

A portion of each fraction was trimethylsilylated by dissolving in 200 μl pyridine, and treating it with 200 μl of HMDS and 50 μl TMCS for 8 h. The derivatives were dried under nitrogen, and the residue dissolved in cyclohexane and the pyridine hydrochloride precipitate removed by centrifugation.

Gas chromatography

Fractions **A** and **B** from Sephadex LH-20 chromatography were analysed by gas chromatography on a packed OV 1 column or open-tubular OV 101 column prior to GC–MS analysis. Linear temperature programming was used in each case.

Gas chromatography–mass spectrometry

Aliquots of the samples were injected into the GC–MS instrument and the column temperature programmed between 220 and 280°. Spectra were taken repetitively every 8 s thoughout the period of elution of the sample from the gas chromatograph and stored on magnetic tape. On completion of the analysis the data were processed and total ion current recordings viewed on a video display unit. Hard copies of mass spectra were obtained from the stored data when required.

RESULTS

Metabolism of Nilevar and Orabolin in the marmoset monkey

It was considered likely that the small concentration of endogenous steroids in marmoset urine would not interfere with analysis of drug metabolites, thus enabling the routes of drug metabolism to be readily determined and providing mass spectral data to assist in the human studies.

Figure 1 illustrates the gas chromatographic separation of steroids in Sephadex fraction **A** as trimethylsilyl ether derivatives run on packed and open-tubular columns. All the major steroids marked are Nilevar metabolites. No Nilevar metabolites were detected in fraction **B** which normally contains pregnanetriolones (e.g. tetrahydrocortisone) and more polar compounds; therefore it appears that all major metabolites of Nilevar have no more than three hydroxyl groups.

A substantial proportion of administered Nilevar was excreted unchanged (approximately 36% of total identifiable metabolites) together with the metabolites 3α,5α-tetrahydro-Nilevar (16%), 3α,5β-tetrahydro-Nilevar (15%), a hydroxy-Nilevar (20%) and a hydroxy-tetrahydro-Nilevar (12%) (see Table 1).

Figure 2a illustrates the mass spectrum of Nilevar trimethylsilyl ether. The base peak, at *m/e* 157, is formed by cleavage of the D-ring between C-13 and C-17 and between C-14 and C-15 [4]. The ion *m/e* 144 results from fragmentation of the C-13/C-17 and C-15/C-16 bonds. (These two fragment ions should be present in the spectra of all Nilevar and Orabolin metabolites where there has been no hydroxylation in the D-ring or ethyl side-chain). The parent ion is at *m/e* 374 and major ions, found at *m/e* 345 (M − 29) and *m/e* 255 (M − 90 − 29) are formed by the loss of the ethyl side-chain. An intense ion at *m/e* 303 (M − 71) probably arises by loss of the D-ring ethyl group (−29 mass units), and carbon atoms at C-2 and C-3 together with the C-3 oxo group (−42 mass units). A loss of 42 mass units is commonly observed in 3-oxo-4-ene steroids (e.g. progesterone and androstenedione).

Figure 2b shows the spectrum of urinary 3α,5α-tetrahydro-Nilevar (M = 450) with the base peak at *m/e* 157 and prominent ions formed by loss of the ethyl group (M − 29) and ethyl group plus trimethylsilyl grouping (M − 29 − 90).

The mass spectrum of the hydroxylated Nilevar (M = 462) is illustrated in Fig. 2c. The major fragment ions at *m/e* 420 (M − 42) and *m/e* 157 indicate that the hydroxyl group is not in the A or D-rings or side-chain, although the position of the additional hydroxyl group was not ascertained. The mass spectra of the hydroxytetrahydro-Nilevar has a parent ion at *m/e* 538 with a prominent ion at *m/e* 509 (M − 29). Further studies are required to establish the position of the hydroxyl functions.

No metabolites were detected which gave a base peak at *m/e* 245, indicating that Nilevar was not metabolised by side-chain or D-ring hydroxylation.

Orabolin was metabolised to Nilevar, the two tetrahydro metabolites of Nilevar, hydroxy-Nilevar and hydroxy-tetrahydro–Nilevar. Trace amounts of Orabolin and a hydroxy-Orabolin were tentatively identified.

* Permission for this investigation to be carried out was given by Northwick Park Hospital Ethical Committee.

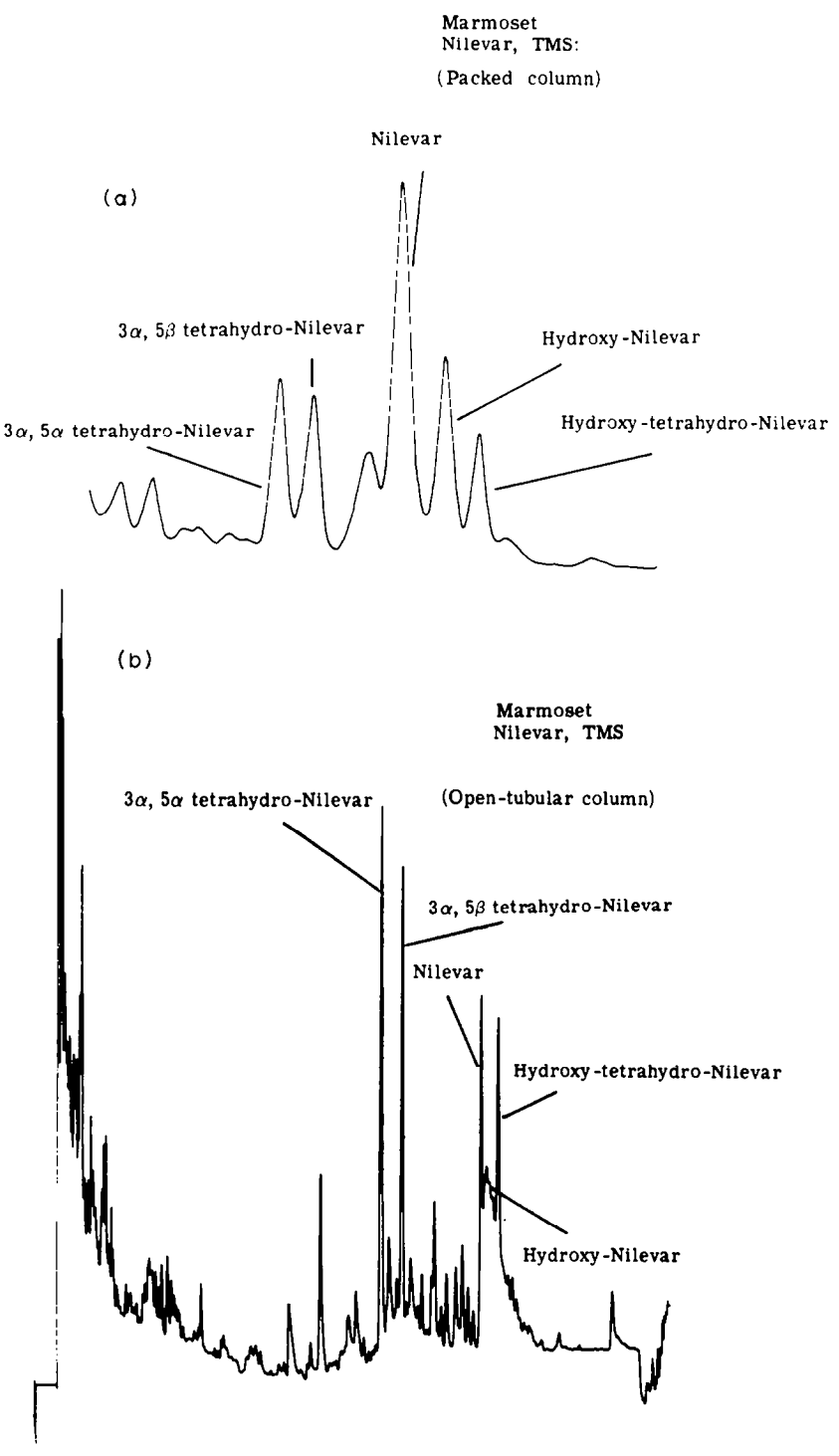
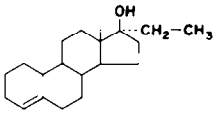
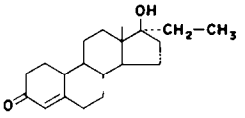
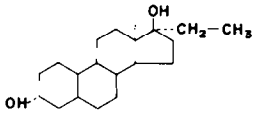
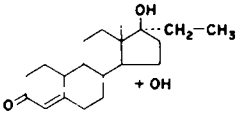
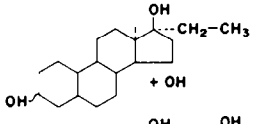
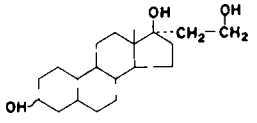


Fig. 1. Gas chromatographic traces from (a) packed, and (b) open-tubular capillary columns of steroid fractions from marmoset urine after administration of Nilevar. The misshapen Nilevar peak on the capillary column was caused by heavy absorption due to the free carbonyl group. Derivatisation of the carbonyl group or use of a newer capillary column with few "absorbative sites" normalises the peak shape.

Metabolism of Nilevar and Orabolin in man

Figure 3 illustrates selected ion chromatograms obtained from repetitive scanning GC–MS of urinary steroids excreted following ingestion of Nilevar. As previously mentioned metabolites of Nilevar without a hydroxyl group in the D-ring or side-chain give characteristic ions at *m/e* 144 and 157 (Fig. 1) and peaks 1 and 2 were identified as 3α,5β-tetrahydro-Nilevar and Nilevar. The peak immediately prior to 1 was identified as the endogenous urinary steroid, 11β-hydroxy-aetiocholanolone. The *m/e* 157 response is given by the isotope peak of the *m/e* 156 ion which

Table 1. Structure of drugs and metabolites

Structure	Name	M+ TMS Derivative	Detected Man	Detected Marmoset
	Orabolin	360		
	Nilevar	374	+	+
	3α,5α-tetrahydro-Nilevar	450	+	+
	Hydroxy-Nilevar	462		+
	Hydroxy-tetrahydro-Nilevar	538		+
	19-nor-5ξ,17α-pregnane-3ξ,17β,21-triol	538	+	

is the base peak in this steroid. Peak 3 is a metabolite of Nilevar hydroxylated in the D-ring or side-chain. Hydroxylation in this part of the steroid molecule results in a shift of the *m/e* 157 fragment to *m/e* 245. The mass spectrum of peak 3 (Fig. 4) is consistent with the structure 19-nor-5ξ,17α-pregnane-3ξ,17β,21-triol (19-nor-pregnanetriol). The molecular ion is at *m/e* 538 and prominent ions are formed by loss of the side-chain *m/e* 421 (M − 117), the side-chain plus trimethylsilanol *m/e* 331 (M − 117 − 90) and the side-chain plus C-17 and the C-17 hydroxyl group which must be at position 21. Additional confirmation of the structure of this 19-nor-pregnanetriol was obtained from the spectra of its perdeutero-trimethylsilyl derivative.

From the selected ion chromatogram, Fig. 3, it can be seen that an endogenous steroid, 5-pregnene-3β,16α,20α-triol, has a very similar retention time to the major metabolite of Nilevar and Orabolin and also has fragment ions (e.g. *m/e* 144, 157) which are of the same mass values. However, it was possible to resolve the components of this multiple peak either by capillary column chromatography or by further processing of the mass spectral data. This latter approach involves the resolution of ions by considering only the ions which maximise in a spectrum [5].

During this study urine was also obtained from a cancer patient being administered Nilevar (15 mg/day) for therapeutic reasons. The metabolism was found to differ from that of the normal adult. Large amounts of Nilevar were excreted unchanged and 19-nor-pregnanetriol was only a minor metabolite. A *hydroxy*-Nilevar was observed although it was different from the *hydroxy*-Nilevar found in marmoset urine (Fig. 2c). The parent ion is at *m/e* 462 and base peak at *m/e* 157 indicating that no metabolism of the D-ring or side-chain had occurred. An ion corresponding to the *m/e* 303 ion of Nilevar (Fig. 2a) is seen at *m/e* 391 and is probably formed by the same mechanism, loss of C-2 and C-3 and the carbonyl group. The altered metabolism in the cancer patient may either reflect altered metabolism during the period the patient was on the drug (since the urine sample was obtained several days after a daily regime of Nilevar had been started), or altered hepatic metabolism due to the cancer condition.

The metabolism of Orabolin in the normal adult was virtually identical to that of Nilevar since the same compounds were detected in approximately the same amount. No 3-deoxy metabolites of Orabolin were detected, neither were steroids showing evidence of de-ethynylation. This latter finding is significant since de-ethynylation has been shown to occur for other synthetic steroids [6].

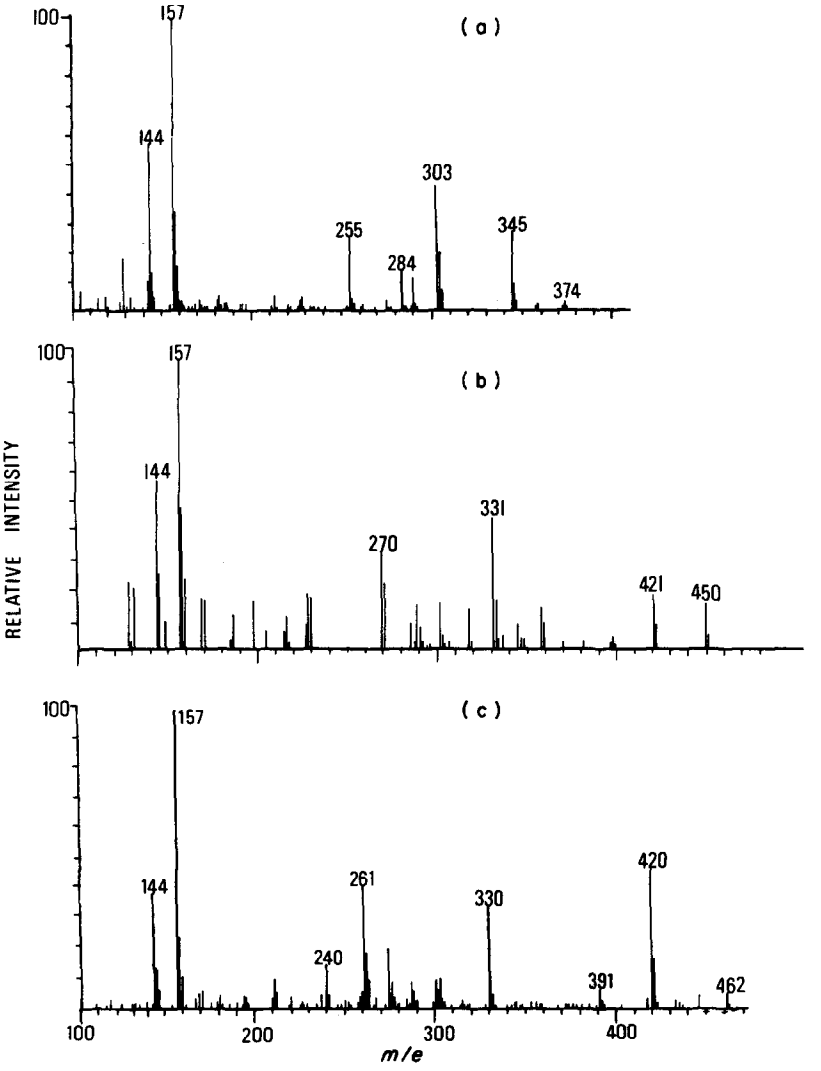


Fig. 2. Mass spectra of the trimethylsilyl derivatives of (a) Nilevar, (b) tetrahydro-Nilevar, and (c) a hydroxy-Nilevar.

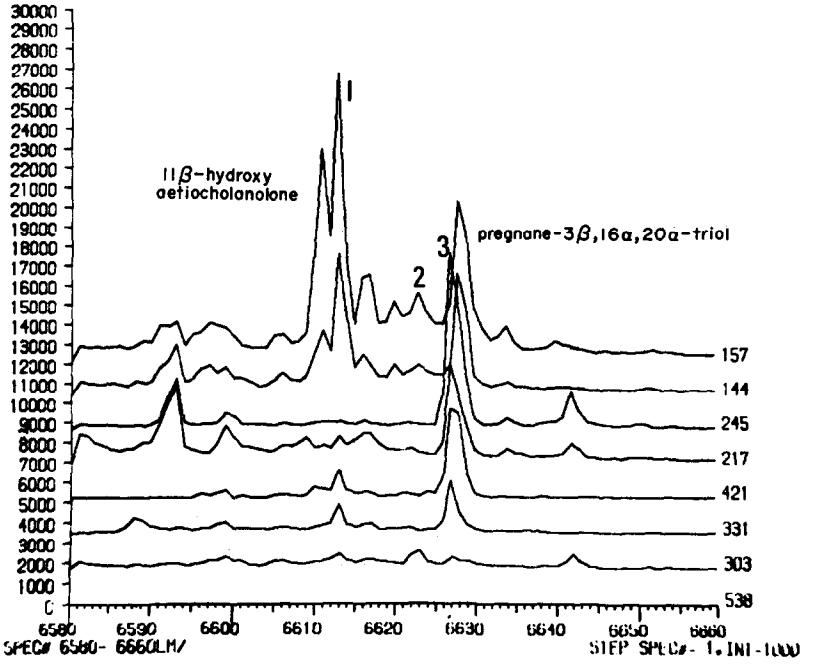


Fig. 3. Selected ion plots obtained from the repetitive mass spectral scan data showing responses from ions which are present in the trimethylsilyl derivatives of Nilevar and its metabolites: **1** is 3α,5β-tetrahydro-Nilevar, **2** is Nilevar and **3** is 19-nor-pregnanetriol.

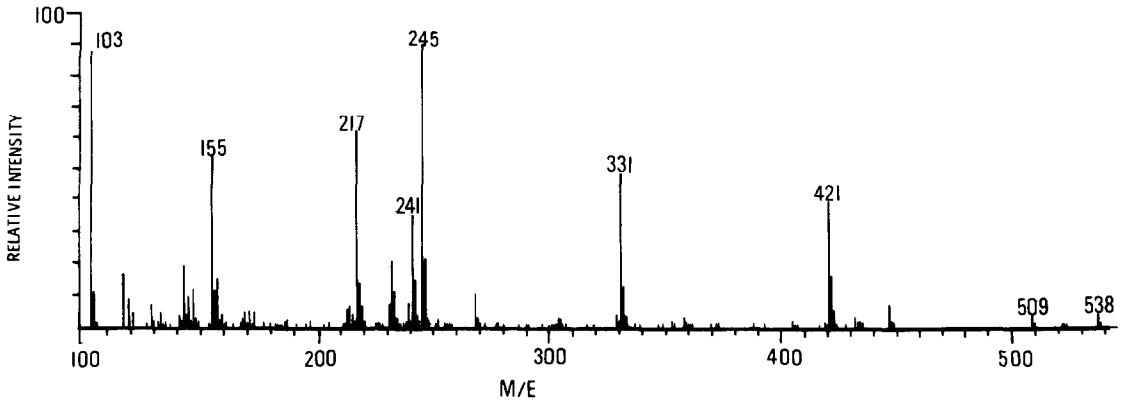


Fig. 4. Mass spectrum of the principal metabolite of Nilevar and Orabolin-19-nor-5ξ,17α-pregnane-3ξ,17β,21-triol.

DISCUSSION

The urinary metabolites of ingested Nilevar in man have been previously studied by Brooks and co-workers [7], and Adhikary and Harkness[8]. The former identified by GC–MS two "tetrahydro" metabolites and a "triol" which was considered to have a hydroxyl group in the ethyl side-chain. Our studies confirmed these findings and the mass spectrum of the "triol" suggested that its structure was 19-nor-3ξ,17β,21-trihydroxy-5ξ,17α-pregnane (19-nor-pregnanetriol). In addition unchanged Nilevar was detected as well as a metabolite with a hydroxyl group probably in the B-ring (position 6 or 7). No metabolites containing more than three hydroxyl groups were found.

The marmoset monkey metabolised Nilevar principally to the tetrahydro derivative, a hydroxy-Nilevar and a hydroxy-tetrahydro-Nilevar: there was no evidence for hydroxylation in the side-chain to the 19-nor-pregnanetriol metabolite found in man. The metabolism of Orabolin in man was very similar to that of Nilevar. The major metabolite had a mass spectrum and retention time identical to the 19-nor-pregnanetriol identified as a Nilevar metabolite. Nilevar and tetrahydro-Nilevar were also detected in urine following ingestion of Orabolin. Neither unchanged Orabolin nor hydroxylated 3-deoxy metabolites of Orabolin were detected although small amounts of these compounds were found in the marmoset urine. It therefore seems probable that the major pathway of Orabolin catabolism is via Nilevar. The conversion of Orabolin to Nilevar has also been demonstrated in rabbit liver slices by Okada *et al.*[9].

The metabolism of Orabolin is very similar to that of another synthetic 3-deoxy steroid, lynestrenol (17α-ethynyl-19-nor-4-androsten-17β-ol). Kamyab *et al.*[10] studied the metabolism of [^{14}C]-lynestrenol in women and concluded that it was mainly metabolised to polar compounds, probably containing at least two hydroxyl groups, but absolute identification of metabolites was not achieved. This investigation was followed by demonstration of the conversion of [^{14}C]-lynestrenol to norethisterone (17α-ethynyl-19-nor-testosterone) by liver homogenates [11]. Both Orabolin and lynestrenol are almost certainly oxidised to 3-hydroxy-4-ene steroids prior to conversion to Nilevar and norethisterone respectively.

Although from our studies it was shown that there is extensive hydroxylation of the ethyl side-chain of Orabolin and Nilevar there is little or no oxidation of the ethynyl side-chain of lynestrenol, norethisterone or other steroids containing this group [6, 12–14]. These ethynyl compounds metabolise to give steroids hydroxylated in the 1β,2α,6α,10β,16α and 16β positions [6, 14, 15]. It is of interest that Williams and co-workers showed that de-ethynylation of labelled ethynyl-oestradiol can occur *in vivo* but the present study failed to reveal the presence of de-ethynylated metabolites of Orabolin and Nilevar (e.g. 19-nor-androsterone and 19-nor-aetiocholanolone). Thyssen[16] partially characterised two of the urinary metabolites of allylestrenol administered to non-pregnant women. The original 17β-hydroxyl group was acetylated and the alkyl side-chain altered, probably reduced and hydroxylated. He observed no oxidation at C-3.

The present study was undertaken to develop methods for the specific and sensitive detection of anabolic steroid metabolism in urine from athletes suspected of drug abuse [2]. We have come to the conclusion that the 19-nor-pregnanetriol is the most suitable analyte for Orabolin and Nilevar detection by selected ion recording GC-MS, since the major ions in the trimethylsilyl ether of tetrahydro-Nilevar would conflict with ions in the trimethylsilyl ethers of endogenous urinary steroids (e.g. 11β-hydroxyandrosterone, 11β-hydroxy-aetiocholanolone and 5-pregnene-3β,16α,20α-triol).

CONCLUSIONS

1. In both man and the marmoset monkey Orabolin is hydroxylated at position 3 and converted to Nilevar prior to further metabolism.

2. In man Orabolin and Nilevar are converted to the same major metabolites—tetrahydro-Nilevar and 19-nor-pregnanetriol.

3. The marmoset monkey excretes Nilevar unchanged or as tetrahydro-derivatives or hydroxylated. It does not excrete Nilevar or Orabolin metabolites with a hydroxyl group in the D-ring or side-chain.

4. The most suitable metabolite for routine examination of human urine for Nilevar and Orabolin metabolites is 19-nor-pregnanetriol.

Acknowledgements—We wish to thank Dr. C. R. Hetherington of the Animal Division, Clinical Research Centre, for organising the collection of urine specimens from the marmoset monkey after drug administration, Mr. M. Chu and Mr. M. Madigan for technical assistance with mass spectrometry, Mr. A. C. Thomas and Mr. M. Bhasin for computer programming, and the Organon Laboratories and G. D. Searle who donated the steroid drugs.

The work was supported by a grant from the Sports Council.

REFERENCES

1. Brooks R. V., Firth R. J. and Sumner A. A.: *Br. J. Sports Med.* **9** (1975) 89–92.
2. Ward R. J., Shackleton C. H. L. and Lawson A. M.: *Br. J. Sports Med.* **9** (1975) 93–97.
3. Shackleton C. H. L.: *J. steroid Biochem.* **6** (1975) 1429–1432.
4. Middleditch B. S., Vouros P. and Brooks C. J. W.: *J. pharm. Pharmac.* **25** (1973) 143–149.
5. Biller J. E. and Biemann K.: *Analyt. Lett.* **7** (1974) 515–528.
6. Williams M. C., Hetton E. D. and Goldzieher J. W.: *Steroids* **25** (1975) 229–246.
7. Brooks C. J. W., Thawley A. R., Middleditch B. S., Anthony G. M. and Stillwell W. G.: *J. chromatogr. Sci.* **9** (1971) 35–43.
8. Adhikary P. M. and Harkness R. A.: *Acta endocr., Copenh.* **67** (1971) 721–732.
9. Okada H., Amatsu M., Ishihara S. and Tokuda G.-I.: *Acta endocr., Copenh.* **46** (1964) 31–36.
10. Kamyab S., Fotherby K. and Klopper A. I.: *J. endocr.* **42** (1968) 337–343.
11. Mazaheri A., Fotherby K. and Chapman J. R.: *J. endocr.* **47** (1970) 251–252.
12. Fotherby K., Kamyab A., Littleton P. and Klopper A.: *Biochem. J.* **99** (1966) 14.
13. Kamyab S., Fotherby K. and Klopper A. I.: *J. endocr.* **41** (1968) 263–272.
14. Sisenwine S. F., Kimmel H. B., Liu A. L. and Ruelius H. W.: *Acta endocr., Copenh.* **73** (1973) 91–104.
15. Layne D. S., Golab T. and Arai K.: *Biochem. Pharmac.* **12** (1963) 905–911.
16. Thyssen J. H.: Thesis to University of Utrecht (1967).