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STEROID MONO- AND DIHEPTAFLUOROBUTYRATES; PREPARATION, PURIFICATION AND ESTIMATION

J. R. G. CHALLIS AND R. B. HEAP
A.R.C. Institute of Animal Physiology, Babraham, Cambridge (Great Britain)
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SUMMARY

Reaction conditions have been determined for the quantitative formation of steroid mono- and diheptafluorobutyrates with hydroxyl and 3-enol substituents. The percentage conversion at the low concentrations found in peripheral plasma (10^{-10} to 10^{-12} moles) was greater than 90 %. Identification of these esters was established by gas chromatography on different stationary phases and by combined gas chromatography–mass spectrometry. Purification by gel filtration on small columns of Sephadex LH-20 permitted the separation of heptafluorobutyrates of oestrogens and 20β-dihydroprogesterone from the parent compounds without any detectable breakdown. These techniques, which are being employed for the assay of progesterone and oestradiol in peripheral plasma, are discussed in relation to other highly sensitive procedures.

INTRODUCTION

The preparation of steroid heptafluorobutyrates was described by CLARK AND WOTIZ[1] in 1963, and techniques have been developed subsequently for the estimation of steroids as these derivatives using gas–liquid chromatography with an electron capture detector[2–6]. In order to achieve high sensitivity and to assay circulating levels of ovarian steroids, we have studied in some detail the preparation and purification of diheptafluorobutyrate derivatives, which have greater electron-capturing properties than monoheptafluorobutyrates[3,7], pentafluorophenylhydrazones[8] or corresponding chloroacetates[9]. The diheptafluorobutyrates, unlike the monoheptafluorobutyrates, are relatively unstable. Chromatographic purification prior to assay has usually resulted in breakdown of these derivatives[9] but the use of gel filtration on Sephadex LH-20, swollen in organic solvents, has allowed their purification[10]. This paper describes the reaction conditions necessary to prepare high yields of steroid diheptafluorobutyrates compared with those for monoheptafluorobutyrates, the gel filtration of these derivatives on Sephadex LH-20, and their gas chromatographic behaviour on different liquid phases. Molecular weights have been determined by gas chromatography–mass spectrometry.

EXPERIMENTAL

Trivial names of steroids

Testosterone = 17β-hydroxyandrost-4-en-3-one; oestradiol-17β = oestra-1,3,5(10)-triene-3,17β-diol; oestradiol-17α = oestra-1,3,5(10)-triene-3,17α-diol; oestriol = oestra-1,3,5(10)-triene-3,16α,17β-triol; oestradiol-3-methyl ether = 3-methoxyoestra-1,3,5(10)-trien-17β-ol; dehydroepiandrosterone (DHA) = 3β-hydroxyandrost-5-en-17-one; oestrone = 3-hydroxyoestra-1,3,5(10)-trien-17-one; progesterone = pregn-4-ene-3,20-dione; 20α-dihydroprogesterone = 20α-hydroxypregn-4-en-3-one; 20β-dihydroprogesterone = 20β-hydroxypregn-4-en-3-one.

Chemicals

Sephadex LH-20 was obtained from Pharmacia Ltd., Uppsala, Sweden. All solvents were A.R. grade and were normally used within one week of redistillation. Nanograde benzene (Mallinckrodt Co.) was used directly. Heptafluorobutyric anhydride (HFBA) was prepared by refluxing a large excess (2 moles) of phosphorus pentoxide with 1 mole of heptafluorobutyric acid (Koch-Light Ltd.) overnight. A fraction that distilled at 108° was collected under anhydrous conditions, redistilled, and stored at 4° over silica gel where it was stable for several months.

Radioactive steroids

These were obtained from the Radiochemical Centre, Amersham, and had the following specific activities: [7α-^{3}H]progesterone, 8.5 Ci/mmole; [4-^{14}C]progesterone, 58.5 mCi/mmole; [6,7-^{3}H]oestradiol, 31.7 Ci/mmole; [6,7-^{3}H]oestrone, 500 mCi per mmole.

Radiochemical purity was checked at frequent intervals by radiochromatogram scanning after paper chromatography in the following systems: light petroleum–methanol–water (10:9:1) and petroleum ether–benzene–methanol–water (66:33:80:20); only those solutions which revealed a single radioactive peak corresponding to authentic steroid were used.

Labelled 20β-dihydroprogesterone was prepared from [7α-^{3}H]progesterone by conversion with 20β-hydroxysteroid dehydrogenase[11] and purified by paper chromatography[12].

Gas–liquid chromatography

Electron capture detection was performed on a Pye Series 104, Model 74 GLC apparatus, with a ^{63}Ni electron capture detector operated in the pulsed mode with an interval of 500 μsec and using a 1% XE-60 or 1% SE-30 column. The column oven temperature was 195°, and the detector oven temperature 235°. The carrier gas (argon–methane, 90:10) flow was 50 ml/min, and no purge gas was used. Samples applied in hexane to glass sample holders were also analysed using a Pye Series 104 Model 94 gas–liquid chromatograph equipped with an "Auto-Solids" injection system. The column oven was operated at 200°, and the flash heater 20–30° higher; carrier gas (argon–methane, 90:10) flow was 75 ml/min. GLC with flame ionisation detection was carried out on a Model 400 F and M Scientific Corporation gas chromatograph. Stationary phases used were 1% XE-60, 1% SE-30 and 1% QF-1. The 6-ft. columns were operated under the following conditions: column temperature 185–195°, detector

temperature 220°, flash heater temperature 240°; gas flows, argon (carrier) 50 ml/min, hydrogen 40 ml/min, and air 300 ml/min.

Radioactivity

Samples were counted on a Packard Tri-Carb liquid scintillation counter, Model 2003, in scintillation fluid (4 g PPO* + 0.1 g POPOP**/l toluene). The counting efficiency at optimum instrument settings was 38 % for tritium and 80 % for carbon-14. Quench corrections were found to be unnecessary.

Gas chromatography–mass spectrometry

This was performed on an LKB 9000 gas chromatograph–mass spectrometer in the Department of Chemistry, University of Glasgow. Chromatography was on a 1 % XE-60 column operated under conditions similar to those used in our own laboratory, and with 5α-cholestane as reference steroid.

Cleaning of glassware

New glassware was washed in chromic acid, acetic acid (10 %), hot tap water and finally distilled water. Subsequently it was washed in hot water and soaked overnight in 2 % Decon 75 and finally rinsed thoroughly with hot and distilled water and oven dried. In addition, tubes for heptafluorobutyrate esterification were rinsed in freshly double-distilled acetone, and allowed to air dry.

Preparation of steroid heptafluorobutyrates

The steroid solution was evaporated to dryness in small test tubes with ground glass joints under a stream of nitrogen at 53°. The optimal concentrations for the preparation of mono- and diheptafluorobutyrates were investigated by using different amounts of HFBA and benzene. The tubes were heated at 70° for 30 min on an aluminium block, then immediately placed in a desiccator at room temperature for 15 min. A fine stream of nitrogen was used to remove all traces of reagents from the tubes, and samples were taken up in a small volume (50 or 100 μl) of solvent. The percentage conversion of steroids to their heptafluorobutyrates was assessed chromatographically by GLC, and by gel filtration (Sephadex LH-20).

Purification

Sephadex LH-20 was equilibrated for at least 24 h in organic solvents (hexane, ethyl acetate, acetone or mixtures of these) and transferred to glass columns plugged with glass wool. Column dimensions were 200 × 2.5 mm (I.D.), 60 × 5 mm, 180 × 4 mm or 200 × 20 mm.

The effect of bed volume and solvent polarity on steroid and heptafluorobutyrate elution patterns was examined. Samples were applied to columns of known flow rates in three transfers of 100 μl solvent and timed fractions collected. After use, columns were stored at 4° under the appropriate solvent and were suitable for repeated use with negligible changes in flow rates for at least seven months.

Estimation

Heptafluorobutyrates were estimated by gas chromatography with electron

* PPO = 2,5-diphenyloxazole.
** POPOP = 1,4-bis[2-(5-phenyloxazolyl)benzene].

capture detection using a peak height ratio method after the addition of a suitable internal standard[13]. A known amount of labelled steroid added at the beginning of the experiment was used to correct for procedural losses.

RESULTS

Preparation of derivatives

The conditions required for the preparation of micro quantities of the derivatives are given in Table I. The substitution of a non-phenolic hydroxyl group required milder conditions of esterification (HFBA–benzene, 1–5:5–50) than those needed for the formation of an enol monoheptafluorobutyrate or of di- and tri-substituted compounds. The precise duration and temperature of the reaction was not critical and of the various conditions tested, most reproducible results were obtained by heating for 30 min at 70°.

TABLE I

CONDITIONS OF PREPARATION AND GAS CHROMATOGRAPHIC DATA OF STEROID HEPTAFLUOROBUTYRATES

Derivative	*Esterification*[a] *in HFBA (µl)–benzene (µl)*	*RRT*[b] *of steroid HFB*		ΔR_{mr}[c]	
		1% SE-30	*1% XE-60*	*1% SE-30*	*1% XE-60*
Monoheptafluorobutyrates					
(i) Hydroxyl group					
Testosterone	5:5	0.33	1.83	−0.16	−0.68
20β-Dihydroprogesterone	1:50	0.61	5.76	−0.15	−0.24
20α-Dihydroprogesterone	1:50	n.d.[d]	6.71	n.d.	−0.27
Oestradiol-17β	1:50	0.34	4.32	−0.16	−0.39
Oestradiol-17α	1:50	n.d.	3.95	n.d.	−0.41
Oestradiol-3-methyl ether	5:5	0.37	1.18	0.00	−0.42
Dehydroepiandrosterone	1:50	0.28	1.51	−0.08	−0.42
Oestrone	5:5	0.33	1.83	−0.16	−0.68
Pregnenolone	5:5	0.53	n.d.	−0.08	n.d.
(ii) Enol					
Progesterone	25:25	0.60	2.00	n.d.	−0.80
Diheptafluorobutyrates					
(i) Hydroxyl group					
Oestradiol-17α	5:5	n.d.	0.67	n.d.	−0.59
Oestradiol-17β	5:5	0.34	0.79	−0.08	−0.56
(ii) Hydroxyl and enol groups					
Testosterone	5:5	0.34	0.63	−0.07	−0.50
20α-Dihydroprogesterone	10:50	n.d.	0.81	n.d.	−0.59
20β-Dihydroprogesterone	25:25	0.60	0.83	−0.08	−0.55
Triheptafluorobutyrates					
Oestriol	10:5	0.49	1.24	−0.10	−0.33

[a] Reaction carried out at 70° for 30 min.

[b] RRT = relative retention time when 5α-cholestane = 1.0.

[c] $\Delta R_{mr} = \frac{\log r_{HFB} - \log r_{free}}{\text{number of substituted groups}}$, where r = relative retention time.

[d] n.d. = not determined.

The percentage conversion of microgram and submicrogram amounts was assessed by gas–liquid chromatography using flame ionisation detection and Sephadex LH-20 gel filtration. With the reaction conditions given above, greater than 95 % conversion was observed for all steroids except oestriol, which gave a 75 % yield. Mono- and di-substituted steroids were stable for up to six months if kept in acetone at −15°.

Gas chromatography

A better separation of steroid heptafluorobutyrates from the free compound was achieved on 1 % XE-60 columns than on 1 % SE-30 (Table I). The value ΔR_{mr} (see legend, Table I) was calculated and on XE-60 the following order of change was observed: progesterone ($\Delta R_{mr} = -0.80$), oestrone (−0.68), diheptafluorobutyrates of testosterone and the epimers of oestradiol and 20-dihydroprogesterone, monoheptafluorobutyrates of oestradiol and dehydroepiandrosterone (−0.39 to −0.42), the triheptafluorobutyrate of oestriol (−0.33), and the monoheptafluorobutyrates of testosterone and 20-dihydroprogesterone epimers (−0.22 to −0.27). Hence in compounds forming a diheptafluorobutyrate, the ΔR_{mr} values were very similar, the addition of a second group giving a relatively larger increase in ΔR_{mr} than was found in the mono-substituted compounds.

Gas chromatography–mass spectrometry

Definitive identification of steroid heptafluorobutyrates was ascertained by GC–MS, and the *m/e* values for the principal peaks of the spectrum are recorded in Table II. The base peak of all spectra was observed at *m/e* values which corresponded to the calculated parent molecular ion. For oestriol triheptafluorobutyrate (expected M^+, *m/e* 876) an accurate assignment was not possible under the conditions used. Only a small parent peak at *m/e* 484 was observed for DHA monoheptafluorobutyrate, a larger peak occurring at *m/e* 270, and corresponding to $[M-214]^+$, the loss of a heptafluorobutyryloxy residue (mass = 213) and hydrogen at C_8 of the parent molecule. For most steroids, peaks of *m/e* values were observed corresponding to the loss of one, two, or, for oestriol, three heptafluorobutyryloxy moieties.

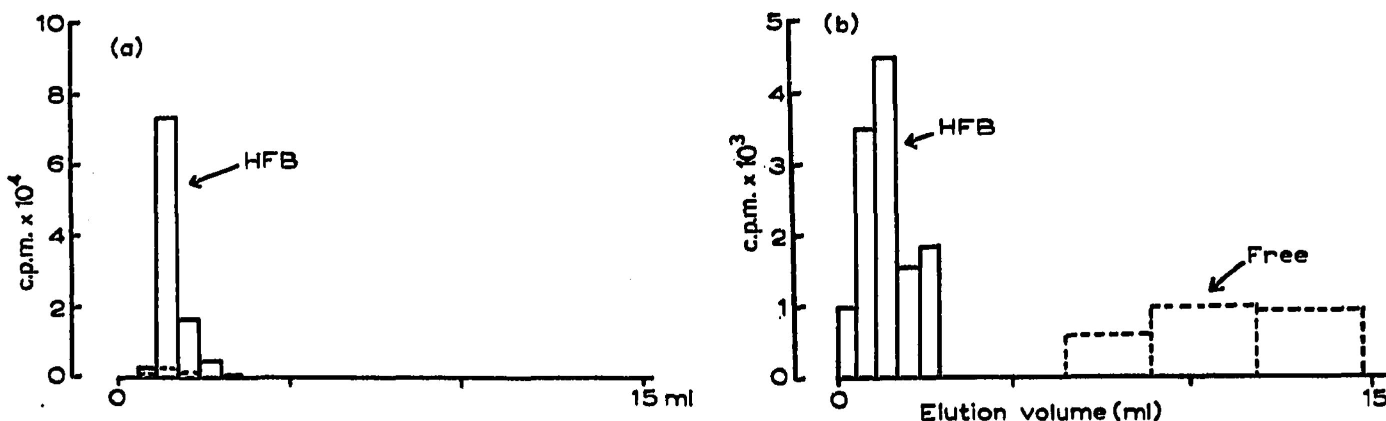

Fig. 1. Oestrogen elution pattern on Sephadex LH-20 swollen in hexane: (a) Oestradiol-17β, (b) oestrone. Column size, 180 × 2.5 mm. Flow rate, 0.62 ml/min. Solid bars, heptafluorobutyrate ester (HFB): dotted bars, parent steroid.

TABLE II

GAS CHROMATOGRAPHY–MASS SPECTROMETRY OF STEROID HEPTAFLUOROBUTYRATES

Derivative	*Formula*	*Molecular weight*	*Mass spectrometry*		*Corresponds to*
			Main peaks (m/e)	*Mass loss*	
Monoheptafluorobutyrates					
(i) Hydroxyl group					
Oestrone	$C_{22}H_{21}O_3F_7$	466	466	—	molecular ion
			253	213	−HFB
Oestradiol-17β	$C_{22}H_{23}O_3F_7$	468	468	—	molecular ion
			254	214	−HFB + H
Oestradiol-3-methyl ether	$C_{23}H_{25}O_3F_7$	482	482	—	molecular ion
			269	213	−HFB
Testosterone	$C_{23}H_{27}O_3F_7$	484	484	—	molecular ion
			399	85	$-C_5H_9O$
Dehydroepiandrosterone	$C_{23}H_{27}O_3F_7$	484	(484)	—	molecular ion (very small)
			270	214	−HFB + H
20β-Dihydroprogesterone	$C_{25}H_{31}O_3F_7$	512	512	—	molecular ion
(ii) Enol					
Progesterone	$C_{25}H_{29}O_3F_7$	510	510	—	molecular ion
			495	15	$-CH_3$
Diheptafluorobutyrates					
(i) Hydroxyl groups					
Oestradiol-17β	$C_{26}H_{22}O_4F_{14}$	664	664	—	molecular ion
			451	213	−HFB
			237	427	−2 HFB + H
Oestriol	$C_{26}H_{22}O_5F_{14}$	680	680	—	molecular ion
			466	214	−HFB + H
(ii) Hydroxyl, enol					
Testosterone	$C_{27}H_{26}O_4F_{14}$	680	680	—	molecular ion
			467	213	−HFB
			253	427	−HFB + H
20β-Dihydroprogesterone	$C_{29}H_{30}O_4F_{14}$	708	708	—	molecular ion
			495	213	−HFB
Triheptafluorobutyrates					
Oestriol	$C_{30}H_{21}O_6F_{21}$	876	850	—	molecular ion
			663	213	−HFB
			450	426	−2 HFB
			237	639	−3 HFB
			234	642	−3 HFB + 3 H

Purification

Mono- and di-substituted compounds were purified and separated from unconverted steroid by gel filtration on Sephadex LH-20. Experiments were carried out with radioactive steroids to establish the elution patterns of free steroids and their separation from heptafluorobutyrate esters (Fig. 1). It was found with Sephadex columns (180 × 2.5 mm) swollen in hexane that nanogram amounts of oestrone and oestradiol heptafluorobutyrates were eluted immediately after the void volume (V_e values: oestrone, 1.5; oestradiol, 1.5; where V_e is elution volume, in ml, to position of maximum of peak). Recoveries of 17β-oestradiol diheptafluorobutyrate and oestrone monoheptafluorobutyrate were greater than 90 % in the first 3 ml while less than 3 %

of the free oestrogens eluted in the same volumes. Although free oestrone (V_e 10.8) was well separated from its heptafluorobutyrate (Fig. 1), free oestradiol, which is sparingly soluble in hexane, was eluted very slowly. On columns swollen in solvents of greater polarity (hexane–ethyl acetate, ethyl acetate, acetone) free oestradiol was more readily eluted, although its separation from the heptafluorobutyrate was less complete. A good separation of free and esterified oestradiol was achieved when the bed volume was increased (180 × 4 mm or 200 × 20 mm) but these columns were not suitable for routine assays.

In contrast, the mono- and diheptafluorobutyrates of progesterone and 20β-dihydroprogesterone, respectively, were less well resolved from the free compounds (Fig. 2). The best separation was obtained on Sephadex columns (180 × 4 mm) swollen in ethyl acetate from which the heptafluorobutyrates were eluted in a relatively discrete fraction (V_e 1.80–1.85) compared with the free compound (V_e 2.5–3.0). Recoveries of known amounts of free steroids and their esters were greater than 85 %.

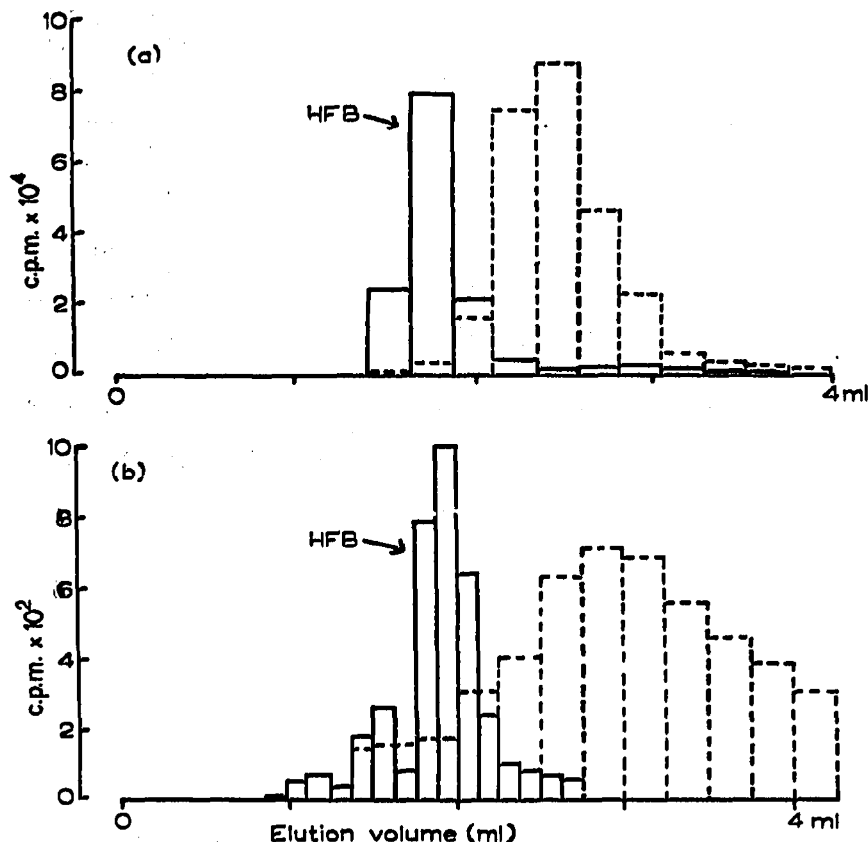

Fig. 2. Progesterone (a) and 20β-dihydroprogesterone (b) elution patterns on Sephadex LH-20 swollen in ethyl acetate. Column size, 180 × 4.0 mm. Flow rate, 0.12 ml/min. Solid bars, heptafluorobutyrate ester (HFB); dotted bars, parent steroid.

The amount of free steroid overlapping into the heptafluorobutyrate fraction was 14.6 % but since in assays at least 90 % of the steroid was usually esterified, the radioactivity derived from any unconverted free steroid would be negligible.

The stability of mono- and diheptafluorobutyrates during the purification

procedure was investigated. When a known amount (12.5 ng) of 17β-oestradiol was esterified, purified through Sephadex LH-20 and estimated by gas chromatography using dehydroepiandrosterone monoheptafluorobutyrate as internal standard, a recovery of 11.8 ± 1.2 ng was obtained. No additional peaks, indicative of breakdown, were observed on the GLC trace.

Estimation

Crystalline DHA and progesterone monoheptafluorobutyrates for use as internal standards were prepared by techniques previously described[9,14]. Purity was confirmed by melting point determinations, and by gas chromatography of high concentrations using flame ionisation and electron capture detectors. The derivatives were stored at −15°, and were stable for at least three months under anhydrous conditions. Solutions in acetone or benzene were stable for up to twelve months.

Calibration lines for progesterone, 20β-dihydroprogesterone and 17β-oestradiol were constructed. After esterification and gel filtration heptafluorobutyrates were taken up in hexane (100 μl) containing an internal standard, DHA heptafluorobutyrate (2 μg/ml) for 17β-oestradiol diheptafluorobutyrate and progesterone monoheptafluorobutyrates, or progesterone heptafluorobutyrate (13 μg/ml) for 20β-dihydroprogesterone diheptafluorobutyrate. There was a linear relationship between peak height ratios (steroid estimated: internal standard) and concentration for amounts up to 1 ng injected onto the column (Fig. 3).

The stability of esterified progesterone and 17β-oestradiol was investigated by

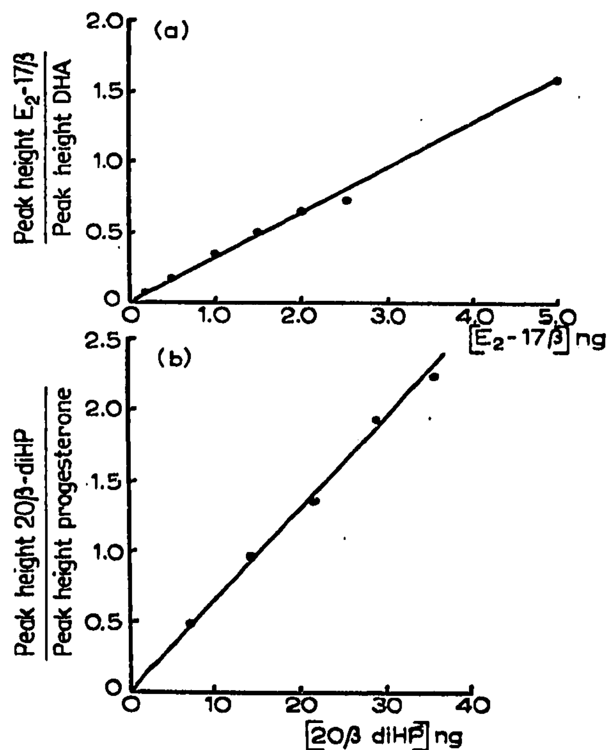

Fig. 3. Calibration lines for (a) oestradiol-17β (E_2-17β) and (b) 20β-dihydroprogesterone (20β-diHP). For GLC conditions, see text.

TABLE III

THE STABILITY OF PROGESTERONE MONOHEPTAFLUOROBUTYRATE

Samples of progesterone (5, 10, 25, 35, 50 ng) were esterified and applied in liquid injections to gas chromatography after taking up in hexane containing dehydroepiandrosterone monoheptafluorobutyrate (2 μg/ml). Day 1 = day of preparation.

Statistic	*Day*			
	1	*2*	*3*	*4*
Coefficient of linear regression, r	0.999	0.999	0.994	0.989
t-Value	79.923	47.973	16.230	11.949
Slope of linear regression, b	0.0321	0.0310	0.0301	0.0280
$S.E._b$	0.0003	0.0006	0.0018	0.0023

repeated assays of the same samples stored at $-15°$ on four consecutive days (Table III). The coefficient of the linear regression was highly significant in all instances, but for progesterone heptafluorobutyrate there was evidence of slow hydrolysis judged by a decrease in slope of the line, and an increased variation. The calibration line for oestradiol 17β-diheptafluorobutyrate was constant for at least four days.

Standard solutions of all three steroids were also measured using a solid injection system for applying samples to the column. After the samples had been applied in hexane to glass "slugs" and allowed to evaporate in air at ambient temperature, repeated chromatography showed that the compounds were stable for at least 24 h in a dry state and in an inert atmosphere (progesterone, coefficient of linear regression for five standards: $r = 0.993 \pm 0.003$, $S.E._b \pm 0.0032$, $n = 3$).

DISCUSSION

The reaction conditions required to form hydroxyl and 3-enol substituted mono- or diheptafluorobutyrates have been determined for several steroids at concentrations expected in peripheral plasma of different species (10^{-10} to 10^{-12} moles). These conditions are similar to those described for the preparation of the two testosterone heptafluorobutyrates[3]. With the exception of DHA monoheptafluorobutyrate, definitive identification of both mono- and di-substituted esters was obtained by GC–MS since the M^+ of the mass spectra was observed at an m/e corresponding with the theoretical molecular ion of the steroid heptafluorobutyrate. The GC–MS results for monosubstituted compounds confirm those of other workers[9].

An objection to the use of the high electron-capturing diheptafluorobutyrates for steroid assays has been the inability to purify these relatively unstable compounds by chromatography[7,9]. Consequently direct assay by gas chromatography may result in a false estimation of tracer recoveries[4]. We have found, however, that by the careful standardisation of anhydrous reaction conditions, high yields of these esters were obtained, and in instances where this was not achieved, gel filtration on Sephadex LH-20 allowed separation of the ester from unconverted steroid[15]. With regard to oestradiol diheptafluorobutyrate, gel filtration aids mainly in the removal of electron capturing impurities before GLC rather than in separation from the free compound, since unesterified steroid has a low solubility in the volume of hexane employed.

Stable crystalline preparations of DHA and progesterone monoheptafluorobutyrates were used as internal standards for GLC, but attempts to crystallise oestradiol diheptafluorobutyrate were unsuccessful. In assays, calibration lines were constructed from different concentrations of oestradiol esterified simultaneously with unknown samples. The slope of these standard lines remained constant for at least four days provided the samples were kept in hexane at −15°. Low concentrations of progesterone monoheptafluorobutyrate or 20β-dihydroprogesterone diheptafluorobutyrate, however, showed a gradual breakdown over a period of four days. It was significant that both mono- and diheptafluorobutyrates were stable for up to 24 h in the inert atmosphere of the loading magazine of the "Autosolid" injection system which should give considerable improvement in sensitivity and rapidity of such GLC assays.

Despite the preparation of derivatives with greater electron capturing properties than even the diheptafluorobutyrates[15,16] far greater sensitivity, necessary for the measurement of some steroids, may be achieved by competitive protein-binding assays[17,18]. Problems such as solvent impurities are common to both types of procedures, but although the technique of electron capture is both more expensive and time-consuming than that of protein binding, it is still essential for those steroids for which a specific receptor protein has not yet been isolated. In addition it is difficult by displacement analysis using radioisotopes to obtain precise estimations of mass in those kinetic experiments where the isolated steroids are already labelled. Moreover, the extensive chromatographic purification and derivative formation required to establish radiochemical purity offsets the advantages of protein-binding techniques.

In our laboratory, assays employing gas chromatography with electron capture detection have been developed for the routine estimation of plasma progesterone, oestradiol-17β and oestrone. Thin-layer chromatography has been used for purification, thereby achieving a 50–60% recovery of added radioactive oestradiol-17β. It is possible to measure this steroid as its 3,17β-diheptafluorobutyrate at concentrations as low as 30 pg/ml plasma.

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