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GLC Determination of Medroxyprogesterone Acetate in Plasma

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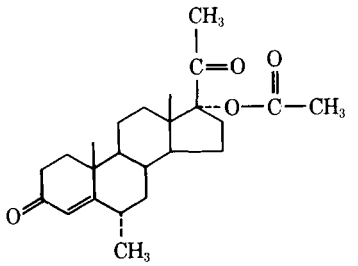
Abstract □ To study the absorption, metabolism, and excretion of medroxyprogesterone acetate in animals and man, a method was developed for measurement of the intact drug in plasma based on: *(a)* cyclohexane extraction of the specimen, *(b)* formation of the 3-enol heptafluorobutyrate ester, and *(c)* quantification *via* GLC using an electron-capture detector. The assay is quantitative above 1 ng medroxyprogesterone acetate/ml of plasma, and overall precision is approximately ±10% *(SD)* in the range of 5–20 ng/ml. The procedure was successfully applied to absorption studies in dogs after intramuscular drug administration. Plasma drug concentrations, as measured by radioimmunoassay, were five to 10 times greater as compared to the GLC assay, indicating that drug-related materials in addition to intact medroxyprogesterone acetate were in peripheral circulation.

Keyphrases □ Medroxyprogesterone acetate—GLC analysis in plasma, compared to radioimmunoassay technique, absorption in dogs after intramuscular administration □ GLC—analysis, medroxyprogesterone acetate in plasma, compared to radioimmunoassay technique

Medroxyprogesterone acetate[1] (17α-acetoxy-6α-methylprogesterone, I), a synthetic progestogen (1, 2), is an effective agent for the control of fertility (3) and for the treatment of malignancies. Investigations in animals (4, 5) and humans (6–8) indicated that, after subcutaneous or intramuscular drug administration, I and drug-related materials were slowly absorbed from the injection site, resulting in prolonged activity.

To study the absorption, metabolism, and excretion of I in animals and man as well as to aid in determining the specificity of the radioimmunoassay (7) for measurement of I, a simple, sensitive, and specific GLC method of analysis was developed for the intact drug in plasma.



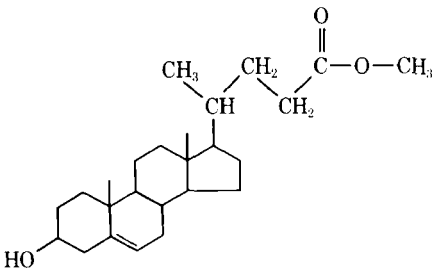
I

EXPERIMENTAL

Reagents and Materials—Compound I and the methyl ester of 3β-hydroxy-5-cholenic acid[2] (II), used as internal standard, were synthesized. Acetone, benzene, and cyclohexane, distilled in glass, were used as supplied[3]. Spectral grade acetonitrile[4] and heptafluorobutyric anhydride (1-ml ampuls)[5] were used without further purification. Aqueous stock solutions of I (10 μg/ml) were prepared by dissolving 1 mg of drug in 1 ml of acetone. The solution was diluted to a final volume of 100 ml with distilled water. Aliquots were diluted with distilled water to final concentrations of 12.5, 25, 50, and 100 ng/ml. The stock solution of II (10 μg/ml) was prepared in 50% (v/v) acetonitrile in acetone. All stock solutions were stored in glass containers. Silicone gum rubber (OV-17) on 80–100-mesh Gas Chrom Q (10% w/w) was used as supplied[6].

Instrumentation—A two-speed reciprocating shaker[7] was used for shaking the samples in the horizontal position. A mixer[8] was used to aid in preparing the heptafluorobutyrate esters. GLC measurements were made with a gas chromatograph[9] equipped with a hydrogen flame-ionization detector, a nickel-63 electron-capture detector, and a −0.2 to 1.0-mv recorder[10]. All cylinders of gases for chromatography (*i.e.*, helium, hydrogen, oxygen, and 10% methane in argon) were fitted with filters containing molecular sieve 4A.

Chromatographic Conditions—All analytical chromatography was conducted on U-shaped glass columns [0.61-m (2-ft) × 3-mm i.d.] of 10% (w/w) OV-17 on 80–100-mesh Gas Chrom Q. All newly



II

[1] Provera, The Upjohn Co.

[2] Methyl ester of 3β-hydroxy-5-cholenic acid, supplied to The Upjohn Co. by Dr. L. C. King, Evanston, Ill.

[3] Burdick and Jackson Labs., Muskegon, Mich.

[4] Matheson, Coleman and Bell, Milwaukee, Wis.

[5] Pierce Chemical Co., Rockford, Ill.

[6] Applied Science Labs., State College, Pa.

[7] Eberbach and Sons, Ann Arbor, Mich.

[8] Vortex model K-500, Scientific Industries, Queen's Village, N.Y.

[9] F & M model 400, Hewlett-Packard Co., Avondale, Pa.

[10] Honeywell Electronik 15, Honeywell, Philadelphia, Pa.

Table I—Effect of pH on the Extraction of Medroxyprogesterone Acetate-7-^{3}H from Water and Dog Plasma Using Cyclohexane as the Solvent for Extraction[a]

Matrix	Solution pH	Amount Added[b], ng	Amount Found, ng	Recovery, %
Water	1.5	200.0	195.8	97.9
	7.2	200.0	185.0	92.5
	10.0	200.0	204.0	102.0
Plasma	1.5	200.0	179.8	89.9
	7.2	200.0	190.6	95.3
	10.0	200.0	185.6	92.8

[a] Ratio of organic solvent to biological specimen was 2:1 (v/v). [b] Total amount of medroxyprogesterone acetate-7-^{3}H added to 5-ml sample (equivalent to 40 ng/ml).

prepared columns were preconditioned at 275° for 1 hr without carrier gas flow and for 16 hr with a carrier gas flow of 10 ml/min. During analysis, the column, injection port, and electron-capture detector were maintained isothermally at 250, 270, and 310°, respectively. Flow rates of 10% methane in argon, used as carrier and purge gas, were maintained at 60 ml/min. The pulse interval for the electron-capture detector was 150 μsec. Under these conditions, the heptafluorobutyrate esters of I and II had retention times of 9.6 and 13.6 min, respectively (Fig. 1).

In ancillary studies, using the flame-ionization detector, chromatography was conducted on a U-shaped glass column [0.61-m (2-ft) × 3-mm i.d.] of 1% (w/w) OV-17 on 80–100-mesh Gas Chrom Q. The column, injection port, and detector block were maintained isothermally at 240, 245, and 245°, respectively. Helium, hydrogen, and oxygen flow rates were 60, 40, and 400 ml/min, respectively. The retention times for I-heptafluorobutyrate ester, 5α-cholestan-3-one (utilized as GLC standard), and I were 1.6, 3.4, and 5.4 min, respectively (Fig. 2).

Synthesis of Standard Materials—Standard materials were prepared for identification by dissolving 200 mg of I or II in 5 ml

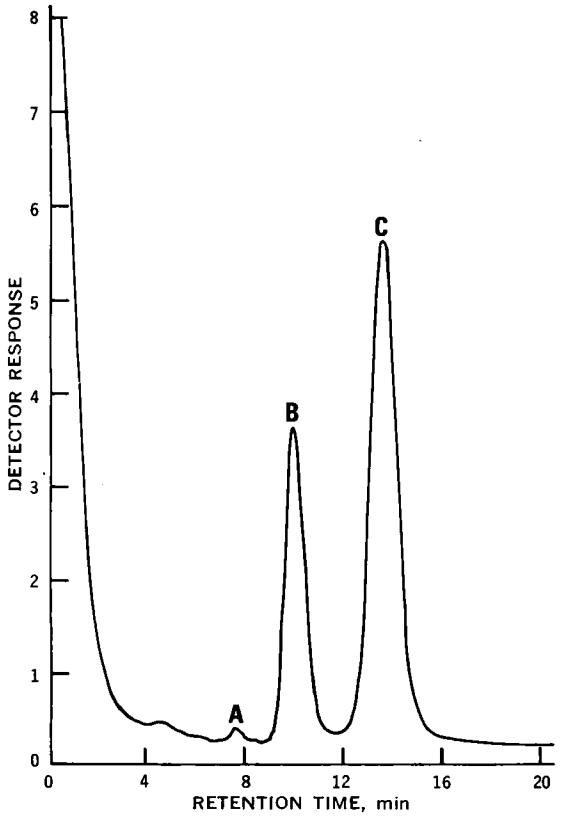


Figure 1—*Gas–liquid chromatogram (electron-capture detector) of medroxyprogesterone acetate and internal standard heptafluorobutyrates. Key: A, III-heptafluorobutyrate; B, I-heptafluorobutyrate; and C, II-heptafluorobutyrate.*

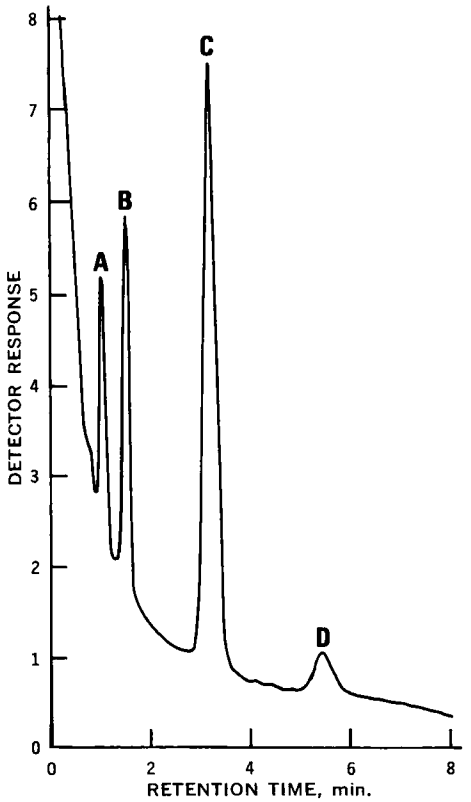


Figure 2—*Gas–liquid chromatogram (flame-ionization detector) of medroxyprogesterone acetate-3-enol-heptafluorobutyrate reaction mixture (reaction temperature = 90°, and reaction solvent = benzene). Key: A, III-heptafluorobutyrate; B, I-heptafluorobutyrate; C, 5α-cholestan-3-one; and D, I.*

50% (v/v) acetonitrile in acetone. Heptafluorobutyric anhydride (1 ml) was added, and the mixture was allowed to react at 25° for 1 hr. The reaction mixture was evaporated to dryness under a gentle stream of nitrogen gas. The I residue was reconstituted in 5 ml ether and washed with 10 ml water. The resulting material was filtered, dried, and crystallized from acetone–commercial hexane[11]. The residue from reaction of II was dissolved in ether, washed with water, and decolorized with activated charcoal. The resulting material was filtered, dried, and crystallized from methanol (9).

Assay Procedure—*Preparation of Plasma Standards*—Pipet 1.0-ml aliquots of the I aqueous stock solutions equivalent to 12.5, 25, 50, and 100 ng into glass-stoppered centrifuge tubes. Add 4.0 ml of control plasma to each centrifuge tube and mix well with the mixer. Prepare an appropriate blank. Extract all standards in the same manner as described below for the plasma specimens.

Preparation of Samples—Place 5.0 ml of plasma in a glass-stoppered centrifuge tube. For plasma specimens less than 5 ml, dilute to a final volume of 5.0 ml with distilled water. Add 10.0 ml cyclohexane and shake in the horizontal position for 10 min. Centrifuge for 10 min at 2000 rpm. Transfer a 9.0-ml aliquot of the cyclohexane layer to a fresh glass-stoppered centrifuge tube and evaporate to dryness under a gentle stream of nitrogen gas. Wash down the walls of the centrifuge tube with 0.5 ml cyclohexane and evaporate to dryness with nitrogen. Add 0.2 ml of the II stock solution and 50 μl heptafluorobutyric anhydride. Mix thoroughly and allow to react at 25° for 60 min. Evaporate to dryness with a gentle stream of nitrogen gas. Reconstitute the residue in 50 μl benzene. Inject a 1-μl aliquot of the solution for analysis into the chromatograph.

Calculations—The peak heights for the heptafluorobutyrate esters of I and II are measured. Peak height ratios are obtained by dividing the peak height of I-heptafluorobutyrate by the peak height of II-heptafluorobutyrate. Calibration curves for known concentrations of I in plasma are prepared by plotting peak

[11] Skellysolve B.

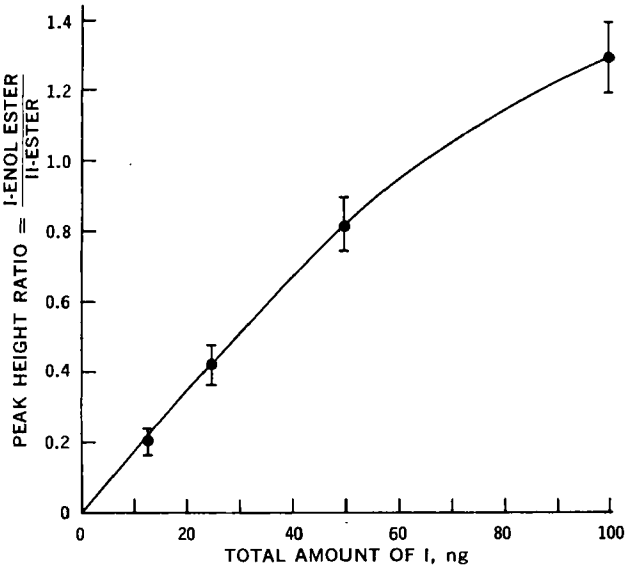


Figure 3—*Calibration curve; relationship between ratio of peak height of medroxyprogesterone acetate-3-enol-heptafluorobutyrate/internal standard heptafluorobutyrate and amount of medroxyprogesterone acetate in plasma. Each point represents the mean* ± SD *for eight determinations.*

height ratios *versus* total free I in the standard, expressed as nanograms. Values for unknown concentrations of I in plasma specimens, calculated in the same manner, are then read directly from the nonlinear standard curve (Fig. 3). The total I is adjusted for the initial sample volume and expressed as nanograms I per milliliter plasma.

Preparation of Animals—In studies designed to determine the utility of the analytical methodology, two female beagle dogs (E-102 and G-101) weighing 8.25 and 8.10 kg, respectively, received a single intramuscular dose of 75 mg I/kg body weight. The drug was administered as two separate 2-ml injections of an aqueous suspension (150 mg I/ml)[12] into the gluteus muscle of each animal. The animals were housed in holding cages with free access to food[13] (300 g/day) and water. All blood specimens (10 ml) were withdrawn in heparinized syringes from the jugular veins at preselected time intervals from 0 to 59 days postdrug administration. The plasma was harvested and stored at −18°. All plasma specimens were analyzed for I using the GLC assay.

In a second study, the aqueous suspension of I was administered (75 mg/kg im) to two female beagle dogs (G-403 and K-202) weighing 9.8 and 11.8 kg, respectively. An equivalent volume of physiological saline (~5 ml) was administered intramuscularly to a third female beagle dog (G-303) weighing 10.6 kg, which served as an untreated control. Blood specimens were withdrawn *(vide supra)* at predetermined time intervals from 0 to 108 days postdrug administration. All plasma specimens were analyzed using the GLC assay and the radioimmunoassay (7).

RESULTS AND DISCUSSION

Selection of Solvent for Extraction of Plasma Specimens—Studies designed to determine the extraction efficiency of known amounts of tritium-labeled I (specific activity, 32 μCi/μg)[14] from simple aqueous solution and dog plasma showed that the compound was quantitatively extracted with cyclohexane, ether, or methyl ethyl ketone. Cyclohexane was selected as the solvent of choice, since: *(a)* no volume changes were observed during the extraction of I from plasma or water, *(b)* it evaporated rapidly under a gentle stream of nitrogen gas, and *(c)* less endogenous background materials (*viz.*, cholesterol), as measured by GLC using an electron-capture detector, were extracted from the plasma specimens.

Table II—Recovery of Medroxyprogesterone Acetate from Dog Plasma

Added, ng	Found[a], ng	Recovery[b], %
5.0	4.57	91.4 ± 14.6
12.5	12.00	96.0 ± 12.1
25.0	23.55	94.2 ± 10.0
50.0	47.65	95.3 ± 7.4
75.0	74.02	98.7 ± 9.5
100.0	102.70	102.7 ± 5.8

[a] Means of five replicate samples. [b] Mean ± *SD*.

Selection of pH for Extraction of Plasma Specimens—A series of plasma and aqueous specimens (5 ml) containing known amounts of tritium-labeled I were adjusted to pH 1.5, 7.2, and 10.0 with 0.1 *N* aqueous sulfuric acid or sodium hydroxide. All samples were extracted with 10 ml cyclohexane. The results (Table I) showed that the pH of the samples had little, if any, effect on the extraction efficiency of I. GLC of the extract residues, after reaction with heptafluorobutyric anhydride, indicated that less endogenous materials were extracted from the plasma specimens at neutral pH (*i.e.*, 7.2–7.4) as compared to acidic or basic pH.

Selection of Internal Standard—Ideally, an internal standard should have physical and chemical properties similar to the compound to be analyzed. The two materials should be adequately separated from each other by GLC. During this study, 5α-cholestan-3-one and the monoheptafluorobutyrate derivatives of various steroids were investigated as potential internal standards. As the monoheptafluorobutyrate derivative, only II was adequately separated from I and the endogenous background materials present in plasma extracts. Since II was not efficiently extracted from plasma into cyclohexane, it was added to the extract residue prior to derivative formation. Consequently, II served only as a quantitative measure of heptafluorobutyrate derivative formation and the amount of material injected into the chromatograph.

Ester Formation with Heptafluorobutyric Anhydride—The reaction of steroid 4-en-3-ones with heptafluorobutyric anhydride, in an equal volume of benzene, was reported (10, 11) to produce nearly quantitative yields of the 3-enol heptafluorobutyrate es-

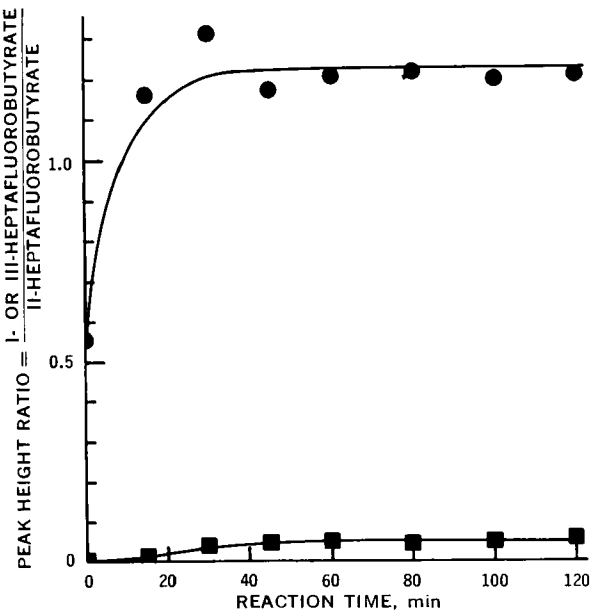


Figure 4—*Effect of reaction time on formation of medroxyprogesterone acetate-3-enol-heptafluorobutyrate [reaction temperature = 25°, and reaction solvent = 50% (v/v) acetone in acetonitrile]. Key:* ●—●, *I-heptafluorobutyrate; and* ■—■, *III-heptafluorobutyrate.*

[12] Depo-Provera, The Upjohn Co., is a sterile aqueous suspension of medroxyprogesterone acetate.
[13] Purina Lab Chow, Ralston Purina Co., St. Louis, Mo.
[14] Medroxyprogesterone acetate-7-^{3}H, prepared by Dr. R. C. Thomas, The Upjohn Co., Kalamazoo, Mich.

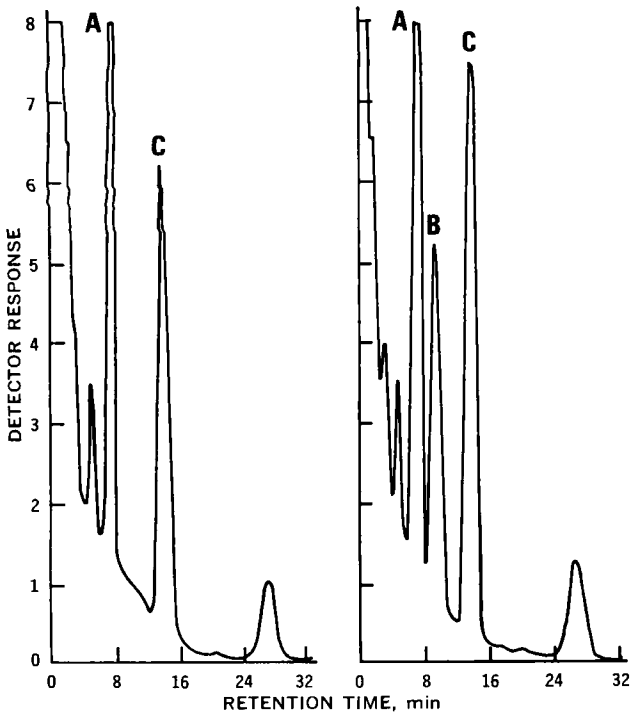


Figure 5—*Gas–liquid chromatogram (electron-capture detector) of dog plasma extracts. Left: normal plasma specimen. Right: plasma specimen from dog at 24 days after single-dose intramuscular administration of 75 mg medroxyprogesterone/kg body weight. Key: A, cholesterol heptafluorobutyrate; B, I-heptafluorobutyrate; and C, II-heptafluorobutyrate.*

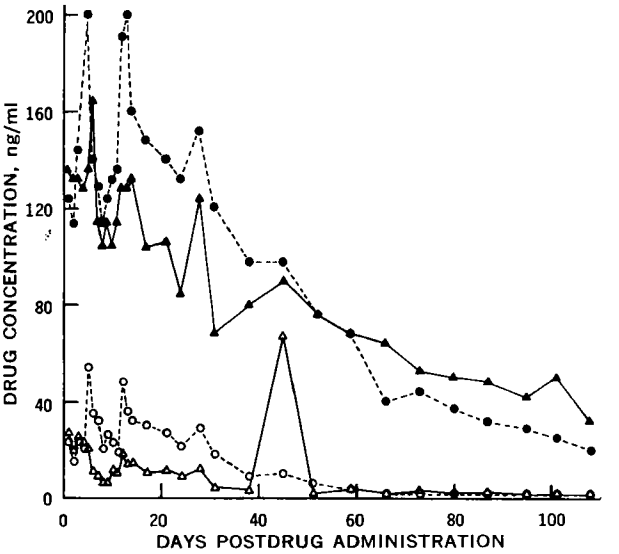


Figure 6—*Plasma drug concentrations* versus *time in dogs after single-dose intramuscular administration of 75 mg medroxyprogesterone acetate/kg body weight. Key: ▲—▲, Dog G-403, radioimmunoassay; △—△, Dog G-403, GLC; ●—●, Dog K-202, radioimmunoassay; and ○—○, Dog K-202, GLC.*

ters. Studies of the formation of I-heptafluorobutyrate *versus* time, using GLC with a flame-ionization detector and microgram amounts of I, indicated that at least two products were formed (Fig. 2). At 90°, the half-time for disappearance of I starting material (R_T = 5.4 min) from the reaction mixture was estimated to be less than 10 min. The I-enol-heptafluorobutyrate (R_T = 1.6 min) appeared very rapidly, reached a maximum concentration at about 1–2 hr, and then decreased with additional reaction time. An initial lag time was observed in the formation of the second reaction product (R_T = 1.2 min), designated III-heptafluorobutyrate. A maximum concentration was found at 3 hr, after which it decreased until termination of the experiment (*i.e.*, 6 hr). Results from studies conducted at reaction temperatures of 70 and 50° indicated that a single reaction product (as measured by GLC) could not be obtained.

Subsequent studies, using GLC in conjunction with mass spectrometry, confirmed that I-enol-heptafluorobutyrate was being chromatographed intact and provided evidence suggesting that the second reaction product (*viz.*, III-heptafluorobutyrate) was an isomer of I-enol-heptafluorobutyrate formed by migration of double bonds. Intact I-enol-heptafluorobutyrate and III-heptafluorobutyrate had identical molecular ions (*m/e* 582) and similar fragmentation patterns. The data provided no indication that diheptafluorobutyration or thermal cleavage (10, 12) at C-17 had taken place. These results were consistent with the studies described for heptafluorobutyration of progesterone (13) and testosterone-17β-acetate (14).

Tetrahydrofuran, methylene chloride, acetone, acetonitrile, and a mixture of 50% (v/v) acetonitrile in acetone were evaluated as potential solvents to replace benzene in the esterification. All reactions were conducted for 1 hr at 25°, using 4:1 ratio of organic solvent to heptafluorobutyric anhydride. Based upon *(a)* volatility of the solvent, *(b)* solubility of the internal standard, and *(c)* minimum formation of III-heptafluorobutyrate, 50% (v/v) acetonitrile in acetone was selected. Studies of the formation of I-heptafluorobutyrate *versus* time indicated that the reaction was completed within 60 min at 25° (Fig. 4). Less than 5% of III-heptafluorobutyrate was formed during the reaction, based upon peak area ratios. Similar studies with II showed that heptafluorobutyration was complete in less than 15 min. These results were consistent with the milder conditions required for esterification of free hydroxyl groups (15).

Synthesis of standard material indicated that the heptafluorobutyrate ester of I was a crystalline material (mp 138–140°) at room temperature. GLC (using a solid sample injector and flame-ionization detector) indicated that the material submitted for elemental analysis was greater than 98% pure.

Anal.—Calc. for $C_{28}H_{33}F_7O_5$: C, 57.73; H, 5.71; F, 22.83. Found: C, 57.87; H, 5.96; F, 23.36.

The heptafluorobutyrate ester of II was isolated as fine white crystals (mp 81–83°). GLC (using a solid sample injector and flame-ionization detector) indicated that the material submitted for elemental analysis was greater than 99% pure.

Anal.—Calc. for $C_{29}H_{39}F_7O_4$: C, 59.58; H, 6.72; F, 22.75. Found: C, 59.59; H, 6.75; F, 23.21.

IR and mass spectrometric analyses, before and after GLC, supported the proposed structures and confirmed that the heptafluorobutyrate esters of I and II chromatographed as the intact molecules.

Assay Sensitivity and Specificity—At a sensitivity of 3.2×10^{-10} amp/mv, 0.0036 μg of I-heptafluorobutyrate produced a full-scale response. Under the assay conditions described, the lower limit of detection sensitivity for I in extracts of plasma is 0.001 μg/ml of the original sample aliquot. This value is based on a sample signal equivalent to 2% of full-scale response. Using peak height ratios or peak area ratios, a nonlinear relationship between detector response and concentration is obtained from I over the range of 0–0.1 μg/ml. Peak height ratios are obtained facilely, and quantification from a standard curve is adequate. Analysis of plasma specimens from drug-treated animals, using GLC in conjunction with mass spectrometry, showed that the material responding to the assay is identical to known I-heptafluorobutyrate.

Recovery Experiments—Known amounts of I in aqueous solution were placed in centrifuge tubes, and water or plasma was added. The samples were thoroughly mixed and extracted with cyclohexane. All extract residues were esterified and analyzed chromatographically. The results (Table II) indicated that recovery of I from plasma ranged from 91.4 ± 14.6 to 102.7 ± 5.8% as compared to simple aqueous samples. In the range of 5–20 ng I/ml plasma, overall precision was approximately ±10% *(SD)*.

Identification of Cholesterol—During analysis of dog plasma extracts for I, a major peak (R_T = 7.5 min) was observed in the gas–liquid chromatograms of specimens from both drug-treated and control animals (Fig. 5). Mass spectrometric analysis of the material(s) in the chromatographic peak suggested that the major component was the heptafluorobutyrate ester of cholesterol. GLC

and mass spectrometric analyses of aqueous and plasma specimens, containing known amounts of cholesterol, indicated that nanogram amounts of cholesterol were extracted and measured as the heptafluorobutyrate ester. Additional studies, based upon measurement of the free sterol, confirmed the identification of the endogenous material as cholesterol. These results were consistent with the serum cholesterol concentrations observed in normal dogs (*i.e.*, 1.4–2.2 mg cholesterol/ml) (16, 17).

Plasma Levels of Medroxyprogesterone Acetate in Dogs—Results from the measurement of plasma I concentrations in female beagle dogs, after single-dose intramuscular administration of 75 mg I/kg body weight, demonstrated the utility of the analytical methodology. In the first study, peak plasma I concentrations of 29 and 14 ng/ml were observed at 11 and 14 days postdrug administration, respectively, indicating slow drug release from the injection site. Measurable amounts of intact I (1.7–6.0 ng/ml) were found in peripheral circulation on Day 59.

In the second study, peak plasma I concentrations of 27 and 54 ng/ml (as measured by GLC) were observed at 1–5 days postdrug administration in Dogs G-403 and K-202, respectively (Fig. 6). Beyond Day 95, plasma I concentrations were below the level of assay detection sensitivity (*i.e.*, less than 1 ng/ml). As measured by radioimmunoassay, peak plasma drug concentrations of 200 and 164 ng I equivalents/ml were found at 5 and 6 days, respectively, in Dogs K-202 and G-403. Measurable amounts of drug-related materials (*i.e.*, 20–30 ng I equivalents/ml) were found in peripheral circulation on Day 108. Using either GLC or radioimmunoassay, the observed plasma drug concentrations showed approximately a two- to threefold variation between animals and a cyclic variation within each animal. Plasma drug concentrations, as measured by radioimmunoassay, were five to 10 times greater as compared to GLC. The radioimmunoassay–GLC ratio increased with time, suggesting that drug-related materials other than intact I were circulating in plasma. Since the plasma specimens analyzed by radioimmunoassay were not extracted or fractionated, it is concluded that drug-related materials in addition to intact I were being measured.

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