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ANALYSIS OF PROFILES OF UNCONJUGATED STEROIDS IN RAT TESTICULAR TISSUE BY GAS CHROMATOGRAPHY–MASS SPECTROMETRY

S. H. G. ANDERSSON and J. SJÖVALL*
Department of Physiological Chemistry, Karolinska Institutet, Box 60 400, S-104 01 Stockholm, Sweden

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Summary—A gas chromatographic–mass spectrometric (GC–MS) method for analysis of unconjugated steroids in a rat testis is described. A combined solvent-solid extraction procedure, utilizing Lipidex 1000 and Sep-Pak C_{18}, gives a 25-fold purified extract. Steroids in this extract are fractionated by straight phase high-performance liquid chromatography (HPLC) on a LiChrosorb DIOL column in *n*-hexane–2-propanol, 92:8 (v/v). Four fractions are collected and the steroids are converted to *tert*-butyldimethylsilyl (TBDMS), 3-enol-TBDMS, and mixed TBDMS-trimethylsilyl (TMS) derivatives using TBDMS- and TMS-imidazole with sodium formate as catalyst under conditions suitable for the steroids present in the respective fractions. The derivatives are purified by reversed phase HPLC in 100% methanol and are analyzed by GC–MS, using selected ion monitoring of the major ions of high mass. For quantification, a mixture of known amounts of ten ^{14}C-labelled steroids, [^{3}H]estradiol and [$^2H_3$]estradiol are added to the testis homogenate. The mean concentrations (ng/g wet wt) of the twelve steroids determined were: 4-androstene-3,17-dione, 4.0; testosterone, 127; 17β-hydroxy-5α-androstan-3-one, 4.5; 5α-androstane-3α,17β-diol, 5.7; 5α-androstane-3β,17β-diol, 1.5; progesterone, 5.5; 17α-hydroxyprogesterone, 14.4; 3β-hydroxy-5-androsten-17-one, 0.07; 5-androstene-3β,17β-diol, 0.25; 3β-hydroxy-5-pregnen-20-one, 10.3; 3β,17β-dihydroxy-5-pregnen-20-one, 0.95; and estradiol, 0.025. Variations between animals were large whereas testes from the same animal in most cases had similar steroid concentrations.

INTRODUCTION

Acute and chronic intake of ethanol decreases the testicular production of testosterone in man and animals [1]. There may be a number of mechanisms behind this effect, and the relative importance of direct and indirect actions of ethanol on testosterone biosynthesis and metabolism *in vivo* is far from clear [1]. In order to investigate possible metabolic blocks or side reactions in testicular steroid biosynthesis, a method was needed for characterization and quantitative analysis of steroid patterns in the rat testis. Radioimmunoassay would not provide the flexibility required of an "unbiased" analysis, and would not permit metabolic studies using stable isotopes. Thus, gas chromatography–mass spectrometry (GC–MS) had to be used. This paper describes a method that is reasonably general for unconjugated steroids of the types expected as intermediates and metabolites in the pathway from cholesterol to testosterone. It is based on purification and derivatization methods described in previous papers [2, 3] with some modifications to improve sensitivity and reproducibility. Isotopically labelled internal standards are used and the method has been applied to the analysis of twelve steroids in the rat testis.

*To whom correspondence should be addressed.

EXPERIMENTAL

Solvent, reagents, chromatographic materials

Solvents were of reagent grade and were redistilled in a spinning band distillation apparatus. Trimethylsilylimidazole (TSIM) was distilled *in vacuo* prior to use and *tert*-butyldimethylsilylimidazole (TBDMSIM) was synthesized according to Blair and Phillipou [4]. Imidazole was recrystallized from toluene and dried *in vacuo* at 80°C, and *tert*-butyldimethylsilyl chloride was sublimated before use in the synthesis. Sodium formate was recrystallized from water. Lipidex 1000 and 5000 (Packard Instruments Co., Downers Grove, IL, U.S.A.) were washed in ethanol and aqueous ethanol and stored at 4°C in methanol. Sep-Pak C_{18} cartridges (Waters Assoc., Milford, MA, U.S.A.) were washed with 5 ml of methanol followed by 5 ml of water immediately before use. Radioactivity was determined in a Packard Trace 7140 radioactivity flow monitor (Packard Instruments Co., Downers Grove, IL, U.S.A.) connected to a high-performance liquid chromatography (HPLC) apparatus (LDC, Riviera Beach, FL, U.S.A.).

Steroids

Unlabelled steroids were from Steraloids Inc. (Wilton, NH, U.S.A.), Sigma Chemical Co. (St Louis, MO, U.S.A.) and Makor Chemicals Ltd

(Jerusalem, Israel). Steroids labelled with ^{14}C were from the Radiochemical Center (Amersham, U.K.). 5α-[4-^{14}C]Androstane-3α,17β-diol ([^{14}C]3αA) and 5α-[4-^{14}C]androstane-3β,17β-diol ([^{14}C]3βA) were synthesized from 17β-hydroxy-5α-[4-^{14}C]androstane-3-one ([^{14}C]DHT). About 2.5 μg of [^{14}C]DHT was stirred for 40 min with 5 mg $NaBH_4$ in 1 ml of methanol followed by addition of 200 μl 1 M HCl. The methanol was evaporated, 1 ml of water was added and the products were extracted with diethyl ether. The ether was removed, the sample was dissolved in 200 μl of 70% methanol and the 3α and 3β isomers were separated by HPLC. A 250 × 4 mm reversed phase column Spherisorb ODS, 7 μm (Jones Chromatography, Llanbradach, U.K.) was used with a pre-column (RP-18, 10 μm, 15 × 3.6 mm i.d., Brownlee Labs Inc., Santa Clara, CA, U.S.A.), and the mobile phase was 70% methanol at a flow rate of 1 ml/min. 5-[4-^{14}C]Androstene-3β,17β-diol ([^{14}C]A[5]) was synthesized from 3β-hydroxy-5-[4-^{14}C]androsten-17-one ([^{14}C]DHEA) in the same way using the same HPLC system for purification of the product. All radioactive steroids were checked for isotopic purity by HPLC. [11,11,12-^{2}H]estradiol ([^{2}H]E_2) was obtained by reduction of labelled estrone from a previous synthesis [5].

Animals

Sprague–Dawley rats weighing about 370–390 g were used. They were killed by cervical dislocation, the testes were immediately taken out and held in liquid nitrogen for 30–40 s and were then stored at −70°C until analyzed.

Analytical procedure

A flow scheme of the method is shown in Fig. 1.

Extraction and purification. The testis (1.3–1.5 g) was homogenized in 20 ml of *n*-hexane–2-propanol, 3:2 (v/v), using an Ultra Turrax TP 18/10 homogenizer (Jancke and Kunkel, IKA-Werk, Staufen, F.R.G.). To the homogenate was added a standard mixture (Table 1) of labelled steroids in 250 μl toluene–ethanol, 95:5 (v/v). The homogenate was shaken and ultrasonicated for 5 min after addition of the standard mixture and was then filtered into a 100 ml round-bottomed flask. The residue was washed 3 times with 3 ml of *n*-hexane–2-propanol. Lipidex 1000, 4 ml of gel equilibrated in *n*-hexane–2-propanol (3:2) over night, was added and solvents were evaporated *in vacuo* at 25°C on a Rotavapor (Büchi, Switzerland). The dried gel was transferred in 3 × 3 ml of water to a chromatography column (10 mm i.d., about 30 cm long with a sintered glass disc to hold the gel) having a washed Sep-Pak C_{18} cartridge attached to the effluent end [2]. Prior to the transfer of the gel, a few ml of water were injected through the Sep-Pak cartridge from below and into the glass column to remove air and avoid air bubbles in the gel. The water from the sample was allowed to

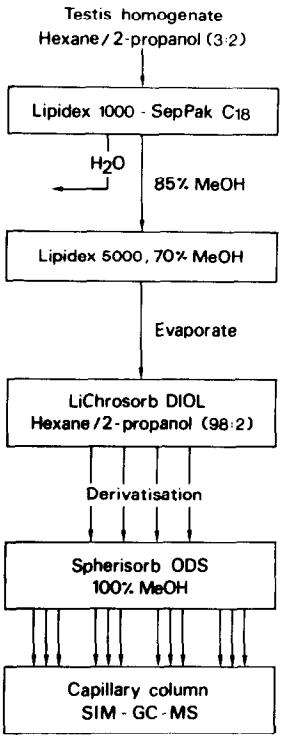

Fig. 1. Flow scheme of the analytical procedure.

Table 1. Mixture of steroids added as internal standards to the homogenized testis and their fractionation by straight phase HPLC

Steroid	Abbreviation	Amount added (ng)	Amount added (cpm)	Retention (vol. ml)	Collected in fraction*
3β-hydroxy-5-[4-^{14}C]pregnen-20-one	[^{14}C]P5	23.7	10910	5.4	II
4-[4-^{14}C]pregnene-3,20-dione	[^{14}C]P4	37.2	17250	4.8	I
17α-hydroxy-4-[^{14}C]pregnene-3,20-dione	[^{14}C]17P4	31.1	19370	9.4	III
3β-hydroxy-5-[4-^{14}C]androsten-17-one	[^{14}C]DHEA	1.41	590	6.6	II
4-[^{14}C]androstene-3,17-dione	[^{14}C]A4	11.0	4880	5.6	II
5-[4-^{14}C]androstene-3β,17β-diol	[^{14}C]A5	9.81	4070	9.2	III
17β-hydroxy-4-[4-^{14}C]androsten-3-one	[^{14}C]T	44.5	19400	8.1	II
17β-hydroxy-5α-[4-^{14}C]androstan-3-one	[^{14}C]DHT	5.41	2570	7.0	II
5α-[4-^{14}C]androstane-3β,17β-diol	[^{14}C]3βA	5.53	2330	9.0	III
5α-[4-^{14}C]androstane-3α,17β-diol	[^{14}C]3αA	5.37	2260	9.0	III
[11,11,12-$^{2}H_3$]estradiol	[^{2}H]E_2	0.033	—	n.d.†	IV
[1,2,6,7-^{3}H]estradiol	[^{3}H]E_2	—	56000	14.6	IV

*See text, analytical procedure.
†Not determined.

drain at a rate of about 0.5 ml/min, followed by a wash with 20 ml of water at the same rate. The steroids were then eluted with 15 ml of methanol–water, 85:15 (v/v), at a flow rate of about 0.5 ml/min. The eluate was diluted with 2 ml of water and passed through a column of Lipidex 5000 (80 × 4 mm) packed in methanol–water, 70:30 (v/v), followed by a wash with 5 ml of the latter solvent mixture at a flow rate of about 0.3 ml/min. The solvents were evaporated *in vacuo* and the residue was ultrasonicated and transferred in 3 × 1 ml *n*-hexane–2-propanol (3:2) to a test tube. The solvents were evaporated, 200 μl of *n*-hexane–2-propanol, 92:8 (v/v), was added and the mixture was ultrasonicated. This sample was injected into a 1 ml sample loop of an HPLC system consisting of a LiChrosorb DIOL column (10 μm, 250 × 4 mm, i.d., Merck Darmstadt, F.R.G.) fitted with a silica guard column (7 μm, 15 × 3.6 mm i.d., Brownlee Labs Inc.) and *n*-hexane–2-propanol, 92:8 (v/v), as mobile phase at a flow rate of 1 ml/min. Radioactivity in the effluent was determined with a radioactivity flow monitor. Four fractions (I–IV) were collected using the four major radioactivity peaks ([^{14}C]P^4, [^{14}C]T, [^{14}C]17P^4, [^{3}H]E$_2$, see Table 1) as markers to change fractions. The solvents were evaporated.

Derivatization

tert-Butyldimethylsilyl (TBDMS) and mixed TBDMS-trimethylsilyl (TMS) derivatives were formed in 1 ml micro reaction vessels with Teflon-lined screw-cap seals (Supelco Inc., Bellefonte, PA, U.S.A.). The vessels were treated with 6.5 M sodium hydroxide overnight and then washed thoroughly before use, in order to create a polar glass surface. Sodium formate, 1 mg in 100 μl of water, and sodium hydroxide, 1 μg in 100 μl of water, were added to the vessels. The water was evaporated under a stream of nitrogen followed by heating for 30 min at 120°C. With this procedure an even coating of sodium formate was obtained on the part of the vessel wall to be in contact with reagents and samples. The fractions were transferred to the reaction vessels in 3 × 0.4 ml *n*-hexane–2-propanol, 92:8 (v/v), and the solvents were evaporated under a stream of nitrogen. Twenty μl of TBDMSIM and 100 μl *n*-heptane were added, the vials were purged with nitrogen, closed and placed in a reaction block (Reacti-Therm™ Pierce Chemical Company, Rockford, IL, U.S.A.) at 100°C. To the vial containing 17α-hydroxyprogesterone (17P^4) and 17α-hydroxypregnenolone (17P^5) (fraction III) was added 20 μl of TSIM after 4 h, and heating was continued for another 2 h at the same temperature. The other vials were heated for 5 h. After cooling, the heptane was evaporated and 200 μl of methanol was added. Each sample was then injected into a 1 ml sample loop of an HPLC system consisting of a reversed-phase column (Sperisorb ODS 5μ, 250 × 4.6 mm i.d., Jones Chromatography, Llambradach, U.K.), and a pre-column (RP-18, 10 μm, 15 × 3.6 mm, i.d., Brownlee Labs Inc.). The mobile phase was 100% methanol at a flow rate of 1 ml/min. Radioactivity was measured continuously on the radioactivity flow monitor. Three fractions were collected. Fraction 1 (4.5–10.5 ml) contained mono-TBDMS and mixed mono-TBDMS-mono-TMS derivatives, fraction 2 (10.5–40 ml) contained di-TBDMS and mixed di-TBDMS-mono-TMS derivatives and fraction 3 (40–60 ml) contained tri-TBDMS derivatives and C$_{27}$ steroids with two TBDMS groups. In the analysis of the estradiol fraction, only the peak of [^{3}H]estradiol di-TBDMS ether was collected. The methanol was evaporated *in vacuo* and the derivatized samples were transferred to test tubes with 3 × 0.5 ml *n*-hexane.

Gas chromatography–mass spectrometry

Steroids were determined by GC–MS using selected ion monitoring. A VG 7070E double focusing mass spectrometer connected to a DANI 3800 gas chromatograph (VG Analytical, Manchester, U.K.) was used, with a fused silica column (25 m × 0.32 mm i.d.) coated with a 0.25 μm film of cross-linked methyl silicone (QUADREX Corp., New Haven, CT, U.S.A.) and fitted with an all-glass falling-needle injection system. The outlet end of the column extended into the ion source. The ionization energy was 22.5 eV and the trap current 200 μA. The samples were dissolved in 200 μl of hexane and 3–30 μl were injected with the oven temperature set to 280°C and the ion source temperature to 260°C. Selected ions were focused by switching the accelerating voltage with a hardware multiple ion detection unit.

The identification of a steroid was based on the appearance of peaks in the chromatograms of two major ions having the same intensity ratio and retention time as those given by the reference steroid (Table 2). The quantification was based on the heights of one of these peaks (Table 2) and the corresponding peak given by the labelled standard. The concentrations of endogenous steroids were calculated from the following equation:

$$\text{Steroid concentration [ng/g wet weight]} = \frac{A}{W} \times \frac{(R_S - R_L)}{(1 - R_S \times R_U)};$$

A = amount [ng] of internal standard added
W = wet weight of testis [g]
R_S = ratio of unlabelled to labelled ions in the sample steroid
R_L = ratio of unlabelled to labelled ions in the labelled standard
R_U = ratio of "labelled" to unlabelled ions in the unlabelled reference steroid.

The amounts of ^{14}C-labelled steroids in the internal standard mixture were determined in the same way by addition of known amounts of the unlabelled steroids. Since no labelled 17P^5 was available, [^{14}C]17P^4 was used as internal standard for

Table 2. Retention indices and characteristic ions of TBDMS and TBDMS–TMS derivatives of steroids in rat testis

Steroids[a]	Derivative	R.I.[b]	Characteristic ions (m/z) for Identification[c]	Quantification[d]
DHEA	3-TBDMS	2854	387 (M-15) 345 (M-57)	345 (347)
A^4	3-enol-TBDMS	2901	400 (M^+) 385 (M-15)	400 (402)
P^5	3-TBDMS[e]	2979 3017	373 (M-57) 298 (M-132)	373 (375)
P^4	3-enol-TBDMS[e]	3021 3067	428 (M^+) 413 (M-15)	428 (430)
3αA	3,17-diTBDMS	3068	463 (M-57) 387 (M-133)	463 (465)
$17P^5$	3-TBDMS,17-TMS	3117	518 (M^+) 475 (M-43)	475[f]
$17P^4$	3-enol-TBDMS,17-TMS	3182	516 (M^+) 473 (M-43)	473 (475)
A^5	3,17-diTBDMS	3187	503 (M-15) 461 (M-57)	461 (463)
3βA	3,17-diTBDMS	3192	463 (M-57) 387 (M-133)	463 (465)
DHT	3-enol,17-diTBDMS	3214	518 (M^+) 461 (M-57)	518 (520)
E_2	3,17-diTBDMS	3223	500 (M^+) 443 (M-57)	443 (446)
T	3-enol,17-diTBDMS	3254	516 (M^+) 459 (M-57)	516 (518)

[a]For abbreviations see Table 1. [b]Kovats index, determined in the GC–MS analyses. [c]Origin of ion given in parenthesis. M^+, molecular ion. [d]m/z value for the labelled standard in parenthesis. [e]Two isomers, the minor one having a 17α-oriented side chain and the shorter retention time. [f]Quantified using m/z 475 of [^{14}C]$17P^4$ as the reference peak.

quantification of this steroid. The ratio between the heights of the peaks given by the two steroids in the chromatogram of m/z 475 (Table 2) was determined and a response factor was calculated by analysis of mixtures of known amounts of $17P^5$ and [^{14}C]$17P^4$.

RESULTS AND DISCUSSION

Analytical method

Extraction and purification was performed essentially as described previously [2]. The recovery of ^{3}H-labelled testosterone and progesterone in the effluent from the combined Lipidex 1000 and Sep-Pak C_{18} column was 92–95%. Following removal of cholesterol by passage of the effluent through Lipidex 5000, the recovery was about 90% and the weight of the final extract was about 1 mg, i.e. a 20–25-fold purification from the original lipid extract [2].

This material was sufficiently pure for fractionation on an analytical HPLC column provided with a guard column. A straight phase system was employed to avoid the use of an aqueous mobile phase and to simplify evaporation of the solvents. Furthermore, in this way the most polar steroids, which were present in low concentrations, were widely separated from contaminating material eluted near the front. A comparison was made of LiChrosorb Si60 and LiChrosorb DIOL columns and the latter were found to give the best separation for the present purpose. All steroids were not separated, but fractions could be collected in such a way that splitting of a steroid between two fractions was minimized. The total recovery of radioactivity from the mixture of labelled steroids added to the homogenized testis was about 90%, i.e. including prepurification and HPLC. The possible occurrence of selective losses was not studied in detail but there were no obvious signs of such losses.

Derivatization.

The type of derivatives used was chosen for the following reasons. (1) Enol ether derivatives of 3-oxosteroids have better GLC properties than methyloximes. They are thermally more stable and show less tendency to adsorption. (2) TBDMS derivatives give mass spectra with intense ions of high mass suitable for selected ion monitoring [reviewed in refs 6, 7]. (3) TBDMS derivatives give relatively predictable mass spectra so that a search can be made for steroids with a given molecular weight. (4) TBDMS derivatives are chemically stable and non-polar. This permits purification by reversed phase HPLC in nonaqueous methanol. The stability also makes storage possible.

Reaction conditions were chosen on the basis of a previous study [3]. The aim was to obtain a single derivative of each steroid in the fraction from the HPLC column. Relatively long reaction times were required to ensure complete derivatization of groups reacting with TBDMSIM. Depending on the type of steroid, the yields of derivatives of the ^{14}C-labelled steroids listed in Table 1 were between 70 and 100% including purification by reversed phase HPLC. In order to obtain reproducible yields of 3-enol-TBDMS

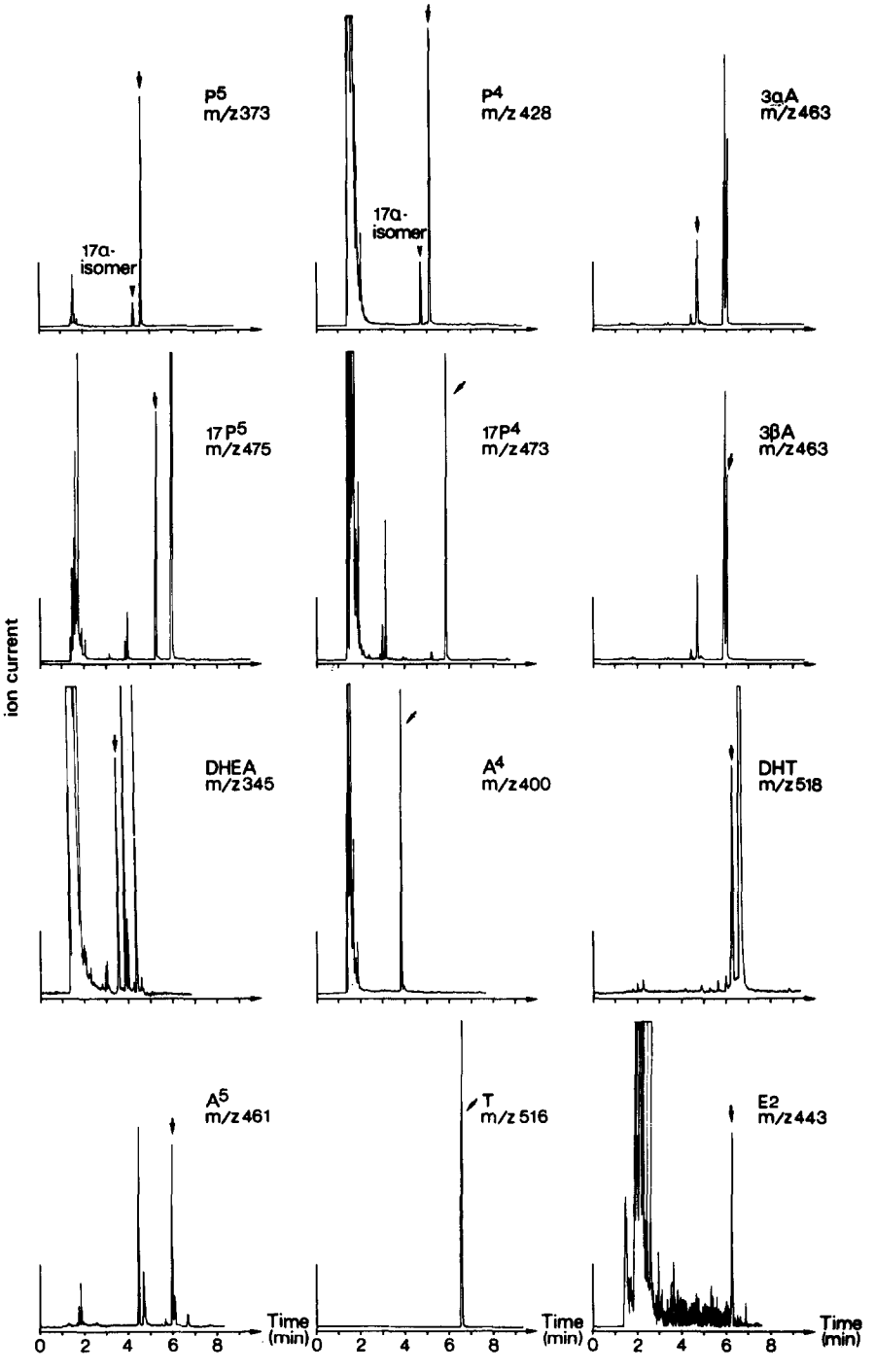

Fig. 2. Selected ion chromatograms obtained in the GC–MS analysis of twelve steroids in a rat testis. For abbreviations see Table 1.

derivatives it was necessary to treat the reaction vessels with strong aqueous sodium hydroxide prior to use. This ensured a uniform coating of the glass walls with sodium formate. A siliconized glass surface prevented this coating. The yields of enol-TBDMS derivatives were also slightly improved when 1 μg of sodium hydroxide was added to the sodium formate catalyst. This effect was studied with 0.1 μg–1 mg of sodium hydroxide per mg sodium formate. Addition of more than 10 μg was found to have a negative effect on the reaction.

The use of ^{3}H-labelled instead of ^{14}C-labelled steroids as internal standards was also studied. Under the derivatization conditions used, ^{3}H was lost

by enolization which prevented a calculation of yields. Comparisons of the conversion of [1,2,6,7-$^3H_4$]testosterone, [4-^{14}C]testosterone and unlabelled testosterone into the 3-enol-TBDMS-17-TBDMS derivative showed that 20% of the ^{3}H was lost from the first-mentioned steroid. There was no loss of ^{3}H in the absence of TBDMSIM and since 20.1% of the ^{3}H was present in the 6α position (according to the manufacturer) it appears likely that the 6α-^{3}H was lost by migration to the nucleophilic nitrogen on the imidazole ring of the reagent in a concerted type of reaction.

Reversed phase HPLC of the derivatives using methanol as the mobile phase did not produce any degradation products and the yields were the same as when a Lipidex 1000 column in hexane was used [3]. The reaction mixture could be directly injected into the HPLC system and intermediate removal of reagents, e.g. on Lipidex, was not required. The fractions were very clean and contained no contaminants disturbing quantification by GC–MS. This is illustrated by the selected ion chromatograms shown in Fig. 2.

GC–MS analysis

The precision of the selected ion monitoring was tested by repeated analyses of known amounts of the twelve steroids studied. The response was linear for all steroids, giving a mean correlation coefficient of 0.9997 ± 0.0004. The coefficient of variation in 17 analyses of 20 ng amounts of six steroids was 1.3%.

The selected ion chromatograms of the ions listed in Table 2 showed peaks at the correct retention times without interference by other peaks (Fig. 2). The use of purified solvents, reagents and chromatographic media was essential for high sensitivity. For example, the detection limit (signal/noise ratio 2) was 0.6 pg for the derivative of estradiol.

Steroid concentrations in testis

The identified steroids and their concentrations in 10 testes from 5 animals are given in Table 3. Only testosterone and pregnenolone have previously been determined by GC–MS of pooled testicular samples, and similar concentrations were found as in the present study [8].

All of the steroids have previously been measured by radioimmunoassay (RIA). In most cases only a few steroids have been analyzed and only two studies include simultaneous determinations of eight [9] and eleven [10] steroids. The agreement between our values and those obtained in previous studies is variable. The concentrations of dehydroepiandrosterone and 5-androstene-3β,17β-diol are more than 10 times lower and that of 17α-hydroxypregnenolone three times lower than those found in testes of 1-year old Wistar rats [10]. Similarly, the values for 5α-androstane-3α,17β-diol and 5α-androstane-3β,17β-diol are 3 and 6 times lower, respectively, than those reported for 12 month old Sprague–Dawley rats [11]. The reported levels of estradiol are highly variable [9, 10] and our values belong to the lower range [9]. The large differences between reported concentrations of many steroids in testis may be due to strain and age factors and to dietary and other conditions of animal housing. In some cases, when the GC–MS values are markedly lower, the specificity of the RIA method may not have been sufficient.

Table 3. Identified steroids and their concentrations in 10 testes from 5 rats

Steroid*	Steroid concentration, ng/g wet weight	
	Mean ± SD	Range
A[4]	3.97 ± 2.47	2.13–9.84
T	127 ± 96.2	71.8–313
DHT	4.45 ± 2.91	1.96–8.5
3βA	1.68 ± 1.43	0.54–4.67
3αA	5.65 ± 4.41	2.23–15.7
P[4]	5.53 ± 4.04	2.54–13.4
17P[4]	14.4 ± 14.1	6.37–42.4
DHEA	0.07 ± 0.04	0.03–0.14
A[5]	0.25 ± 0.13	0.10–0.43
P[5]	10.3 ± 6.07	4.97–21.9
17P[5]	0.95 ± 0.48	0.35–1.78
E_2	0.025 ± 0.008	0.015–0.038

*For abbreviations see Table 1.

The variations in steroid concentrations were higher in our study than in previous studies [9–11]. This variation was between animals, and the differences between concentrations in testes from the same animal were much smaller. Thus, the mean difference was only 5% for testosterone and pregnenolone, while the mean difference for other steroids varied between 10 and 35%. In most animals the differences were smaller, but occasional large differences increased the mean values. The biological significance of these findings remains to be established.

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