Journal of Chromatography, 167 (1978) 117–124
© Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 11,167

GAS CHROMATOGRAPHIC AND CAPILLARY COLUMN GAS CHROMATOGRAPHIC–MASS SPECTROMETRIC DETERMINATION OF SYNTHETIC ANABOLIC STEROIDS

I. METHANDIENONE AND ITS METABOLITES

H. W. DÜRBECK, I. BÜKER, B. SCHEULEN and B. TELIN
Institute of Chemistry, Institute 4, Applied Physical Chemistry, Nuclear Research Centre (KFA) Jülich, P.O. Box 1913, D-5170 Jülich (G.F.R.)

SUMMARY

The determination of methandienone (I) (17α-methyl-17β-hydroxyandrosta-1,4-dien-3-one) in human urine by gas chromatography and capillary column gas chromatography–mass spectrometry has been studied. After oral administration to man two major metabolites were detected, the structures of which have been identified as 17-epi-methandienone (II) and 6β-hydroxy-17-epi-methandienone (III). These metabolites are exclusively excreted in the unconjugated form. At least two more metabolites are extractable from the free fraction of the urine but no measurable amounts of I itself were found. The rate of metabolism and urinary excretion seems to be reasonably fast. The total amount of recovered I in the form of the metabolites II and III is about 5%. Extraction and clean-up procedures and chromatographic details are presented.

INTRODUCTION

In recent years, a series of synthetic anabolic steroids have been used for the stimulation of the biosynthesis of proteins[1–8].

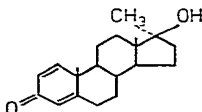
It is well established, however, that the stimulating anabolic effect of these drugs is always accompanied by undesirable androgenic effects which are more or less pronounced, depending on structural differences of the various synthetic steroids[9,10]. Moreover, dangerous side effects[11–13], such as liver damage, have been reported[6] after the application of massive doses of anabolic hormones with a 17α-alkyl group. Even small amounts administered over a long period may influence the metabolism of the natural androgens[14].

In spite of these well known facts, little information is available on the determination and metabolism of synthetic steroids in man, although a knowledge of metabolism is necessary in order to understand the rationale of drug toxicity.

Moreover, many important parameters such as metabolism, excretion rate, total amount excreted, possible fat storage or other accumulation processes must still be determined in order to establish a scientifically sound basis for the legal control of the administration of anabolic drugs.

Parallel to previously reported high-performance liquid chromatographic (HPLC) studies[15,16], some sensitive and generally applicable methods for the determination of synthetic anabolic steroids and their metabolites have been developed in which gas–liquid chromatography (GLC) and capillary column gas chromatography–mass spectrometry (GC–MS) are employed.

In the first paper of a series, we describe the general clean-up procedure for unconjugated steroids in urine and the basic instrumental requirements. Because of its particular significance as one of the most frequently used synthetic anabolic steroids, methandienone (I) (17α-methyl-17β-hydroxy-androsta-1,4-dien-3-one, also termed methandrostenolone, Dianabol, Nerobol) (Fig. 1), was selected as the first drug to be investigated.



I

Fig. 1. Structure of methandienone (I).

EXPERIMENTAL

Before the beginning of the drug administration, two urine samples are collected as control specimens. After the application of a single dose of 10 mg of methandienone, urine samples are collected every 3 or 4 h for at least 3 days.

Clean-up procedure

This procedure is the crucial part of the analysis and should therefore be performed with maximum care. All reagents used should be of analytical-reagent grade or better. A critical suitability test for every reagent is recommended on account of possible interferences by impurities.

A 50-ml volume of urine is adjusted to pH 5.0 $\pm$ 0.1 with glacial acetic acid. The unconjugated steroids are removed from the sample by extracting four times with 20 ml of dichloromethane. The combined extracts are washed with 15 ml of 0.1 *N* sodium hydroxide solution and then with 15 ml of 0.02 *N* acetic acid. Trace amounts of water are removed from the organic phase by filtration through a soft paper filter (the normal drying procedure with anhydrous sodium sulphate should be omitted to avoid massive contamination with phthalates or silicones). The dichloromethane extract is then evaporated to dryness at ambient temperature. The dry residue is dissolved in 2 ml of methanol and the precipitating waxes are separated by filtration. After evaporation to dryness the residue must be absolutely free from water and methanol.

Silylation

On the basis of detailed preliminary experiments on the silylation of sterically hindered hydroxy groups in steroids, the following procedure is recommended.

A 100-μl volume of N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) and 10 μl of trimethylchlorosilane (TMCS) are dissolved in 1 ml of pyridine, 150 μl of this reagent are added to the dry urine extract and the mixture is kept at room temperature for approximately 30 min. The solution is then ready for gas chromatography.

Gas chromatography with packed columns

The gas chromatographs were (1) a Hewlett-Packard Model 5754 B and (2) a Hewlett-Packard Model 5700 A. The columns were (1) a 2 m × 2 mm I.D. glass column, coated with 3% OV-101 on Gas-Chrom Q (80–100 mesh) and (2) a 2 m × 2 mm I.D. glass column, coated with 3% OV-25 on Gas-Chrom Q (80–100 mesh). Prior to the OV-25 coating the solid support was deactivated with 0.5% Dexil 300 according to the procedure of Donike[17]. The carrier gas was helium at flow-rates of (1) 32 ml/min and (2) 40 ml/min.

The temperature programmes were (1) 4 min isothermal at 180°, then programmed at 6°/min to 270°, and (2) 4 min isothermal at 170°, then programmed at 8°/min to 270°.

Data acquisition and processing were effected with a Hewlett-Packard Model 2108 microcomputer, 3352 B chemical software and laboratory-designed BASIC programs.

Capillary column gas chromatography–mass spectrometry

The gas chromatograph was a Carlo Erba Model 2900, with a 40-m glass capillary column coated with SE-54 (purchased from H. u. G. Jaeggi, Trogen, Switzerland). The carrier gas was helium at a flow-rate of 3 ml/min at 200°.

The temperature programme was: 6 min isothermal at 30°, rapid heating to 200°, then programmed at 2.5°/min to 260°. Sample application was effected by splitless injection according to Grob and Grob[18].

The mass spectrometer was a Varian-MAT CH 7 A, the ion source being electron impact at 25 eV, 600 μA and 250°, equipped with an all-glass interface as described recently[19,20]. The temperature was 270° and the carrier gas flow-rate was about 2 ml/min. Data processing was carried out with a Varian Spectro System 100.

To protect the interface and ion source from the silylating solvent, the column and interface were disconnected for the first 6 min after sample injection.

RESULTS

Typical chromatograms of a urine sample which was collected 7 h after the administration of 10 mg of methandienone to a healthy man aged 24 years are presented in Figs. 2, 3 and 4 for three columns of different size and polarity. The peaks of interest are numbered from 1 to 6. Peaks 1, 3 and 5 are also present in the control specimens and therefore most suitable as reference compounds. From MS measurements they were identified as (1) phthalate (probably di-*n*-octyl phthalate), (3) squalene and (5) cholesterol. Peaks 2 and 4 are found only after the administration of the anabolic steroid and are thus most valuable for a rapid screening procedure of drug application. On the basis of computer-aided multiple-ion mass fragmentography and by comparison of the relative retention data (Table I), peak 2 was identified as 17-

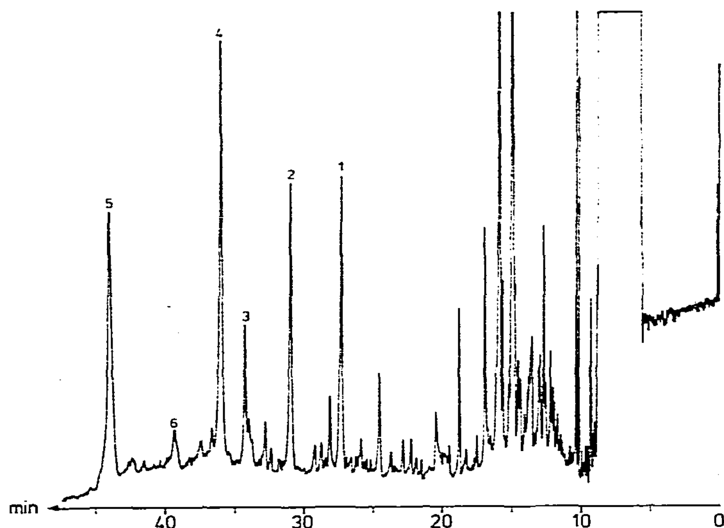


Fig. 2. GC–MS chromatogram of a urine sample after the administration of methandienone, using a glass capillary column coated with SE-54. (1) Phthalate; (2) 17-epi-methandienone; (3) squalene; (4) 6β-hydroxy-17-epi-methandienone; (5) cholesterol; (6) 6β-hydroxy-methandienone.

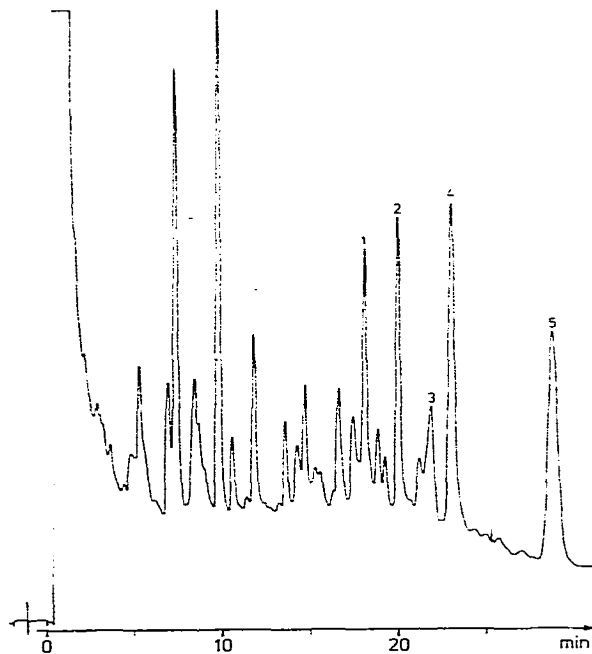


Fig. 3. Chromatogram of the same urine sample as in Fig. 2, using a packed column coated with OV-101. Peaks as in Fig. 2.

epi-methandienone (II). By the same instrumental means of identification the more polar metabolite (peak 4) is considered to be 6β-hydroxy-17-epi-methandienone (III) (see Fig. 5). In much smaller quantities at least two more metabolites may be found, the structures of which, however, have not been fully confirmed. It is noteworthy

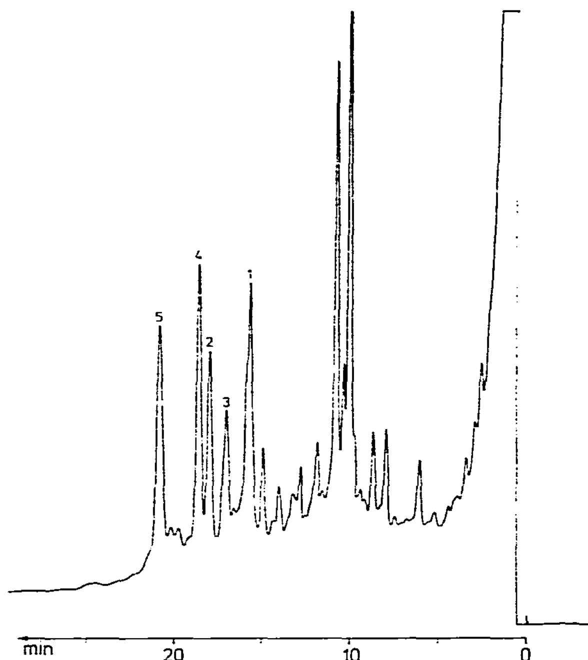


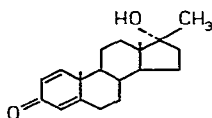
Fig. 4. Chromatogram of the same urine sample as in Figs. 2 and 3, using a packed column coated with OV-25. Peaks as in Fig. 2.

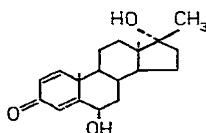
TABLE I

RELATIVE RETENTION TIMES OF SOME METHANDIENONE METABOLITES

R.S.D. ± 0.002 (reference: cholesterol).

Compound	*Column*		
	SE-54	*OV-25*	*OV-101*
Methandienone	0.772	0.935	0.784
Metabolite II	0.702	0.860	0.714
Metabolite III	0.817	0.889	0.816
Compound 6	0.895	0.947(?)	–





II III

Fig. 5. Structures of 17-epi-methandienone (II) and 6β-hydroxy-17-epi-methandienone (III).

that there is no evidence for the excretion of the administered drug in any of the "free" urine fractions examined.

Qualitative aspects

As at least two specific metabolites of methandienone (peaks 2 and 4) are completely separated from all other compounds in all chromatograms, the drug can be detected by a simple screening procedure. A computer programme has been developed which compares the relative retention times of the two metabolites (II and III) with those of the three reference compounds (peaks 1, 3 and 5). If all of these cross-references, a total of 18 events, are positive, the sample is automatically classified as containing methandienone. For the determination of therapeutic doses, the evaluation of two chromatograms is usually sufficient.

Quantitative aspects

The GC methods described are capable of determining 10 ng of either metabolite with a standard deviation of $\pm 8\%$. This amount is equivalent to a concentration of 30 μg/l in urine. The calibration graph for methandienone is linear from 30 ng to 1 μg, showing a slight deviation between 10 and 30 ng. The sensitivity can be increased by a factor of 10 or more by different methods of pre-concentration or by applying single-ion MS. This technique, however, is not sufficiently specific and not suitable for rapid screening procedures.

DISCUSSION

Although methandienone is one of the most frequently used synthetic anabolic steroids, little has been reported on its chromatographic behaviour or any other simple and reliable screening procedure. The same applies to the examination of its metabolism. In two papers[7,21] the detection of methandienone in the "free" fraction of urine by GC on packed columns was described and the procedure was claimed to be specific for the detection of drug administration. However, the chromatograms presented exhibited only a few peaks and poor resolution.

On the other hand, it was shown by carbon skeleton chromatography[22,23] and by single-ion mass spectrometry[24] that two metabolites occur in the "free" fraction, one of which was considered to be 6β-hydroxymethandienone[24,25], while the other was identified as 17-epi-methandienone[26], *i.e.* the conformation at C-17 has changed.

The results from the chromatograms presented in this paper indicate that at least two major metabolites (peaks 2 and 4) can be detected. The less polar compound (peak 2) has a mass spectrum very similar to that of methandienone. However, the chromatographic properties are different, because on non-polar columns the retention time of silylated methandienone is very close to that of squalene (peak 3). Accordingly, the first metabolite is 17-epi-methandienone, which is in agreement with the literature[26]. If the urine extract is subjected to GLC on non-polar columns without silylation, the retention times of methandienone and its 17-epimer nearly coincide. This could be the reason for the misinterpretation of the chromatograms in previous papers[7,21].

From our extensive MS measurements it is evident that the second metabolite (peak 4) has two silylated hydroxy groups (one at C-6 and the other at C-17) and

a methyl group at C-17. These data indicate the structure of 6β-hydroxymethandienone. However, the same typical fragments concerning the substitution at C-6 and C-17 have been found for peak 6, which is excreted only in small amounts[27]. If we assume that on efficient non-polar columns compounds with a 17α-methyl-17β-hydroxy configuration are generally eluted after their corresponding 17-epimers, which has been proved for methandienone and 17-epi-methandienone, peak 4 could be identified as 6β-hydroxy-17-epi-methandienone (III) and peak 6 (see Fig. 2) as 6β-hydroxymethandienone.

Consistent with these assignments is the fact that no measurable amounts of unchanged methandienone could be detected in any urine fraction. This means that the epimerization is the initial and predominant step of the metabolic process and that the reaction rate is reasonably fast. The mechanism of this interesting biochemical pathway is still unknown, however it seems to be the reason for the fact that even the major part of the 6β-hydroxy metabolite is excreted as the 17-epimer.

In general, the total carbon skeleton of methandienone remains unchanged during the metabolic process[28]. This is in agreement with the results obtained after the administration of other $\Delta^{1,4}$-3-keto-steroids, such as prednisone and prednisolone[29].

Owing to the stability of the dienone group in the ring A, the normally occurring reduction of the C-4 double bond and the 3-keto group seems to be suppressed[29]. It is considered that this resistence to reduction is responsible for the unusual formation of appreciable amounts of 6β-hydroxy derivatives.

As to the possibility of conjugation of the metabolites investigated, these compounds were all detected in the "free" fraction of the urine samples. After enzymatic hydrolysis with β-glucuronidase[30], the examination of the "conjugated" fraction showed no measurable amounts of any of these metabolites[15]; therefore these fractions were not studied further.

First approaches to the quantitation of the metabolic process indicate that only 5% of the applied dose is recovered as the two major metabolites identified. Hence the fate of the remaining 95% is still unknown and requires further investigation. The rate of excretion of both metabolites reaches the maximum between 5 and 10 h after application. About 3 days after administration they are no longer traceable, which is in agreement with observations on other synthetic steroids.

CONCLUSIONS

The results presented show that on the basis of well established GC techniques, either with packed columns or with glass capillaries, the administration of methandienone can be detected with sufficient sensitivity. In combination with automated sampling and data-processing devices, the methods used offer a reliable and rapid screening procedure. They can be supplemented by independent analysis based on the previously reported HPLC methods.

The synthetic steroid drugs examined are not excreted unchanged in urine. Detection of their administration therefore depends on finding the metabolites in urine. Since the structure and the chromatographic behaviour of many of these metabolites are unknown, further research will be necessary, utilizing GLC and GC–MS as instrumental techniques.

For detailed studies of the metabolism of such compounds and for the elucidation of their structure, capillary column GC–MS in combination with computer-aided multiple-ion mass fragmentography appears to be the method of choice.

REFERENCES

1 K. P. Littmann, *Internist*, 14 (1973) 621.
2 J. R. Bierich, *Monatsschr. Kinderheilkd.*, 114 (1966) 444.
3 B. O'Mally, *Trans. N.Y. Acad. Sci.*, 31 (1969) 478.
4 V. Wynn, J. London and V. H. T. James, *J. Endocrinol.*, 25 (1962) 199.
5 K. S. Clarke, *J. Maine Med. Ass.*, 61 (1970) 55.
6 A. Mader, *Leistungssport*, 7 (1977) 136.
7 M. Donike, *Sportarzt Sportmed.*, 26 (1975) 1.
8 D. Freed, A. J. Banks and P. Langson, *Brit. Med. J.*, 3 (1972) 761.
9 H. L. Krueskemper, *Anabole Steroide*, Georg Thieme, Stuttgart, 1969.
10 H. Ippen, *Index Pharmacorum*, Georg Thieme, Stuttgart, 1970, p. 240.
11 H. J. Arndt, *Münch. Med. Wschr.*, 116 (1974) 1715.
12 B. H. Kilshaw, R. A. Harkness, B. M. Hobson and A. W. Smith, *Clin. Endocrinol.*, 4 (1975) 537.
13 T. Khosla, *Brit. Med. J.*, 3 (1975) 158.
14 E. L. Schäfer and R. Buchholz, *Fehler und Gefahren der Hormontherapie*, Georg Thieme, Stuttgart, 1974.
15 C. G. B. Frischkorn and H. E. Frischkorn, *J. Chromatogr.*, 151 (1978) 331.
16 H. W. Dürbeck, C. G. B. Frischkorn and H. E. Frischkorn, *Deut. Zh. Sportmed.*, 29 (1978) 97.
17 M. Donike, *Chromatographia*, 6 (1973) 190.
18 K. Grob and G. Grob, *Chromatographia*, 5 (1972) 1.
19 H. W. Dürbeck, I. Büker and W. Leymann, *Chromatographia*, 11 (1978) 295.
20 H. W. Dürbeck, I. Büker and W. Leymann, *Chromatographia*, (1978) submitted for publication.
21 T. Féher and E. H. Sárfy, *J. Sports Med.*, 16 (1976) 165.
22 P. M. Adhikary and R. A. Harkness, *Anal. Chem.*, 41 (1969) 470.
23 P. M. Adhikary and R. A. Harkness, *Acta Endocrinol.*, 67 (1971) 721.
24 A. M. Lawson and C. J. W. Brooks, *Biochem. J.*, 123 (1971) 25p.
25 E. L. Rongone and A. Segaloff, *Steroids*, 1 (1963) 179.
26 B. S. MacDonald, P. J. Sykes, P. M. Adhikary and R. A. Harkness, *Biochem. J.*, 122 (1971) 26p.
27 H. W. Dürbeck and I. Büker, in preparation.
28 K. Fotherby, S. Kamyab, P. Littleton and A. Klopper, *Biochem. J.*, 99 (1966) 14p.
29 F. Galetti and R. Gardi, *Steroids*, 18 (1971) 39.
30 C. G. B. Frischkorn and I. Ohst, *Z. Lebensm.-Unters.-Forsch.*, in press.