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Determination of estrone and 17β-estradiol in human hair by gas chromatography–mass spectrometry

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An efficient procedure is described for the determination of estrone and 17β-estradiol in hair by gas chromatography–mass spectrometry (GC-MS). The method involves alkyloxycarbonylation with isobutyl chloroformate (isoBCF) of phenolic hydroxy groups after alkaline digestion of hair samples. The resulting isobutyloxycarbonyl derivatives of estrone and 17β-estradiol are extracted with hexane and subjected to chlorodifluoroacetyl derivatization in order to protect the remaining alcoholic hydroxy groups. When GC-MS with selected ion monitoring (SIM) was used, the quantitative ions were at *m/z* 270 and 384 in the electron ionization mass spectra for estrone and 17β-estradiol, respectively. The detection limits for SIM of the steroids were 1 and 2 pg, respectively, and the SIM responses were linear with correlation coefficients varying from 0.991 to 0.994 in the concentration range 0.2–4.0 ng g^{-1} for the estrogens studied. The detection of estrone and 17β-estradiol in hair samples was possible in the concentration range of 0.24–1.30 ng g^{-1}. The concentrations of the two estrogens detected were different in male and female hair samples.

1. Introduction

The steroidal compounds estrogens contain an aromatic A ring, and thus have a phenolic character, and are mostly represented by estrone and 17β-estradiol. These are synthesized from the androgenic precursor androstenedione and testosterone by the cytochrome P-450 enzyme aromatase, located primarily in granulosa cells, the adipose stromal cell and the placenta.[1] Their metabolism has been implicated in the risk of hormone-dependent diseases, such as breast cancer,[2,3] and has demonstrated physiological significance.[4]

Plasma and urine are the most useful samples for the identification and quantification of drugs and for the interpretation of data of toxicological significance. The major inconveniences with plasma and urine are rapid fluctuations of the plasma concentration of most hormones and lack of specificity. However, hair as a non-invasive matrix, is easy to collect and offers a wider period of time during which the effects of an exogenous or an endogenous environment could be identified, since hair grows at approximately 1 cm per month.[5]

Recently, we reported the determination of testosterone and 5α-dihydrotestosterone, which is converted from testosterone by 5α-reductase, from human head hair using gas chromatography–mass spectrometry (GC-MS).[6] Therefore, as the cytochrome P-450 aromatase is present in human hair follicles,[7] we carried out investigations to confirm the presence of estrone and 17β-estradiol in human hair.

GC-MS of estrone and 17β-estradiol requires a pure chromatographic signal. However, co-eluting substances often lead to asymmetric chromatographic signals and impure mass spectra of estrone and 17β-estradiol when using polar solvents in the extraction step. To remove many polar substances which co-elute endogenous components, we used an efficient extraction step with hexane after alkyloxycarbonylation (AOC) of phenolic hydroxy groups, as one of the specific extraction methods to make polar compounds extractable into organic solvents.[8,9]

The present study was undertaken to identify estrone and 17β-estradiol in human hair, in the context of the intense current interest in the pathways of steroid synthesis in hair follicles and related tissues.

2. Experimental

2.1. Chemicals

Estrone (1,3,5[10]-estratrien-3-ol-17-one), 17β-estradiol (1,3,5[10]-estratriene-3,17β-diol) and nandrolone (4-estren-17β-ol-3-one) as an internal standard (IS) were purchased from Sigma (St. Louis, MO, USA). The derivatization reagents chlorodifluoroacetic anhydride (CDFAA) and isobutyl chloroformate (isoBCF) were purchased from Acros Organics (Geel, Belgium).

2.2. Preparation of standard solutions

Stock standard solutions of estrone, 17β-estradiol and nandrolone were prepared at concentrations of 100 μg ml^{-1} in methanol. These solutions were used to prepare working standard solutions of various concentrations (1–100 ng ml^{-1}) in methanol.

2.3. Sample preparation and pre-treatment

Human hair samples were obtained by cutting a certain length off the end of the head hair of 10 subjects (five women and five men, aged 28–44 years) while they were having a haircut. The pre-wash procedure was based on the liquid–extraction method that is well-established for sample preparation in hair studies.[10] Briefly, to prevent contamination with estrogens from sweat, sebum, *etc.*, the hair was washed first with ethanol, then water and finally acetone. 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. A 100 pg amount of the IS (10 μl × 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} sodium phosphate buffer (pH 7.0) and 50 μl of isoBCF were added for AOC, along with 5 ml of hexane. The mixture was mechanically shaken for 30 min and centrifuged at 2400 rpm for 5 min, then the organic phase was transferred to a test-tube. The organic layer was evaporated under a stream of nitrogen. The isoBCF-treated residue was further dried in a vacuum desiccator over P_2O_5–KOH for about 30 min, and then subjected to the derivatization as described above.

2.3. Derivatization

CDFAA (20 μl) and hexane (100 μl) were added to the residue and the mixture was allowed to stand at 50 °C for 30 min. After the excess reagent had been evaporated under a stream of nitrogen, 40 μl of hexane were added to the residue. Approximately 2 μl of the isoBOC–CDFA-derivatized sample solution was injected into the GC-MS system.

2.5. Precision and accuracy

Samples for intra- and inter-day assays and for recovery tests were prepared at two different concentrations, such as 10 and 20 ng g^{-1}, using hair samples whose estrone and 17β-estradiol levels had been pre-determined. They were analyzed in a day for intra-day assays and every other day for inter-day assays. Calibration samples were prepared using 1 ml of 1 mol l^{-1} NaOH, added with increasing amounts (0.2 to 4.0 ng ml^{-1}) of estrogen standards in the same manner as described for hair.

2.6. Gas chromatography–mass spectrometry

The GC-MS system (Model 5973 mass-selective detector (MSD) combined with a Model 6890 Plus gas chromatograph, Hewlett-Packard, Avondale, PA, USA) was used in both scan and selected ion monitoring (SIM) modes. The electron energy was 70 eV and the ion source temperature was 230 °C. The gas chromatograph was equipped with a 30 m × 0.25 mm id × 0.25 μm film thickness capillary column coated with cross-linked 5% phenyl–methylsilicone gum phase (DB-5; J & W Scientific, Folsom, CA, USA). 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, 240 °C (held for 3 min); program rate 5 °C min^{-1} to 280 °C and 10 °C min^{-1} to a final temperature of 320 °C, held for 5 min.

2.7. Data acquisition

In the SIM mode, the base ions for estrone and 17β-estradiol and a molecular ion for nandrolone were selected as quantitative ions (*m/z* 270, 384 and 498, respectively). 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 the area ratios matched with derivatized steroid standards (Table 1). A dwell time of 120 ms and a relative electron multiplier (EM) voltage of 400 V higher than that in the scanning mode were chosen for each ion monitored.

2.8. Calculation

Mixed standard solutions in the range 0.1–10.0 ng g^{-1} were prepared for determining the detection limits for the overall procedure. 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. Calibration standards were obtained by spiking suitable amounts of each of these standards into 1 ml of water. Mixtures containing absolute amounts of several concentrations of each steroid were pre-treated, derivatized and analyzed in triplicate. A least-squares regression analysis was performed on the measured peak area ratios against the increasing weight ratios of estrogens to the IS in order to obtain the linearity of SIM responses and to plot calibration curves for the quantitative measurement of estrogens.

3. Results

3.1. GC-MS characteristics

Estrogens have a phenolic hydroxy and one or more polar groups, such as alcoholic hydroxy and/or ketone. In order to stabilize compounds and improve GC properties, the present study was therefore carried out to examine the new derivatization method using the isoBCF and CDFAA. The estrone and 17β-estradiol studied here confirmed the formation of 3-isobutyloxycarbonyl (3-isoBOC) and 3-isoBOC-17β-CDFA derivatives, respectively. In addition, nandrolone, as the IS, formed the 3,17-di-CDFA derivative (Fig. 1). The π-electrons of the conjugated double bonds are displaced towards the electron-attracting C-3 oxygen, and the migratory aptitude of the C-6 hyperconjugated hydrogen is enhanced; therefore, 3,5-diene formation occurred.[11]

Upon reaction with CDFAA at 50 °C in hexane to achieve better GC properties, the underivatized alcoholic hydroxy groups were chlorodifluoroacetylated to *O*-CDFA groups within 30 min, yielding a single derivative for each compound. Similarly to the fragmentation patterns of general derivatives, including a chloride, each derivative except estrone showed an isotope effect pair of M and M + 2 ions. As a result, this allowed the rapid confirmation of compounds possessing alcoholic hydroxyl groups.

The most intense peaks of estrone and 17β-estradiol derivatives were $[M - 100]^+$ ions resulting from the cleavage of isoBOC. This will affect the isoBOC derivative owing to the tendency for expulsion of a dimethylmethyloxycarbonyl radical as the most stable tertiary radical in the EI mode. This stabilized

Table 1 GC-SIM-MS data for estrone and 17β-estradiol as their isoBCF–CDFA derivatives

Estrogen	M_r	RRT[a]	Characteristic ions (*m/z*)[b]	Detection limit/pg	Calibration range/ng g^{-1}	Linearity[c] (r^2)
Estrone	370	1.31	370, <u>270</u>, 213	2	0.2-4.0	0.994
17β-estradiol	484	1.40	484, <u>386</u>, <u>384</u>	1	0.2–4.0	0.991
Nandrolone	498	1.00	<u>498</u>, 369, <u>222</u>	—	—	—

[a] Retention time relative to that of nandrolone as IS (11.32 min). [b] Quantitative ions are underlined. [c] Linearity described by linear correlation coefficients for calibration curves.

charge results in increased fragmentation, which is, however, an advantage for identification when an unknown compound is present. As shown in Fig. 1, the minor ions $[M - 128]^+$ detected in the mass spectra of CDFA-derivatized estrone (*m/z* 242) and 17β-estradiol (*m/z* 356) were derived from the losses of CO_2CF_2Cl from the molecular ions, except for nandrolone, which is derived from the loss of *m/z* 129 (suggesting a hydrogen transfer to the ring system), and cleavage of each ring was found in all spectra.

3.2. Validation of the method

The detection limits of estrone and 17β-estradiol for SIM were 1 and 2 pg on-column, respectively. The GC-SIM-MS responses were linear with correlation coefficients varying from 0.991 to 0.994 in the concentration range 0.2–4.0 ng g^{-1} for the estrogens studied (Table 1).

The precision of the method was assessed by analyses of replicate aliquots of hair specimens spiked with two different concentrations of each of the estrogens. The intra- and inter-day assay variances obtained by GC-MS analyses with SIM are given in Table 2. The recoveries ranged from 84.6 to 98.2% with good, overall precision that appears to be satisfactory for the quantification of estrone and 17β-estradiol in unknown hair samples.

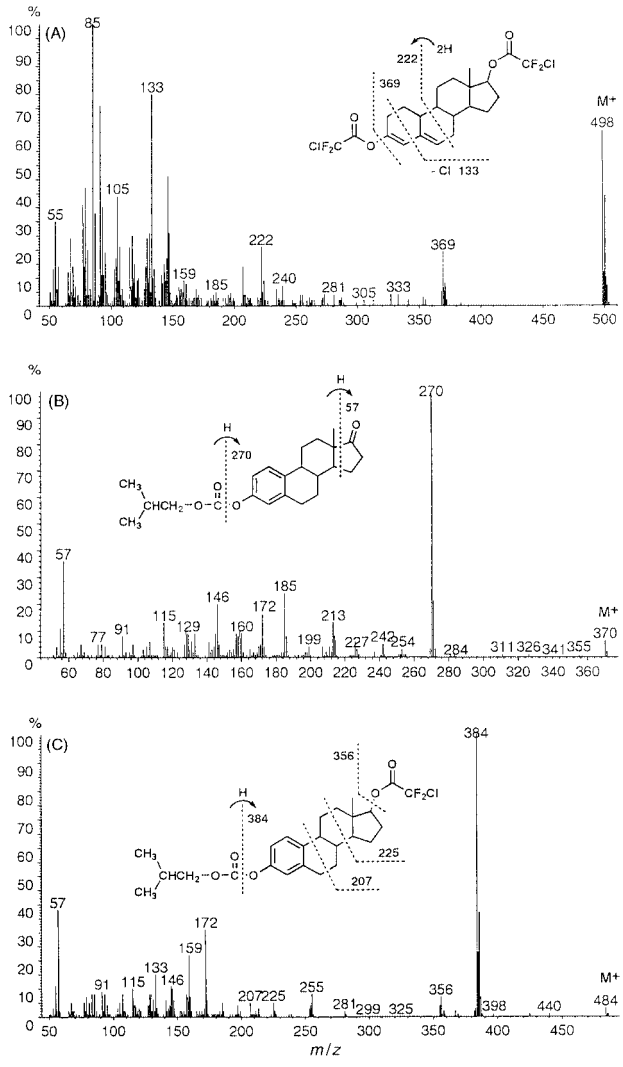

Fig. 1 EI (70 eV) mass spectra of (A) di-CDFA-nandrolone, (B) isoBOC-estrone and (C) isoBOC-CDFA-17β-estradiol.

3.3. Screening of estrogens in human hair

When applied to hair samples from healthy subjects (five women and five men, ages 28–44 years), the present GC-SIM-MS technique provides a sensitive method for the quantification of estrone and 17β-estradiol in 200 mg of hair (Fig. 2). Most of the interfering backgrounds were diminished, and estrone and 17β-estradiol were sensitively detected from the chromatogram with good overall precision and accuracy within the normal concentration range of 0.24–1.30 ng g^{-1}. Each estrogen detected in 10 hair specimens was quantitatively determined (Table 3).

The estrone and 17β-estradiol contents in male scalp hair were about 50% lower than those in female scalp hair. These results agree with those reported by Sawaya,[7] who determined that the cytochrome P-450 aromatase content in women's frontal hair follicles was sometimes greater than that in men's frontal hair follicles.

4. Discussion

We achieved the quantification of estrone and 17β-estradiol in human head hair using GC-MS. Highly sensitive detection methods are required to identify estrone and 17β-estradiol in hair, owing to their low concentrations. Therefore, the correct choice of the appropriate extraction technique is essential in

Table 3 Concentrations of estrone and 17β-estradiol found in hair samples

	Estrone/ng g^{-1}		17β-Estradiol/ng g^{-1}	
Sample No.	Male	Female	Male	Female
1	0.32	0.58	0.24	0.49
2	0.44	0.66	0.31	0.61
3	0.38	0.91	0.41	0.74
4	0.41	1.21	0.29	0.78
5	0.52	1.30	0.33	0.66
Median	0.41	0.91	0.31	0.66
Range	0.32–0.52	0.58–1.30	0.24–0.41	0.49–0.78
Mean ± *s*	0.41 ± 0.07	0.93 ± 0.32	0.32 ± 0.06	0.66 ± 0.11

Table 2 Intra- and inter-day assay tests with a hair spiked with known amounts of estrone and 17β-estradiol[a]

		Intra-day (n = 3)		Inter-day (n = 3)	
Estrogen	Amount added/ ng g^{-1}	Amount found/ng g^{-1}) (mean ± s)[b]	Recovery (%)	Amount found/ ng g^{-1} (mean ± s[a])	Recovery (%)
Estrone	10	10.8 ± 1.5 (13.8)	93.2	10.3 ± 1.2 (11.6)	98.2
	20	19.3 ± 2.4 (12.4)	91.0	21.5 ± 4.4 (20.5)	84.6
17β-Estradiol	10	10.4 ± 0.8 (7.7)	94.6	10.4 ± 1.1 (10.6)	93.6
	20	21.2 ± 3.7 (17.5)	89.8	20.5 ± 1.9 (9.3)	91.8

[a] Analyzed on a DB-5 capillary column (30 m × 0.25 mm id × 0.25 μm film thickness) in the SIM mode with a dwell time of 120 ms and relative EM voltage of 400 V higher than that in the scanning mode. The hair used as the basal level is female sample 3 (see Table 3). [b] s = standard deviation for n = 3; values in parentheses are relative standard deviations (%).

removing the background from the matrix effects. Extraction with hexane after isobutyloxycarbonylation with isoBCF is a very effective method for improving the detection of estrone and 17β-estradiol by removing disturbing polar substances. In contrast to previous studies on related topics,[12,13] the most significant advantage of this technique is that it demonstrates the minimization of the background noise level without an additional purification step, such as solid-phase extraction with Sephadex LH-20 or DEAE-Sephadex. Therefore, the extraction after the AOC with isoBCF of estrogens in aqueous biological samples provides a rapid, simple, and accurate isolation technique with high and reproducible yields.

Under the present AOC conditions, only phenolic hydroxy groups gave isoBOC formation, and did not lead to oxycarbonylation at alcoholic hydroxy groups. Unlike the AOC derivatives of phenolic hydroxyl groups reported in the literature,[14] substitution of *O*-isoBOC by *tert*-butyldimethylsilylation (TBDMS) for a second derivatization was not observed under the present *O*-CDFA derivatization condition.

The choice of the optimum derivatization method is of primary concern not only for GC analysis but also because it plays an essential role concerning sensitivity, and specificity of mass fragmentation. Since the introduction by Clark and Wotiz[15] of perfluoroacylates as steroid derivatives with high electron affinity, considerable interest has been shown in these compounds in many papers.[16–18] For the two estrogens studied,

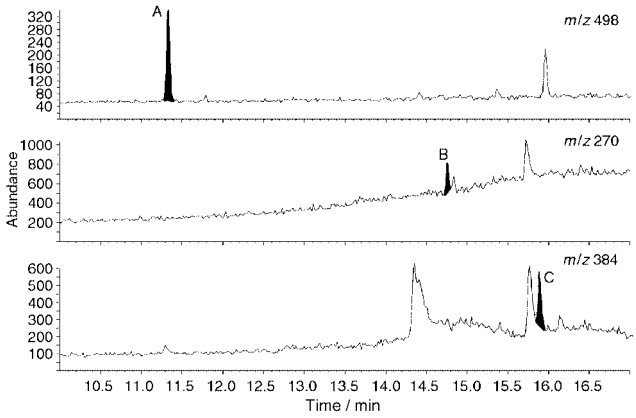

Fig. 2 Extracted ion chromatograms of (A) nandrolone, (B) estrone and (C) 17β-estradiol as their derivatives from a female subject (estrone, 1.21 ng g^{-1}; 17β-estradiol, 0.78 ng g^{-1}) separated on DB-5 fused-silica capillary column (30 m × 0.25 mm id × 0.25 μm film thickness).

mixed isoBOC–CDFA derivatization was excellent for GC-MS assay using an MSD because it gives rise to high mass increments, thus diminishing the background noise. In the present work, the detection of estrone and 17β-estradiol in hair samples was possible in the concentration range 0.24–1.30 ng g^{-1}. On comparing the concentrations of estrone and 17β-estradiol detected in male and female hair, higher concentrations of the two were found in female hair.

We previously reported the measurement of steroids related to androgen biosynthesis, such as pregenolone, dehydroepiandrosterone, testosterone and dihydrotestosterone in human hair.[6] Also, we report here that estrone and 17β-estradiol are converted from the androgens by cytochrome P-450 aromatase. Because our results are so consistent and reliable, this study may be the starting point for further studies in the field of estrogen analysis, and could lead to diverse applications in biomedical research.

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