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Measurement of testosterone and pregnenolone in nails using gas chromatography–mass spectrometry

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Abstract

An efficient method for the determination of testosterone and pregnenolone in human nails using gas chromatography–mass spectrometry (GC–MS) with d_3-testosterone as an internal standard is described. The method involves alkaline digestion and liquid–liquid extraction, with subsequent conversion to mixed pentafluoropenyldimethylsilyl-trimethylsilyl (flophemesyl-TMS) derivatives for sensitive analysis in the selected-ion monitoring (SIM) mode. The limit of detection (LOD) and limit of quantification (LOQ) were lowered to 0.1 and 0.2 pg/g, respectively, when 100 mg of nail-clippings were used. The mean recoveries of testosterone and pregnenolone were 89.8 and 86.7%, respectively, while good overall precision (% C.V.; 4.5–9.5) and accuracy (% bias; 3.9–8.4) were demonstrated. Linearity as a correlation coefficient was 0.9913 (testosterone) and 0.9965 (pregnenolone). When applied to fingernail and toenail samples from seven healthy men and nine healthy women, testosterone and pregnenolone were positively detected in the concentration range of 0.24–5.80 ng/g. The levels of two steroids studied in the nails were found to be higher in the male subjects than in the female subjects, and except for the toenails of the females, the levels of testosterone were higher than those of pregnenolone.

Keywords: Testosterone; Pregnenolone

1. Introduction

Pregnenolone is a naturally occurring metabolite of cholesterol that acts as a precursor to a major androgen testosterone. It is metabolized by practically every tissue in the body to a large variety of related steroids. Some reproductive disorders in men are associated with abnormal concentrations of pregnenolone and testosterone [1–3], and testosterone analysis could have application as a means of detecting steroid abuse in athletes.

Non-invasive biological fluids are the most extensively investigated for steroid analysis. A major limitation of investigating non-invasive biological fluids is the relatively short time these samples are available after delivery. Also, because a large difference (10-fold) exists between the serum testosterone levels of males and females, we were prompted to

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search for another type of non-invasive sample. Keratinized samples, such as hair fibers or nail-clippings, provide a greater retrospective time window for detecting drug exposure. Recently, hair has been tested and recognized in the biomedical field [4–6] as an invaluable non-invasive biological specimen.

For many years, nails have been subjected to elemental analysis in a variety of applications, including forensic investigations [7–9], clinical diagnosis of various disease states [10,11], and therapeutic monitoring [12,13]. Recently, a report was also written on imbalances in the concentrations of trace elements in the nails of patients with Alzheimer's disease [14]. Nail analysis offers some advantage over hair analysis because nails continually grow and they survive after delivery. Nail-clippings are also easy to sample in suitable amounts. One major limitation of nail analysis is that nail-clipping are often difficult to analyze in newborn babies since the nails are so small and only a small amount can be retrieved at time. In contrast to hair [15,16], no attempts have yet been made to measure steroid levels in nails.

Gas chromatography–mass spectrometry (GC–MS) is a technique widely used for the detection of steroids in biological matrices, usually as their alkylsilyl ether [17–19] or perfluoroacyl ester [20–22]. Selecting the best and most appropriate choice of derivatization is of primary concern, not only for GC properties, but also for the selection of good quantitation ions in order to detect the trace amount in the selected-ion monitoring (SIM) mode. Therefore, we introduced a highly sensitive technique of mixed pentafluorophenyldimethyl-silyl-trimethylsilyl (flophemesyl-TMS) derivatization, which formed an intense molecular ion and diminished background noise without the additional purification steps [16,23].

In continuation of steroid analysis to investigate their biochemical roles, the present study was undertaken to combine flophemesyl-derivatization with subsequent trimethylsilylation of the hydroxyl and ketone groups, respectively, by GC–SIM-MS. The objective of this work was to determine the steroid contents of nails with a simple and accurate method. Such a method would make the possibility of their metabolism and the improvement of detecting xenobiotic compounds in nails as trace analyses.

2. Experimental

2.1. Chemicals

Testosterone (4-androsten-17β-ol-3-one) and pregnenolone (5-pregenen-3β-ol-20-one) were purchased from Sigma (St. Louis, MO, USA). A deuterated internal standard (I.S.), 16,16,17-$^{2}H_3$-testosterone, was obtained from Cologne Laboratory (Institute of Biochemistry, German Sports University, Germany). Pentafluorophenyldimethylsilyl chloride (flophemesyl chloride), *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA), ammonium iodide (NH_4I), and dithioerythritol (DTE) were purchased from Sigma (St. Louis, MO, USA).

2.2. Derivatization

The flophemesyl chloride (50 μl) was added to the dried 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, trimethylsilyating reagent (40 μl, MSTFA/NH_4I/DTE, 1000:4:5, 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 derivatized sample solution was injected into the GC–MS.

2.3. Sample preparation and pretreatment

Tip portions of the representative fingernail and toenail-clippings were carefully collected from seven healthy males (aged 28–43) and nine healthy females (aged 24–39). To avoid contamination with steroids from, e.g. sweat, sebum etc., the nail-clipping was washed with methanol by vortexing for 15 s. This wash step was repeated twice, and the methanol wash was immediately transferred to a test-tube. After they were dried (60°C), nail-clippings were cut into short lengths of ~1–2 mm, and amounts of 100 mg were weighed in a test tube. The pretreatment was achieved based on alkaline digestion and liquid–liquid extraction, which is well-established for steroid detection in hair [16]. Then 10 μl of d_3-testosterone solution (1 μg/ml) and 1 ml of 1 *M* NaOH were added, and the solution was heated at 80°C for 1 h. Finally, 1 ml of 0.1 *M* phosphate buffer (pH 7.0)

was added, and the pH was adjusted to 10–11 by adding 0.3 ml of 2 *M* HCl, along with 5 ml of *n*-pentane. The mixture was mechanically shaken (10 min) and centrifuged (2400 rpm, 5 min), and the organic layer was separated by placing it in a dry ice–acetone bath. 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 derivatization procedure.

2.4. Method validation

A stock solution of testosterone, pregnenolone, and d_3-testosterone was prepared by dissolving the pure standard in methanol (0.1 mg/ml). The solution was stored at 4°C and used within 2 months. The stock solution was used to obtain a working solution of varying concentrations (1–100 ng/ml) in methanol. Spiked 1 *M* NaOH solutions containing increasing concentrations of testosterone and pregnenolone: 0.1, 0.2, 0.5, 1, 5, and 10 ng/ml and a fixed concentration of an I.S. (10 μl×1 μg/ml) were analyzed according to the procedure described above. Linearity was checked by performing linear regression analysis of the peak area ratios of each steroid/d_3-testosterone against two steroid concentrations.

Samples for precision ($n=3$) and accuracy ($n=5$) assays as well as recovery tests were added at two different concentrations, such as 2 and 5 ng/g, using the pooled nail-clippings from a healthy man for 6 months whose testosterone and pregnenolone levels had been pre-determined. They were analyzed daily for precision, and every other day for accuracy assays. The precision was expressed as a relative standard deviation (coefficient of variation; % C.V.), and peak areas were considered for the calculation of the concentration and to establish the precision. Also, these samples were pretreated and analyzed by GC–MS, as described. Accuracy was expressed as a percentage bias (% bias) of each steroid recovered. The absolute recovery of the present method was measured as the response of a extracted nail sample spiked before extraction expressed as a percentage of the response of an extracted nail sample to which analyte has been added at the same nominal concentration just before derivatization. Their quantitative results were related to the exact concentrations added.

The limit of detection (LOD) and the limit of quantification (LOQ) were calculated by measuring the analytical background response. The signal-to-noise (S/N) ratio was used to determine the LOD, and it was estimated as the concentration of testosterone and pregnenolone in 1 *M* NaOH solutions that generated a peak with an area at least three times higher than the baseline noise. LOQ was considered to be ten times the standard deviation of the five NaOH solutions analyzed using the maximum sensitivity allowed by the system. The LOQ was subsequently validated by analysis of six nail samples known to be near the LOQ.

2.5. Gas chromatography–mass spectrometry

The GC–MS system (Model 5973MSD combined with a Model 6890 plus gas chromatograph; Hewlett-Packard, Avondale, PA) 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 I.D.×0.11-μm film thickness capillary column coated by a cross-linked methyl silicone gum phase (Hewlett-Packard). The carrier gas was helium at a column head pressure of 121 kPa. The split (1:5) method of injection was used. The temperature program was as follows: initial temperature 220°C (2 min); program rate 4°C/min to 240°C (5 min); 15°C/min up to a final temperature of 310°C, where it was held for 3.33 min.

2.6. Data acquisition

In the scanning mode, the mass range was 50–650 u at a rate of 0.48 scans/s. In the SIM mode, three characteristic ions for each steroid were used for peak identification, as listed in Table 1, while one ion, underlined in the table, was selected for quantification. Each peak in the nail samples was identified by matching the area ratios of three ions with those of steroid standards. The underlined quantitation ions correspond to $[M]^+$ ions for testosterone and d_3-testosterone, and $[M\text{-}15]^+$ ions for pregnenolone.

The start time for SIM was programmed from 5.0 to 18.0 min to set up nine ions to be monitored, and a dwell time of 80 ms was chosen. The relative voltage of the electron multiplier was set to 400 V

Table 1
GC–SIM-MS and validation data for the analysis of testosterone and pregnenolone as their flophemesyl-TMS derivatives[a]

Steroids	MW	Retention time (min)	Ion selected (m/z)	LOD[b]	LOQ[c]
GC–SIM-MS data					
Testosterone	288	13.12	584, 569, 327	0.1	0.2
Pregnenolone	316	14.91	612, 597, 157	0.1	0.3
d_3-Testosterone	291	13.08	587, 572, 330	NA[d]	NA
	Recovery (%)	Linearity (r)[e]	Range (ng/g)		
Validation data					
Testosterone	89.8	0.9907	0.04 –1		
Pregnenolone	86.7	0.9989	0.04 –1		
d_3-Testosterone	NA	NA	NA		

[a] Analyzed on a Ultra-1 (17 m×0.20 mm I.D., 0.11-μm film thickness) fused-silica capillary column initially at 220°C (2 min), and then raised to 240°C (5 min) at 4°C/min, and then to a final temperature of 310°C (3.33 min) at 15°C/min, in SIM mode for three ions for each steroid for peak identification and one ion (underlined) for quantification at dwell time of 80 ms. Relative voltage of electron multiplier was set to 400 V higher than that in the scanning mode for each ion monitored.
[b] Limit of detection.
[c] Limit of quantification.
[d] Not applicable, used as an internal standard.
[e] Linearity was described with linear correlation coefficients for calibration curves.

higher than that in the scanning mode for each ion monitored.

3. Results and discussion

3.1. Gas chromatography–mass spectrometry

Testosterone and pregnenolone have two ionizable positions as hydroxyl and ketone groups. In order to stabilize compounds and improve GC properties, the present method was selected to demonstrate the mixed-derivatization method using flophemesyl chloride and a mixture of MSTFA/NH_4I/DTE. The testosterone and pregnenolone that were displayed confirmed the formation of flophemesyl-TMS derivatives with good GC–MS properties, as noted in our previous reports [16,23].

3.2. Method validation

The analytical performance parameters assessed for the overall assay were selectivity, linearity, precision and accuracy, recovery, LOD, LOQ, and stability. The identity of the chromatographic peak was confirmed not only by its retention time but also by its three characteristic ions, and testosterone and pregnenolone were well resolved and free from interference peaks in the SIM mode. The response of testosterone and pregnenolone was checked in the range of application of the analytical method by linear regression analysis with the least-squares method of peak area ratios of testosterone/d_3-testosterone and pregnenolone/d_3-testosterone against different concentrations of two steroids. The linear responses to testosterone and pregnenolone were obtained in the range of 0.04–1.0 ng/ml with correlation coefficients varying from 0.9913 and 0.9965, respectively.

In testosterone, the precision expressed as % C.V. ranged from 4.5 to 8.6% at two different concentrations with 2 and 5 ng/g, indicating that the analytical method is repeatable. The mean recoveries at 2 and 5 ng/g were 87.6 and 91.9%, respectively, while the % bias varied from 3.9 to 8.4% for three different runs (n=5). The accuracy of the present method was then determined at each concentration by assessing the agreement between the measured

and nominal concentrations of the analytes in the spiked nail sample whose testosterone and pregnenolone levels had been pre-determined. The LOD and LOQ were 0.1 and 0.2 ng/g, respectively, on the basis of a S/N ratio of 3. In pregnenolone, the % C.V. ranged from 5.1 to 9.5% at two different concentrations with 2 and 5 ng/g, while the % bias varied from 4.8 to 6.9% for three different runs ($n=5$). The mean recoveries at 2 and 5 ng/g were 79.8 and 93.6%, respectively, and the LOD and LOQ were 0.1 and 0.3 ng/g, respectively.

In the course of a prior study [24], an excellent stability in flophemesyl-derivatized steroids was observed. The flophemesyl-TMS derivatives in a vial capped at room temperature stabilized the testosterone, pregnenolone, and d_3-testosterone for at least 2 weeks. These conditions allowed a large number of samples to be extracted and stored for later GC–MS analysis.

3.3. Testosterone and pregnenolone in fingernails and toenails

After being washed sequentially with methanol, the nail samples collected from seven healthy males and nine healthy females were treated with 1 *M* NaOH solution to extract steroids from nail matrices. The two steroids were not detected in the methanol wash. When the present method was applied to the aqueous extracts of fingernails and toenails, an excellent separation of testosterone and pregnenolone was achieved with no significantly interfering background peaks. This excellent separation is demonstrated in a typical selected ion current chromatogram (Fig. 1).

Testosterone and pregnenolone were positively detected in 100-mg aliquots of all the nail samples studied, and their concentrations varied from 0.24 to 5.80 ng/g (Table 2). From subject to subject, wide

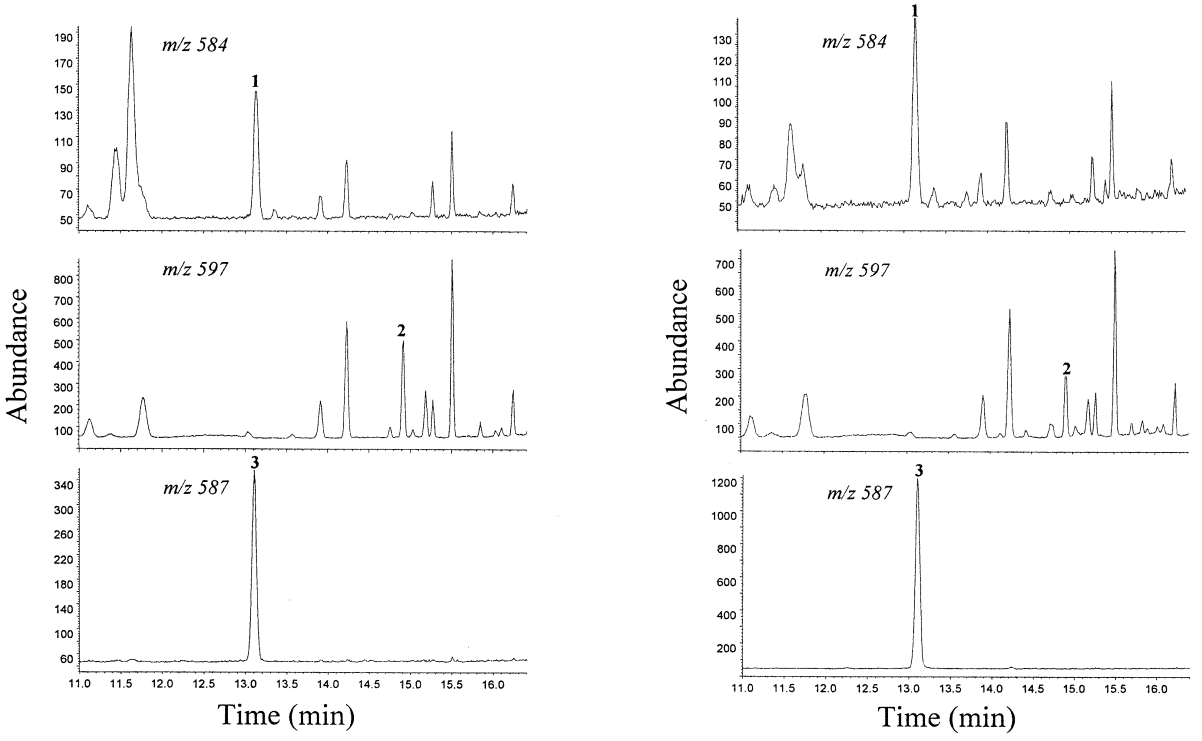

Fig. 1. Selected ion current chromatograms of a fortified fingernail extract to which is added 2.0 ng of testosterone and pregnenolone, respectively, (left) and a male fingernail extract (right) separated on an Ultra-1 (17 m×0.2 mm I.D.×0.11 μm) fused-silica capillary column. 1, testosterone; 2, pregnenolone; 3, d_3-testosterone.

Table 2
Testosterone and pregnenolone levels (ng/g) in nail samples from seven men and nine women[a]

Steroid	Male nails (n=7)		Female nails (n=9)	
	Fingernail	Toenail	Fingernail	Toenail
Testosterone	3.98±1.14 (4.08, 2.05–5.80)	2.20±1.02 (2.44, 0.45–3.11)	1.08±0.47 (0.98, 0.24–1.69)	1.84±0.71 (1.51, 0.87–2.98)
Pregnenolone	3.17±0.65 (3.10, 2.11–4.21)	2.01±0.97 (2.01, 0.30–3.41)	0.90±0.33 (0.76, 0.48–1.53)	3.06±1.03 (3.56, 1.06–4.33)

[a] *n*-Pentane extracts of hair (100 mg) collected from seven healthy male (aged 28–43) and nine healthy females (aged 24–39) was subjected to alkaline digestion with subsequent flophemesyl-TMS derivatization for the GC–SIM-MS analysis. GC–SIM-MS conditions are described in Table 1. Concentration data are mean±SD (median, range).

variations in the levels of testosterone and pregnenolone were observed. In mean amounts, the levels of the two steroids studied in the nails were found to be higher in the male subjects than in the females; however, the range for the males and females ranges overlapped. We speculate that this sex difference might reflect a correlation between androgen concentrations in nails and serum.

In comparison with hair [16] as the keratinized specimens, the concentrations for testosterone and pregnenolone corresponded to some degree. However, the concentrations of pregnenolone in nails were 10-fold less than hair pregnenolone (mean: 21.38 ng/g; range: 8.45–38.60 ng/g).

4. Conclusion

The measurement of pregnenolone and testosterone levels in biological specimens is widely employed to evaluate the androgenic status of the subject under investigation. In contrast to the large number of methods published to determine steroids in biological fluids, there are few studies that analyze non-invasive specimens, such as hair and nail. In this study, we have achieved the measurement of pregnenolone and testosterone in human fingernail and toenail specimens.

Nails are derived from the same cells as are the epidermis and hair, and consist of hard, dead, keratinous cells. The mechanism of drug entry and incorporation into the nail matrix is unknown. However, it is assumed that the dividing cells responsible for nail formation also enable drug entry. During nail formation, drugs may enter continuously or as a single event. The benefit of nail analysis, over plasma and urine analysis, is that it can show varying exposure over weeks and months [25].

The sensitive, specific, and reproducible method demonstrated appears to be suitable for the detection and quantification of testosterone and pregnenolone from an aqueous solution using *n*-pentane as a non-polar organic solvent. Within 1 h of the derivatization steps, clean nail extracts could be obtained with excellent peak shapes, higher response, and shorter analysis time. Moreover, the combination of flophemesyl and the TMS-derivatization of two ionizable positions, such as the hydroxyl and ketone groups, on the steroid enhanced the GC–SIM-MS properties. It enhanced these properties by lowering the LOD and LOQ down to 0.1 and 0.2 ng/g, respectively. An extension of the present method for the study of steroid hormonal-related diseases is underway.

References

[1] F.E. Govier, R.D. McClure, D. Kramer-Levien, J. Urol. 156 (1996) 405.
[2] H.M. Behre, S. Kliesch, E. Leifke, T.M. Link, E. Nieschlag, J. Clin. Endocrinol. Metab. 82 (1997) 2386.
[3] T. Noriko, T. Junko, F. Aya, K. Yoshiharu, T. Toru, F. Shuji, K. Kanji, T. Hisato, A. Nobuyuki, Clin. Chem. 46 (2000) 523.
[4] L.N. Jones et al., J. Invest. Dermatol. 106 (1996) 461.
[5] M.H. Choi, Y.S. Yoo, B.C. Chung, J. Invest. Dermatol. 116 (2001) 57.
[6] M.H. Choi, K.R. Kim, Y.T. Kim, B.C. Chung, Clin. Chem. 47 (2001) 145.
[7] H.A. Shapiro, J. Forensic Med. 14 (1967) 65.
[8] D. Garside, J.D. Ropero-Miller, B.A. Goldberger, W.F. Hamilton, R.M. Maples, J. Forensic Sci. 22 (1998) 974.
[9] D.A. Engelhart, E.S. Lavins, C.A. Sutheimer, J. Anal. Toxicol. 22 (1998) 314.

[10] F.W. Sunderman Jr., Hum. Pathol. 4 (1973) 549.
[11] G. Skopp, L. Pötsch, Ther. Drug Monit. 19 (1997) 386.
[12] A.Y. Finlay, Br. J. Dermatol. 126 (1992) 28.
[13] D.T. Roberts, Dermatology 194 (1997) 37.
[14] D.E. Vance, W.D. Ehmann, W.R. Markesbery, Neurotoxicology 9 (1988) 197.
[15] M.J. Wheeler, Y.-B. Zhong, A.T. Kicman, S.B. Coutts, J. Endocrinol. 159 (1998) R5.
[16] M.H. Choi, B.C. Chung, Analyst 124 (1999) 1297.
[17] D. de Boer, E.G. de Jong, R.A.A. Maes, J.M. van Rossum, J. Steroid Biochem. Mol. Biol. 42 (1992) 411.
[18] W. Schänzer, M. Donike, Anal. Chim. Acta 275 (1993) 23.
[19] T. Kim, M.H. Choi, B.H. Jung, B.C. Chung, Bull. Kor. Chem. Soc. 19 (1998) 194.
[20] V. Ferchaud, B. le Bizec, M.P. Montrade, D. Maume, F. Monteau, F. Andre, J. Chromatogr. B 695 (1997) 269.
[21] M.H. Choi, J.Y. Kim, B.C. Chung, Anal. Lett. 32 (1999) 1313.
[22] M.H. Choi, B.C. Chung, W. Lee, U.C. Lee, Y. Kim, Rapid Commun. Mass Spectrom. 13 (1999) 376.
[23] M.H. Choi, J.Y. Kim, B.C. Chung, Analyst 124 (1999) 675.
[24] E.D. Morgan, C.F. Poole, J. Chromatogr. 89 (1974) 225.
[25] N.P. Lemos, R.A. Anderson, J.R. Robertson, J. Anal. Toxicol. 23 (1999) 147.