STABLE FLUORINATED DERIVATIVES OF ESTRADIOL-17β, ESTRONE AND OTHER HYDROXY-STEROIDS SUITABLE FOR ELECTRON CAPTURE DETECTION - GAS CHROMATOGRAPHY

by

A. Zmigrod (1), Shoshana Ladany and H.R. Lindner

Department of Biodynamics, The Weizmann Institute of Science, Rehovot, Israel.

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ABSTRACT

Pentafluorobenzoyl derivatives of estrone, estradiol-17β and testosterone were prepared and characterized by their melting point, infrared spectrum, fluorine content and R_F values on thin-layer chromatograms. The stability, gas chromatographic behavior and electron affinity of these derivatives were investigated. In contrast to the 3-heptafluorobutyrates of estrone and estradiol, the corresponding pentafluorobenzoates were shown to be stable esters. The electron affinity of the 3-pentafluorobenzoates and the 3-heptafluorobutyrates of estrogens was of the same order. In the 17-position of estradiol and testosterone, introduction of the pentafluorobenzoyl group was markedly more effective in enhancing electron capturing capacity than was heptafluorobutyration. Conditions for pentafluorobenzoylation were defined under which the reaction is specific for phenolic hydroxyl groups. Of the derivatives investigated, estrone pentafluorobenzoate and estradiol-3-pentafluorobenzoate-17-heptafluorobutyrate were found to be most suitable for determination of estrone and estradiol, respectively. A method for the preparation, purification by thin-layer chromatography, and quantitation by electron capture detection -gas chromatography of these two estrogen derivatives on a sub-nanogram scale is described.

INTRODUCTION

The low concentrations of steroid hormones in body fluids, especially in plasma, require extremely sensitive methods for their determination. Recently, several methods for ultra-microdetermination of steroid hormones by gas chromatography have been described, using

halogenated derivatives for electron capture detection. Of the various derivatives investigated, heptafluorobutyrates received particular attention, owing to their high electron affinity (2). A method for measurement of plasma estrogens as their heptafluorobutyrates was published by Wotiz (3). Although this method is very sensitive, its main disadvantage is the instability of phenolic heptafluorobutyrate esters in the presence of moisture or hydroxylic solvents, which renders their purification by thin-layer or paper chromatography impossible (3). Chromatographic purification of the steroid derivative before gas chromatography is most desirable for two reasons: first, because many residual substances accompanying the steroid throughout the isolation procedure react with the halogenated reagent to give electron capturing compounds which may seriously interfere with the detection. Secondly, losses due to incomplete esterification of the steroid or decomposition of the derivative cannot be accounted for, unless the derivative is chromatographically separated from any free steroid that may be present.

The aim of the present study was to find derivatives of phenolic and other hydroxysteroids which are stable enough to be purified by conventional chromatography and yet show electron absorbing ability of the same order as the heptafluorobutyrates. It was expected that pentafluorobenzoates would fulfill both demands: since in esters of pentafluorobenzoic acid the carbonyl group is stabilized by conjugation with the benzene ring, these esters should be less susceptible to hydrolysis than esters of saturated acids, while the presence of five fluorine atoms in the molecule should give rise to high electron affinity.

EXPERIMENTAL

Reagents and Apparatus

Steroids were purchased from Ikapharm, Ramat-Gan, Israel. All solvents were of analytical grade and were redistilled before use. Triethylamine was of analytical grade and was refluxed over KOH and distilled before use. Pentafluorophenylhydrazine was purchased from Peninsular Chemresearch Inc., Gainsville, Florida. Pentafluorobenzoyl chloride was purchased from Aldrich Chemical Co. Inc. and was redistilled under reduced pressure. Heptafluorobutyric anhydride was purchased from Peninsular Chemresearch Inc. It was refluxed over P_2O_5, distilled, and the fraction boiling at 109°C was collected (3).

Thin-layer chromatography was performed on 5 x 20 cm glass plates which were coated with a 0.25 mm layer of silica gel GF 254 (Merck AG, Darmstadt, Germany). The plates were washed by prerunning in methanol, and were then activated at 110° C for one hour. A Packard Radiochromatogram Scanner Model 7201 was used for scanning radioactivity on chromatograms.

Melting points were determined on a Fisher-Johns Apparatus and are corrected. Infrared spectra were obtained on a Perkin-Elmer 237 Grating Spectrophotometer, using potassium bromide micro-pellets.

Gas liquid chromatography.- A Packard 7821 Gas Chromatograph was used with either a Model 811 hydrogen flame ionization detector or a Model 810 ^{63}Ni electron capture detector. Two-meter-long coiled silanized glass columns were used; columns of 4 mm internal diameter in conjunction with the hydrogen flame ionization detector, and 2 mm columns with the electron capture detector. Columns were packed with 1% XE-60, 1% QF-1 or 3% SE-30 on Gas Chrom Q mesh 100/120 (Applied Science Labs. Inc.) and were operated at 200-240°C.

For electron-capture detection, the carrier gas used was argon/methane (90 : 10) at 33 ml/min. (inlet pressure 31 p.s.i.). The gas was purified by sequential passage through activated molecular sieves of types 5A and 13X (Linde Air Products Co.). High inlet pressures and high column temperatures were required to shorten the retention times of the relatively non-volatile pentafluorobenzoates. To preserve gas flow rates compatible with maximal detector response under these conditions, small diameter columns were used. These also minimize bleeding rate and on-column absorption, and hence enhance sensitivity. Pulses of 12 V amplitude and 11 μsec width (Hewlett-Packard Model 212A pulse generator) were applied at 230 μ sec intervals to the detector, giving a standing current of 2.2×10^{-9} A. Detector response was linearly related to mass when the decrease in current induced by passage of the electron-capturing compound did not exceed 3×10^{-10} A. Under the conditions used, this applied over the range of 20-500 pg for those compounds listed in Table II whose molar

response was equal to or higher than that of estrone heptafluorobutyrate. [For larger amounts, the transformation proposed by Wentworth et al. (4) and Simmonds et al. (5) becomes necessary to linearize the response]. Peak areas were measured with a mechanical planimeter. Molar responses were calculated from the linear portion of the curves obtained by plotting peak areas vs. mass.

Detector temperature was varied from 200 - 300°C : for most of the compounds tested 248°C was found to be optimal, but for estradiol-3-methyl ether-17-heptafluorobutyrate, estrone-3-pentafluorobenzoate and testosterone-17-chloroacetate 280°C was optimal and was used for quantitative determinations.

Preparation of Derivatives

Pentafluorobenzoylation of phenolic hydroxyls.- The steroid or steroid derivative (100 mg) was dissolved in 30 ml benzene, 1.5 ml triethylamine and 0.15 ml pentafluorobenzoyl chloride were added, and the mixture was allowed to stand overnight at room temperature in the dark. Subsequently 3 ml of 3N HCl was added, and the mixture was left at room temperature for 30 minutes. The aqueous phase was then removed. The benzene solution was washed once with 1/10 volume of 3N HCl, twice with 1/10 volume 1N Na_2CO_3, twice with 1/10 volume water, and was evaporated. The residue was crystallized three times from acetone-methanol. Under these conditions no significant esterification of non-phenolic hydroxyls occurred.

Pentafluorobenzoylation of 17β-hydroxyls.- Esterification of 17β-hydroxyl groups, which are less acidic than phenolic hydroxyls, required higher concentrations of reactants and longer reaction times. The steroid or steroid derivative (100 mg) was dissolved in 4 ml of a 20% solution of triethylamine in benzene and 0.15 ml pentafluorobenzoyl chloride was added. After standing for 48 hours at room temperature in the dark, 3 ml of 3N HCl and 6 ml benzene were added, and the mixture was kept at room temperature for 30 min. From here on the procedure was similar to that for preparation of phenolic pentafluorobenzoates.

Estrone pentafluorobenzoate was prepared from estrone (3-hydroxy-estra-1, 3, 5(10)-trien-17-one) by the method for pentafluorobenzoylation of phenolic hydroxyls described above. The crystalline product obtained consisted of needles melting at 165-166°C . Fluorine analysis: Calcd. for $C_{25}H_{21}F_5O_3$: 20.47% , found: 20.66% . The infrared spectrum showed two carbonyl absorption bands at 1730 cm^{-1} and at 1747 cm^{-1} , and the OH stretching band at 3360 cm^{-1} - present in the spectrum of estrone - was absent.

Estradiol-3-pentafluorobenzoate was prepared from estradiol (estra-1, 3, 5(10)-triene-3, 17β-diol) by the procedure described for pentafluoro-

benzoylation of phenolic hydroxyls. It crystallized in needles, melting at 127-129°C . Fluorine analysis: calcd. for $C_{25}H_{23}F_5O_3$: 20.38% , found: 20.06% . The infrared spectrum showed an ester carbonyl absorption band at 1752 cm^{-1} and an OH stretching band at 3390 cm^{-1} . The ΔR_{Mr} value (see ref. 6,7 for definition) for pentafluorobenzoate formation from estradiol was 0.44 on an XE-60 gas chromatography column, and was practically identical with the corresponding value for estrone (0.45), but different from the ΔR_{Mr} (0.64) for pentafluorobenzoylation of testosterone (17β-hydroxyandrost-4-en-3-one), thus indicating that the product was estradiol-3-pentafluorobenzoate and not estradiol-17-pentafluorobenzoate. The location of the ester group was further established by conversion to estrone pentafluorobenzoate: an aliquot of the esterification product was oxidized with CrO_3 in dry pyridine (8). The oxidation product was subjected to thin-layer chromatography using system A (Table I), and located on the plate by its ultraviolet light absorption. Its mobility was identical with that of authentic estrone pentafluorobenzoate. Upon elution with ethyl acetate and evaporation of the solvent, a crystalline material was obtained which melted at 160-163°C . Its infrared spectrum and its retention time on an XE-60 column were identical with those of estrone pentafluorobenzoate.

Estradiol-bispentafluorobenzoate was prepared from estradiol by the procedure for pentafluorobenzoylation of 17β-hydroxyl groups described above, except that twice the amount of pentafluorobenzoyl chloride was used. The product melted at 140-141°C . Fluorine analysis: calcd. for $C_{32}H_{22}F_{10}O_4$: 28.79% , found: 29.07% . The infrared spectrum showed two ester carbonyl bands at 1743 cm^{-1} and at 1762 cm^{-1} and no OH stretching band was present.

Estradiol-3 methyl ether-17-pentafluorobenzoate was prepared from estradiol-3-methyl ether by the method for pentafluorobenzoylation of 17β-hydroxyl groups described above. It crystallized in needles, melting at 147-148°C . Fluorine analysis: calcd. for $C_{26}H_{25}F_5O_3$: 19.79% , found: 19.50% . The infrared spectrum showed an ester carbonyl band at 1740 cm^{-1} , and no OH stretching band was present.

Testosterone pentafluorobenzoate was prepared from testosterone by the method for pentafluorobenzoylation of 17β-hydroxyl groups described above. It melted at 169-170°C . Fluorine analysis: calcd. for $C_{26}H_{27}F_5O_3$: 19.70% , found: 18.96% . The infrared spectrum showed an ester carbonyl band at 1735 cm^{-1} , and no OH stretching band was present.

Estradiol-3-methyl ether-17-heptafluorobutyrate was prepared from estradiol-3-methyl ether according to the method of Nakagawa et al. (9) for testosterone heptafluorobutyrate. The product crystallized from methanol in long needles melting at 62-63°C . Fluorine analysis: calcd. for $C_{23}H_{25}F_7O_3$: 27.59% , found: 26.70% . The infrared spectrum showed an ester carbonyl band at 1775 cm^{-1} , and no OH stretching band was present.

Table I.

Thin layer chromatography of fluorinated derivatives of steroids

Compound*	R_F Values			
	System A	System B	System C	System D
Estrone	0.17			
Estrone PFBe	0.53			
Estradiol		0.00	0.00	0.30
Estradiol-3-PFBe				0.59
Estradiol-3-PFBe-17-HFBu		0.44	0.75	
Estradiol-bis-PFBe		0.38	0.51	0.95
Estradiol-3-ME		0.14	0.07	
Estradiol-3-ME-17-PFBe		0.54	0.66	
Testosterone				0.28
Testosterone PFBe				0.69

System A: Benzene/acetone (97:3).
System B: Cyclohexane/acetone (96:4).
System C: Petroleum ether (b.p.40-60°C)/benzene (1:1).
System D: Benzene/acetone (9:1)

* PFBe, pentafluorobenzoate; HFBu, heptafluorobutyrate; ME, methyl ether.

Estradiol-3-pentafluorobenzoate-17-heptafluorobutyrate was prepared from estradiol-3-pentafluorobenzoate according to the method of Nakagawa et al. (9) for testosterone heptafluorobutyrate. It crystallized from acetone-methanol in plates melting at 125-126°C . Fluorine analysis: calcd. for $C_{29}H_{22}F_{12}O_4$: 34.4% , found: 34.6% . The infrared spectrum showed two ester carbonyl bands at 1748 cm^{-1} and at 1778 cm^{-1} and no OH stretching band was present.

Testosterone heptafluorobutyrate was prepared as described by Nakagawa et al. (9). Melting point: 86-88°C [literature (9) 86-88°C]. Fluorine analysis: calcd. for $C_{23}H_{27}F_7O_3$: 27.48%, found: 27.23% . The infrared spectrum was identical with that published by Nakagawa et al. (9).

Estrone-3-methyl ether-17-pentafluorophenylhydrazone was prepared according to the method of Attal et al. (10). The melting point was 188-189°C [literature (10) 189-190°C]. The infrared spectrum was identical with that published by Attal et al. (10).

Testosterone chloroacetate was prepared as described by Brownie et al. (11). Melting point: 123-124°C [literature (11) 124-125°C]. The infrared spectrum was identical with that published by Neudert and Röpke (12).

Each of the derivatives gave a single peak when chromatographed on two different columns (1% XE-60, 1% QF-1 or 3% SE-30) and detected with a hydrogen flame ionization detector.

Estrone heptafluorobutyrate and estradiol-bisheptafluorobutyrate were prepared in nanogram amounts by the method of Wotiz (3).

Micro-preparation of estrone-pentafluorobenzoate and estradiol-3-pentafluorobenzoate-17-heptafluorobutyrate (10^{-10} - 10^{-6} g) was conducted as follows: 50 µl of a 0.1% solution of pentafluorobenzoyl chloride in benzene and 50 µl of a 2% solution of triethylamine in benzene were added to the dry steroid; the latter contained tritiated steroid as tracer. The mixture was left overnight at room temperature in the dark. In the case of estrone, the reaction mixture was then evaporated to dryness under a stream of nitrogen. For preparation of the estradiol derivative, 20 µl of a 10% solution of heptafluorobutyric anhydride in hexane was added at this stage, the mixture was kept at 60°C for 30 minutes, and was then taken to dryness under a stream of nitrogen. The dry residues, containing either estrone pentafluorobenzoate or estradiol-3-pentafluorobenzoate-17-heptafluorobutyrate were chromatographed on thin-layer, using system A and system B (Table I), respectively. Following chromatography, the thin-layer plates were scanned for radioactivity and the radioactive zones were eluted in 5 ml ethyl acetate. The eluates were evaporated, the dry residue was dissolved in a known volume of ethyl acetate, and an aliquot was injected into the gas chromatograph.

RESULTS AND COMMENTS

As anticipated, pentafluorobenzoylation of the phenolic hydroxyls of estrone and estradiol yielded stable esters. These esters, in contrast to the corresponding heptafluorobutyrates (3), can be purified by thin-layer chromatography, and can be prepared in crystalline form. The higher

stability of pentafluorobenzoates of estrone and estradiol was also evident from the fact that neither prolonged contact with 3N HCl (30 minutes at room temperature) nor washing of solutions of the derivatives in organic solvents with 1N Na_2CO_3 resulted in any cleavage of the phenolic ester bond.

The retention times of the pentafluorobenzoates are considerably longer than those of the corresponding heptafluorobutyrates; however, the molar responses they elicit are of the same order or even higher (see Table II).

Another advantage of the pentafluorobenzoates lies in the fact that specificity for phenols can be achieved under appropriate reaction conditions: using a low concentration of triethylamine, under the conditions outlined for micropreparation of the estrogen derivatives, pentafluorobenzoylation of phenolic hydroxyl groups is quantitative, but the less acidic 17-hydroxyl groups do not react. This was demonstrated in several experiments in which tritiated estrone, estradiol, estradiol-3-methyl ether and testosterone were treated individually as described, and the products were chromatographed on thin-layer in parallel with the respective authentic free steroids and pentafluorobenzoates. Scanning of the chromatograms for radioactivity revealed that estrone and estradiol had been quantitatively converted to the corresponding 3-pentafluorobenzoates, whereas estradiol-3-methyl ether and testosterone remained unchanged (less than 3% conversion).

Estrone pentafluorobenzoate and estradiol-3-pentafluorobenzoate-17-heptafluorobutyrate, when prepared on a nanogram or subnanogram scale,

were sufficiently pure for electron capture detection - gas chromatography. Thus, when 100 picogram of estrone or estradiol were converted to these derivatives, chromatographed on thin-layer and a 1/5 aliquot (equivalent to 20 picogram of free steroid), was injected into the gas chromatograph, a clearly defined peak of the derivative was obtained (Fig. 1), while a similarly treated reagent blank showed no peak in the corresponding areas.

Table II shows the relative retention times and relative molar responses of various halogenated steroid derivatives. Estrone pentafluorobenzoate was found to be most satisfactory for quantitation of estrone. Its molar response was 2.6 times higher than that of estrone heptafluorobutyrate and 6.5 times higher than that of estrone-3-methyl ether-17-pentafluorophenylhydrazone, while the preparation of the latter is more time consuming.

Estradiol-3-pentafluorobenzoate, as such, was not used for electron capture detection of estradiol, since it is desirable to esterify the second hydroxyl group, to prevent absorption on the gas chromatography column. On the other hand, estradiol-3, 17-bispentafluorobenzoate had an excessively long retention time, and was therefore not suitable for gas chromatography. As steroid 17-heptafluorobutyrates, in contrast to phenolic heptafluorobutyrates, are stable (13), the gas chromatographic behavior of estradiol-3-pentafluorobenzoate-17-heptafluorobutyrate seemed worth exploring. This derivative had a relatively short retention time, its molar response was equal to that of estradiol-bisheptafluorobutyrate, and it could successfully be prepared in sub-microgram amounts (14).

Table II

Relative retention times and relative molar responses of various steroids and fluorinated derivatives.

Compound*	Relative retention times**			Relative molar response*** at optimal detector temp.
	QF-1 230°C	SE-30 230°C	XE-60 240°C	
Estrone	0.30	0.58	0.74	
Estrone HFBu				1.0
Estrone PFBe	1.90	2.65	2.10	2.6 †
Estrone-3-ME	0.22	0.50	0.31	
Estrone-3-ME-17-PFPh	1.00	3.87	2.02	0.4
Estradiol	0.20	0.61	0.68	
Estradiol-3-PFBe	1.20	2.81	1.88	
Estradiol-bis-PFBe	7.07		8.13	
Estradiol-3-PFBe-17-HFBu	1.30	2.59	1.06	2.5
Estradiol-bis-HFBu				2.6
Estradiol-3-ME	1.47	0.54	0.29	
Estradiol-3-ME-17-PFBe	0.87	2.61	1.14	2.2
Estradiol-3-ME-17-HFBu				0.2 †
Testosterone	0.47	0.66	0.65	
Testosterone PFBe	3.22	3.34	2.89	3.1
Testosterone HFBu				1.2
Testosterone chloroacetate	1.58	1.79	1.87	0.1 †

* PFBe, pentafluorobenzoate; HFBu, heptafluorobutyrate; ME, methyl ether; PFPh, pentafluorphenylhydrazone.

** Retention times relative to progesterone. (Retention times of progesterone were 9.0 min., 7.2 min., and 4.5 min. on QF-1, SE-30 and XE-60 , respectively).

*** Molar responses, relative to estrone heptafluorobutyrate, were calculated from the slopes of the linear portion of the mass/response curves (see Experimental section). The detector response to estrone HFBu was 4.4×10^3 A x sec./mole. Column used was XE-60.

† Detector temperature 280°C ; for all other compounds the optimal temperature was 248°C .

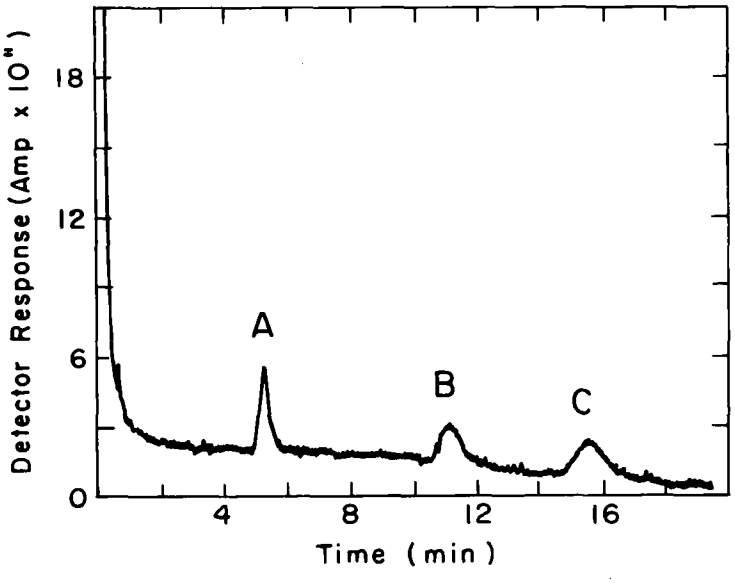

Fig. I. Gas chromatogram of fluorinated derivatives of steroids on XE-60 at 235°C, using an electron capture detector. A, estradiol-3-pentafluorobenzoate-17-heptafluorobutyrate, equivalent to 20 picogram free estradiol; B, estrone-3-pentafluorobenzoate, equivalent to 25 picogram free estrone; C, testosterone-pentafluorobenzoate, equivalent to 28 picogram free testosterone.

Estradiol-3-methyl ether-17-pentafluorobenzoate had an electron affinity similar to both estradiol-3-pentafluorobenzoate-17-heptafluorobutyrate and estradiol-bis-heptafluorobutyrate, but its preparation is somewhat more laborious. Estradiol-3-methyl ether-17-heptafluorobutyrate, although a stable derivative (a 17-heptafluorobutyrate), showed an extremely low electron affinity, much lower than the analogous testosterone heptafluorobutyrate. We can offer no explanation for this apparent dis-

crepancy, but our findings are in accordance with those of Exley (13).

Testosterone pentafluorobenzoate had a 2.6 times higher molar response than testosterone heptafluorobutyrate, and may be the derivative of choice for testosterone assay.

It is intended to utilize the new derivatives, estrone pentafluorobenzoate and estradiol-3-pentafluorobenzoate-17-heptafluorobutyrate, for the development of a sensitive method for estimation of estrone and estradiol in mammalian plasma, employing sequential thin-layer chromatography and electron capture detection - gas chromatography.

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