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# INVESTIGATIONS OF ANABOLIC DRUG ABUSE IN ATHLETICS AND CATTLE FEED

## II. SPECIFIC DETERMINATION OF METHANDIENONE (DIANABOL®) IN URINE IN NANOGRAM AMOUNTS*

C. G. B. FRISCHKORN and H. E. FRISCHKORN

*Institute of Chemistry, Institute for Applied Physical Chemistry, Nuclear Research Centre (KFA) Juelich (G.F.R.)*

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SUMMARY

A high-performance liquid chromatographic method for the determination of the free excreted anabolic drug methandienone in pharmacokinetic studies is described. After extraction of the free steroids from urine, separation on reversed-phase columns leads to quantitative determination of this drug down to 5 ng, and to qualitative detection of less than 1 ng amounts. Because the anabolic drugs and their metabolites are eluted later than the other normally excreted constituents this method is also useful for the routine surveillance of anabolic drug abuse in the sports in general.

## INTRODUCTION

Steroids with anabolic effects are normally used as therapeutic drugs to improve the nitrogen retention in humans suffering from protein-consuming diseases[2–4]. But as shown at the Olympic Games at Montreal 1976, and in the public discussions before and after, they are widely abused in athletics[5]. The improved nitrogen retention results in additional build-up of muscles under the conditions of modern power-training. Anabolic drugs are taken as typical training dope over long periods in high doses. This chronic abuse involves the risk of irreversible changes in hormone metabolism, changes in mineral metabolism, and physical damage[4,6]. Psychological effects such as drug addiction phenomena are sometimes reported. To prevent these harmful effects on athletes, and for ethical reasons, the International Olympic Committee has blacklisted all anabolic drugs[7].

The anabolic drugs are also widely used to fatten cattle, despite prohibition[8], but the amount of contaminated meat on the market is unknown. Because no fast and sensitive method for the routine determination of anabolic drugs in body fluids has

* For Part I, see ref. 1.

hitherto been available it has been impossible to control adequately the compliance with prohibition both in sports and in cattle and poultry feeding.

Many methods for the analysis of urinary steroids and steroidal drugs have been discussed[3,9,10], but they generally lack specificity or are too time-consuming. We have therefore developed an efficient method during our pharmacokinetic and metabolic studies on anabolic drugs. It is also useful for the routine analysis of anabolic drugs and their metabolites in the presence of the natural steroids excreted in the urine. The method permits the qualitative and quantitative changes in steroid excretion patterns to be followed in the urine during and after the intake of anabolic substances.

The separation and analysis of steroid hormones by high-performance liquid chromatography (HPLC) has been reported elsewhere[11–14]. It has been applied mainly to synthetic mixtures of pure steroids in attempts to develop basic concepts in the field of HPLC but not much has been achieved hitherto in the application of this method to the analysis of natural steroids in biological fluids, such as urine or blood plasma.

This paper reports the simultaneous determination of methandienone and its possible metabolites: methyltestosterone, isomethyltestosterone and 17-methyldihydrotestosterone (5$\alpha$) in the presence of natural urinary steroids. The HPLC method described here is sensitive, reproducible, and uncomplicated. It can be applied to the determination of most of the anabolic drugs used in sports and in animal feeding.

## INSTRUMENTATION AND METHODOLOGY

A Spectraphysics Liquid Chromatograph 3500 B (Spectraphysics, Darmstadt, G.F.R.) was used, equipped with a gradient programmer, a constant flow-rate regulator, and the UV-Detector 230 (cell, 12 $\mu$l; wavelength 254 nm). The samples were introduced by a sample loop (10 $\mu$l).

### *Columns*

A 300 × 3 mm I.D. stainless steel column (Latek, Heidelberg, G.F.R.) was used, packed by the balanced-slurry method with either Nucleosil CN, 10 $\mu$m (CN-Column) or Nucleosil $Cj_8$, 10–13 $\mu$m ($C_{18}$-Column). Both columns have now been used for more than 1000 separations without loss of efficiency. The ready-for-use columns now available are nearly of the same quality, obviating the time-consuming and somewhat difficult packing procedure. Useful columns are: $\mu$-Bondapack-CN (Waters, Königstein, Taunus, G.F.R.), 7.5 $\mu$m, 300 × 3 mm I.D.; and Hibar-RP-8 (E. Merck, Darmstadt, G.F.R.), 7 $\mu$m, 250 × 2.5 mm I.D.

### *Mobile phases*

These were degassed in an ultrasonic bath shortly before use and if necessary, also during operation. The two used were: (i) CN-Column, linear gradient: water–methanol (8:2), containing from 0 to 60% water–methanol (2:8) within 10 min; flow-rate, 1.0 ml/min at *ca.* 1600 p.s.i.; (ii) $C_{18}$-Column, slightly concave gradient: water–methanol (6:4) from 10 to 50% water–methanol (1:9) within 5 min; flow-rate, 0.8 ml/min at *ca.* 2400 p.s.i.

*Materials*

All solvents were "Uvasol" grade (E. Merck); the steroids, obtained from Steraloids (Wilton, N.H., U.S.A.) were chromatographically pure [two-dimensional thin-layer chromatography (TLC) on silica gel plates, 20 × 20 cm (E. Merck). First dimension: triple development with diisopropyl ether; second dimension: chloroform–cyclohexane–ethyl acetate (75:10:15); detection either under UV light (254 nm) or by spraying the plates with 50% sulphuric acid in methanol then warming them during 10 min to 110°].

*Extraction method*

The following method is used for the extraction of the free and neutral steroids from urine together with methandienone.

Urine (25 ml) is brought to pH 5.2 with acetic acid and any sediments filtered or centrifuged. The sample is then extracted with 4 × 20 ml of methylene chloride. If an emulsion forms, it is centrifuged and the lower phase collected. The combined methylene chloride extracts are washed successively with 2 × 10 ml of 0.1 *M* sodium hydroxide, 10 ml of 0.02 *M* acetic acid, and 10 ml of distilled water, dried with anhydrous sodium sulphate, and evaporated to dryness at 40°. The residue is taken up in 2.5 ml of methanol and aliquots of 10 $\mu$l are injected into the HPLC sample loop.

*Identification*

Identification of the excreted methandienone is based on comparison of the retention time in HPLC with the authentic substance and on TLC of the product isolated from the appropriate peak. For TLC, the combined peak-fraction from 10 injections is saturated with sodium chloride and extracted with methylene chloride. After drying and evaporation, the residue is taken up in methylene chloride.

## RESULTS

The extraction and chromatographic separation of the free steroids, both presented for the special case of the determination of methandienone, afford the possibility of a sensitive and fast method for detecting and determining anabolic drugs and their metabolites in routine work and in pharmacological investigations.

With a sensitivity of 0.005 a.u.f.s., 5 ng of the $\Delta^{1,4}$-3-ketosteroid methandienone can be determined quantitatively on the $C_{18}$-Column. Qualitative identification of methandienone and other $\Delta^4$-3-ketosteroids is possible down to 1 ng using an UV-detector at a wavelength of 254 nm. The $C_{18}$-Column loses sensitivity during continuous use after about 50 determinations. For the recovery of the original sensitivity it must be washed free of adsorbed impurities successively with propan-2-ol, chloroform and methanol. Quantitative results can be obtained by comparison of either the peak height or the peak area of methandienone separated from urine with a methandienone standard solution, injected after each sample.

The mean standard deviation obtained from five injections each of three different urine extracts with different methandienone contents was 0.07, and the mean day-to-day coefficient of variation 8.3%; both indicate good reproducibility and precision, considering the difficulties of steroid analysis in biological material due to thermal and chemical lability.

Figs. 1 and 2 show typical patterns of the free neutral steroids excreted in the urine of a healthy athlete, before and after treatment with a single dose (10 mg) of methandienone. Whereas methandienone is cleanly separated from the other urine constituents on the $C_{18}$-Column (Fig. 1b, Peak B), interference with other substances occurs on the CN-Column (Fig. 2b, Peak B). For calculating the recovery of free methandienone in the extraction and HPLC, amounts from 1 to 10 $\mu$g (final quantity for the injection 4–40 ng per 10 $\mu$l) were added to 25 ml of a blank urine sample and the peak height of the recovered methandienone was compared with that of a standard solution (10 ng methandienone per 10 $\mu$l methanol). An average recovery of 91% was obtained.

For Peak B (Fig. 1b), retention time in HPLC and spot colour and position in TLC were in full agreement with authentic methandienone, whereas Peak B separated on the CN-Column contained, besides methandienone, an unknown substance, which is identical with that of Peak D in Fig. 1b.

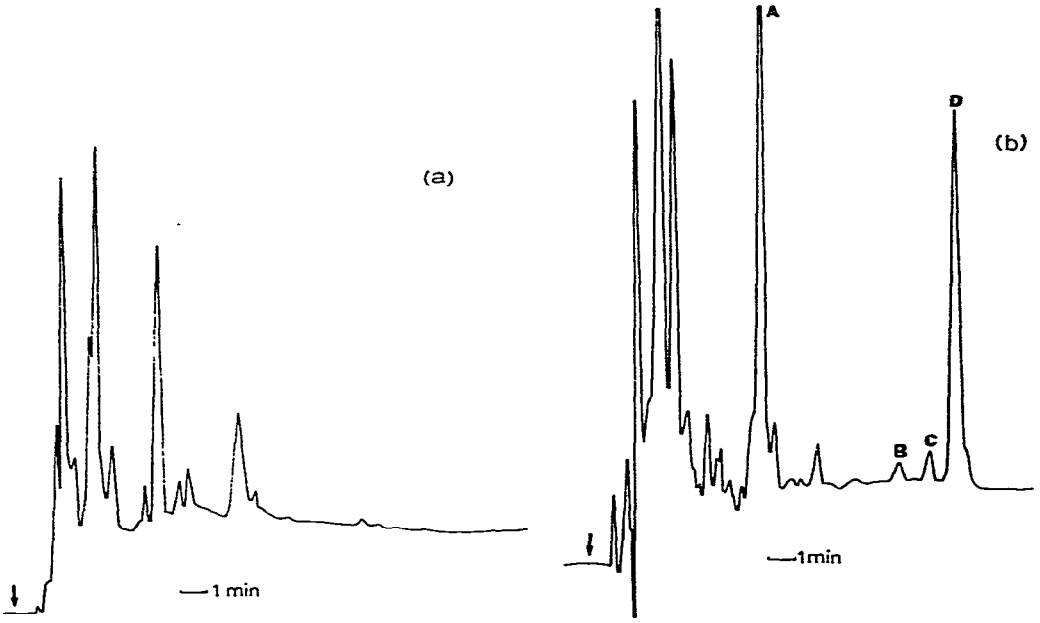


Fig. 1. (a) Neutral free urinary steroids of a healthy athlete. (b) After a single dose (10 mg) of methandienone. A, probably cortisone; B, methandienone; C and D, unknown metabolites of methandienone. Conditions: Nucleosil $C_{18}$, 10–13 $\mu$m, 3 × 300 mm I.D.; 0.8 ml/min (*ca.* 2400); water–methanol (6:4), slightly concave gradient from 10% water–methanol (1:9) within 5 min; UV 254 nm, 0.02 a.u.f.s.

The method, if adapted, can be used for the determination of methandienone and applied for screening tests of other anabolic drugs, as shown in Fig. 3.

## DISCUSSION

Usually, steroids are predominantly excreted in urine as their glucuronide or sulphate conjugates and only in low proportion as free steroids. In contrast to this, the

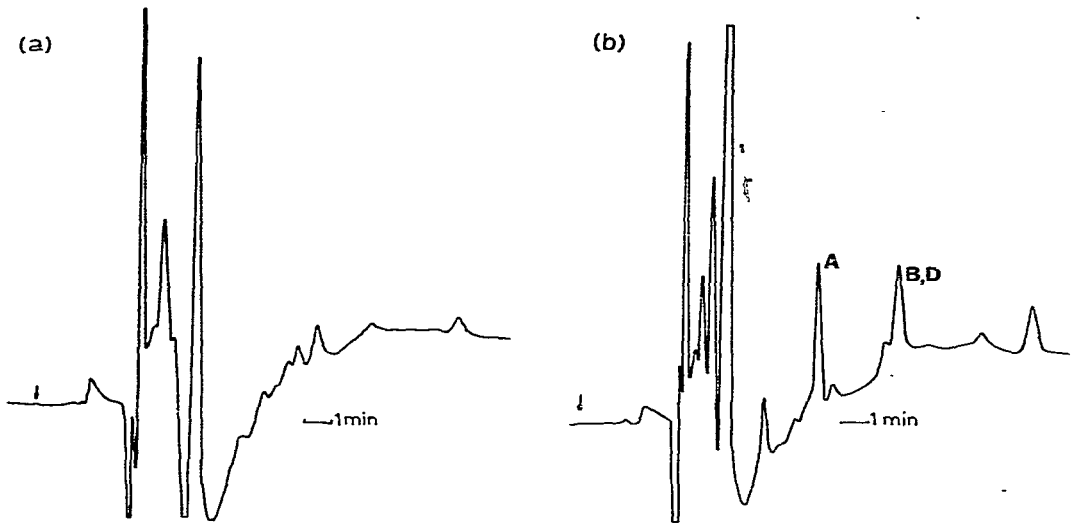


Fig. 2. (a) Neutral free urinary steroids of a healthy athlete. (b) After a single dose (10 mg) of methandienone. A, probably cortisone; B, methandienone; D, unknown metabolite of methandienones. Conditions: Nucleosil CN, 10 $\mu$m, 3 × 300 mm I.D.; 1.0 ml/min (*ca.* 1600); water–methanol (8:2) linear gradient from 0 to 60% water–methanol (2:8) within 10 min; UV 254 nm, 0.02 a.u.f.s.

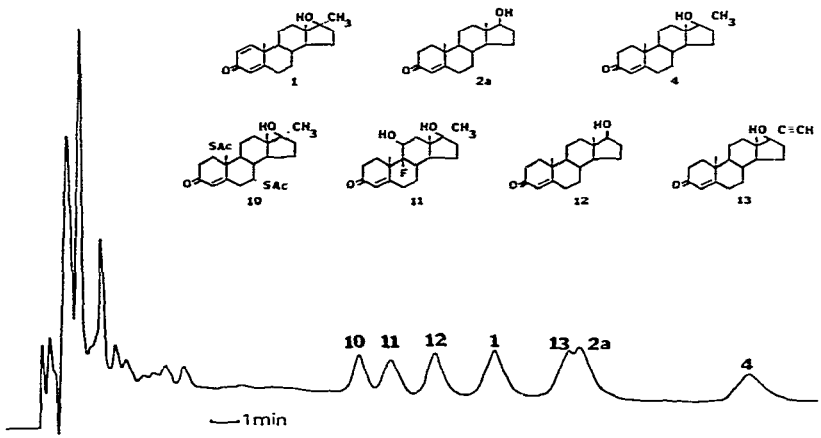


Fig. 3. Anabolic drugs: artificial mixture added to an extract of urinary steroids. Conditions as in Fig. 1a, except slightly concave gradient water–methanol (6:4) from 0 to 40% water–methanol (1:9) within 10 min. 1, Methandienone (Ciba); 2a, testosterone; 4, methyltestosterone; 10, Emdabol (Merck); 11, Ultandren (Ciba): 12, nortestosterone (Schering); 13, ethisterone (Progestin P, Organon).

anabolic steroids are present in human urine predominantly in unconjugated form, because the 17-hydroxy group is sterically hindered and difficult to esterify. Thus, as demonstrated in Fig. 1b, and also in Fig. 3, anabolics can be detected after a simple extraction step that isolates the anabolic drug and its metabolites together with free and neutral urinary steroids.

However, the anabolics as well as the other steroids should be excreted also

and possibly in larger quantities in the conjugated form than free. For more extensive metabolic investigations and more information about pharmacokinetic properties, hydrolysis is recommended. Hydrolysis can be performed by a new, time-saving method[1] that requires no more time for the determination of the total steroid excretion than for the simple extraction method used here. Chromatograms obtained from the free steroid fraction are not very complicated. They show most of the characteristic changes in the excretion pattern seen in the more complicated ones obtained from the total (free and conjugated) excreted steroids. Thus, for routine work it is easier to use the free steroid fraction.

HPLC produces a fast separation of androgenic steroids. The chemically bonded reversed-phase column material is more stable and shows more reproducible results than the adsorbents used in other studies for the separation of steroids[14]. We examined two liquid stationary phases, both chemically bonded, a non-polar octadecyl silyl material ($C_{18}$-Column) and a more polar phase containing terminal nitrile groups (CN-Column). The latter is recommended by manufacturers[15,16] for separation of androgenic steroids. Both materials were tested for applicability to experiments on the pharmacokinetics and metabolism of methandienone, and the results are shown in Figs. 1, 2 and 4.

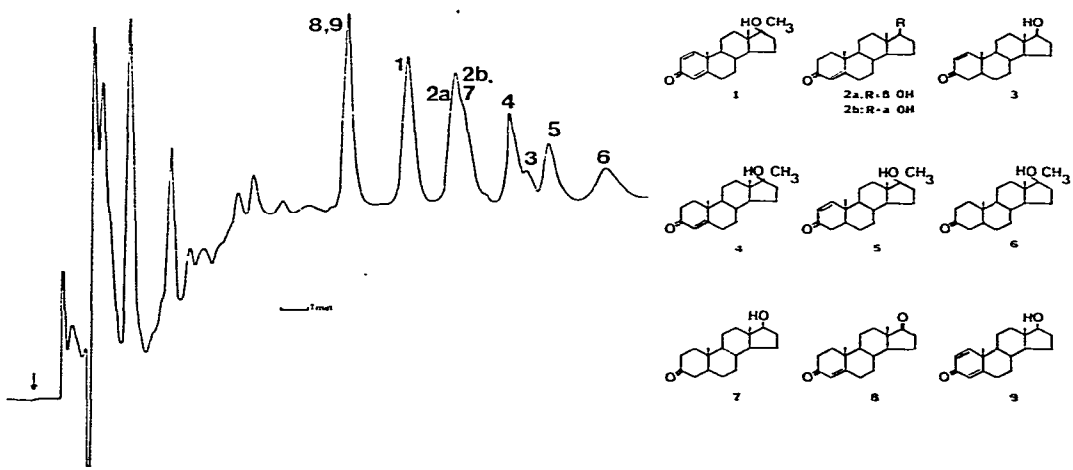


Fig. 4. Androgens: artificial mixture added to an extract of urinary steroids. Conditions as in Fig. 1a, except UV 280 nm, 0.01 a.u.f.s. Numbers refer to steroid structures in Table I.

Typical patterns of the free neutral steroids excreted in the urine of a healthy athlete before and after a single treatment (10 mg) with methandienone are shown in Figs. 1a, 1b, and Figs. 2a, 2b, respectively. As Fig. 1b shows, methandienone is excreted mainly in form of its metabolites, only small amounts emerging unchanged. As has recently been shown, total metabolism is also to be expected for all anabolic steroids related to methandienone. Thus, detection of this particular drug and other anabolic steroids depends mainly on finding metabolites in urine.

The different separations produced by the two columns are also demonstrated in Table I, which contains the net retention times, $t'$, of some androgenic steroids of relative similar structure. On the $C_{18}$-Column, methandienone itself interferes with

neither natural steroids nor with some of its possible metabolites. Significant interference has been found only for testosterone (2a) with epitestosterone (2b) and dihydrotestosterone (7) and an unknown metabolite of methandienone (assigned Peak D in Fig. 1b). For satisfactory resolution and quantitative determination, the latter is separated sufficiently from the testosterones (2a, 2b, 3) by using a weaker elution gradient system. The other interferences shown in Fig. 4 are not important practically. The presence of both "isotestosterone" (3) and 17β-hydroxy-androsta-1,4-dien-3-one (9) in urine has not yet been reported. Apart from the insufficient separation of the testosterones, the $C_{18}$-Column is the column of choice for the separation of methandienone and its metabolites from natural steroids present in urine. It is to be preferred in every respect to the CN-Column, which is sometimes recommended for the separation of urinary steroids.

TABLE I

NET RETENTION TIMES $t'$ AND $k'$-VALUES OF SOME RELATED 17-HYDROXY-ANDROSTANES, SEPARATED ON DIFFERENT COLUMNS AND UNDER CONDITIONS LEADING TO SEPARATION FROM OTHER NEUTRAL SUBSTANCES IN URINE
Conditions: A, MeOH–$H_2O$ (6.5:3.5), flow-rate 1.0 ml/min (2500 p.s.i.); B, as given in Figs. 1 and 4; C, MeOH–$H_2O$ (2:8), flow-rate 2.4 ml/min (2750 p.s.i.); D, as given in Fig. 2.

| | *$C_{18}$-Column* | | | | *CN-Column* | | | |
|---|---|---|---|---|---|---|---|---|
| | *A* | | *B* | | *C* | | *D* | |
| | *t' (min)* | *k'* | *t' (min)* | *k'* | *t'* | *k'* | *t'* | *k'* |
| Methandienone (1) | 2.55 | 2.83 | 11.70 | 6.16 | 15.1 | 13.39 | 7.85 | 2.31 |
| Testosterone (2a) | 2.85 | 3.16 | 13.30 | 7.11 | 15.2 | 13.10 | 7.80 | 2.33 |
| Epitestosterone (2b) | | | 13.60 | 7.44 | 15.2 | 13.10 | 7.90 | 2.36 |
| "Isotestosterone (5α)" (3) | 3.40 | 3.78 | 14.00 | 7.37 | 19.0 | 15.83 | | |
| Methyltestosterone (4) | 3.60 | 4.00 | 15.40 | 8.11 | 19.0 | 17.27 | 8.40 | 2.51 |
| "Isomethyltestosterone (5α)" (5) | 4.30 | 4.77 | 16.75 | 9.31 | 24.3 | 20.17 | 9.55 | 2.85 |
| 17-Methyldihydrotesto-sterone (5α) (6)* | 4.95 | 5.50 | 18.95 | 9.97 | 28.1 | 23.50 | | |
| Dihydrotestosterone (5α) (7)* | 3.40 | 3.78 | 15.70 | 8.49 | 22.0 | 18.30 | | |
| Androstan-4-ene-3,17-dione (8) | 1.97 | 2.06 | 9.48 | 4.99 | 11.3 | 6.95 | | |
| 17β-Hydroxyandrosta-1,4-dien-3-one (9) | 1.95 | 2.05 | 9.45 | 4.97 | 11.3 | 6.95 | | |

* UV detector at 280 nm.

The specificity of the HPLC for methandienone was tested by TLC, as mentioned in the experimental part. TLC served also to identify the substances corresponding to the various peaks in HPLC. As expected from the retention times given in Table I, no other substance was found in the spot corresponding to the collected eluates of methandienone and its possible metabolites (indicated in Fig. 1b as A, C, D), separated on a $C_{18}$-Column from the neutral free steroids. The substance corresponding to Peak D, which in HPLC interferes with epitestosterone and testosterone, differs in TLC in every respect from both these steroids. On the basis of HPLC and TLC data, Peak A is probably due to cortisone, which has been reported to be excreted after stimulation by anabolics[17]. The substances corresponding to Peaks C and

D have not yet been identified as natural steroids by HPLC or TLC. They may be hydroxylated metabolites of methandienone.

Chromatographic interference of methandienone and its metabolites with drugs other than steroids has not been studied, as no drugs were taken by the subjects of this study. From an inspection of the chromatograms of urines obtained from humans without or with methandienone treatment, and a consideration of Table I and Fig. 4, it may be concluded that for treated humans the neutral part of the excreted steroids is eluted in two main groups on the $C_{18}$-Column. The 17-hydroxysteroids with the androstane or norandrostane skeleton emerge significantly later than the other neutral urinary constituents under our experimental conditions (Fig. 4). Because all known anabolic steroids belong to one of these two groups, treatment with anabolic drugs may be taken for granted when a peak appears in the chromatogram of the neutral part of urine after *ca.* 8 min ($k' < 5$). The natural steroids with a 17-hydroxy group that could interfere with one of the synthetic anabolic drugs are normally excreted in the free form in such small amounts (*e.g.*, testosterone, 1.1 $\mu$g per 24 h) that they are not detectable without further concentration. Thus, the method outlined for the detection of methandienone and its metabolites in urine is not restricted to this particular anabolic drug, but should be generally applicable to the analysis of other androgenic anabolics and their metabolites in urine, as work in progress has revealed recently.

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