STEROID MONOCHLOROACETATES. PHYSICAL-CHEMICAL CHARACTERISTICS AND USE IN GAS-LIQUID CHROMATOGRAPHY.

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Synthesis and physical-chemical characteristics (melting points, infrared-, visible- and ultraviolet spectra, paper-, thin-layer- and gas-liquid chromatographic behaviour) of monochloroacetate derivatives of steroids representing the androstane-, pregnane-, estrane- and cholestane series are described. The usefulness of these derivatives in gas-liquid chromatography is demonstrated. Through their strong electron absorbing properties the quantitation of nanogram amounts of these derivatives by electron capture detection is possible.

INTRODUCTION

Shortly following the preliminary introduction of the electron capture detector in gas-liquid chromatography[1], the extreme sensitivity of this detector for chlorinated compounds was used in the field of pesticides[2]. The general observation that introduction of halide atoms in non-electron absorbing compounds might produce positive centers for electron attachment[3], prompted LANDOWNE AND LIPSKY[4] to prepare a series of cholesteryl-haloacetates[5]. They demonstrated, that the electron capture detector was far more sensitive towards the chlorinated than the free compounds. The sensitivity of electron capture detection of the halo-acetates was highly increased as compared with the sensitivity of flame and argon ionization detection of the free and halo-acetylated compounds. Whereas chloroacetates in general proved to be more sensitive for electron capture detection than bromo- and fluoro-acetates, the detection of monochloroacetates proved to be more sensitive than detection of the dichloro- and trichloro-acetates. We have been able to confirm this finding in the series of chloroacetates[6].

Methods for the estimation of testosterone[7] and progesterone[8] in human peripheral plasma, based on the quantitation of nanogram amounts of the monochloro-acetate derivatives of testosterone and 20-reduced progesterone have been developed. Since all reactive hydroxyl-groups in steroids might be expected to form chloroacetates equally well[7], the use of this derivative might find general application, allowing the quantitation of nanogram amounts of steroids by electron capture detection following gas-liquid chromatography. To illustrate this principle further, we have prepared the monochloro-acetates of several steroids, representing the androstane, pregnane, estrane and cholestane series.

MATERIALS

Free steroids were obtained from commercial sources; they were recrystallized before use.
Solvents were analar reagents of Britisch Drug Houses Ltd. and redistilled before use.
Monochloroacetic anhydride was obtained from K and K Laboratories, Inc. (Plainview, N.Y.) and kept dry in a desiccator.
Pyridine was refluxed over barium oxide for 4-6 hrs and fractionated through a Vigreux column. The fraction boiling at 115°C was collected and stored in a desiccator.
Tetrahydrofuran (Merck) was refluxed for 3 hrs with potassium hydroxide pellets and subsequently distilled off sodium. It was stored over sodium in a dark brown bottle.
Silica gel GF_{254} (Merck) according to Stahl containing an ultraviolet fluorescent indicator was used for thin-layer chromatography.

SYNTHESIS OF CHLOROACETATES

The preparation of milligram amounts (100-200 mg) of the chloroacetates derivatives was for all steroids first tried according to the methods described [4,7] for the synthesis of cholesterol- and testosterone-chloroacetates.

1 gram chloroacetic anhydride and 0.2 ml pyridine were added to 100-200 mg of the steroid dissolved in 5 ml tetrahydrofuran. After standing overnight in the dark, 5 ml of distilled water were added and the solution was extracted with 5 ml diethyl ether 3 times. The combined ether extracts were washed once with 5 ml 6 N hydrochloric acid and twice with 5 ml distilled water. The was-

hed ether extract was filtered through anhydrous sodium sulphate and taken to dryness.

This method was successful for all steroids tried, except 3β, 17α-dihydroxypregn-5-en-20-one. This steroid did not dissolve sufficiently in tetrahydrofuran and had to be dissolved in chloroform. Reaction with chloroacetic anhydride and pyridine was carried out in the chloroform solution and after addition of water, the chloroform layer separated and was treated as the ether extract.

Recrystallization of the residues was in most cases done from aqeous acetone; in a few cases this was unsuccessful and other solvents had to be used (see: Table 1). If a solution showed a yellow color during recrystallization, this solution was repeatedly treated with activated charcoal (boiled and filtered) until a colorless solution was obtained.

Melting points of the pure steroid chloroacetates are given in Table 1. The melting points of the two steroid chloroacetates, that were previously described (cholesterol-chloroacetate [4,7]: 161-163°C and testosterone-chloroacetate [7,9]: 124-125°C) are indentical with those found in the present investigation.

From some of the steroid chloroacetates the carbon, hydrogen and chlorine contents were estimated [10]. The results presented in Table 2 show a good agreement between the estimated and calculated values.

INFRARED SPECTRA

Infrared spectra were recorded with a Beckman IR-8 grating spectrophotometer. The accuracy of the wavelength calibration (at 2000 cm^{-1}) was approximately 10 cm^{-1}. To obtain a spectrum the steroids were dispersed in potassiumbromide pellets of approximately 1 mm thickness and a diameter of 13 mm. In each case approximately 1.5 mg of the steroid was mixed with 300 mg potassiumbromide.

The spectra of free, acetylated and chloroacetylated testosterone, estrone and pregnanediol (figures 1,2 and 3) represent characteristic examples of the specific spectral changes due to derivative formation.

TABLE 1

MELTING POINTS OF FREE STEROIDS AND STEROID CHLOROACETATES

	melting point (°C)		solvent for recrystallization of Cl-acetate
	free steroid	steroid Cl-acetate	
Estradiol-3-Me-ether	117	117[xx)]	acetone-H_2O
Pregnanolone	148	102	methanol-H_2O
Cholesterol	148	160-161	acetone-H_2O
Dehydroepiandrosterone	149	160-161	,,
Testosterone	151	124-125	,,
20α-OH-pregn-4-en-3-one	167	167[xxx)]	,,
20β-OH-pregn-4-en-3-one	174	166	,,
Estradiol	177	129-130[x)]	hexane-benzene
Androsterone	185	134	acetone-H_2O
Pregnanediol	235	155[x)]	,,
Estrone	260	157-158	,,
3β,17α-di-OH-pregn-5-en-20-one	272	202	methanol

x) di-chloroacetates

xx) mixed melting point with free compound: 95°C

xxx) mixed melting point with free compound: 137°C

The infrared spectra of the acetates showed the well known [11,12,13)] absence of the hydroxyl-bands in the 3 μ and the 8-10 μ regions, and the introduction of the carbonyl absorption at approximately 5.8-5.9 μ due to the introduction of the acetyl-group. In the cases of testosterone- and estrone-acetate these new bands were clearly distinguished from the carbonyl absorption resulting from the keto group in the steroid nucleus. Furthermore, acetylation resulted in the appearance of the more or less characteristic ester bands in the 8 and 10 μ area.

The spectra of the steroid chloroacetates exhibited in general the same characteristic differences from the spectra of

TABLE 2

ELEMENTAL ANALYSIS OF SOME STEROID CHLOROACETATES

Compound		%C	%H	%Cl
Pregnanediol-di-chloroacetate ($C_{25}H_{38}O_4Cl$)	calculated	63.46	8.02	14.99
	found	63.19	7.90	14.84
Estradiol-3-Me-ether-chloroacetate ($C_{21}H_{27}O_3Cl$)	calculated	69.44	7.44	9.78
	found	69.06	7.33	9.65
20β-OH-pregn-4-en-3-one-chloroacetate ($C_{23}H_{33}O_3Cl$)	calculated	70.24	8.40	8.93
	found	71.22	8.60	8.88
Testosterone-chloroacetate ($C_{21}H_{29}O_3Cl$)	calculated	69.06	7.95	9.73
	found	69.42	8.09	9.84
3β,17α-dihydroxy-pregn-5-en-20-one-chloroacetate ($C_{23}H_{33}O_4Cl$)	calculated	67.48	8.07	8.68
	found	67.32	8.32	8.31

the free compounds as the steroid acetates: disappearance of the hydroxyl absorptions and introduction of carbonyl and ester bands. Possibly as a result of the chlorine atom the carbonyl absorption of the chloroacetate group at 5.8 μ was shifted as compared to the carbonyl absorption of the acetate group. In the series of steroids, that we have investigated, we have not been able to detect a general rule for this shift. Going from the acetates to the chloroacetates for testosterone, estrone and pregnanediol, the shift of the carbonyl band for example was from 1735 → 1707, 1762 → 1759 and 1725 → 1752 cm^{-1} respectively.

As seen from the spectra in Figures 1-6, the carbonyl absorption of the chloroacetyl group of the investigated steroids

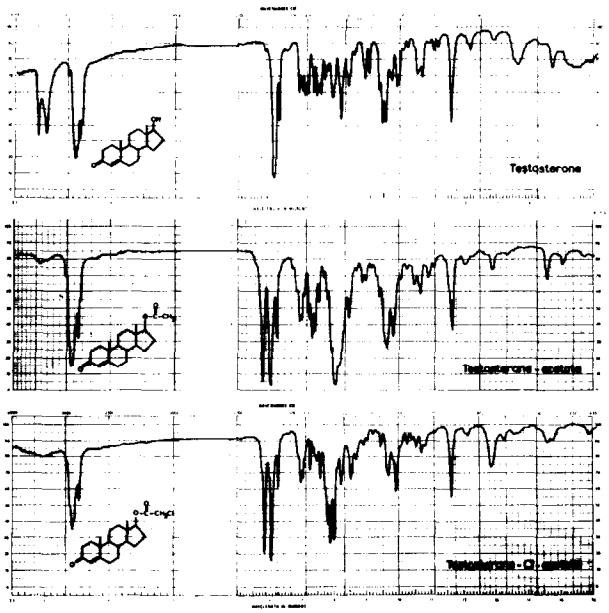

FIGURE 1.

Infrared spectra of Testosterone, Testosterone acetate and Testosterone chloroacetate.

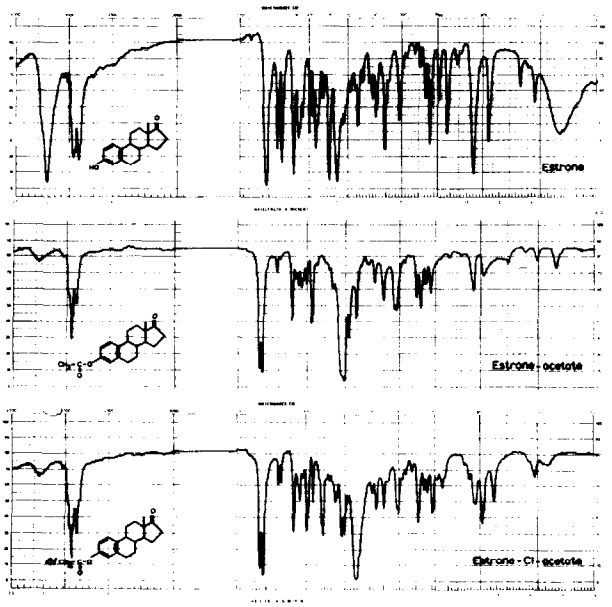

FIGURE 2.

Infrared spectra of Estrone, Estrone acetate and Estrone chloroacetate.

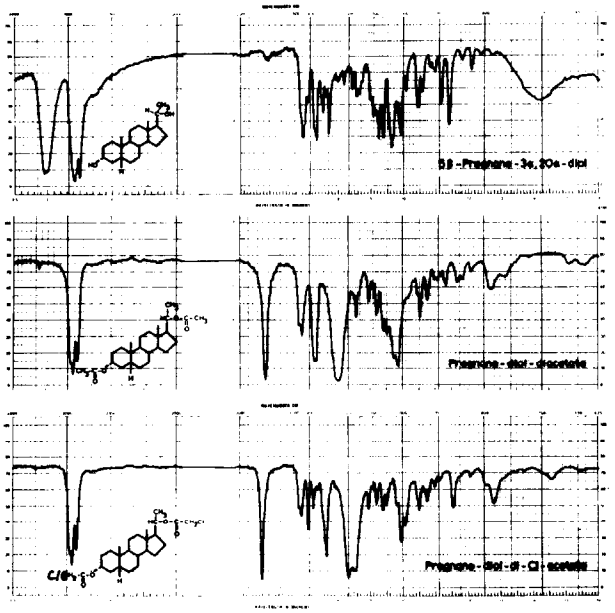

FIGURE 3.
Infrared spectra of Pregnanediol, Pregnanediol diacetate and Pregnanediol di-(chloroacetate).

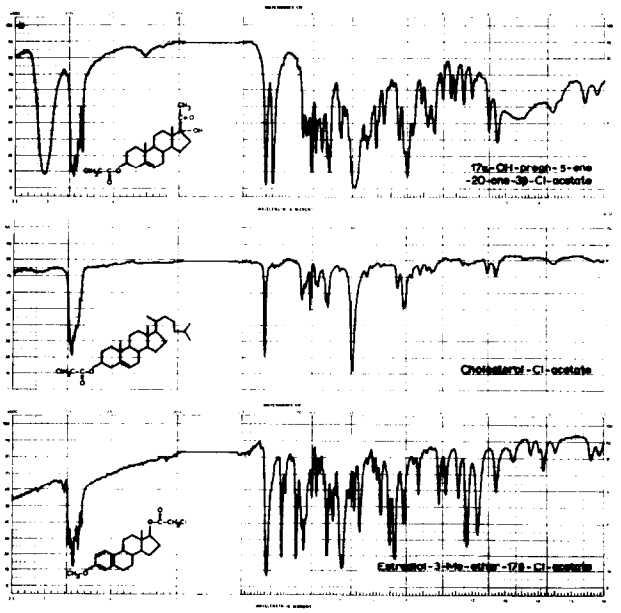

FIGURE 4.
Infrared spectra of 17α-Hydroxypregn-5-en-20-one-3β-chloroacetate, Cholesterol chloroacetate and Estradiol-3-methyl-ether-17β-chloroacetate.

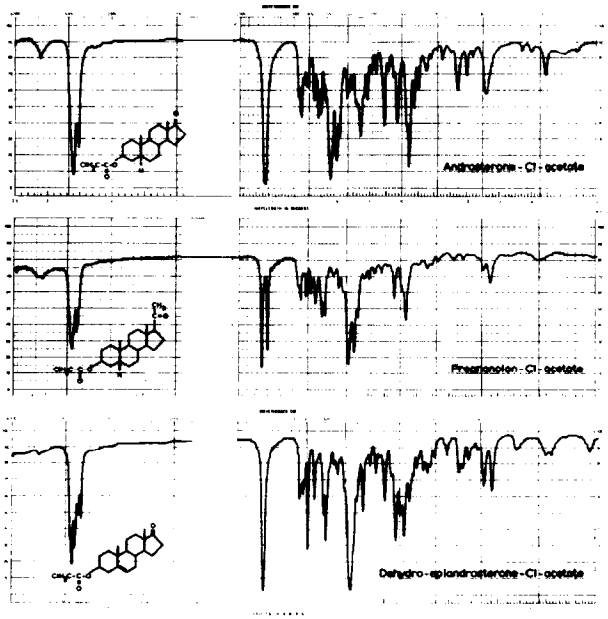

FIGURE 5.
Infrared spectra of Androsterone chloroacetate, Pregnanolone chloroacetate and Dehydroepiandrosterone chloroacetate.

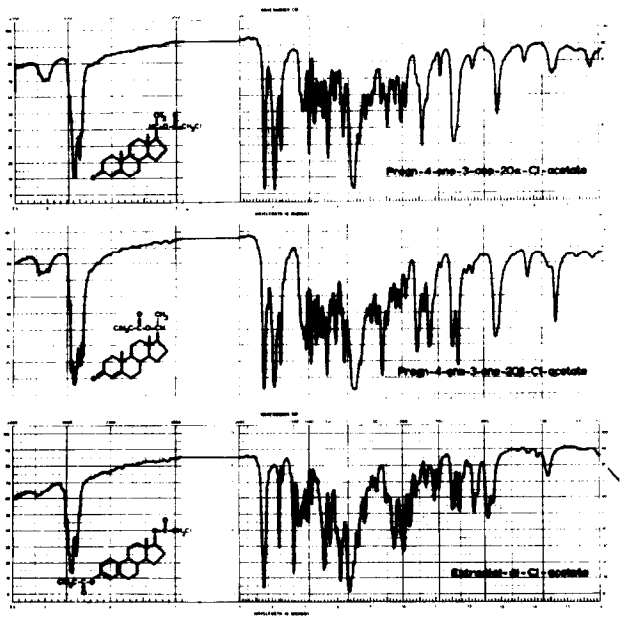

Figure 6.
Infrared spectra of Pregn-4-en-3-one-20α-chloroacetate, Pregn-4-en-3-one-20β-chloroacetate and Estradiol di(-chloroacetate).

fell, with the exception of testosterone chloroacetate, in the range from 1730-1750 cm^{-1}. The differences between the spectra of the steroid acetates and the corresponding steroid chloroacetates must undoubtedly be related to the presence or absence of the chlorine atom. These differences, though rather small and difficult to correlate with specific structural changes, were clear enough to distinguish the acetates from the chloroacetates using proper reference spectra.

The infrared spectra of the chloroacetate derivatives of pregnanediol (Figure 3) and estradiol (Figure 6) clearly showed the absence of any hydroxyl absorption in the 3μ area, indicating that most likely a di-monochloroacetate derivative had been formed. This was confirmed by the results of the elemental analysis (Table 2). The chloroacetyl derivative of 3β,17α-dihydroxypregn-5-en-20-one (Figure 4) still exhibited a hydroxyl absorption. The results of the elemental analysis (Table 2) indicated, that chloroacetylation of this steroid has resulted in the esterification of only one of the hydroxyl groups, that are attached to the steroid nucleus. In analogy with the difficult acetylation of the highly hindered tertiary 17α-hydroxyl group [27], we presume, that the 17α-hydroxyl group is still present and that chloroacetylation has only taken place in the 3β-position.

ULTRAVIOLET AND VISIBLE SPECTRA

The chloroacetate derivatives of steroids had the same spectroscopic properties and gave the same color reactions as the corresponding free steroids and steroid acetates.

The chloroacetates of the ultraviolet absorbing Δ4-3-keto steroids (testosterone, 20α- and 20β-hydroxypregn-4-en-3-one) showed in methanolic solution maximal absorption at 240 mμ. The molercular extinction coefficients were identical to those of the free steroids [14] (20β-hydroxypregn-4-en-3-one-chloroacetate: λ^{max}_{MeOH} = 241 mμ; ε = 16.733; testosterone chloroacetate: λ^{max}_{MeOH} = 241 mμ; ε = 16.144).

Chloroacetates of the 17-ketosteroids and pregnanolone gave the usual color development in the Zimmermann reaction[15].

Pregnanediol di-monochloroacetate gave a yellow color with concentrated sulphuric acid (λ^{max} = 420 mμ) [16]. The chloroacetates of dehydroepiandrosterone and 3β,17α-dihydroxy-pregn-5-en-20-one gave with a 2-1 mixture of concentrated sulphuric acid and ethanol the chromogen characteristic for the steroid Δ^5-3β-OH configuration (λ^{max} = 403 mμ) [17].

Estrogen chloroacetates gave the typical color development in the Kober reaction [18] and the Barton reaction [19].

PAPER AND THIN-LAYER CHROMATOGRAPHY

The results obtained in paper chromatography and thin-layer chromatography clearly demonstrated the low polare character of the chloroacetate derivatives as compared to the free steroids. Using the Bush A system Ligroin-Methanol-Water 100-70-30 and the Methylcyclohexane/Propylenglycol system for paper chromatography on Whatman No.1 paper and mixtures of benzene and ethylacetate as developing solvent for thin-layer chromatography on silica gel plates, it was generally impossible to separate corresponding steroid acetates and steroid chloroacetates when they were chromatographed in a mixture.

GAS-LIQUID CHROMATOGRAPHY

For gas-liquid chromatography an F and M model 400 gas chromatograph equipped with a flame ionization and an electron capture detector was used. Pure dry nitrogen was used as carrier gas (outlet velocity: 75 ml/min). With the electron capture detector a purge gas stream of argon containing 10% methane (outlet velocity: 225 ml/min) was added to the carrier gas stream just before entering the detector. The electron capture detector was operated in a pulsed way; pulses of approximately 50 Volts/cm were applied to the detector every 150 μsec during 0.75 μsec.

Columns of XE-60 and SE-30 on Gas Chrom P (80-100 mesh) were prepared as described by HORNING et al. [20]. For flame ionization detection samples were introduced onto the column dissolved in n.hexane. Using the electron capture detection samples were dissolved for injection in either n.hexane or benzene.

Qualitative behaviour

Retention times of the free steroids, the steroid acetates and the steroid chloroacetates were estimated relative to the retention time of cholestane. Table 3 shows the results obtained with the non-selective SE-30 phase; Table 4 shows the results obtained with the selective XE-60 phase. On both types of stantionary phases the steroid chloroacetates have markedly increased retention times as compared to the free steroids and the steroid acetates. This behaviour is clearly reflected in the Separation factors [21] and the ΔR_{M-r}-values [22] (SE-30 phase: mean $\Delta R_{M\text{-chloroacetylation}} = 0.46$ versus mean $\Delta R_{M\text{-acetylation}} = 0.16$; XE-60 phase: mean $\Delta R_{M\text{-chloroacetylation}} = 0.50$ versus mean $\Delta R_{M\text{-acetylation}} = 0.03$).
The ΔR_{M-r}-values for acetylation on the SE-30 phase are in the same range as those reported by others [22]. On the XE-60 phase the estrone acetate and estradiol diacetate differ from the other steroids acetates in that they have a shorter retention time than the free steroids. The ΔR_{M-r}-values for acetylation as well as for chloroacetylation of estrone and estradiol are strikingly smaller than corresponding values for the other steroids that were investigated.

Sensitivity of detection

Figure 9 illustrates the difference between the sensitivity of electron capture detection and flame ionization detection following chromatography of free testosterone, testosterone acetate and testosterone chloroacetate. Presumably as a result of the presence of the chlorine atom the sensitivity of detection of steroid chloroacetates with the flame ionization detector is generally a little lower, though in the same order of magnitude as the sensitivity of detection of free steroids and steroid-acetates. The sensitivity of the electron capture detection of chloroacetate derivatives, however, is much higher than the sensitivity of detection of the free steroids (Figures 7 and 8) and the steroid acetates (Figure 9).

To obtain an impression about the relative sensitivity of

TABLE 3

GAS CHROMATOGRAPHIC DATA (6 ft 1% SE-30 column)

	Relative[x] retention			Separation		ΔR_{Mr}	
	free	acetate	Cl-acet.	acetate / free	Cl-acet. / free	acetylation	Cl-acetylation
Androsterone	0.36	0.47	1.00	1.31	2.78	0.12	0.44
Dehydroepiandrosterone	0.36	0.53	1.18	1.47	3.28	0.17	0.52
Estradiol-Me-ether	0.40	0.61	1.28	1.53	3.20	0.18	0.50
Estrone	0.44	0.62	1.27	1.41	2.88	0.15	0.46
Estradiol	0.46	1.00	3.80	2.18	8.30	0.17	0.46
Testosterone	0.54	0.80	1.70	1.48	3.15	0.20	0.50
Pregnanolone	0.57	0.90	1.62	1.58	2.84	0.17	0.45
Pregnanediol	0.62	1.36	4.80	2.19	7.65	0.17	0.45
20β-OH-pregn-4-en-3-one	0.98	1.28	2.41	1.29	2.52	0.12	0.39
20α-OH-pregn-4-en-3-one	1.05	1.34	2.65	1.31	2.48	0.11	0.40
Cholesterol	1.92	2.71	5.45	1.41	2.84	0.15	0.45

x) Cholestane = 1
Retention time cholestane 225°C: 3.4 minutes
,, ,, ,, 205°C: 6.8 minutes

TABLE 4

GAS CHROMATOGRAPHIC DATA (3 ft 1% XE-60 column)

	Relative retention[x]			Separation		ΔR_{Mr}	
	free	acetate	Cl-acet.	acetate / free	Cl-acet. / free	acetylation	Cl-acetylation
Estradiol-Me-ether	2.39	2.55	8.3	1.07	3.1	0.03	0.54
Androsterone	2.71	2.96	8.3	1.09	3.4	0.04	0.54
Dehydroepiandrosterone	3.02	3.22	10.9	1.07	3.6	0.03	0.56
Pregnanediol	3.90	5.10	39.0	1.31	8.8	0.06	0.50
Pregnanolone	4.01	4.22	13.0	1.06	3.0	0.03	0.51
Cholesterol	5.10	5.73	16.7	1.12	2.7	0.03	0.52
Testosterone	6.10	6.60	21.4	1.14	3.6	0.06	0.54
Estradiol	6.15	5.61	45.6	0.91	7.4	-0.04	0.44
Estrone	7.50	5.88	17.5	0.79	2.4	-0.10	0.37
20β-OH-pregn-4-en-3-one	7.80	9.88	29.7	1.27	3.7	0.10	0.58
20α-OH-pregn-4-en-3-one	9.22	10.90	33.2	1.18	3.4	0.07	0.56

x) Cholestane = 1
Retention time free cholesterol 220°C: 2.9 minutes
,, ,, ,, ,, 205°C: 7.0 minutes

electron capture detection for the different compounds, we have arbitrarily chosen testosterone chloroacetate as a reference. Solutions of the steroids were chromatographed on a 3 ft 1% XE-60 column and the observed peak areas on the recorder

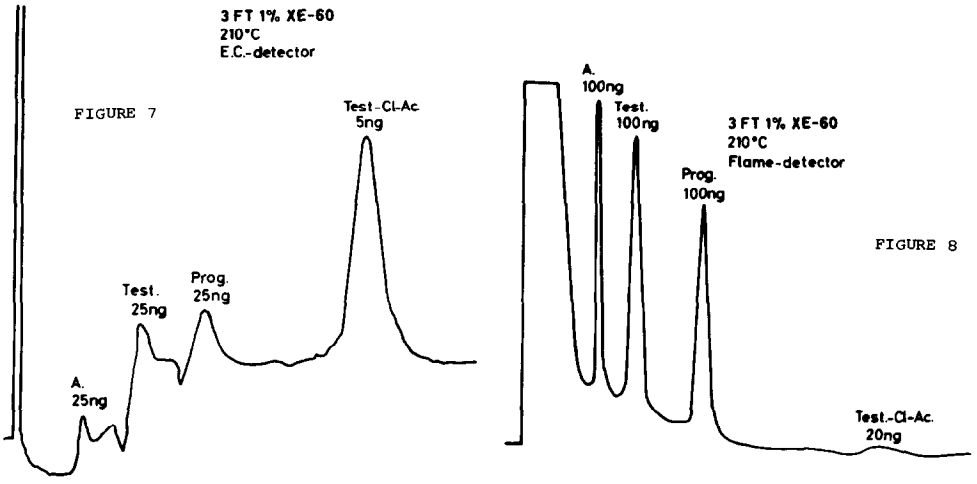

FIGURE 7.

Comparative sensitivity of electron capture detection for Androsterone (A), Testosterone (Test.), Progesterone (Prog.) and Testosterone chloroacetate (Test-Cl-Ac.) following gas-liquid chromatography.
A mixture of 25 nanograms each of androsterone, testosterone and progesterone and 5 nanograms of testosterone chloroacetate was chromatographed on a 3 ft. 1% XE-60 column; the column temperature was kept at 210°C; nitrogen (outlet velocity: 75 ml/min) was used as carrier gas and argon, containing 10% methane (outlet velocity: 225 ml/min) was used as purge gas.

FIGURE 8.

Comparative sensitivity of flame ionization detection for Androsterone (A), Testosterone (Test.), Progesterone (Prog.) and Testosterone chloroacetate (Test-Cl-Acet.).
A mixture of 100 nanograms each of androsterone, testosterone and progesterone and 20 nanograms of testosterone chloroacetate was chromatographed on a 3 ft. 1% XE-60 column; the column temperature was kept at 210°; nitrogen (outlet velocity: 75 ml/min) was used as carrier gas.

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chart were compared with the peak area of simultaneously chromatographed testosterone chloroacetate. Results are presented in Table 5. We wish to emphasize, that in this procedure no corrections were made for losses during chromatography as was done by LOVELOCK et al.[23] in a similar type of experiment. For each compound, however, several estimations were done with amounts varying from 1-100 nanograms. Standard deviations of

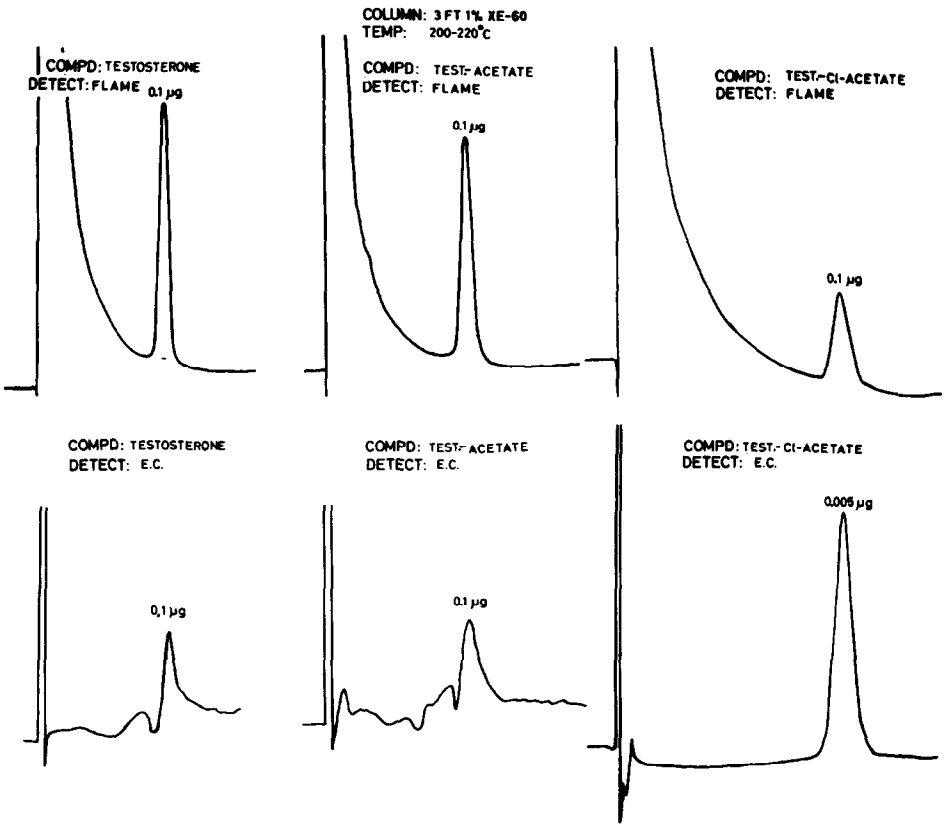

FIGURE 9.

Sensitivity of electron capture and flame ionization detection for Testosterone, Testosterone acetate and Testosterone chloroacetate.
Chromatography was done on a 3 ft. 1% XE-60 column under the conditions described in this paper. The amounts of compounds that were chromatographed are given in the figure.

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the peak areas relative to those of testosterone chloroacetate varied between 3 and 9%. This indicates in our opinion, either that losses of all compounds over a wide range of concentrations were proportional to losses of testosterone chloroacetate, or that very small or no losses occurred.

The absolute sensitivity of the electron capture detector under the described conditions was such, that 1 nanogram of testosterone chloroacetate had a peak area of 2.5 cm^2 at an arbitrary attenuation setting of 1/32 of the maximal sensitivity; the peak to peak noise level at this setting never exceeded 1%

TABLE 5

MOLECULAR ELECTRON ABSORBING ACTIVITIES

	M.W.	E.A.
Estrone Cl-acetate	347	109
20β-OH-pregn-4-en-3-one Cl-acetate	393	102
Testosterone Cl-acetate	365	100
Androsterone Cl-acetate	367	78
20α-OH-pregn-4-en-3-one Cl-acetate	393	61
Pregnanolone Cl-acetate	395	40
Pregnanediol di-(Cl-acetate)	473	38
Dehydroepiandrosterone Cl-acetate	365	37
Estradiol di-(Cl-acetate)	425	35
Estradiol-3-Me-ether Cl-acetate	363	29
Cholesterol Cl-acetate	463	9
Progesterone	314	3
Testosterone	288	1
Androsterone	290	0.5

M.W. = Molecular Weight

E.A. = molecular electron absorbing activity. All values are given in peak areas relative to the peak area of testosterone chloroacetate = 100.

of the possible full scale deflection. For qualitative purposes 0.1 nanogram (10^{-9} grams) could be detected; at an arbitrary attenuation setting of 1/4 of the maximal sensitivity the peak height of 0.1 nanogram was approximately 3 times the peak to peak noise level; the peak to peak noise level at this setting was sometimes, however, as high as 10% of the full scale deflection.

DISCUSSION

The main purpose of the present work was the further investigation of monochloroacetates as stable derivatives of steroids, that could easily be prepared and might allow sensitive detect-

ion using electron capture following gas-liquid chromatography.

The chloroacetate derivatives are as easily prepared in macro amounts (for standardization purposes) as in micro amounts[24].

With the exception of the chloroacetate derivative of 3β,17α-dihydroxypregn-5-en-20-one, the stability of the steroid chloroacetates during gas-liquid chromatography appears to be excellent. Within the temperature range that we have used for gas chromatography of these derivatives (up to 225°C), we have never observed indications of decoposition as judged by the observation of a single symmetrical peak without any appreciable "leading" or "tailing". After several weeks of storage of solutions of steroid chloroacetates in methanol or ethanol at 20°C or 4°C it is, however, often possible to detect traces of the corresponding free steroids in these solutions. We prefer to prepare solutions of steroid chloroacetates in hexane, benzene or tetrahydrofuran; even after several months of storage we have never observed the presence of free steroids in such solutions.

One disadvantage of the use of the steroid chloroacetates in gas-liquid chromatography might be their relative long retention on specific columns like an XE-60 column. This makes it necessary to operate such columns at relatively high temperatures, very close to the highest permissible operating temperature (220°C) of the presently available electron capture detectors. Consequently, we have routinely operated our 3 feet 1% XE-60 columns at 220°C with the electron capture detector at 200°C; over a period of almost two years we have never had any indication of contamination of the detector.

The sensitivity of electron capture detection of the steroid chloroacetates under the conditions described in this paper was good enough to estimate them in the order of 1-10 nanograms (10^{-9}-10^{-8} grams) with adequate precision. Besides in methods for the estimation of plasma testosterone[7] and progesterone[8], the chloroacetate derivatives have in our hands also proved to be very useful for the estimation of several other steroids, that were isolated in small amounts from plasma and other biological sources.[25]

CLARK AND WOTIZ[26] have reported about the use of steroid heptafluorobutyrates for electron capture detection following gas-liquid chromatography. This derivative seems to combine a high volatility with an excellent sensitivity, which will permit the detection of steroids in amounts of 10^{-9}-10^{-10} grams[28].

We have encountered several difficulties with the preparation and application for gas-liquid chromatography of some derivatives that might be expected to capture electrons. For example, the preparation of steroid trichloro- and pentachlorophenylhydrazones on a milligram scale was unsuccessful and the volatility of steroid dinitrophenylhydrazones and thiosemicarbazones is too low to permit gas chromatography at reasonable temperatures (maximum 250°C) within a time (30 minutes) useful for routine analytical purposes[29].

Finally, the usefulness and application of any steroid derivative for quantitative analytical purposes following gas-liquid chromatography may eventually not be limited by detector sensitivity alone. Other factors (adsorption onto glass or columns, contaminations, etc.) inherent with the handling of nanogram or subnanogram amounts of steroids, may well prove to raise more serious problems than detector sensitivity.

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5. The following trivial names and abbreviations have been used throughout this paper:
chloroacetate, monochloroacetate; androsterone, 3α-hydroxy-5α-androstane-17-one; androstenedione, androst-4-ene-3,17-dione; cholestane, 5α-cholestane; cholesterol, cholest-5-en-3β-ol; dehydroepiandrosterone, 3β-hydroxyandrost-5-en-17-one; estradiol, estra-1,3,5(10)-triene-3,17β-diol; estrone, 3-hydroxyestra-1,3,5(10)-trien-17-one; estriol, estra-1,3,5(10)-triene-3,16α,17β-triol; estra-diol-3-Me-ether, 3-methoxyestra-1,3,5(10)-trien-17β-ol; pregnane-diol, 5β-pregnane-3α,20α-diol; pregnanolone, 3α-hydroxy-5β-pregnan-20-one; testosterone, 17β-hydroxyandrost-4-en-3-one; 20α-OH-pregn-4-en-3-one, 20α-hydroxypregn-4-en-3-one; 20β-OH-pregn-4-en-3-one, 20β-hydroxypregn-4-en-3-one; 3β,17α-diOH-pregn-5-en-20-one, 3β,17α-dihydroxypregn-5-en-20-one.

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24. The procedure routinely used in our laboratory for the preparation of chloroacetates of microgram or nanogram amounts of steroids is similar to the method described previously [7]:

Samples are throughly dried in a vacuumdesiccator; to the dried samples are added 0.5 ml of a solution of monochloroacetate anhydride in tetrahydrofuran (10mg/ml) and 0.1 ml pyridine. The reaction is carried out overnight in the dark in a desiccator. On milliliter of water is added and the solution is extracted with 1 ml ethylacetate 3 times. The combined ethylacetate extracts are washed once with 1 ml 6 N hydrochloroic acid and twice with 1 ml distilled water. After evaporation of the ethylacetate, the residue is further proceeded by thin-layer or paper chromatography.

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28. A sample of pure crystalline Testosterone heptafluoro butyrate was kindly provided to us by Dr. Vermeulen (Gent). Amounts in the order of 0.1 nanograms (10^{-10} grams) could be quantitated with good precision. Retention time relative to cholestane on a 3 ft 1% XE-60 column under the conditions described in this paper, column temperature 180°C, was: 2.8.

The Molucular electron absorbing activity relative to testosterone chloroacetate (see: Table 5) was approximately 1500.

29. During the preparation of this manuscript we obtained through the courtesey of Dr. E. Menini (Edenburgh) samples of androst-4-ene-3,6,17-trione and pregn-4-ene-3,

6,20-trione. These compounds, that can be obtained through chromic oxide oxidation of corresponding Δ5,3β-hydroxysteroids, could be detected by electron capture with excellent sensitivity. Amounts in the order of 0.1 - 0.2 nanograms (10^{-10} - 2.10^{-10} grams) could be quantitated.
Retention times (relative to cholestane) on a 3 ft. 1% XE-60 column under the conditions described in this paper were 18.0 for androst-4-ene-3,6,17-trione and 25.0 for pregn-4-ene-3,6,20-trione. Molecular electron absorbing activities relative to testosterone chloroacetate (see: Table 5) were approximately 550 for pregn-4-ene-3,6,20-trione and 500 for androst-4-ene-3,6,17-trione.