STEROID ANALYSIS OF HUMAN APOCRINE SECRETION

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Received 2-20-79

ABSTRACT

Analysis of the secretion of the human apocrine gland has shown the presence of dehydroepiandrosterone and androsterone sulfates, two androgen steroids previously identified in axillary sweat. These steroid sulfates were characterized by the gas chromatographic/mass spectrometric analysis of the odorous steroids formed on direct injection of the apocrine secretion into the hot gas chromatographic injector. No spectral evidence was found for the presence of the Δ^{16}-androgen steroids which have axillary-like odors and have also been reported in axillary sweat. Cholesterol was the major steroid component of the secretion.

INTRODUCTION

Bacterial breakdown of apocrine secretion results in the formation of the unique "human axillary odor" [1]. Recent psychological studies have sought to determine what role the axillary odors may play in human olfactory communication [2]. However, no documented behavioral or endocrine changes by volatiles produced in the axillae have been reported to occur in humans. Previous studies have shown that androgen steroids are present in the axillary area. Julesz found androsterone sulfate (AS) and dehydroepiandrosterone sulfate (DHAS) in addition to high levels of cholesterol on thin-layer chromatographic (TLC) analyses of extracts of axillary hairs [3]. Brooksbank demonstrated that, following injections of radioactive pregnenolone or progesterone, steroid secretion was concentrated in the axillary area. By using cotton-wool pads to collect axillary surface materials and analyzing the

extract by TLC and gas chromatography with gas fraction collection, he showed the following steroids to be present: androst-4-ene-3, 17-dione, dehydroepiandrosterone (DHA), and pregnenolone [4]. By extraction and analysis using gas chromatography/mass spectrometry (gc/ms) of collected pads which had been worn in the axillae by 12 men over 5-7 day periods, Brooksbank et al. also showed the presence of 5α-androst-16-en-3α-ol 1 but not 5α-androst-16-en-3-one 2 in the axillary area [5]. In a separate study also using pads followed by extraction and gc/ms analysis, Gower was able to demonstrate the presence of 5α-androst-16-en-3-one in one subject [6]. Claus and Alsing, using radioimmunoassay on pad extracts in thirteen subjects, showed an average secretion rate of 14 ng/hr/armpit for 5α-androst-16-en-3-one [6b]. The latter two Δ^{16}-steroids, particularly 5α-androst-16-en-3-one, have unique axillary-like odors [7]. They are present in boar saliva where they act as sex pheromones, causing estrous sows to lordose when exposed to these compounds [6a, 8].

The 'axillary sweat' collected in these previous studies from the skin surface represents a mixture of materials from the apocrine, eccrine and sebaceous glands in addition to desquamating epidermal cells. The authors cited above [3-6] suggest that the observed steroids originate from the secretion of the apocrine gland. In addition, Karunakaran et al. have reported that gas chromatographic analysis of sebaceous secretions shows the presence of only trace amounts of androgens [9]. In this report we conclusively demonstrate that at least two androgen steroids, AS and DHAS, in addition to cholesterol, are found in the 'pure' apocrine secretion.

Our analyses are based on the observation that heating of sterile,

odorless apocrine secretion to temperatures in excess of 150° produces a strong axillary-odor. This odor is evident on the needle after injection of the neat secretion into the gas chromatograph's hot injector. Analysis of the odor produced by this thermal process led to the identification of AS and DHAS in the secretion.

EXPERIMENTAL

Collection of apocrine secretion: Seven young healthy male adults served as paid volunteers, and informed consent was obtained prior to each experiment. The subjects rested in a supine position to minimize emotionally elicited eccrine sweating. The entire area of the axillary vault was shaved, washed with a nonionic detergent (0.1% Triton X-100), thoroughly rinsed with water, blotted dry and finally washed with hexane. By this procedure desquamated horny cells, most of the cutaneous flora, and skin surface lipids are removed, yielding apocrine secretion with minimal contamination by bacteria, cell debris, and sebum. To stimulate the apocrine glands, 0.1 ml of 1:2000 Adrenalin (in physiologic saline) was intradermally injected. Droplets of apocrine secretion, which emerged from the ductal orifices, were collected with 10µl micropipettes. The micropipettes containing the samples were stored in a freezer prior to analysis. With carefully selected subjects, 1-3µl of secretion could be obtained at each collection.

Gas Chromatography (GC) and GC/MS Analysis: Organic compounds arising from the heating of apocrine secretion were separated and identified by GC and GC/MS. The instrumentation employed was a Perkin-Elmer 990 gas chromatograph interfaced to a Perkin-Elmer Hitachi RMU-6L mass spectrometer by a Watson-Beimann separator [10]. The mass spectrometer conditions included: ionizing voltage at 70ev; source temperature at 200°; and temperature of the GC/MS interface at 260°. The columns employed for separation were the following: a 6 ft. x 2mm pyrex 3% SE-30 on 80/100 Gas Chrom. Q, initial temperature 180° (4 min.), followed by temperature programming to 300° at 8°/min. (condition a); and a 10 ft. x 2mm pyrex 3% XE-60 on 80/100 Gas Chrom. Q, initial temperature 185° (16 min.), followed by temperature programming to 230° at 4°/min. (condition b). Relative gas chromatographic retention times of apocrine odor constituents as well as authentic compounds were calculated using 5α-cholestane as a standard (5α-cholestane 20.6 min., condition b) [6a, 11]. Structural assignments were based upon mass spectral comparisons with authentic samples as well as relative retention times (rrt). Dehydroepiandrosterone (DHA), dehydroepiandrosterone sulfate (DHAS), androsterone sulfate (AS), androsterone, 5α-androst-16-en-3α-ol, and sulfatase were obtained from Sigma Chemical Co. 5α-androst-16-en-3-one was provided by International Flavors and Fragrances, Union Beach, N.J.

Cholesterol levels were quantitatively determined by comparing peak areas with cholesterol standards of similar concentrations.

Heating of Apocrine Secretion and Synthetic Steroid Sulfates: Three

methods were used to produce odor, including: (1) direct injection of the secretion (0.1µl) into the chromatograph; (2) transfer of an aliquot of secretion to a tube containing Tenax (Applied Science) which is connected to the chromatographic injector, flowing nitrogen through the tube to remove water, then rapidly heating the tube to produce the odor; and (3) placing the glass capillaries used for collection of secretion in a stainless steel tube connected to the injector port and heating in a helium stream. The odor generated by these methods were chromatographed using conditions a and b; in addition, similar chromatographic profiles were obtained from each procedure. Procedure 1 was used for most of the analyses.

5α-androst-2-en-17-one 3 and androstadien-17-ones 4 were prepared by pyrolysis of AS and DHAS, respectively, using heating procedure 1 or 2. With procedure 2, the sulfate (1.0mg) was mixed with Tenax (ca. 30mg) and the pyrolysis products condensed on a glass capillary, rinsed with chloroform, and analyzed.

Extraction of Apocrine Secretion: Apocrine secretion was shaken with either hexane or chloroform solvents. The solvent was removed, dried and concentrated for GC/MS analysis.

RESULTS

GC/olfactory analyses of thermally activated apocrine secretion from all subjects showed the presence of at least four compounds which had axillary-like odors (Figures 1, 2). Mass spectral analysis identified the odorants as androgen steroids including 5α-androst-2-en-17-one 3, androsta-3,5-dien-17-one 4a, androsta-2,4-dien-17-one 4b and an isomeric-dien-17-one 4c.

5α-Androst-2-en-17-one (rrt = 0.62, b) shows a base peak at m/z 218 which arises from the presence of the double bond in the C-2 position. This double bond directs a retro Diels-Alder fragmentation of the A ring with charge retention on the remaining steroid moiety [12]. The spectra of 4a (rrt = 0.80, b; Fig. 3) and 4b (rrt = 0.68 b; ref [13]) were very similar with the parent ion of m/z 270 being the most intense peak. The isomer of shortest time 4c (rrt = 0.47 b) possibly androsta-2,5-dien-17-one, has a base peak of m/z 121 which distinguishes it from the other isomers (Fig. 4).

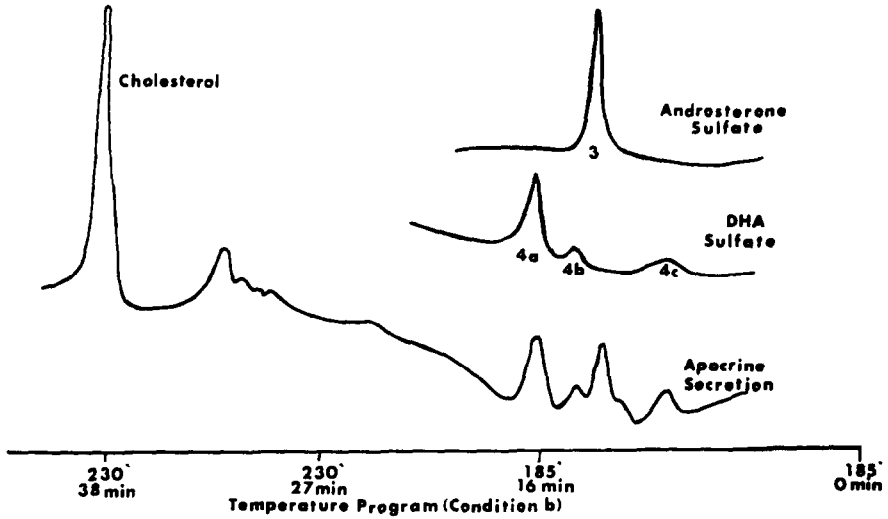

Figure 1 Comparison of Androgen Sulfates and Apocrine Secretion by Direct Injection into Gas Chromatograph

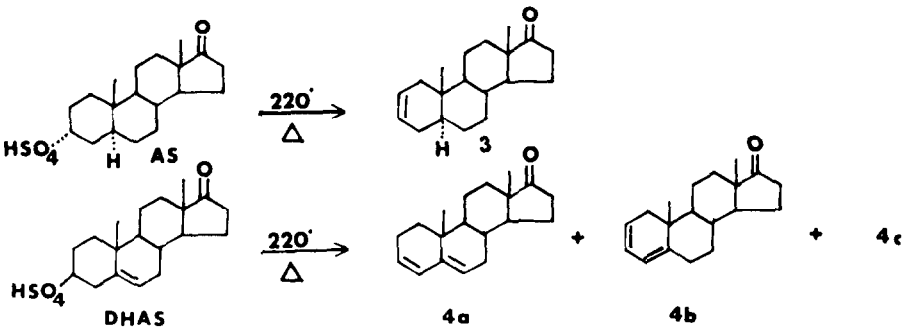

Figure 2 Products Observed from Direct Injection of Steroid Sulfates into Gas Chromatograph

Pyrolysis of DHAS gave three isomeric androstadien-17-ones all identical in retention times (Fig. 1) and mass spectra data (Figs. 3-6) to those found in injected apocrine secretion. Similarly, pyrolysis of AS by direct injection gave only 5α-androst-2-en-17-one 3 (Figs. 1,2). Comparison of chromatographic peak heights of pyrolysis from injection of standard solutions of both DHAS and AS with apocrine secretion gives

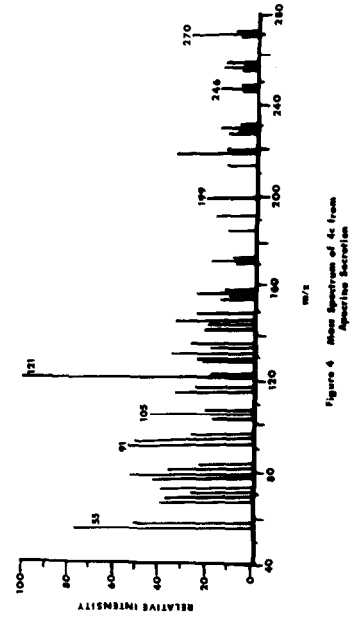

Figure 4 Mass Spectrum of 4c from Aporrhiza Secretion

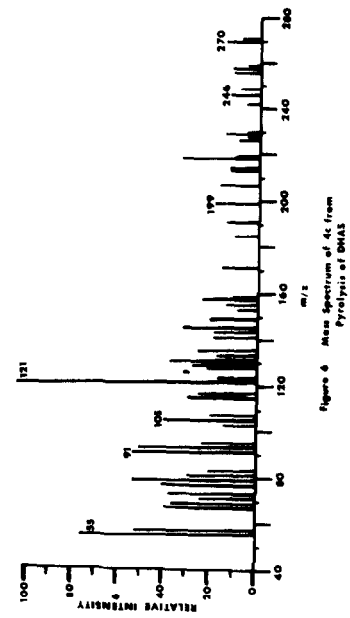

Figure 4 Mass Spectrum of 4c from Pyrolysis of DHAS

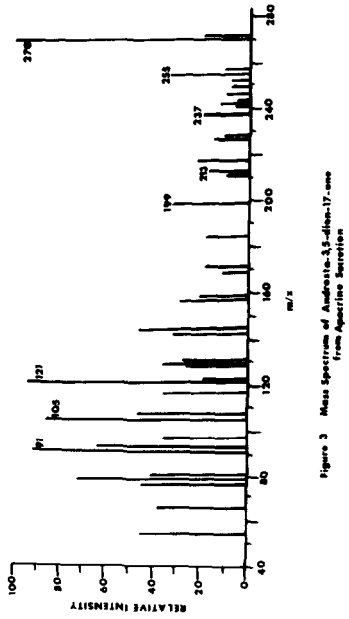

Figure 3 Mass Spectrum of Androsta-3,5-dien-17-one from Aporrhiza Secretion

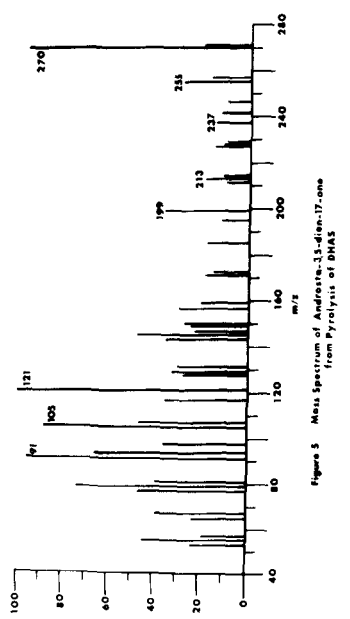

Figure 5 Mass Spectrum of Androsta-3,5-dien-17-one from Pyrolysis of DHAS

concentrations of approximately 0.8μg/μl of secretion (range 0.6 - 1.3) for DHAS and 0.4μg/μl of secretion (range 0.2 - 0.8) for AS. Within the group of subjects studied a consistent chromatographic profile was obtained.

Neither free androsterone or dehydroepiandrosterone could be found in hexane or chloroform extracts of apocrine secretion. Since these steroids are detectable at levels > 0.05μg, this result suggests that they are mainly present in a conjugated form.

In both the direct injection of the apocrine secretion and the extraction experiments the chromatographed constituents were closely examined for the presence of 5α-androst-16-en-3-one and 5α-androst-16-en-3α-ol which have relative retention times of 0.68 and 0.47 respectively (condition b). Although odorous peaks are found at these retention times when apocrine secretion is injected, spectral data failed to confirm the presence of these steroids and indicated only compounds 4b and 4c at these retention times.

The chromatograms from each of the pyrolysis experiments, as well as from chloroform extracts of the secretion, showed that cholesterol was present in apocrine secretion. Quantitative analysis using procedure 1 demonstrated that ca. 1% of the collected secretion, by weight, is cholesterol.

DISCUSSION

We have demonstrated that two androgens, DHA and androsterone, which had been previously found on the axillary skin surface, are secreted as their sulfates by the apocrine gland. In addition, cholesterol is also present in the secretion of this gland.

The steroid sulfates and cholesterol were analyzed by direct

injection of the aqueous natural secretion into the GC. Normal pyrolysis/GC techniques employing rapid heating of samples to 1000° have been used to analyze steroids by the pattern of fragment molecules formed [14]. In our work, the injection port temperature of 250° was sufficient to cause sulfate elimination and introduction of a double bond. This results in a single en-one 3 from AS and three isomeric dien-ones 4 from DHAS.

DHA is the major adrenal androgen and stimulates sebaceous gland activity [15]. Both DHA and androsterone can be metabolized to other more active androgens such as dihydrotestosterone by human axillary skin [16]. In addition, urinary DHA levels have been found to increase in stressed individuals [17]. Apocrine secretion is delivered to the skin surface under conditions of emotional stress [18]. Thus, the release of DHA by a stress-activated gland may be a part of the body's general response to emotional stress. The monitoring of DHA levels in urine and/or in the axillae may have diagnostic value.

In boar testes preparations, the odorous Δ^{16}-androgens are biosynthesized from pregnenolone and progesterone with trace amounts arising from DHA and testosterone in some cases [6a]. A steroid dehydratase system capable of transforming testosterone to androsta-4, 16-dien-3-one has been isolated from cow ovaries [6a]. Whether apocrine secretion or skin bacteria have such an enzyme system available is unknown.

5α-Androst-16-en-3α-ol is found in human urine and its oxidation product, 5α-androst-16-en-3-one, has been designated by Amoore et al. as the primary urinous odor [7]. Both of these steroids have been reported in the human axillae [5, 6]. At a 5α-androst-16-en-3-one

concentration of ca. 0.8ng, which is ca. 100 times below the sensitivity of our gc/ms system for this compound, the odor level would be ca. 400 times above threshold and would have imparted a detectable odor to the collected secretion [7]. Consequently, the apocrine secretion collected by us must contain < 0.002 ng/μl of 5α-androst-16-en-3-one, which is the olfactory threshold in humans. This is in agreement with the report by Kingsbury and Brooksbank that after injection of radioactive 5α-androst-16-en-3-ol or 5α-androst-16-en-3-one neither could be detected at the axillary skin surface [19]. Olfactory and gc/ms studies of both boar fat extracts and spermatic vein plasma have shown 5α-androst-16-en-3-one to be responsible for the boar taint odor [8, 20]. Our preliminary examinations of hexane extracts of apocrine secretion found no indication of free androgen steroids upon chromatographic analysis and no odor on the heated needle after injection. Consequently, the odor from the heated secretion is probably due to the identified pyrolysis products (3, 4a-c) which when heated, yield axillary-like odors similar to 5α-androst-16-en-3-one [21].

The ability to detect 5α-androst-16-en-3-ol and 5α-androst-16-en-one from the skin surface of the axillae [5, 6] and not in freshly collected apocrine secretion suggests that these compounds are formed in the axillae by the action of enzymes or bacteria.

ACKNOWLEDGEMENTS

This research was supported in part by grant number 1 R01 AM22023-01 from the National Institute of Arthritis, Metabolism and Digestive Diseases.

REFERENCES

1. Shehadeh, N. and Kligman, A., J. Invest. Dermatol., 40, 61 (1963)
2. Comfort, A., Pheromones, p. 386-396, ed., M.C. Brich, North-Holland Publishing Co. (1974); Doty, R. Chemical Signals in Vertebrates,

p. 273-286, ed., D. Müller-Schwarze and M. Mozell, Plenum Press, (1977)
3. Julesz, M., Acta Med. Acad. Sci. Hunger., 25, 273 (1968)
4. Brooksbank, B.W.L., Experentia, 26, 1012 (1970)
5. Brooksbank, B.W.L., Brown, R. and Gustafsson, J.A., Experentia, 30, 864 (1974)
6.a. Gower, D.B., J. Ster. Biochem. 3, 45 (1972); b. Claus, R. and Alsing, W., J. Endocr., 68, 483 (1976)
7.a. Amoore, J.E., Pelosi, P. and Forrester, J.L., Chemical Senses and Flavor 2, 401 (1977); b. Amoore, J., Chemical Senses and Flavor 2, 267 (1977)
8. Melrose,D.R., Reed, H.C.B. and Patterson, R.L.S., Brit. Veterin. J., 127, 487 (1971)
9. Karunakaran, M.E., Pochi, P.E., et al., J. Invest. Dermatol., 60, 121 (1973)
10. Watson, J.T. and Biemann, K., Anal. Chem., 37, 844 (1965)
11. Gower,D.B., Harrison, F.A. and Heap, R.B., J. Endocr., 47, 356 (1970)
12. Budzikiewicz, H. and Linscheid, M., Org. Mass Spectrom., 9, 88 (1974)
13. Phillipou,G. and Seamark, R.F., Steroids, 25, 673 (1975)
14. Menzer, F.M., et al., Anal. Chem. 50, 1135 (1978)
15. Strauss, J.S. and Pochi, P.E., Excerpta Medica Int. Congr., 83, 407 (1964)
16. Hay, J.B. and Hodgins, M.B., J. Endocr., 59, 475 (1973)
17. Spiteller, G., Pure Appl. Chem., 50, 205 (1978)
18. Hurley, J. and Shelley, W., The Human Apocrine Gland in Health and Disease, Thomas, Springfield, Illinois, (1960)
19. Kingsbury, A. and Brooksbank, B.W.L., Hormone Res., 9, 254 (1978)
20. Patterson, R.L., J. Sci. Food Agr., 19, 33 (1968)
21. Kloek, J., Psychiat. Neurol. Neurochir., 64, 309 (1961)

Steroid Nomenclature:

androsterone sulfate (AS): 17-oxo-5α-androstan-3α-yl sulfate;
dehydroepiandrosterone sulfate (DHAS): 17-oxo-5-androsten-3β-yl sulfate;
pregnenolone:3β-hydroxy-5-pregnen-20-one
dehydroepiandrosterone: 3β-hydroxy-5-pregnen-17-one
dihydrotestosterone: 17β-hydroxy-5αandrostan-3-one.