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ANALYSIS OF ANABOLIC STEROIDS IN BODY FLUIDS BY CAPILLARY GAS CHROMATOGRAPHY WITH A TWO-CHANNEL DETECTION SYSTEM AND A COMPUTER

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SUMMARY

A method is described for analysis of multi-component mixtures of steroid metabolites in biological fluids by means of capillary gas chromatography with glass and fused-silica columns and simultaneous detection of methoxylamine–trimethylsilyl derivatives with universal flame-ionization and selective nitrogen alkali flame-ionization detectors. A data system was applied to the on-line treatment of the results. Computer programs were designed for precise calculation of Kováts retention indices from the known values for selected natural urinary steroids. The programs allow the selection of nitrogen-containing components, normalized chromatogram plotting for both detection channels and qualitative and quantitative analysis. Results are presented on the detection of metabolites of methandrostenolone, 17α-methyltestosterone, 19-nortestosterone and fluoxymesterone.

INTRODUCTION

Anabolic steroids (AS), which are normally used for the treatment of certain diseases, are also abused in athletics[1] for improving nitrogen retention and build-up of muscles. Because chronic abuse in high doses is harmful to athletes, AS are prohibited in sports[2].

The assay of AS in biological media is usually performed by radioimmunoassay (RIA)[3,4] and gas chromatography–mass spectrometry (GC–MS)[4,5–7]. Advantages of RIA are speed, high productivity and small sample size. Serious disadvantages are that multi-component analysis is impossible and the specificity is not as high as is often believed. In spite of being time consuming and expensive, GC–MS is generally the method of choice for anabolic doping control.

Advances in capillary GC have rapidly increased our knowledge of the metabolism of steroid hormones and their mode of operation in human body. Multi-component GC analysis of steroid metabolites in body fluids[8–11] has become routine in clinical use for characterizing both health and disease[11–18] and emotional stress[19,20], for studies of drug metabolism[5,6] and for human development studies[21–24].

In our opinion, steroid profiling by GC is suitable for the detection of foreign components, such as AS metabolites, in natural mixtures. Many papers in recent

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years have dealt with different aspects of steroid profiling by GC: the development of techniques for sample preparation and derivatization[12,13,25–30], the improvement of capillary column performance[13,31–35], computer automation of GC[43] and GC–MS[44] steroid analysis.

This study was devoted to improvements in the reliability of identification by GC steroid profiling by means of combined selective nitrogen alkali-flame ionization (N–P) and universal flame-ionization detection and the precise on-line calculation of Kováts retention indices with a data system. The latter allows fast treatment of the results under the conditions of automatic routine analysis for AS doping control and clinical use.

EXPERIMENTAL

Materials

Servachrom XAD-2 resin (Serva, Heidelberg, F.R.G.), particle size 100–200 μm, was purified and regenerated according to known procedures[14,36–38]. Enzyme for steroid conjugate hydrolysis, *Helix pomotia* juice, was isolated from the digestive gland of the snail and used after centrifugation at 5800 and 40,000 *g*. Methoxylammonium chloride (Serva) was dissolved in pyridine (silylation grade) (Pierce, Rockford, IL, U.S.A.) to produce a 4% solution. N-Methyl-N-trimethylsilyltrifluoroacetamide (MSTFA)–N-trimethylsilylimidazole (TSIM)–trimethylchlorosilane (TMCS) (Serva) (100:2:5, v/v/v) was used for silylation[28]. A ready-to-use silylation mixture, N,O-bistrimethylsilylacetamide (BSA)–TSIM–TMCS (3:3:2, v/v/v) was purchased from Pierce. Solvents (ethanol, diethyl ether, dichloromethane, chloroform and benzene) were redistilled before use.

Urine samples were obtained from normal subjects to whom anabolic steroid drugs had been administered in therapeutic doses: methandrostenolone (17α-methyl-17β-hydroxy-1,4-androstadien-3-one) (Akrichin, Moscow, U.S.S.R.), retabolil (19-nortestosterone propionate) (Gedeon Richter, Hungary), 17α-methyltestosterone (Moschim Preparaty, Moscow, U.S.S.R.) and fluoxymesterone (9α-fluoro-11β-hydroxy-17α-methyltestosterone) (CIBA, F.R.G.). Urine samples were collected in glass bottles and stored at 5°C.

Sample preparation

The neutral steroids were isolated from urine according to a modified combination of widely used procedures. A 10-ml aliquot of urine was passed through a 6-mm I.D. glass column, packed with XAD-2 resin to a height of 3.5 cm and the loaded column was washed with 20 ml of distilled water. Free and conjugated steroids were eluted with 3 ml of ethanol, the ethanol was evaporated under vacuum in a vortex evaporator and 1 ml of sodium acetate buffer (pH 4.7) was added to the dry residue. Free steroids were extracted with diethyl ether (2 × 3 ml), which was evaporated to dryness. Ether was removed from the buffer solution of conjugated steroids under vacuum and 100 μl of *Helix pomatia* juice were added for hydrolysis either at 37°C overnight (18 h) or at 55°C for 2 h. After being liberated, the steroids were isolated by extraction with diethyl ether (2 × 3 ml) of the solution adjusted to pH 9 with potassium carbonate. The ether was evaporated before derivatization.

Formation of derivatives

To the dry residue of free or liberated steroids 50 μl of a 4% solution of O-methoxyammonium chloride in dry pyridine were added and the mixture was heated at 70°C for 15 min. Pyridine was removed under vacuum at 70°C and 50 μl of silylation mixture (MSTFA–TMCS–TSIM or BSA–TSIM–TMCS) were added. Complete silylation took 18 h at 70°C, but for anabolic steroid monitoring this time was reduced to 30 min. For major routine analysis incomplete silylation of some natural steroids (with C_{17},C_{20}-hydroxy groups) is not essential.

Before GC analysis, the excess of derivatization reagent was removed by adding either chloroform or dichloromethane (0.5 ml) to the samples and washing the organic layer with 0.1 *N* sulphuric acid (0.5 ml) and then twice with distilled water (0.5 ml), followed by evaporation to dryness.

Gas chromatography

Column. Fused-silica capillary columns were used, either 25 m × 0.21 mm I.D. (Hewlett-Packard) or 25 m × 0.32 mm I.D. (Chrompack, Middelburg, The Netherlands), 25 m × 0.23 mm I.D. (Chrompack). A Pyrex glass capillary column (35 m × 0.41 mm I.D.) was prepared according to the method described by Rutten and Luyten[32]. The glass column was deactivated with benzyltriphenylphosphonium chloride (BTPPC) (Aldrich) and then coated with a solution of 0.4% (v/v) SE-30 (Merck) in hexane by the static procedure[39].

Instrumentation. GC was performed on a Hewlett-Packard 5730A chromatograph equipped with a split–splitless capillary column injection port or a moving-needle injection system[40]. A flame-ionization and a nitrogen-phosphorus detector, housed in one detector oven, were fitted to the capillary column outlet via a splitter with make-up gas (helium, flow-rate 10–15 ml/min). The splitter was made of shrinkable PTFE tubing (for fused-silica columns of 0.32 mm I.D.; Chrompack). For nitrogen-phosphorus detection (NPD) helium–hydrogen (10:1) was used as the combustion gas at a flow-rate of 40 ml/min. The conditions of analysis were as follows: injection port and detector oven temperature, 300°C; column temperature, either isothermal at 250°C or programmed from 220°C (2°C/min delay) to 270°C at 2°C/min; inlet column pressure, 0.5–1.5 kg/cm^2, depending on the inner diameter of the column; average splitting ratio, 50:1 with the split-mode injection.

Data system. Calculation of the retention indices, quantification and chromatogram plotting were performed by means of a Hewlett-Packard 3354 B/C data system, consisting of an HP 1000 21-MX E Series minicomputer, HP 7906 disc drive and HP 18652 A analogue-to-digital converters, coupled with HP 5706 A electrometers of the Model 5730A gas chromatographs.

RESULTS AND DISCUSSION

Sample preparation

The procedures for isolation and derivatization of urinary steroids were selected with the aim of obtaining the fastest sample preparation compatible with acceptable efficiency and recovery. Considerable time saving is achieved by the fast procedure for enzyme hydrolysis at 55°C (2 h compared with 18 h at 37°C).

The reasons for selecting the methoxym–trimethylsilyl (MO–TMS) de-

rivatives are their excellent GC properties, the protection of the C-17 side-chain of corticosteroids from thermal breakdown during silylation and the introduction of a nitrogen heteroatom into the steroid molecule, which allows selective and sensitive detection. The reduced time of derivatization that we used does not affect complete conversion of steroids, except for components with a *tert.*-17α-OH group adjacent to a C_{20}-OH group (pregnanetriol, α-cortol). The anabolic steroids studied and their major metabolites produce persilylated products under these conditions.

After derivatization, the samples are usually clean enough for analysis with flame-ionization detection (FID). However application of NPD for the analysis of these samples is difficult owing to a large solvent peak, covering a considerable part of the chromatogram and resulting in reduced sensitivity and detector damage. Simple clean-up procedures eliminate this problem completely. The application of suitable solvents for introduction of samples into the gas chromatograph is also essential. It is necessary to avoid halogen-containing solvents which reduce the NPD performance rapidly. For this reason, we used benzene with a small amount of acetonitrile (0.02%, w/w). The latter was added to synchronize the solvent response for the two detector channels.

Gas chromatography

A simple PTFE splitter at the outlet of the capillary column allows the simultaneous use of two detection channels. It takes only a few minutes to make such a splitter, which is as suitable as those described elsewhere[41,42]. We were able to observe simultaneous registration of peaks by two detectors without a decrease in separation performance when using it at temperatures up to 280°C.

Retention indices of MO–TMS steroid derivatives calculated for the Hewlett-Packard fused-silica SP 2100 column and the laboratory-built glass capillary column, coated with SE-30, were very close [within 1–2 retention index units (i.u.)] to those published by Leunissen[14,15]. The chemically bonded methylsilicone fused-silica columns (Chrompack) were more polar with an increase by 2–5 i.u. The FID channel sensitivity was several nanograms for components injected by the split mode. NPD was 20–50 times more sensitive than FID for MO–TMS steroid derivatives. Application of the moving-needle injector in combination with NPD allowed reliable detection of picogram amounts.

Identification of steroids

Computer programs were developed for the calculation of Kováts retention indices (RI) for both detector channels. For isothermal operation logarithmic retention index values were used, but with temperature programming linear or arithmetic values were used.

Preliminary studies were conducted for the precise calculation of RI values. Urinary steroids in the form of MO–TMS derivatives were injected together with a series of C_{22}–C_{32} *n*-alkanes. Alkanes detected by FID only were used initially for computer calculation of steroid RI values for both channels. Mean RI values were calculated for the most typical individual steroids: androsterone (A), etiocholanolone (E), dehydroepiandrosterone (DHEA), 11β-hydroxyandrosterone (11β-HA), pregnanediol (PD), pregnanetriol (PT), tetrahydrocortisone (THE), α-tetrahydrocortisol (α-THF) and cortisol. Subsequently we no longer used alkanes for RI calculations, as the

steroid metabolites mentioned above could be recognized in the urine samples by the computer and used as reference compounds for both detection channels. The computer memory kept data on alkane retention as a base for the preliminary search for steroid reference compounds. First, the computer selected two references, androsterone and tetrahydrocortisone, the program allowing considerable deviation (40 i.u.) from standard values, and thus providing high flexibility with regard to possible fluctuations of column flow-rate, stationary phase bleeding and column shortening. Other reference steroids could be found, if their RI values corresponded to A and THE within ± 3 i.u. After the reference compounds had been found the computer updated the "hydrocarbon" retention times according to the actual retention of the steroid mixture and calculated RI values with an accuracy of 0.01 i.u. Thus the retention of "alkanes" was corrected in every analysis with the exception of samples where reference steroids could not be found.

The computer started measuring the adjusted retention times from the solvent front. Synchronized solvent response for the two detectors was achieved by using benzene as a sample solvent, spiked with acetonitrile. The RI values of steroid derivatives in natural urine samples were reproducible within 1 i.u. This was the steroid identification window, which allowed printing of their names. Summarizing the two detector channel data, the computer identified the nitrogen-containing compounds with asterisks. Optional chromatogram plotting was available in normalized or enlarged form at any chart speed. A computer program block diagram is presented in Fig. 1.

The free fractions of urinary steroids did not contain reference compounds. The RI values for such samples were determined with the "No Ref." option on the basis of a hydrocarbon calibration graph, adjusted in the previous analysis of conjugated urinary steroids. The accuracy of RI determinations was, of course, lower for free steroids ($\pm$ 3 i.u.).

Fig. 2 shows a computer report on the analysis of urine steroid sample of a 23-year-old healthy man. The report contains analytical conditions, retention times of the separated steroids, their amounts, retention indices, data on the presence of nitrogen and the names of the compounds. Normalized chromatograms in one time scale for the two detectors are convenient for comparison and selection of nitrogen-containing substances. Pregnanediol, pregnanetriol (tri- and di-TMS derivatives), cortolones, cortols and some others are detected by FID only. Fig. 2 shows only major steroids (areas below 200 are rejected). The number of chromatographic peaks calculated can be expanded to 160 without repeating the analysis by adjusting the area rejection option to lower values and calling for a new report. Also, any section of a plot can be enlarged on either the time or response axis.

Anabolic steroid detection

With a few exceptions, anabolic steroid drugs are converted to metabolites in the human body. Below, four examples of their detection in urine by means of the GC analytical system are discussed/described.

Fig. 3 shows the detection of a tetrahydro metabolite of 17α-methyltestosterone, 17α-methylandrostane-3,17β-diol (major isomer), contained in the conjugated fraction. The compound is detected by FID only with RI 2633.5. Note that the natural 5-androstene-3β,17β-diol (see Fig. 2) with RI 2631.4 does not interfere in this

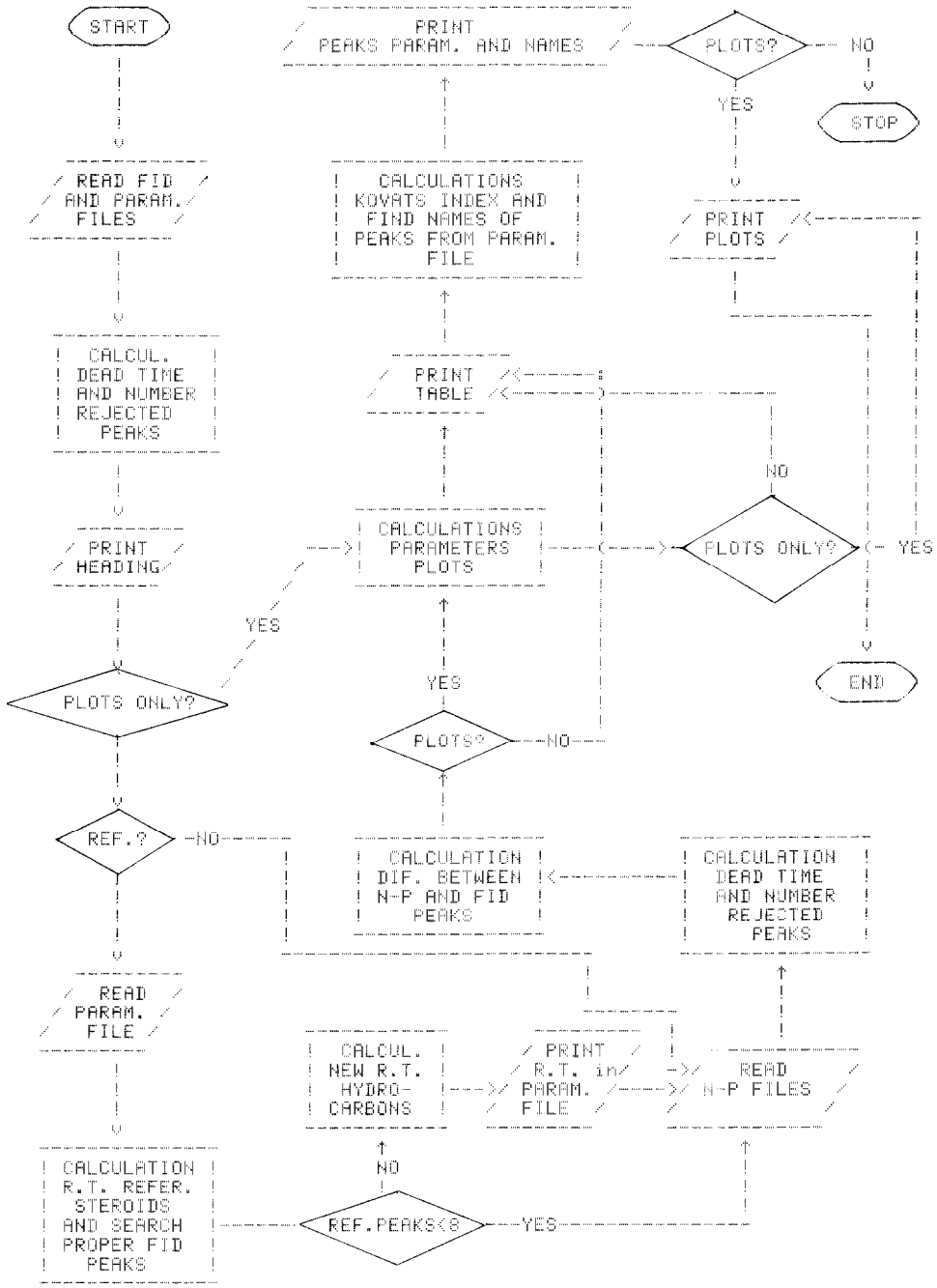


Fig. 1. Block diagram of computer program. N-P = Nitrogen-phosphorus detection; R.T. = retention time.

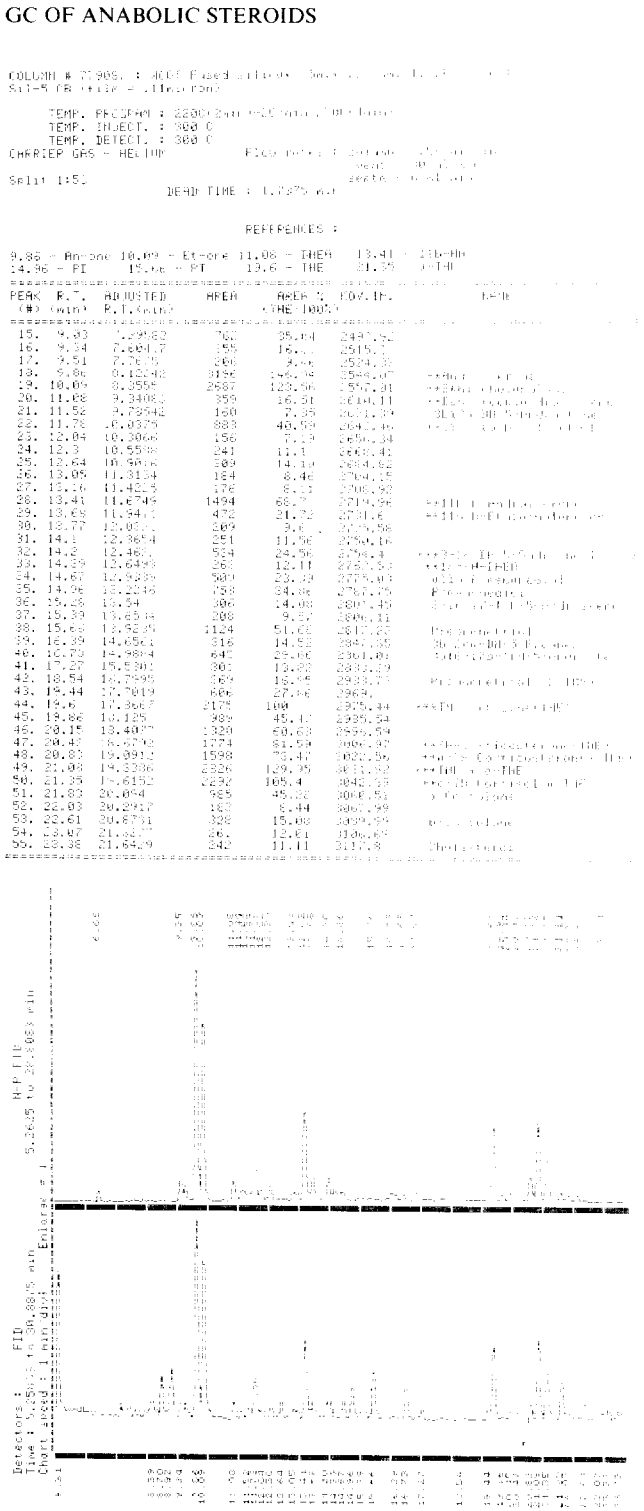
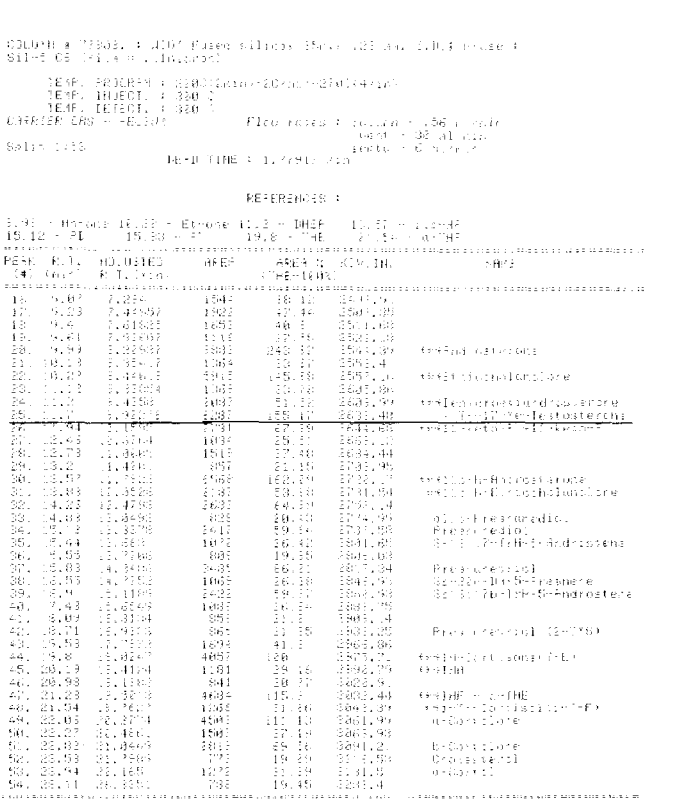


Fig. 2. Computer report on the urinary steroid profile of a healthy 23-year-old man.



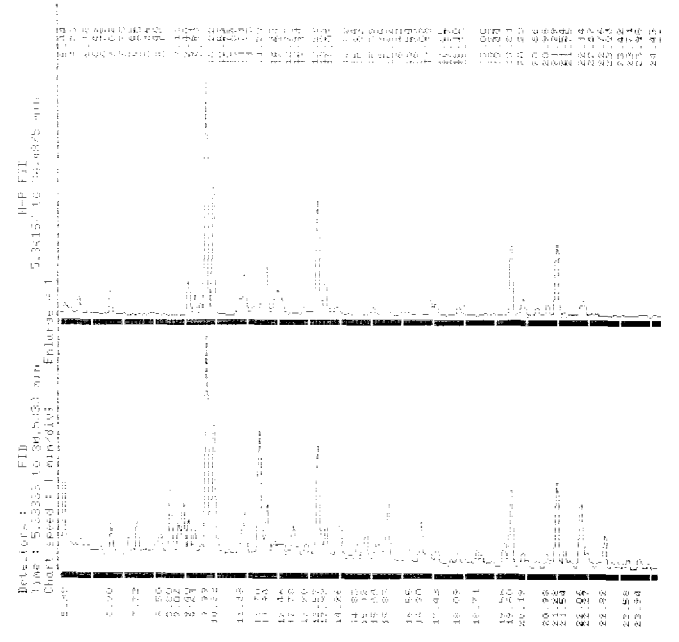


Fig. 3. Computer report on the urinary steroid profile of a 45-year-old man, 24 h after administration o 17α-methyltestosterone (30 mg).

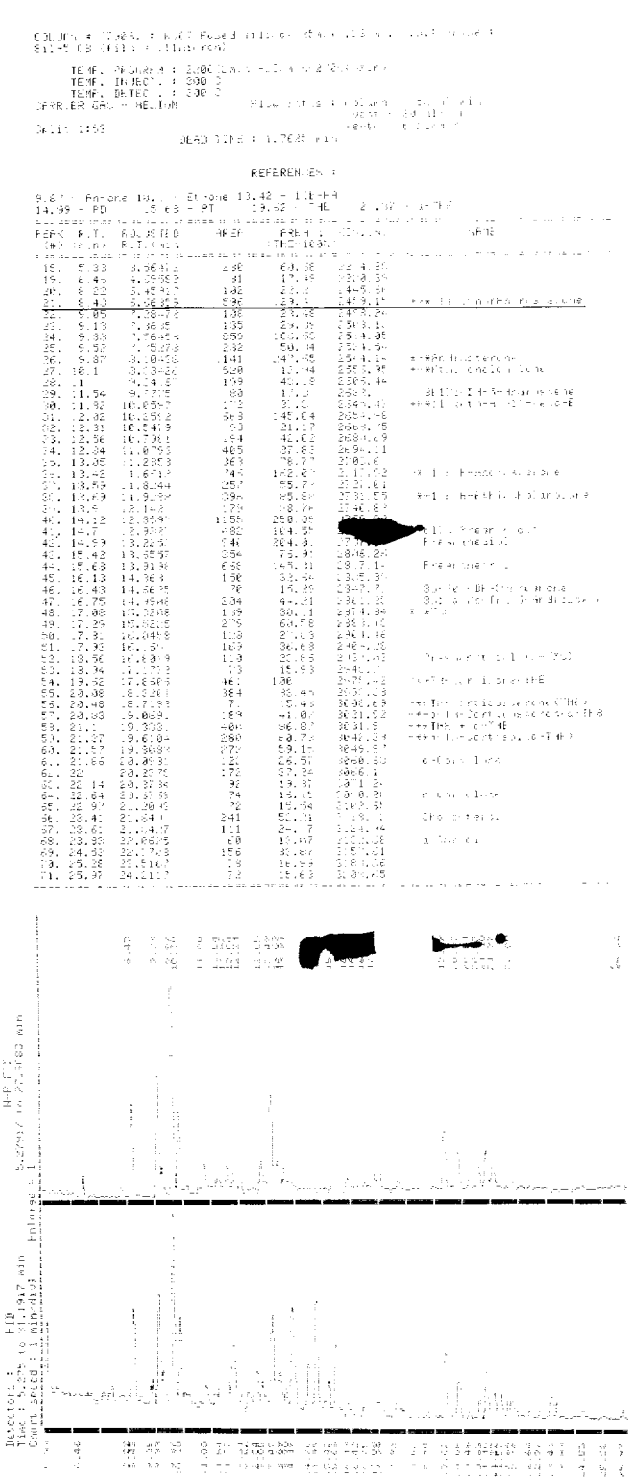


Fig. 4. Computer report on the urine steroid profile of a 19-norandrosterone-containing sample.

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COLUMN # 77908. : WCOT Fused silica, 25m. x .23 mm. I.D.; Phase :
Sil-5 CB (film = .11micron)

     TEMP. PROGRAM : 220C(2min)-2C/min-270C(4min)
     TEMP. INJECT. : 300 C
     TEMP. DETECT. : 300 C
CARRIER GAS - HELIUM              Flow rates : column - .56 ml/min
                                               vent - 30 ml/min
Split 1:53                                     septa - 6 ml/min
                    DEAD TIME : 1.75833 min
```

PEAK (#)	R.T. (min)	ADJUSTED R.T.(min)	AREA	AREA %	KOV.IN.	NAME
7.	5.31	3.55040	1384	8.308	2211.82	
8.	6.35	4.589	530	3.184	2310.58	
9.	9.13	7.3737	617	3.702	2501.52	
10.	11.98	10.2181	514	3.084	2652.24	+ N-P(f = .89)
11.	12.08	10.3241	759	4.559	2657.33	+ N-P(f = .78)
12.	13.37	11.6156	745	4.471	2717.63	+ N-P(f = 1.56)
13.	14.05	12.2878	814	4.887	2746.91	+ N-P(f = 1.25)
14.	15.11	13.3506	509	3.053	2793.21	+ N-P(f = .89)
15.	15.42	13.6641	1204	7.229	2806.45	
16.	15.81	14.0495	1085	6.515	2822.22	+ N-P(f = 1.53)
17.	16.2	14.4425	4552	27.327	2838.3	+ N-P(f = 1.33)
18.	16.95	15.195	3945	23.679	2869.1	+ N-P(f = 1.27)

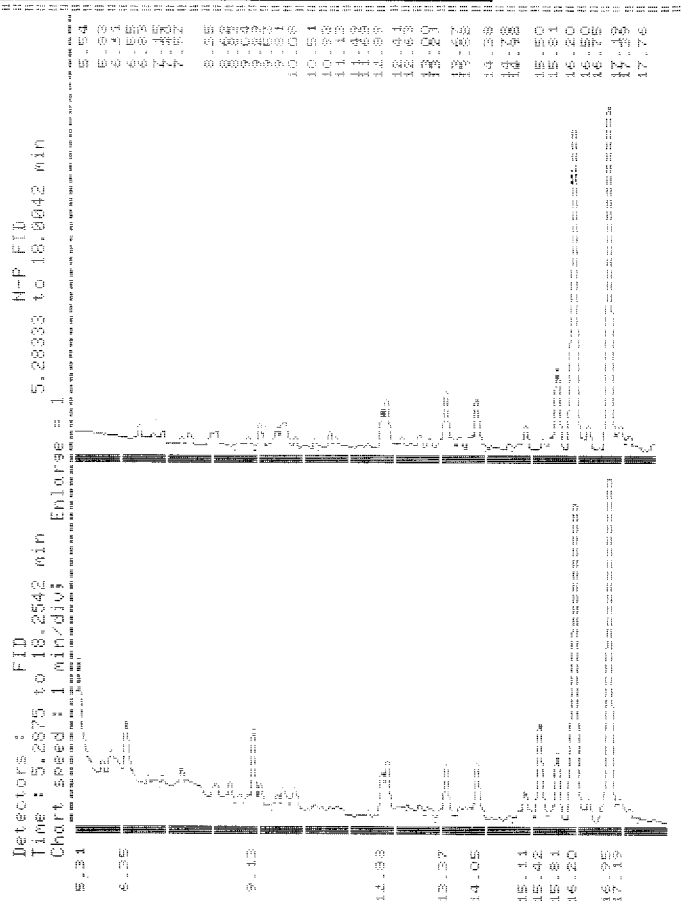

Fig. 5. Fragment of a computer report on a free steroid fraction profile of a male volunteer, 70 h after administration of 30 mg of methandrostenolone. RI 2652.2 and 2657.3: *E*/*Z* isomers of epimethandrostenolone derivatives. RI 2838.3 and 2869.1: 6β-hydroxyepimethandrostenolone and 6β-hydroxymethandrostenolone, respectively.

COLUMN # [illegible] : JCOT Fused silica; 25m.; .33 mm. I.D.; Phase : Sil-5 CB (film = .11micron)

TEMP. PROGRAM : 220C(2min)-2C/min-270C(4min)
TEMP. INJECT. : 300 C
TEMP. DETECT. : 300 C
CARRIER GAS - HELIUM   Flow rates : column - .56 ml/min
                                     vent - 30 ml/min
Split 1:53   septa - 6 ml/min
DEAD TIME : 1.7233 min

PEAK (#)	R.T. (min)	ADJUSTED R.T.(min)	AREA	AREA %	KOV.IN.	NAME
20.	9.11	7.37321	242	1.626	2501.49	+ N-P(f = .23)
21.	9.82	8.08518	491	3.294	2541.38	+ N-P(f = 1.82)
22.	10.05	8.3119	216	1.445	2554.85	+ N-P(f = 1.08)
23.	10.8	9.07083	332	2.225	2596.55	+ N-P(f = 2.04)
24.	11.68	9.948	320	2.144	2639.1	+ N-P(f = 1.44)
25.	11.85	10.1181	140	.941	2647.42	+ N-P(f = 2.72)
26.	12.18	10.4457	400	2.681	2663.18	+ N-P(f = 1.6)
27.	14.61	12.8778	177	1.184	2772.61	
28.	14.88	13.1486	1028	6.896	2784.19	+ N-P(f = .26)
29.	15.38	13.6512	661	4.431	2805.92	
30.	15.97	14.2315	226	1.514	2829.67	+ N-P(f = 4.13)
31.	16.58	14.8465	771	5.171	2854.85	
32.	16.82	15.091	532	3.57	2864.84	
33.	17.32	15.5951	354	2.371	2885.06	+ N-P(f = 3.49)
34.	17.50	15.7575	181	1.215	2893.75	+ N-P(f = 1.41)
35.	17.65	15.9254	612	4.106	2898.99	
36.	18.05	16.3152	216	1.452	2914.26	+ N-P(f = 3.13)
37.	18.54	16.8064	759	5.069	2933.44	+ N-P(f = 1.92)
38.	19.84	18.1079	639	4.289	2984.33	+ N-P(f = 1.5)
39.	20.22	18.4879	1750	11.716	2999.12	+ N-P(f = 1.45)
40.	20.54	18.8032	2635	17.67	3011.2	+ N-P(f = 1.55)
41.	20.72	18.9932	223	1.497	3018.35	
42.	20.91	19.1733	547	3.656	3025.36	+ N-P(f = 1.41)
43.	21.32	19.5864	894	5.999	3041.16	+ N-P(f = 1.33)
44.	21.54	19.8096	144	.966	3049.7	+ N-P(f = 1.03)
45.	21.94	20.2038	420	2.819	3064.78	+ N-P(f = 1.88)

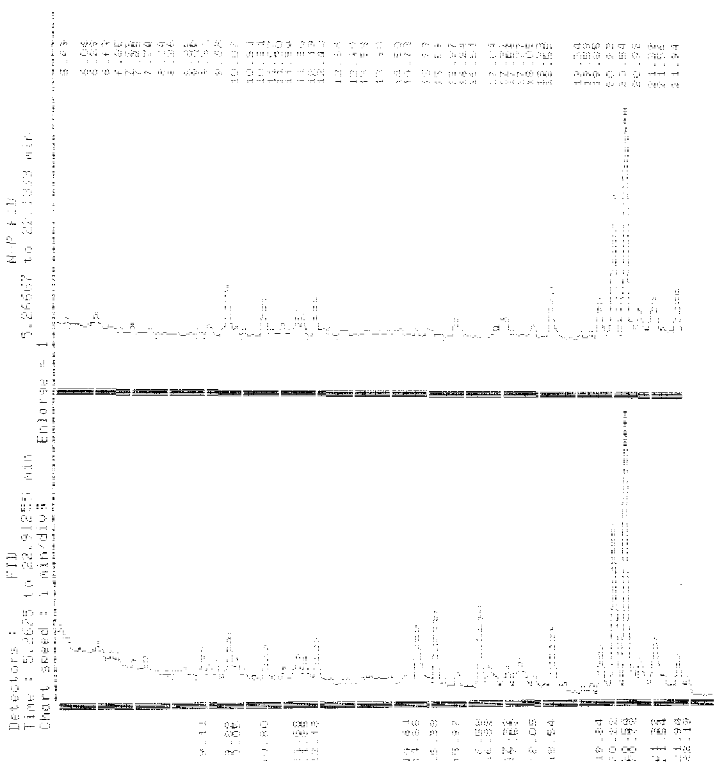

Fig. 6. Fragment of a computer report on a free steroid fraction profile of a male volunteer, 13 h after administration of 10 mg of fluoxymesterone. RI 2639.1 and 2663.1: dehydrofluoxymesterone. RI 2805.9: 9α-fluoro-17α-methyl-4-androstene-3β,6β,11β,17β-tetrol. RI 2829.7: 9α-fluoro-17α-methylandrostane-3β,6β,11β,17β-tetrol. RI 2854.9: 9α-fluoro-17α-methylandrostan-3β,6β,17β-triol-11-one. RI 2639.1 and 2663.2: 6β-hydroxyfluoxymesterone.

analysis, because in our experience its RI value never exceeds 2632. Thus, detection of a peak with RI 2633.5 on the FID channel only is a strong positive indication of a doping substance.

19-Norandrosterone, a metabolite of 19-nortestosterone, is detected on both channels (Fig. 4). Three asterisks indicate a high degree of correlation between the NPD/FID response factors for a standard compound and a serial sample. The reliability of the GC detection of 19-norandrosterone is greatly increased by application of NPD, as this section of the chromatogram often contains background components which interfere with FID.

These two examples of AS determination (with one major metabolite) should be considered as screening analyses for proper sample selection for GC–MS confirmation. This is necessary, as strict measures could be taken against persons guilty of AS abuse, but for most clinical applications GC–MS confirmation is not needed.

Metabolites of 17α-methyl-AS are excreted mostly as free steroids and not as conjugates. This is also true of methandrostenolone[6]. Both detector channels (see Fig. 5) detected four peaks, corresponding to the metabolites of this drug. The first two peaks with RI 2652 and 2657 were *E*/*Z* isomers of the epimethandrostenolone MO derivative and those with RI 2838 and 2869 belong to 6β-hydroxyepimethandrostenolone and 6β-hydroxymethandrostenolone, respectively. Fluoxymesterone metabolites, detected in the free fraction, are shown in Fig. 6. Some of them are detected by FID only: RI 2806, 9α-fluoro-17α-methyl-4-androstene-3β,6β,11β,17β-tetrol; RI 2829.7, 9α-fluoro-17α-methylandrostane-3β,6β,11β,17β-tetrol; and RI 2854.9, 9α-fluoro-17α-methylandrostan-3β,6β17β-triol-11-one. The others were determined on both channels: RI 2639.1 and 2663.2 are the *E*/*Z* isomers of dehydrofluoxymesterone and RI 2999.1 and 3011.2 correspond to 6β-hydroxyfluoxymesterone.

The last two examples provide sufficient data for unequivocal identification of AS, and GC–MS analysis is unnecessary.

CONCLUSION

Data handling in detailed steroid GC profiling and the detection of anabolic steroid metabolites under conditions of routine analysis can hardly be done manually. The GC–computer system described here facilitates studies of metabolism and responses of individuals to drug treatment as well as steroid profiling for problem solving in medicine.

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