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HIGH-RESOLUTION ELECTRON CAPTURE GAS–LIQUID CHROMATOGRAPHY

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SUMMARY

A new method is described for preparation of stable glass capillary columns. The technique utilizes the initial incorporation of unsilanized fumed silicon dioxide on the inner capillary wall (Cab-O-Caps). Silanization with dimethyldichlorosilane, a cross-linking reagent, produces a hydrophobic surface and also inter-bonds the silica particles, forming a thin crust on the capillary surface. Glass capillaries prepared in this manner and coated with OV-17 stationary phase are thermally stable with a life expectancy of several months at temperatures up to 250°. The Cab-O-Caps can be re-used after their efficiency has deteriorated by simply washing with solvents and re-coating. A miniature high-temperature electron capture detector containing a scandium tritide ionization source has been constructed for use with Cab-O-Caps. Their combined performance demonstrates high resolution and sensitivity capabilities with potential also for quantitation of compounds of biological importance.

INTRODUCTION

Since Lovelock's[1,2] first report on the detection of components emerging in an effluent stream of gas by electron attachment processes, the design and operation of an electron capture detector (ECD) has closely paralleled the availability of improved ionization sources[3–5]. Limitations imposed by the temperature and specific activity of radioactive foils has particularly influenced the engineering of a variety of detector geometries.

ECDs with plane parallel geometries containing titanium tritide have been successfully interfaced with capillary columns[6]. Because of the low operating temperature limit their wide applicability to biomedical problems, particularly steroid detection, could not be realized. The ^{63}Ni foil allowed high-temperature analysis (350°) to be performed but the physical size of the foil was large in order to accommodate a high total specific activity for a reasonable standing current and thus the electron capture cell volume was also large (2–4 ml). Conventional packed columns naturally provided the high gas flow required for maintaining column efficiency in the ^{63}Ni electron capture chamber as well as optimum sensitivity. However, the marriage of

the coaxial ^{63}Ni ECD with capillary columns was not feasible because the ECD responds by a concentration-dependent mechanism and the use of a make-up gas to preserve column efficiency would only serve to dilute the solute vapor and lower sensitivity.

Recently, a new high-temperature ionization source (350°) composed of scandium tritide was reported[7,8] which permitted substitution for the conventional ^{63}Ni foil as well as the construction of a low-volume chamber.

Although the combined extraordinary sensitivity of the ECD with high-resolution columns was now feasible, their application to 'sensitive' compounds still awaited improvements in capillary column technology. Major problems associated with glass capillary column preparation appeared to be the development of an appropriate method for treating the inner wall in order to achieve a thermally stable homogeneous thin film of stationary phase. Formation of microdrops and lenses of phase was common and the lifetime of the capillary varied from a few hours to several days[9].

Several technical approaches were developed to overcome this problem. These were: (1) surface corrosion[10–13], (2) carbonization[14–16], (3) application of organic intermediate layers[15,17–20], (4) formation of oriented monolayers[21–26], and (5) chemical modification of silanol groups[27,28]. A summary of these techniques has been reviewed by Novotný and Zlatkis[28]. The most promising method described the incorporation of finely ground ($< 10^{-14}$ cm) and sized diatomaceous earth (Chromosorb W) on inner surfaces of stainless-steel capillaries which provided an irregular surface and prevented film break-up. The special techniques used for introducing the powder yielded the SCOT (support coated open tubular) and PLOT type (porous layer open tubular) capillaries[15,29–32].

A new concept for preparing thermally stable capillaries is based upon a commercially available hydrophobic silica (Silanox 101)[33–36]. Averill and Billeb[33] and Blumer[34] were the first to describe this method using stainless-steel columns, while German *et al.*[35,36] successfully developed Silanox glass capillaries. The silanized fumed silicon dioxide forms a stable colloidal suspension with liquid phases and readily adheres to metal, glass and plastic surfaces.

This paper describes a technique for preparing glass capillary columns containing a thin matrix of chemically inter-bonded silicon dioxide particles (6–10 μ) for maintaining a stable film of liquid phase. The combined performance of a miniature ECD and high-resolution glass capillaries is demonstrated.

EXPERIMENTAL

Apparatus

The ECD designed by Fenimore *et al.*[7] was modified and is depicted in Fig. 1. The entrance and exit orifices (68°) of the coaxial cell were machined to provide a laminar gas flow through the chamber. A cylindrical foil of scandium tritide (240 mCi; U. S. Radium, Bloomsburg, Pa., U.S.A.) served as the ionization source for the detector (450 μl). Capillary column effluent was transported to the electron capture chamber via a 10-cm glass-lined stainless-steel tube (0.158 cm O.D.; Supelco, Bellefonte, Pa., U.S.A.).

An injection port splitter system shown in Fig. 2 was also designed to provide

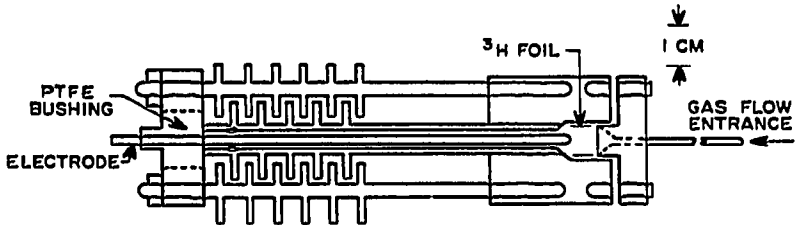

Fig. 1. Miniature electron capture detector for capillary columns.

the capability of selecting any desired split ratio. A standard Fisher/Victoreen stainless-steel injection port was bored to fit a 6-cm length of 5 mm O.D. and 0.75 mm I.D. glass capillary demisting trap. The splitter chamber was constructed from a lowvolume 1/16-in. internal tee (Port No. 009-1291; Perkin-Elmer, Norwalk, Conn., U.S.A.) silver-soldered to the injection port. Glass capillary columns (1–1.5 mm O.D.) were connected to the splitter chamber using high-temperature O-rings (HT-13 rubber;

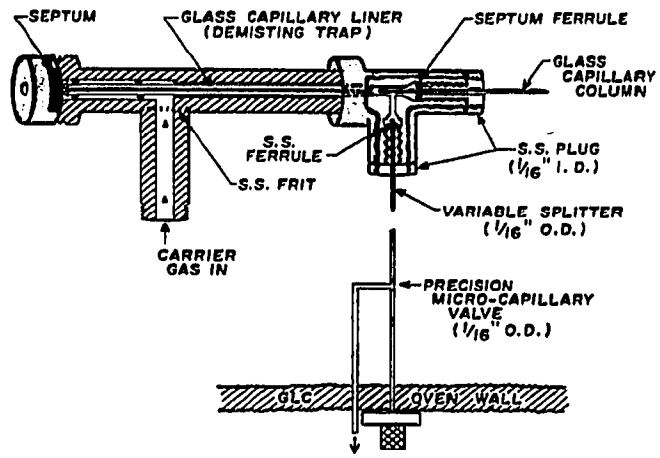

Fig. 2. Injection port splitter system.

Applied Science Labs., State College, Pa., U.S.A.) and 1/16-in.-I.D. male fitting. Adjustment of the splitter arm flow with a high-temperature microcapillary valve (Precision Sampling, Baton Rouge, La., U.S.A.) allowed selection of split ratios. Determination of the split ratio could be made any time during an analysis by monitoring gas flows at the splitter arm and detector exit.

The coaxial detector and injection port system were assembled on a Fisher/Victoreen Series 4400 gas–liquid chromatograph equipped with a programmable oven, vibrating-reed electrometer, electron capture power supply for constant potential or pulse mode operation, and also a flame ionization detector. Electrometer output was displayed on a Varian A-25 strip chart recorder (Varian, Palo Alto, Calif., U.S.A.).

Glass capillary preparation

Prior to drawing glass capillaries, Corning (Corning, N.Y., U.S.A.) Pyrex tubing (1.25 mm length, 8 mm O.D. and 4 mm I.D.) was rinsed with methanol, methylene chloride, 0.25 *N* NaOH, water, methanol, and acetone, and dried in a nitrogen gas stream. Capillaries were drawn into lengths of 30 to 80 m with diameters of 1.0–1.5 mm O.D. and 0.3–0.55 mm I.D. using a coiling apparatus (Hupe and Busch, Groetzingen/Karlsruhe, G.F.R.). Capillaries were washed with methanol and acetone, and dried. The inlet and exit ends were straightened as described by Novotný and Zlatkis[28] to allow attachment to the injection port system and detector.

Capillary columns were connected to a tee system using a zero dead volume 1/16-in. connecting union (HT-13 O-ring seal). One leg of the tee system was connected to a liquid reservoir (PTFE tube, 1.5 cm O.D. and 1.0 cm I.D.; capacity, 6 ml) which was fitted with a precision metering valve (A) (No. 1335G2Y; Hoke, Cresskill, N.J., U.S.A.) upstream from the reservoir. The other leg of the tee system was fitted with a second precision metering valve (B) which regulated only helium flow from a pressure head of 250 p.s.i. Reagent solutions or suspensions were placed in the reservoir with valve B closed; valve A (250 p.s.i. pressure head) was opened until the desired velocity of the liquid was attained through the capillary. After all the liquid had passed from the reservoir to the capillary, valve A was closed and dry helium was used to continue displacing the liquid through the capillary by opening immediately valve B. This technique allowed the capillary to begin drying immediately and aided the deposition of fumed silicon dioxide.

The coating of the inner glass capillary wall with unsilanized fumed silicon dioxide (Cab-O-Sil; Packard, Downers Grove, Ill., U.S.A.) was done as follows. A 0.5% (w/v) suspension of Cab-O-Sil was prepared in 20% acetonitrile – carbon tetrachloride and placed in an ultrasonic bath for 1 min. Prior to introducing the Cab-O-Sil suspension, approximately 25% of the capillary was filled with CCl_4 and then a volume of Cab-O-Sil suspension equivalent to that of the entire capillary was placed in the reservoir. The suspension was immediately forced through the glass capillary at a linear velocity of 3–5 cm/sec. To maintain a constant rate continuous adjustment of valve A was necessary. A buffer capillary of 10–15 m was attached to the exit end of the main column in order to compensate for rapid downstream pressure changes and thus allowing a carefully controlled expulsion rate. The column was dried of excess solvent by passing a stream of helium through it for several hours.

Silanization of the glass capillary wall and fumed silica, as well as cross-linking of silica particles, was achieved in one step by passing a 10% solution of dimethyldichlorosilane in heptane through the capillary at about 2 cm/sec. The volume used was twice the capillary volume. Immediately after expulsion of the main plug of silanizing solution, excess reagent was washed from the capillary using two capillary volumes of heptane, followed by a methanol washing until the effluent had reached neutral pH. The column was dried by passing a stream of helium through it. For a heavier layer of silicon dioxide this process was repeated.

Capillary columns containing silanized, cross-linked Cab-O-Sil particles (Cab-O-Caps) were coated with OV-17 stationary phase (2.5–5% w/v in heptane) using the dynamic method described earlier. The volume of liquid phase solution used was twice the capillary volume. Cab-O-Caps were dried overnight by a 10–20 ml/min flow of helium. The coating step was repeated to produce thick films (lower β term) when desired.

Cab-O-Cap columns were conditioned in the gas-liquid chromatograph with the exit end disconnected from the detector. The oven temperature was raised from ambient to 275° at 2.5°/min using nitrogen carrier flow at 5–10 ml/min. The upper temperature was maintained for a minimum of 18 h prior to coupling to an ECD. The capillary was then silanized *in situ* with 30 μl of Silyl-8 (Pierce, Rockford, Ill., U.S.A.) using 3–5 μl per injection at a 1:5 split ratio. After an additional 2 h at 250° the Cab-O-Cap was ready for use.

The exit end of the Cab-O-Cap column was connected directly to the silanized glass-lined stainless-steel capillary leading to the ECD using a short sleeve of shrinkable PTFE tubing (Pentube Plastics, Clifton Heights, Pa., U.S.A.). The dead volume of the transfer line was less than 50 μl.

When the chromatographic properties (resolution, separation efficiency, etc.) of Cab-O-Caps deteriorated due to continual use (about 1 month) the capillaries were recycled. Cab-O-Caps were washed with several column volumes of acetone, methanol, and acetone, and then dried by a flow of helium. Re-silanization and coating were conducted as previously described.

Preparation of electron capture derivatives

Reactions were carried out in microvials with PTFE cap liners. The compound (10.0 μg) was dissolved in 1 ml acetonitrile containing 50 μl of 5% pyridine in benzene (v/v). After mixing, 50 μl of heptafluorobutyric anhydride were added and the mixture heated for 1 h at 60°. Subsequently the reaction mixture was taken to dryness using a nitrogen stream and the residue was brought to volume with hexane.

Preparation of heptafluorobutyramides was performed similarly except the reaction conditions were 40° for 30 min.

RESULTS AND DISCUSSION

The miniature scandium tritide ECD depicted in Fig. 1 yielded a standing current of 3.1×10^{-8} A, which was approximately an order of magnitude greater than that of ^{63}Ni detectors[7]. A higher standing current was obtained even though the electron capture cell volume was one-eighth to one-tenth of the ^{63}Ni cell, because higher specific activity foils can be produced with ^{3}H. The relative anode position in the thermal electron plasma significantly influenced the magnitude of the standing current and detector sensitivity. For this detector maximum sensitivity was observed with the anode located coaxially 3 mm from the inlet orifice. The current levels are also relatively constant for flow-rates from 1–60 ml/min as previously reported[7]. A direct applied potential *versus* current profile revealed a plateau region beginning at 10 V.

Using the "theoretical plate" concept, the relative performance of several Cab-O-Cap columns was first examined by flame ionization gas–liquid chromatography (GLC). The results for hexadecane as the reference compound are given in Table I. Glass capillary columns prepared by this described procedure yielded plate numbers (n) of greater than 1100 per meter. A comparison of the HETP values (0.7 to 1.0) indicates the consistency of their production over a wide range of lengths (30–78 m) which until recently has been a serious problem in glass capillary column technology.

The combined performance for the miniature ECD and Cab-O-Cap columns

TABLE I

THEORETICAL PLATES FOR OV-17 CAB-O-CAPS

Cab-O-Cap	*Length (m)*	*I.D. (mm)*	*n**	*HETP***
I	30	0.43	38,000	0.8
II	45	0.43	51,000	0.8
III	50	0.43	55,000	0.9
IV	51	0.40	66,500	0.7
V	78	0.43	71,850	1.0

* $n = 5.54\ (t_r/W_h)^2$, in which t_r = retention time and W_h = peak width at half height.
** HETP = Height equivalent to a theoretical plate, L/n.

was examined (Fig. 3). The plate efficiency (>30,000, 30-m OV-17 Cab-O-Cap) for 15 pg of testosterone diheptafluorobutyrate suggests that the detector was compatible with flow-rate requirements for glass capillaries, *i.e.* the volume of the electron capture chamber did not contribute significantly to band broadening. The detection limit was about 2 pg. At low masses (< 5 pg) severe peak tailing and column loss was evident. This problem was not alleviated by additional *in situ* silanization.

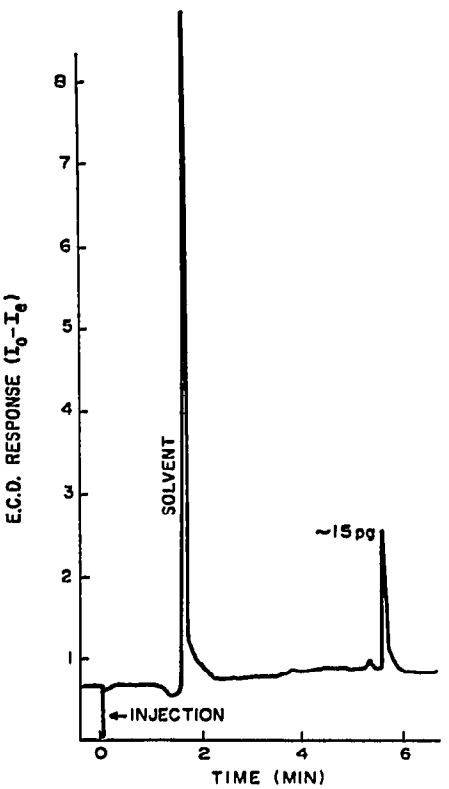

Fig. 3. High-resolution electron capture chromatogram for testosterone diheptafluorobutyrate. 30-m OV-17 Cab-O-Cap; column temperature, 200°; sample size, 1 μl (= 150 pg); split ratio, 1:10; carrier (nitrogen) flow-rate, 5.5 ml/min; pulse, 125 μsec; attenuation, 1.6×10^{-11} a.f.s.; detector temperature, 320°.

A mixture of steroids (heptafluorobutyrates) were subjected to high-resolution EC–GLC (Fig. 4). The resolution of the epimers of 3-hydroxy-5-androstan-17-one (peaks 3, 4, and 5) demonstrates the separation capability for these capillaries. The tailing observed for peaks 3, 4 and 5 was probably due to the polar carbonyl moiety, which provides a severe test for evalution of glass capillary columns. Two alkyl chain isomers of *m*-chlorophenoxypropylamines (as heptafluorobutyramides) were also

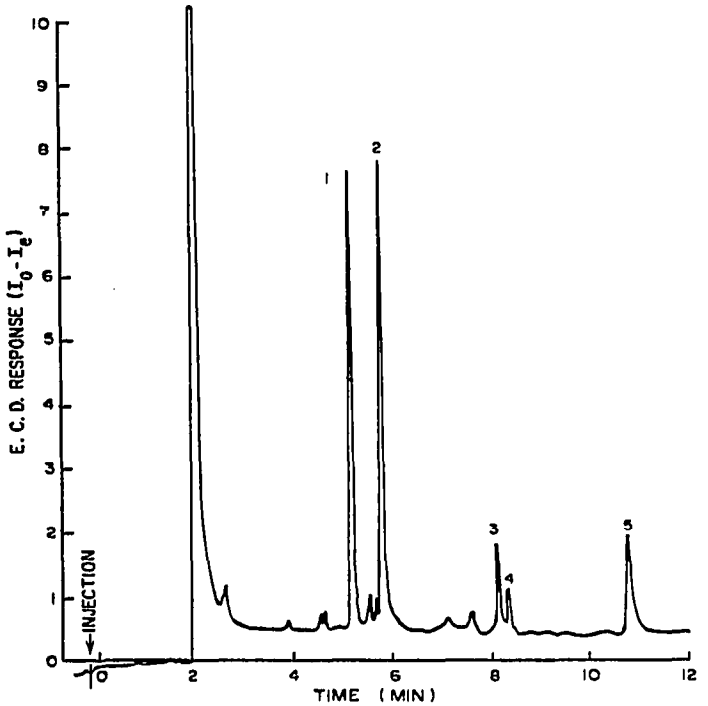

Fig. 4. High-resolution electron capture chromatogram of a mixture of steroid heptafluorobutyrates. 45-m OV-17 Cab-O-Cap; column temperature, 235°; sample size, 1 μl; split ratio, 1:9. 1 = 120 pg of 3α,17β-dihydroxy-5α-androstane diheptafluorobutyrate; 2 = 100 pg of testosterone diheptafluorobutyrate; 3 = 40 pg 3α-hydroxy-5α-androstan-17-one monoheptafluorobutyrate; 4 = 25 pg of 3β-hydroxy-5β-androstan-17-one monoheptafluorobutyrate; 5 = 55 pg of 3β-hydroxy-5α-androstan-17-one monoheptafluorobutyrate. The other conditions were as described in Fig. 3.

examined on a 30-m OV-17 Cab-O-Cap (Fig. 5). From these results the peak resolution (R) and plate number (n) were determined to be 13.7 and 32,000, respectively.

The reproducibility of injection and linearity of response was also assessed. Two concentrations of testosterone —750 pg (series A) and 700 pg (series B)— were repeatedly injected. The peak heights were within $\pm$ 0.8% of the mean for both series (Fig. 6). The detector response to increasing concentration of testosterone was linear from 4 to 200 pg (Fig. 7). These results indicate that the described system —injection port splitter unit, Cab-O-Caps and EC— can be used for obtaining quantitative data.

Glass capillary columns prepared in this manner possessed several features. They were stable at a sustained temperature of 250° for several weeks. An increase in

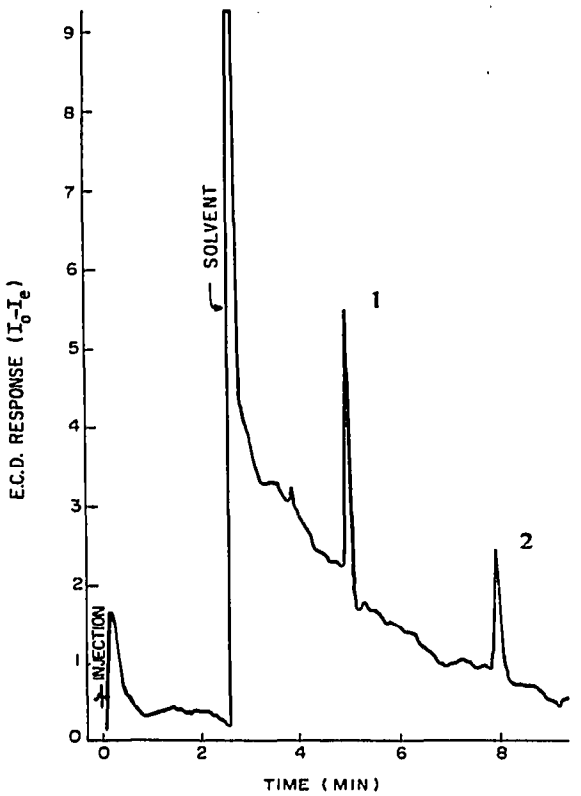

Fig. 5. High-resolution electron capture chromatogram of *m*-chlorophenoxypropylamines as their heptafluorobutyramides. 30-m OV-17 Cab-O-Cap; column temperature, 165°; sample size, 1 μl; split ratio, 1:4; attenuation: 8×10^{-12} a.f.s.; detector temperature, 320°; carrier (nitrogen) flow-rate, 6 ml/min; pulse, 125 μsec. 1 = 6 pg of *m*-chlorophenoxyisopropylamine; 2 = 5 pg of *m*-chlorophenoxy-*n*-propylamine.

surface activity was observed at higher temperatures for prolonged periods of time which probably resulted from pyrolysis of the Si–O bond. *In situ* silanization would restore the column performance. These columns were more resistant to solvent shock than the standard Golay type during flash evaporation and thus up to 0.5 μl of sample could be introduced directly (no splitting).

The life expectancy of Cab-O-Caps was increased by employing a silanized glass demisting trap in the injection port. The periodic removal of the trap containing non-volatile materials was achieved without disturbing the capillary column, since it could be removed through the front of the injection port. A similar technique has been reported[35,36] which can be also used with this injection port splitter unit whereby a 4- to 6-cm packed precolumn is used for reducing aerosol formation.

When the Cab-O-Caps began to lose their high efficiency they were recycled. Since the silicon dioxide particles were chemically inter-bonded, simple washing of the glass capillary and re-coating could be carried out, which avoided repeating the laborious technique of preparing a new column. The 30-m Cap-O-Cap described in this report has been recycled four times during the past year.

The application of high-resolution EC–GLC for qualitative and quantitative investigations on drug metabolism is currently being examined in this laboratory.

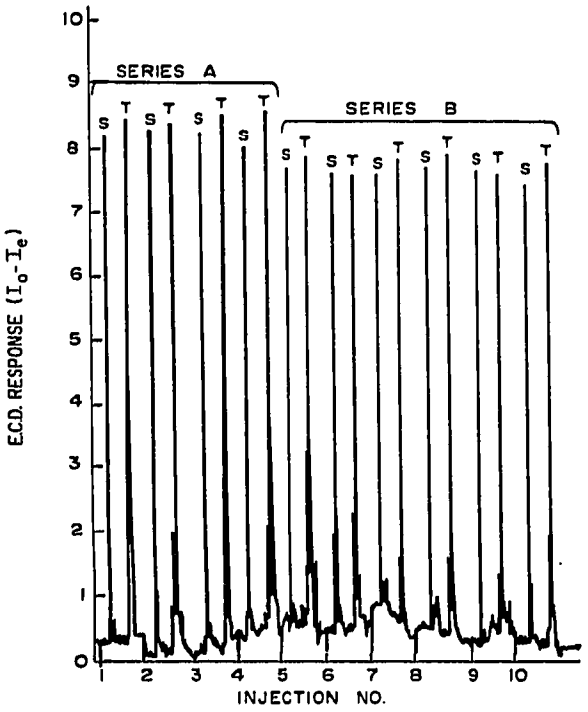

Fig. 6. Reproducibility of sample injection on 65-m OV-17 Cab-O-Cap. Column temperature, 210°; sample size, 1 μl; split ratio, 1:10; carrier (nitrogen) flow-rate, 8.5 ml/min; pulse, 125 μsec; attenuation, 1.6×10^{-11} a.f.s.; detector temperature, 320°. Series A: 750 pg testosterone diheptafluorobutyrate (T). Series B: 700 pg T. S = Solvent.

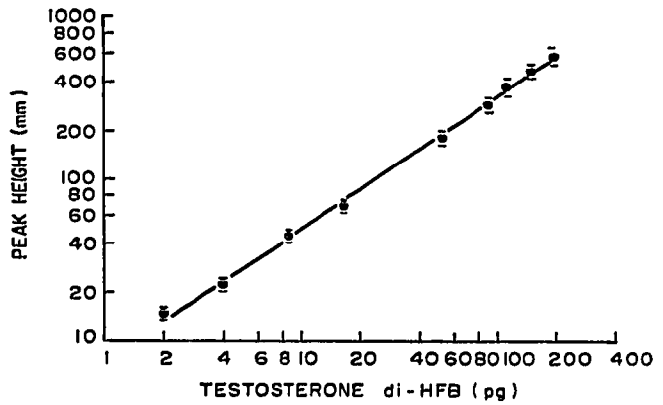

Fig. 7. Response curve for testosterone diheptafluorobutyrate (di-HFB). See the legend to Fig. 3 for parameters.

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