GC-MS determination of steroids related to androgen biosynthesis in human hair with pentafluorophenyldimethylsilyl–trimethylsilyl derivatisation

The Analyst

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An efficient method for the simultaneous determination of eight steroids, androstenedione, dihydrotestosterone (DHT), dehydroepiandrosterone (DHEA), testosterone, androsterone, etiocholanolone, progesterone and pregnenolone, in human hair by gas chromatography-mass spectrometry (GC-MS) using d_3-testosterone as internal standard is described. The method involves alkaline digestion, liquid–liquid extraction and subsequent conversion to mixed pentafluorophenyldimethylsilyl–trimethylsilyl (flophemesyl–TMS) derivatives for sensitive analysis in the selected ion monitoring (SIM) mode. This method showed good overall repeatability and reproducibility of 4.88–11.24 and 3.19–9.58%, respectively. For the first time, the quantification of DHT, DHEA and pregnenolone in human hair has been achieved by GC-MS, testosterone was also quantified. The detection of four steroids in hair samples was possible in the concentration range 0.12–8.45 ng g^{-1}. The other four steroids, androstenedione, androsterone, etiocholanolone and progesterone, were not detected. The detection limits for SIM of the steroids varied in the range 0.02–0.5 ng g^{-1}, and the SIM responses were linear with correlation coefficients varying from 0.991 to 0.996 for most of the steroids studied. The concentrations of the four steroids detected were different in male and female hair samples.

1. Introduction

Steroids related to androgen biosynthesis regulate a variety of biological functions, including reproductive and adaptive responses. Alhough principally synthesized by the adrenal gland, gonads, liver and placenta, the brain is a further site of steroid synthesis and metabolism.

Usually, the measurement of endogenous and exogenous steroids is achieved in urine or plasma.[1,2] In contrast with urine and plasma, the benefit of analysis in hair is that it can show the effect of long-term exposure. To our knowledge, only a few studies have been published in which some anabolic steroids and testosterone have been discovered as androgenic steroids in human hair by gas chromatography-mass spectrometry (GC-MS). These involved the additional purification steps, such as solid-phase extraction or fractional liquid chromatography.[3–5]

Generally, the derivatisation of steroids to mono- or trimethylsilyl ether (TMS) derivatives has been the most common approach.[6,7] Selecting the best and most appropriate choice of derivatisation is of primary concern not only for GC properties, but also for the selection of useful ions for trace quantification in the selected ion monitoring (SIM) mode. Therefore, we introduced mixed pentafluorophenyldimethylsilyl–trimethylsilyl (flophemesyl–TMS) derivatisation, which had been confirmed in a previous study.[8] The significant advantages of this technique include the formation of an intense molecular ion and the minimization of background noise without additional purification steps.

The objective of this work was to determine rapidly and sensitively the steroid contents of hair without additional purification steps using GC-MS, because so far no MS assay has been described for the detection of steroids in hair. Such a method would give the possibility of elucidating their metabolism and improving the detection of xenobiotic compounds in hair by trace analysis.

2. Experimental

2.1. Chemicals

Androstenedione (androst-4-ene-3,17-dione), progesterone (pregn-4-ene-3,20-dione), androsterone (5α-androstan-3α-ol-17-one), etiocholanolone (5β-androstan-3α-ol-17-one), DHEA (dehydroepiandrosterone; 5-androsten-3β-ol-17-one), DHT (dihydrotestosterone; 5α-androstan-17β-ol-3-one), testosterone (androst-4-en-17β-ol-3-one) and pregnenolone (pregen-5-ene-3β-ol-20-one) were purchased from Sigma (St. Louis, MO, USA). The internal standard (IS) was [16,16,17-$^2H_3$]testosterone, obtained from the Cologne Laboratory (Institute of Biochemistry, German Sports University, Cologne, Germany). Pentafluorophenyldimethylchlorosilane (flophemesyl chloride), *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA), ammonium iodide (NH_4I) and dithioerythritol (DTE) were purchased from Sigma.

2.2. Preparation of standard solution

A stock standard solution of eight steroids and d_3-testosterone was prepared at concentrations of 0.1 mg ml^{-1} in methanol. The stock standard solution was used to prepare a working standard solution of varying concentrations (0.01–1.0 μg ml^{-1}) in methanol.

2.3. Derivatisation

Flophemesyl chloride (50 μl) was added to the residue and the mixture was allowed to stand at room temperature for 15 min. After the excess reagent had been evaporated under a stream of nitrogen at 70 °C, the trimethylsilylating reagent [40 μl of

MSTFA–NH_4I–DTE (1000:4:2 v/w/w)] was added to the residue and the mixture was heated at 60 °C for 15 min. Approximately 2 μl of the flophemesyl–TMS derivatised sample solution was injected into the GC-MS system.

Mixed standard solutions in the range 0.01–1.0 ng g^{-1} were prepared for determining the detection limits of the overall procedure. Calibration standard solutions were obtained by spiking suitable amounts of each of these mixed standard solutions into 1 ml of water. Mixtures containing absolute amounts of several concentrations of each steroid were pre-treated, derivatised and analysed in triplicate. To prepare a calibration curve, the ratio of the peak area of steroids to that of d_3-testosterone as IS was plotted *versus* the concentration of the steroids in the calibration standard solution, and a least-squares linear regression analysis was performed. Values of unknown concentrations in hair were determined from the regression line of this calibration curve.

2.4. Sample preparation and pre-treatment

Human scalp hair from five males and five females (aged 28–44 years) was collected during haircutting. To prevent contamination with steroids from sweat, sebum, *etc.*, the hair was washed first with acetone and then with methanol. After drying at 60 °C, the hair samples were cut into short lengths of about 1–2 mm and amounts of 200 mg were weighed into glass test-tubes. The pre-treatment was modified with alkaline digestion, and liquid–liquid extraction is well established for testosterone detection in hair and urine.[5,8] A 10 μl volume of a d_3-testosterone solution (10 ng ml^{-1}) and 1 ml of 1 mol l^{-1} NaOH were added, and the solution was heated at 80 °C for 1 h. Then 1 ml of 0.1 mol l^{-1} phosphate buffer (pH 7.0) was added and the pH was adjusted to 10–11 by adding 0.3 ml of 2 mol l^{-1} HCl and with 5 ml of pentane. The mixture was mechanically shaken (10 min) and centrifuged (2400 rpm, 5 min), and the organic phase was transferred to a test-tube. The organic layer was evaporated to dryness with a rotary evaporator. The residue was dried in a vacuum desiccator over P_2O_5–KOH for at least 30 min before the derivatisation procedure.

2.5. Evaluation of repeatability and reproducibility

The repeatability of the chromatographic analysis was determined in a day by 10 replicate 2 μl injections of a mixture of derivatised standard solution at the 1 ppm level. The reproducibility for hair samples was examined every other day (n = 3) by a 2 μl injection of 10 derivatised extracts obtained from fortified water samples at 10 and 50 ppb levels.

2.6. Gas chromatography-mass spectrometry

The GC-MS system (Model 5973 mass-selective detector combined with a Model 6890 Plus gas chromatograph, Hewlett-Packard, Avondale, PA, USA) was used in both scan and SIM modes. The electron energy was 70 eV and the ion source temperature was 230 °C. The gas chromatograph was equipped with a 17 m × 0.2 mm id × 0.11 μm film thickness capillary column coated with a cross-linked methylsilicone gum phase (Hewlett-Packard). The carrier gas was helium at a column head pressure of 121 kPa. The split (1:10) method of injection was used. The temperature programme was as follows: the initial temperature was 220 °C (2 min), increased at 4 °C min^{-1} to 240 °C (held for 5 min), then at 15 °C min^{-1} to a final temperature of 310 °C (held for 3.33 min).

2.7. Data acquisition

In the SIM mode for quantification, a molecular peak ion for each steroid except pregnenolone was selected as a quantitative

Table 1 GC-MS data of eight steroids as bis-TMS and flophemesyl–TMS derivatives

		m/z (relative intensity, %)							
Steroid	Derivative[a]	$[M]^+$	$[M-15]^+$	$[M-72]^+$	$[M-90]^+$	$[M-105]^+$	$[M-167]^+$	$[M-225]^+$	$[M-257]^+$
Androstenedione	1	430 (100)	415 (18.3)	358 (1.4)	—	325 (7.5)	—	—	—
Progesterone	1	458 (41.2)	443 (36.7)	386 (4.3)	—	353 (5.3)	—	—	
Androsterone	2	586 (43.1)	571 (62.2)	—	496 (1.8)	481 (2.2)	—	361 (3.1)	329 (20.8)
Etiocholanolone	2	586 (49.4)	571 (50.8)	—	—	481 (2.4)	—	361 (1.7)	329 (22.6)
DHEA	2	584 (59.9)	569 (51.0)	—	—	479 (5.3)	—	359 (2.0)	327 (14.2)
DHT	2	586 (47.1)	571 (8.8)	—	496 (3.6)	481 (2.0)	—	—	329 (4.1)
Testosterone	2	584 (92.6)	569 (9.7)	512 (4.1)	—	479 (2.5)	417 (3.6)	359 (4.3)	327 (9.8)
Pregnenolone	2	612 (16.3)	597 (100)	—	522 (9.4)	507 (3.8)	—	—	355 (7.8)

[a] (1) Bis-TMS and (2) flophemesyl–TMS derivatives. The derivatives were analysed on an Ultra-1 capillary column (17 m × 0.2 mm i.d. × 0.11 μm film thickness), with the injector and transfer line temperatures set at 280 and 300 °C, respectively. In the scanning mode, the mass range was *m/z* 50–650 at a rate of 1.42 scans s^{-1}.

Table 2 GC-SIM-MS data for the eight steroids

					Regression line[c]		
Steroid	RRT[a]	Selected ion (*m/z*)[b]	Detection limit/ng g^{-1}	Calibration range/ng g^{-1}	*a*	*b*	Linearity[d]
Androstenedione	0.39	430, 415, 358	0.1	0.5–50	0.0108	0.0005	0.992
Progesterone	0.59	458, 443, 386	0.5	0.5–50	0.0143	0.0011	0.991
Androsterone	0.71	586, 571, 329	0.02	0.5–50	0.0127	0.0007	0.995
Etiocholanolone	0.88	586, 571, 329	0.02	0.5–50	0.0133	0.0008	0.995
DHEA	0.69	584, 569, 327	0.2	1.0–20	0.0052	0.0008	0.996
DHT	0.96	586, 571, 329	0.1	0.1–10	0.0079	0.0008	0.996
Testosterone	1.00	584, 569, 512	0.2	1.0–20	0.0279	0.2155	0.992
Pregnenolone	1.14	612, 597, 522	0.2	0.5–50	0.0125	0.0003	0.996

[a] Retention time relative to that of d_3-testosterone (13.03 min). [b] Quantitative ions are underlined. [c] a = Slope = relative mass response = mean peak area ratio of steroid × mass of IS/mass of steroid; b = y-intercept. [d] Linearity is represented by the linear correlation coefficients for the calibration curves.

ion (*m/z* 430, 458, 584, 586 and 597). The start time for ion monitoring was programmed from 4.1 to 18.0 min to set up one group of 16 ions to be monitored. A dwell time of 150 ms and a relative electron multiplier voltage of 600 V were applied for each ion monitored.

2.8. Calculation

The detection limit for each steroid was calculated based on the weight giving a signal three times the peak-to-peak noise of the background signal. A least-squares regression analysis was performed on the measured peak area ratios against the increasing weight ratios of steroids to IS in order to obtain linearity of the SIM responses and to plot calibration curves for the quantitative measurement of steroids.

3. Results

3.1. Mass spectral analysis

The eight steroids have two ionizable positions with keto and hydroxyl groups. In order to stabilize the compounds and improve their GC properties, an initial effort was made to examine the mixed derivatisation method using flophemesyl chloride and MSTFA–NH_4I–DTE mixture. The six steroids that were displayed confirmed the formation of flophemesyl–TMS derivatives with good GC-MS properties, as noted in our previous report.[8] In contrast, androstenedione and progesterone formed bis-TMS derivatives. Flophemesyl chloride did not lead to the formation of enol ethers and greatly hindered the hydroxyl groups to react.[9]

The eight steroid derivatives were subjected to GC-MS analysis and the electron impact MS data are summarized in Table 1. Similar to our previous report,[8] the intense peaks of six flophemesyl–TMS derivatives were either M^+ or $[M - 15]^+$ ions. Also, in both cases androstenedione and progesterone were identified.

3.2. Validation of the method

To confirm the peak identities, the present method was designed so that three characteristic ions for each steroid were selected on the basis of their mass fragmentation. Each peak was identified by ratios matched with derivatised steroid standards.

When the calibration curve and sensitivity were examined, linear responses for each compound were obtained with correlation coefficients and detection limits varying from 0.991 to 0.996 and from 0.02 to 0.5 ng g^{-1}, respectively (Table 2). The repeatability was evaluated for 1 ppm derivatised mixed standards of eight steroids and the reproducibility was determined with extracts that were fortified with standards of eight steroids at 10 and 50 ppb levels in water. The peak areas of selected ions (quantitative ions) were obtained for the eight steroids. They were quantitated by the ratio of the peak area from the fortified sample to that from the corresponding internal standard (d_3-testosterone), and absolute values were calculated (Table 3).

3.3. Screening of steroids in human hair

The present GC-SIM-MS technique provides a sensitive method for the quantification of steroids in 200 mg of hair. Using this method, at least four steroids, DHT, DHEA, testosterone and pregnenolone, were quantitated. The selected ion chromatogram demonstrates the usefulness of our method in rapid screening for four steroids in scalp hair samples (Fig. 1). Each steroid detected in 200 mg of hair from five male and five female samples was quantitatively determined (Table 4).

4. Discussion

We have achieved the quantification of steroids related to androgen biosynthesis in human scalp hair using GC-MS.

Table 3 Repeatability and reproducibility for the eight steroids

Steroid	Repeatability (%)	Reproducibility (%)	
		10 ppb	50 ppb
Androstenedione	11.24	8.34	7.27
Progesterone	5.92	6.39	6.28
Androsterone	4.88	5.76	6.83
Etiocholanolone	7.45	5.82	4.89
DHEA	8.11	4.22	6.16
DHT	5.90	5.48	9.12
Testosterone	7.39	3.19	9.12
Pregnenolone	6.76	7.86	6.22

All samples were individually prepared as 10 replicates. They were analysed in a day for repeatability and every other day (n = 3) for reproducibility.

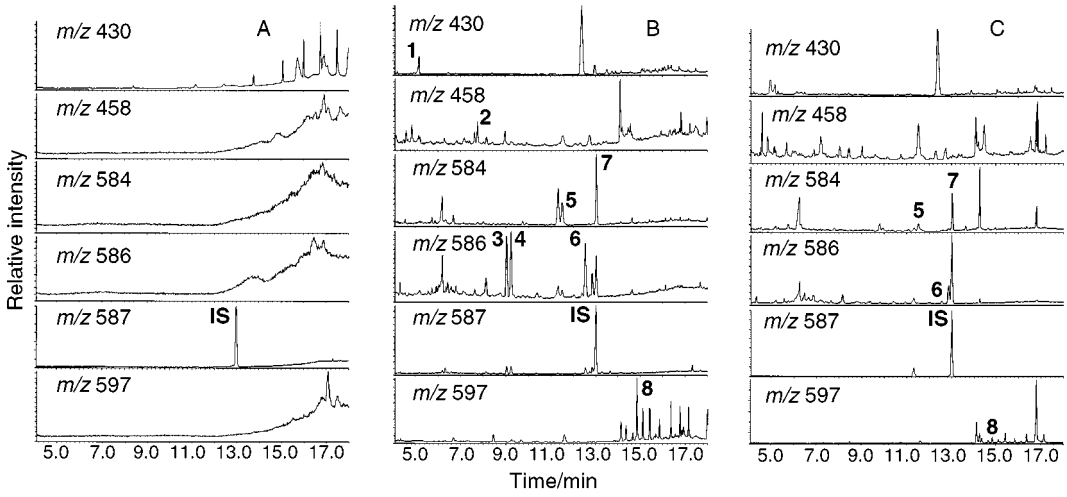

Fig. 1 Selected ion chromatograms of a water blank extract (A), a fortified hair extract concentrated at the 5 ppb level in hair (B) and a male hair extract (C) separated on Ultra-1 (17 m × 0.2 mm id × 0.11 μm film thickness) fused-silica capillary column. Peaks: 1 = androstenedione; 2 = progesterone; 3 = androsterone; 4 = etiocholanolone; 5 = DHEA; 6 = DHT; 7 = testosterone; 8 = pregnenolone.

Table 4 Results of GC-MS quantification of the four steroids detected in human scalp hair

	Male hair/ng g^{-1}			Female hair/ng g^{-1}		
Steroid	Median	Range	Mean ± *s*	Median	Range	Mean ± *s*
DHEA	5.19	2.67–5.84	4.57 ± 0.79	2.18	1.02–3.54	2.25 ± 0.59
DHT	0.50	0.38–0.83	0.57 ± 0.11	0.21	0.12–0.30	0.21 ± 0.04
Testosterone	2.51	2.03–2.54	2.36 ± 0.13	1.41	0.88–1.66	1.32 ± 0.19
Pregnenolone	1.82	0.83–3.75	2.13 ± 0.70	17.09	8.45–38.60	21.38 ± 7.32

Highly sensitive detection methods are required to identify steroids in hair, owing to their low concentrations. Derivatisation to TMS derivatives has been the common approach for the determination of steroids, but the mass increment provided by trimethylsilylation is small. Therefore, mixed flophemesyl–TMS derivatives have been advocated as a better choice for SIM at the higher mass of M^+ or $[M - 15]^+$ ions. In contrast with previous studies[3–5] on related topics, the most significant advantage of this technique is the minimization of the background noise level without additional purification steps such as solid-phase extraction with a C_{18} cartridge or Sephadex LH-20.

The sensitivity achieved with mixed flophemesyl–TMS derivatisation was excellent for GC-MS assay using a mass-selective detector. In the present work, the detection of DHEA, DHT, testosterone and pregnenolone in hair samples was possible in the concentration range 0.12–8.45 ng g^{-1}. In the case of testosterone, the amounts detected in hair agree with those reported by other workers.[4,5] Androstenedione, androsterone, etiocholanolone and progesterone were not detected in this work. On comparing the concentrations of the four steroids detected in male and female hair, a significantly higher concentration of pregnenolone in female hair could be detected.

In conclusion, we have developed a method for the detection of steroids in human hair. This study may be the starting point for further studies in the field of steroid analysis that could lead to diverse applications in biomedical research.

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