DETERMINATION OF TESTOSTERONE BY GAS-LIQUID CHROMATOGRAPHY USING AN ELECTRON CAPTURE DETECTOR

I. RESPONSES OF HALO-ALKYL DERIVATIVES (1)

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INTRODUCTION

The level of testosterone (17β-hydroxy-4-androsten-3-one) in peripheral plasma of normal humans has been reported to vary from 0.02 to 1.0 μg/100 ml. In order to be able to determine plasma testosterone on a sample of convenient volume (10 ml or less), a method which permits quantitation at the nanogram or subnanogram level is necessary. Several methods have already been described; one uses fluorescence techniques (3) and another uses double isotopes labelling procedures (4). Landowne and Lipsky (5) showed that haloacetates of cholesterol give high responses by gas chromatography using an electron capture detector. Van der Molen et al. (6) extended the use of this derivative to other steroids. Brownie et al. (7) applied this procedure for the determination of plasma levels of testosterone using the monochloroacetate. Clark and Wotiz (8) used heptafluorobutyrates for the gas chromatographic-electron capture detection of nanogram level of several steroids including testosterone.

In this study, the responses of several haloalkyl derivatives of testosterone to gas chromatography using the electron capture detector have been compared.

MATERIALS AND METHODS

Reagents and Solvents

Monochloroacetic anhydride (K & K Laboratories, Plainsview, N. Y.)
Monochlorodifluoroacetic anhydride (Pierce Chemical Co., Rockford, Illinois)
Trifluoroacetic anhydride (Eastman Organic Chemicals)
Pentafluoropropionic anhydride (Pierce Chemical Co.)
Heptafluorobutyric anhydride (Peninsular Chemical Research, Gainesville, Fla.)
Heptafluorobutyryl chloride (Peninsular Chemical Research)
Perfluorooctanoyl chloride (Pierce Chemical Co.)
Pyridine, AR (Mallinckrodt Chemical Works)
Benzene, AR (Mallinckrodt Chemical Works)
Testosterone (Nutritional Biochemical Corporation, Cleveland, Ohio, recrystallized)

All anhydrides and chlorides were examined by infrared spectroscopy. All of the anhydrides, except the monochloracetic anhydride and the trifluoroacetic anhydride, were found to contain considerable amounts of the corresponding acids.

Melting points were measured using a Kofler apparatus.

Infrared Spectra were recorded with a Beckman IR-7 Infrared Spectrophotometer using KBr disks.

Molecular weight analyses were performed with a Mechrolab Osmometer Model 301.

Elemental Analyses were done by Schwarzkopf Microanalytical Laboratories, N. Y. or Drs. Weiler and Strauss, Oxford, England.

Preparation of Derivatives:

Testosterone monochloroacetate (I) was prepared according to the method of Landowne and Lipsky (5) for cholesterol monochloroacetate. m.p. 125-126°. Anal. Calcd. for $C_{21}H_{29}O_3Cl$: C, 69.1; H, 8.0. Found: C, 69.2; H, 7.9. Infrared bands at 1730 strong (ester carbonyl), 1665 strong (Δ^4-3-ketone) and 1610 cm^{-1} weak (C=C).

Testosterone heptafluorobutyrate was prepared by reacting testosterone (100 mg) in 100 ml of benzene with 1 ml of heptafluorobutyric anhydride or chloride and 1 ml of pyridine at 60° for 30 minutes. After washing with 5 ml of 6N hydrochloric acid and 50 ml of 2% sodium bicarbonate and evaporation of the solvent, the residue was recrystallized from aqueous methanol goving a product (II). m.p. 86-88°. Anal. Calcd. for $C_{23}H_{27}F_7O_3$: F, 27.5. Found: F, 27.2. Mol. wt. Calcd: 484. Found: 497. Infrared bands at 1773 strong (ester carbonyl), 1679 strong (Δ^4-3-ketone) and 1613 cm^{-1} weak (C=C). When this reaction with the anhydride was carried out without pyridine and in smaller volumes of solvent, recrystallization from methanol gave a product (III). m.p. 90-92°. Anal. Calcd. for $C_{27}H_{26}F_{14}O_4$: F, 39.1. Found: F, 39.2. Mol. wt. Calcd: 680. Found: 668. Infrared bands at 1773 strong (ester carbonyl), 1670 weak and 1637 cm^{-1} weak (C=C). A product, obtained by crystallization from the mother liquor after adding water, was identified as (II).

Testosterone monochlorodifluoroacetate was prepared in a similar manner to give a product (IV). m.p. 100-102°. Anal. Calcd. for $C_{21}H_{27}ClF_2O_3$: C, 62.9; H, 6.8. Found: C, 63.5; H, 6.9. Infrared bands at 1770 strong (ester carbonyl), 1669 strong (Δ^4-3-ketone) and 1614 cm^{-1} weak (C=C). The reaction without pyridine gave, in addition to (IV), a small quantity of a product (V). m.p. 113-115°. Infrared bands at 1783, 1771 strong (ester carbonyl), 1668, 1639 cm^{-1} weak (C=C).

Testosterone trifluoroacetate (VI) was formed similarly, giving a compound, m.p. 108-110°. Anal. Calcd. for $C_{21}H_{27}F_3O_3$: C, 65.6; H, 7.1. Found: C, 65.6; H, 7.3. Infrared bands at 1780 (ester carbonyl), 1669 strong (Δ^4-3-ketone) and 1612 weak (C=C).

Testosterone pentafluoropropionate (VII) was formed similarly to give a product: m.p. 96-99°. Infrared bands at 1770 strong (ester carbonyl), 1669 strong (Δ^4-3-ketone) and 1613 cm^{-1} weak (C=C). The reaction without pyridine gave, in addition to (VII), a product (VIII). m.p. 103-105°. Mol. wt. Calcd. for $C_{25}H_{26}F_{10}O_4$: 580, Found: 580. Infrared bands at 1770 strong (ester carbonyl), 1667, 1631 cm^{-1} weak (C=C).

Testosterone pentadecafluorooctanoate was prepared in a similar manner with the acid chloride, giving a product (IX). m.p. 89-92°. Anal. Calcd. for $C_{27}H_{27}F_{15}O_3$: C, 47.4; H, 4.0. Found: C, 47.9; H, 4.1. Infrared bands at 1777 strong (ester carbonyl), 1672 strong (Δ^4-3-ketone) and 1614 cm^{-1} weak (C=C).

Gas Chromatography:

A F & M Scientific Corporation (Avondale, Pa.) Model 400 series gas chromatograph, equipped with hydrogen flame ionization and electron capture detectors, was used. The 6-foot U-shaped glass column of 1/8 inch internal diameter was packed with 2.0% XE-60 (Applied Science Laboratories, State College, Pa.) on a Gaschrom Q (100-120 mesh, Applied Science Laboratories) and the samples were injected on column. The coating was applied by the filtration technique using 2 g of XE-60 in 100 ml of methylene chloride with 20 g of Gaschrom Q suspended in it. The 2.0% SE-30 column was prepared in a similar manner. The temperature of the column oven was maintained at 200° C, the vaporizer at 260° C and the detector at 205° C. Nitrogen was used as carrier gas at a flow rate of 90 ml/min. No purge gas was used in the detector. The voltage for the electron capture detector was supplied as a pulsating current of interval of 50 μ sec, which was found optimal (9). The recorder was operated at a chart speed of 0.2 inch per minute. The responses were expressed as peak areas, which were calculated as the product of the peak height and the peak width at half the height.

RESULTS

The data on retention times of various derivatives are shown in Table I. Columns 1 and 2 show that the same substance is being detected by the nonspecific and the specific detectors. The molar responses of the above derivatives to electron capture and flame ionization detectors are shown in Table II as the ratios to the responses of the heptafluorobutyrate. Figure 1 shows the calibration

Table I

Relative Retentions

	XE-60 Flame Ionizn	XE-60 Electron Capture	SE-30 Flame Ionizn
Monochloroacetate (I)	6.89	6.90	3.10
Monochlorodifluoroacetate (IV)	2.25	2.25	1.56
Trifluoroacetate (VI)	1.08	1.08	0.82
Pentafluoropropionate (VII)	1.01	1.01	0.90
Heptafluorobutyrate (II)	1.00*	1.00	1.00**
Pentadecafluorooctanoate (IX)	1.22	1.22	1.78
Di-monochlorodifluoroacetate (V)	0.49	--	1.18
Di-pentafluoropropionate (VIII)	0.18	--	0.55
Di-heptafluorobutyrate (III)	0.19	0.19	0.70

*Ratio to cholestane = 3.10 (cholestane = 10 min.)

**Ratio to cholestane = 0.51 (cholestane = 20 min.)

Table II

Relative Responses

	2.0% XE-60 Flame Ionization	2.0% XE-60 Electron Capture
Monochloroacetate (I)	0.99	0.20
Monochlorodifluoroacetate (IV)	1.02	1.41
Trifluoroacetate (VI)	1.00	0.01
Pentafluoropropionate (VII)	1.04	0.28
Heptafluorobutyrate (II)	1.00*	1.00**
Perfluorooctanoate (IX)	1.08	2.10
Di-heptafluorobutyrate (III)	1.11	2.30

*2.7 coulombs/mole

**1.18×10^3 coulombs/mole

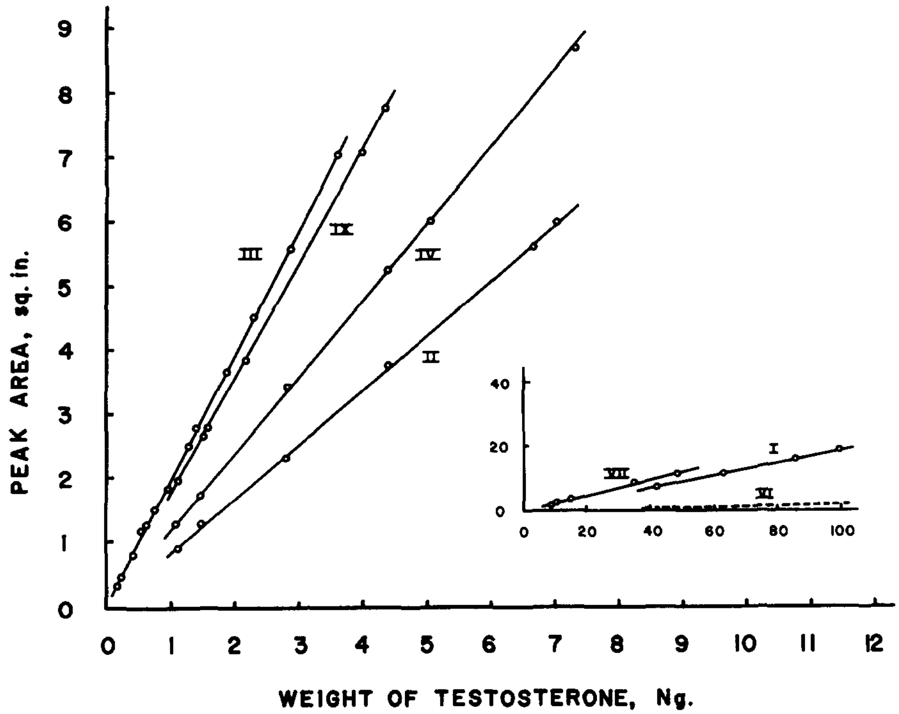

Figure 1

Calibration curves of testosterone halo-alkyl derivatives for the electron capture detector (1 sq. inch = 8 x 10^{-11} ampere minute).

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curves of these compounds. In Figure 2, a chromatogram of a mixture of 4 principal derivatives is shown. Separate injections of each compound gave single peaks in each case identical to those shown in Fig. 2. Figures 3 and 4 show the infrared spectra of the four most sensitive testosterone derivatives.

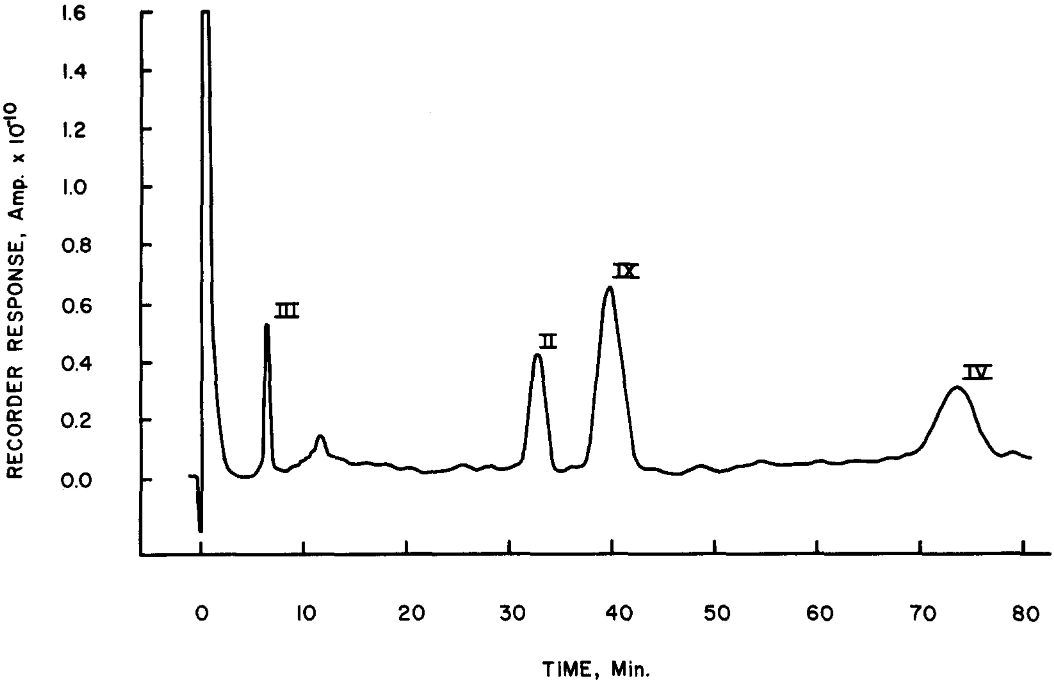

Figure 2

Chromatogram of a mixture of four testosterone halo-alkyl derivatives on 2.0% XE-60: III. Diheptafluorobutyrate, 0.17 ng T: II. heptafluorobutyrate, 1.08 ng T: XI. perfluoro-octanoate, 1.08 ng T: IV. monochlorodifluoroacetate, 1.08 ng T. (T = Testosterone).

DISCUSSION

The retention time of mono-heptafluorobutyrate (monoperfluorobutyrate) (II) shown in Table I does not coincide with that reported by Clark and Wotiz (8). It was found, however, that under some reaction conditions, the reaction with monochlorodifluoroacetic, pentafluoropropionic or heptafluorobutyric anhydride leads to the formation of two products. In each of these three cases, one of the products is thought to be the 3-enol-17-diester as shown by its lack of a conjugated ketone infrared band: the other product is believed to be the 17-ester as

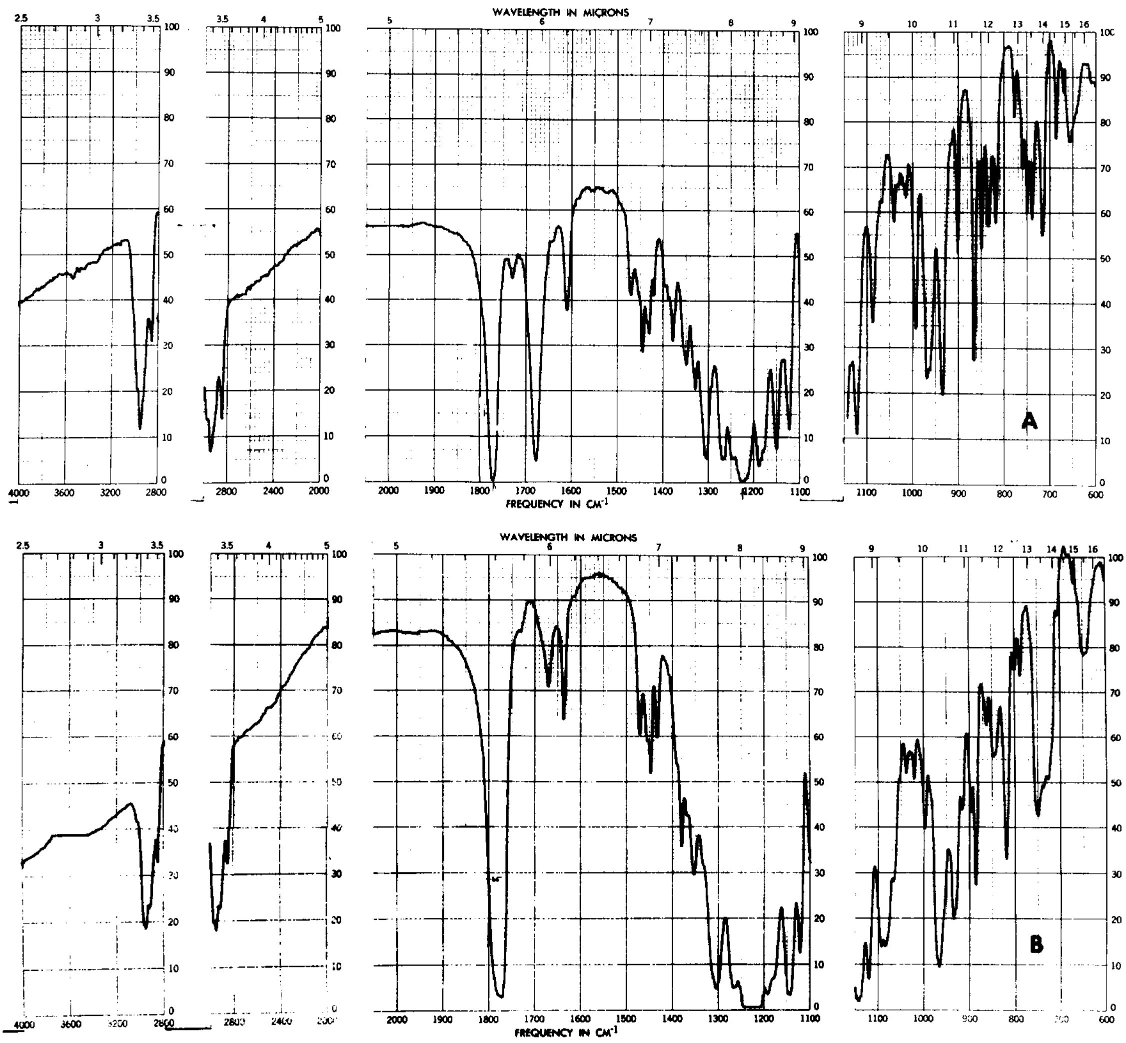

Figure 3

Infrared spectra of testosterone heptafluorobutyrate (A). Testosterone diheptafluorobutyrate (B).

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shown by the presence of a conjugated ketone infrared band. In some of the derivatives, the evidence for their structure was obtained also by elemental analyses and molecular weight determinations. From

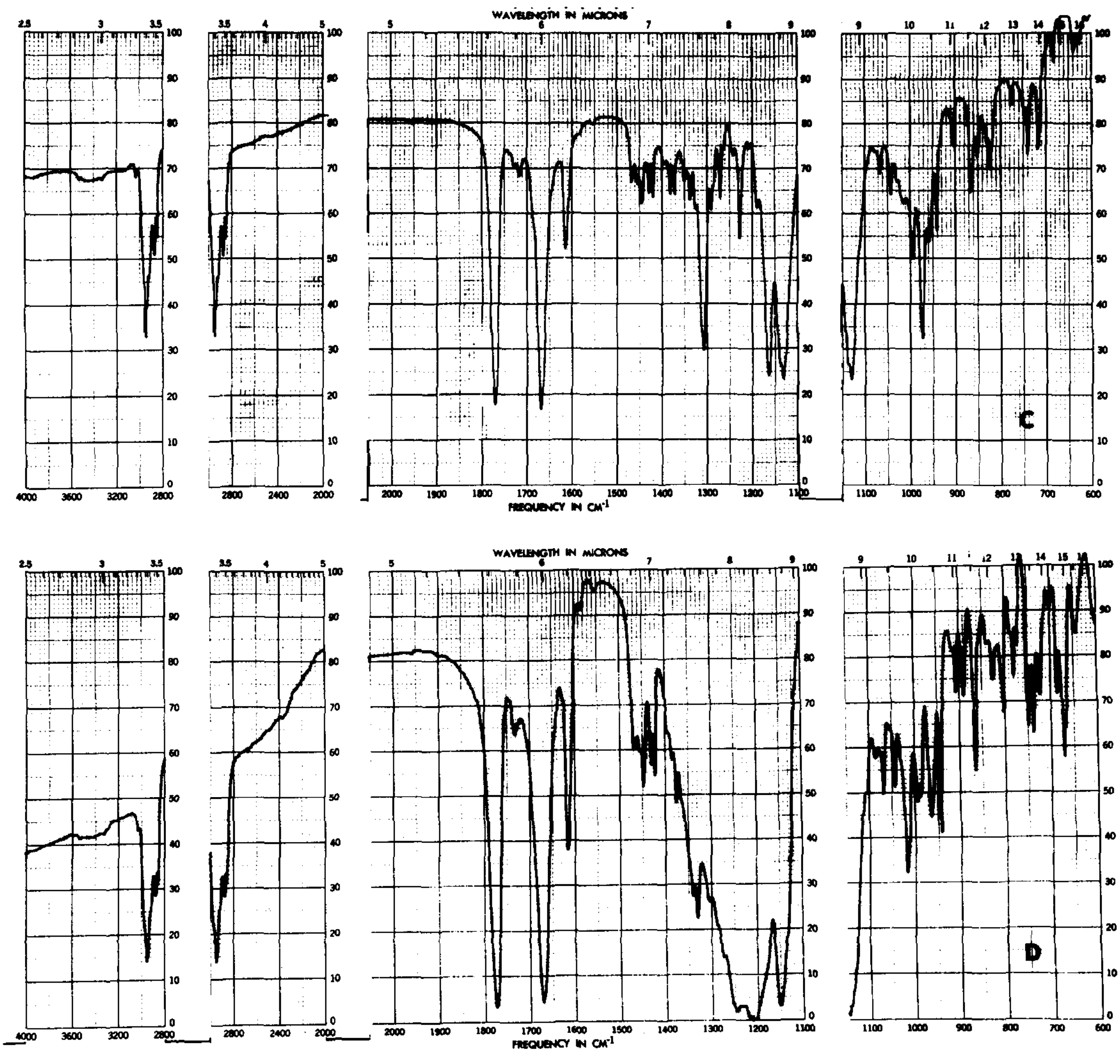

Figure 4

Infrared spectra of testosterone monochlorodifluoro acetate (C). Testosterone pentadecafluorooctanoate (D).

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these results, it seems evident that the derivative which has the relative retention of 0.59 to cholestane (0.19 to heptafluorobutyrate) on an XE-60 column is the di-heptafluorobutyrate and the corresponding mono-ester has a relative retention of 3.10 to cholestane.

Of all the derivatives tested, however, the di-heptafluorobutyrate shows the highest molar response with electron capture detection. This is closely followed by mono-pentadecafluorooctanoate. These two derivatives contain 39.1% and 41.7% fluorine respectively. The mono-heptafluorobutyrate, which contains 27.5% fluorine, shows a response of less than half, while the pentafluoropropionate (21.9% F) shows one tenth, and the trifluoroacetate (14.8% F) shows less than one hundredth of the responses of those two most potent derivatives. A preliminary study on cholesterol derivatives showed a similar trend in this relationship (10). These data seem to show that, in the case of perfluorinated alkyl derivatives, the increasing percentage of fluorine in the molecule leads to an increase in the affinity to electrons. This is in contrast to the report of Landowne and Lipsky (5), which revealed that an increase in the number of chlorine atoms in the acetyl group of cholesterol chloroacetates led to a decrease in response to the electron capture detector.

The diesters were formed as by-products and could not be made in good yields. Moreover, the diesters were more unstable than the monoesters. From these points, in spite of their sensitivity, the diesters were not considered to be suitable derivatives for quantitation.

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1. Supported by World Health Organization Research Training Grant No. M8/181/4N7 awarded to K. Nakagawa and U. S. Public Health Service Grant No. HE-01741.

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9. Our previous experience with a Barber-Colman Model 5000 series gas chromatograph with an electron capture detector showed that the responses and noise levels with direct current and pulsed voltage each at optimum voltage levels were essentially the same. However, in this study, a pulsed voltage source, as supplied with the F. & M. gas chromatograph, was used.

REFERENCES

(continued)

10. Data on cholesterol derivatives using the same 2% x E 60 column as described above.

	Relative Retention Time	Relative Molar response	
		Flame ionizn.	Electron capt.
Trifluoro-acetate	1.04	1.13	0.09
Monochlorodi-fluoroacetate	2.22	0.84	2.08
Heptafluoro-butyrate	1.00*	1.00	1.00**
Perfluoro-octanoate	1.44	0.90	1.85

*Relative retention time to testosterone heptafluorobutyrate 0.72

**Testosterone heptafluorobutyrate 15.1.