# Application of Reverse Phase Partition Chromatography to the Analysis of Testosterone Propionate in Oil Injectables

By EDWARD SMITH

**In the analysis of testosterone propionate in oil injectables, reverse phase chromatography on silanized purified siliceous earth provides a convenient method for separating testosterone propionate from the bulk of the oil. The latter is retained on the column, while the steroid, together with the sterol and triterpenoid components of the oil, is eluted. The testosterone propionate is quantitatively separated from this mixture by direct phase chromatography, with nitromethane as immobile phase and heptane as eluant; the testosterone propionate is then determined spectrophotometrically.**

THE ANALYSIS of steroid in oil solutions has been a problem for many years. Because of the lack of a suitable chemical analysis, the U.S.P. XIV (1) testosterone propionate injection monograph specified a biological assay for the solution in vegetable oil. The assay in the U.S.P. XV (2) and subsequent revisions has been a gravimetric measurement of the semicarbazone derivative, based on the procedure of Madigan *et al.* (3). For samples containing less than 10 mg./ml. of oil, a preliminary liquid–liquid extraction is used to separate the testosterone propionate from the bulk of the oil prior to the formation of the semicarbazone (3). The melting point of the semicarbazone is used as the criterion of identification.

Quantitative procedures have been described (4–6) based on colorimetric determination of the 2,4-dinitrophenylhydrazone after preliminary separation. Umberger (7) used an adsorbent magnesium silicate[1] to chromatographically separate testosterone propionate and progesterone from their oil solution prior to colorimetric determination of their isonicotinylhydrazones. Tappi *et al.* (8) also used the adsorbent magnesium silicate to separate steroids from olive oil.

The application of partition chromatography to the separation of drugs from oil dosage forms was investigated in these laboratories. Levine (9), Heftmann (10), Neher (11), and Bush (12) have reviewed the application of partition chromatography to the analysis of steroids. Jones and Stitch (13) used silicic acid–nitromethane partition columns to separate mixtures of steroids, with 3% $CHCl_3$ in petroleum ether as the mobile phase. Wolff (14) isolated progesterone from its oil injection with nitromethane as the stationary phase on purified siliceous earth[2] and *n*-heptane as the mobile phase. He found that the background ultraviolet absorbance due to the sterol fractions of the oil was thereby reduced to negligible proportions. This procedure was modified in our laboratory (15) and applied to the assay of chlorotrianisene capsules N.F.; the modified procedure is official in the N.F. XII (16). In these separations there is an interval of at least 40 ml. between the complete removal of the oil and the emergence of the desired substance.

Determination of testosterone propionate in oil injectables by this procedure proved to be difficult. The testosterone propionate follows the elution of the oil fraction so closely that sharp separation may not be achieved. The presence of such adjuvants as benzyl alcohol further interferes with this partitioning. No significant improvement was obtained by varying column dimensions or by substituting dimethylsulfoxide for nitromethane as the stationary phase.

Testosterone propionate can be separated from the bulk of the oil in these preparations by reverse phase partition chromatography. In this system, the hydrophobic support retains the nonpolar solvent as stationary phase and a polar solvent is used as the mobile phase. Silanized siliceous earth was first reported by Howard and Martin (17), who used it to separate $C_{12}$ to $C_{18}$ fatty acids. Other workers have used this type of silanized support to separate mixtures of adrenal steroids extracted from blood (18), to purify labeled steroids (19), and to separate these labeled steroids from urine (20). The application of silanized purified siliceous earth to the determination of steroids in oil dosage forms has not been previously described.

Silanized purified siliceous earth provides a convenient method for separating testosterone propionate from the glycerides, which constitute the major portion of the oil. The eluate from the

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[1] Marketed as Florisil by the Floridin Co., Tallahassee, Fla.

[2] Marketed as Celite 545, acid-washed, by the Johns-Manville Corp., New York, N. Y.

reverse phase column contains the testosterone propionate, together with the sterols and triterpenoids from the vehicle. It also contains any free testosterone which may be present. The testosterone propionate is separated from these materials by direct phase partition chromatography as previously described (14, 16).

## EXPERIMENTAL

**Materials**—*Purified Siliceous Earth.*[2]

*Redistilled n-Heptane*—The absorbance *versus* ethanol in 1-cm. cells should be less than 0.500 in the range 250–360 mμ (limit of aromatic content). The residue from the evaporation of 25 ml. of distillate, dissolved in 10 ml. of ethanol, should have an absorbance not greater than 0.01 from 230 to 360 mμ (nonvolatile residue limit).

*Nitromethane*—Reagent grade.

*Silanized Purified Siliceous Earth*—Prepared essentially by the procedure of Howard and Martin (17). Place about 450 Gm. of purified siliceous earth in a large open glass crystallizing dish in a vacuum desiccator containing 30 ml. of silane.[3] Apply vacuum (water aspirator) intermittently for several hours until no liquid silane remains. Float the treated purified siliceous earth on distilled water and gently agitate to allow any uncoated particles to sink. Skim the coated purified siliceous earth off the surface, wash it on a sintered-glass funnel with warm methanol until the filtrate is no longer acidic, and dry at 110°.

*Solvents*—Shake 200 ml. of 90% ethanol with 100 ml. of redistilled *n*-heptane to equilibrate. Use the upper phase as the immobile phase on the reverse phase column and the lower phase as the eluant.

**Assay Preparation**—Dilute an accurately measured sample with *n*-heptane tc obtain a solution containing 0.5 mg. of testosterone propionate per ml.

*Column I (Reverse Phase Column)*—Pack a pledget of fine glass wool in the base of a chromatographic tube (25 × 250 mm. test tube to which is fused a 5-cm. length of 6 or 8-mm. tubing) with the aid of a tamping rod of stainless steel, aluminum, or glass at least 12 in. long and having a disk with a diameter about 1 mm. less than that of the chromatographic tube. To 3.0 Gm. of silanized purified siliceous earth in a 100-ml. beaker add 3 ml. of equilibrated *n*-heptane and incorporate by kneading thoroughly with a flexible spatula blade until the mixture is fluffy. Transfer to a chromatographic tube and tamp gently to compress the material to a uniform mass. In a like manner mix a 2-ml. aliquot of the diluted sample with 3.0 Gm. of silanized purified siliceous earth and pack above the lower layer. Dry-wash the beaker with about 1 Gm. of silanized purified siliceous earth and add this to the column. Place a small pad of glass wool above the column packing.

*Colume II (Direct Phase Column)*—Mix 8.0 Gm. of purified siliceous earth with 9.0 ml. of nitromethane and pack in a column as described above.

**Procedure**—Pass 35 ml. of equilibrated ethanol over column I, collecting the eluate in a 50-ml. beaker or conical flask. Evaporate the eluate just to dryness on a steam bath with the aid of a gentle stream of air. If the sample contains benzyl benzoate, wash down the side of the container with 5 ml. of methanol, add 5 ml. of distilled water, and re-evaporate. Repeat this evaporation step to insure complete removal of the benzyl benzoate.

Dissolve the residue in 5 ml. of *n*-heptane, heating on a steam bath and stirring to dissolve. Cool the solution to room temperature and transfer it quantitatively to column II. Wash the container with 5 ml., followed by 30 ml. of *n*-heptane and then continue the column elution with *n*-heptane. Discard the first 30 ml. of eluate. Collect the next 10-ml. fraction separately as a check[4] and then the next 150 ml. of eluate in a 250-ml. conical flask. Evaporate the 150-ml. fraction to dryness on a steam bath under a gentle stream of air. Dissolve the residue in small portions of 95% ethanol and transfer quantitatively to a 100-ml. volumetric flask. Add 95% ethanol to volume and mix. Concomitantly determine the absorbance of this solution and of a solution of the U.S.P. testosterone propionate reference standard in the same solvent at a concentration of about 10 mcg./ml. in 1-cm. cells at the absorbance maximum at about 240 and at 300 mμ in a suitable spectrophotometer, using 95% ethanol as the blank. Calculate the quantity of testosterone propionate, in milligrams, in the eluate by the formula $0.1\ C_s A_u / A_s$, where $A_u$ is the absorbance of the sample at 240 mμ minus the absorbance of the sample at 300 mμ, $A_s$ is the corresponding value for the standard, and $C_s$ is the concentration of the standard solution in mcg./ml.

**Identification Test**—Evaporate the alcoholic solution of the isolated testosterone propionate to dryness in a suitable container. Transfer the residue obtained to a mullite mortar and prepare a potassium bromide disk in the usual manner. The infrared spectrum obtained should be identical with that obtained from a disk of testosterone propionate reference standard.

## RESULTS AND DISCUSSION

For the application of reverse phase chromatography to the separation of the steroid from the bulk of the oil, several supporting media were investigated. Silanized purified siliceous earth was very effective in achieving the separation; no differences were shown by purified siliceous earth silanized with the combinations of several alkyl chlorosilanes. Nonsilanized purified siliceous earth cannot be used because it does not retain the *n*-heptane as the stationary phase. A fluorocarbon support did not give the desired separations.

During the reverse phase chromatographic partitioning, essentially all of the steroid is eluted in the first 10 ml. of eluate; however, 35 ml. of eluant is used to insure complete recovery of the steroid. Additional eluant up to 150 ml. does not remove any further quantities of ultraviolet-absorbing material from samples containing as low as 10 mg. of testosterone propionate per ml. of oil. This is the

[2] Various silanes used included 15 ml. each of dimethyldichlorosilane and trimethylchlorosilane or 30 ml. of Dow-Corning 1208 (a mixture of methyl trichlorosilane and dimethyldichlorosilane in the approximate weight ratio of 1:2).

[4] This fraction should be free of both testosterone propionate and oil. Evaporate it to dryness and dissolve the residue in 10.0 ml. of 95% ethanol. If the solution has any significant absorbance in the 230–240 mμ range, the column is not functioning properly.

TABLE I—ANALYSIS OF TESTOSTERONE PROPIONATE IN OIL BY COLUMN PROCEDURES

| Concn., mg./ml. of Oil | Vehicle Compn.[a] | Testosterone Propionate mg. Taken | mg. Found | % Recovered | Av. |
|---|---|---|---|---|---|
| 10 | *A* | 1.044 | 1.036 | 99.2 | |
| | | | 1.040 | 99.6 | |
| | | | 1.034 | 99.0 | 99.3 |
| 10 | *A* | 0.994 | 0.997 | 100.1 | |
| | | | 0.983 | 98.7 | |
| | | | 0.989 | 99.3 | 99.4 |
| 25 | *A* | 0.984 | 0.973 | 98.9 | |
| | | | 0.963 | 97.9 | |
| | | | 0.969 | 98.5 | 98.4 |
| 25 | *B* | 1.012 | 1.008 | 99.6 | |
| | | | 1.021 | 100.9 | |
| | | | 0.995 | 98.3 | 99.6 |
| 25 | *B* | 0.994 | 0.984 | 99.0 | |
| | | | 0.982 | 98.8 | |
| | | | 0.979 | 97.6 | 98.5 |
| 50 | *B* | 1.000 | 0.985 | 98.5 | |
| | | | 0.981 | 98.1 | |
| | | | 0.991 | 99.8 | 98.8 |
| 50 | *C* | 0.958 | 0.956 | 99.8 | |
| | | | 0.950 | 99.2 | |
| | | | 0.940 | 99.0 | 99.3 |
| 50 | *D* | 0.958 | 0.964 | 100.6 | |
| | | | 0.952 | 99.4 | |
| | | | 0.940 | 98.1 | 99.4 |
| 50 | *E* | 0.958 | 0.954 | 99.6 | |
| | | | 0.944 | 98.5 | |
| | | | 0.938 | 97.9 | 98.7 |

[a] *A*, Five per cent benzyl alcohol in sesame oil; *B*, 3% benzyl alcohol in sesame oil; *C*, sesame oil: sample heated on water bath for 5 min. to dissolve testosterone propionate; *D*, sesame oil: sealed in ampul, heated at 100° for 2 hr.; *E*, sesame oil: sealed in ampul, heated at 160° for 2 hr.

lowest concentration found in commercial samples, and hence the concentration containing the greatest amount of oil in the aliquot analyzed.

About 99% of the oil, essentially fatty acid glycerides, is retained on the reverse phase column. The sterols and triterpenoid components of the oil are eluted together with the testosterone propionate, as are free testosterone and a number of the common adjuvants. Benzyl alcohol, chlorobutanol, and phenol are readily removed azeotropically during the evaporation of the ethanol eluate from the reverse phase column. Benzyl benzoate is partially removed in this step, but the subsequent evaporation of the 50% methanol added to the residue is necessary to completely volatilize this high boiling liquid. None of the testosterone propionate is lost during this evaporation. The parabens are not removed in this step, but are retained on the direct phase column in the ensuing step of the procedure.

The final separation of the testosterone propionate from the sterols and triterpenoids of the oil and any free testosterone on the purified siliceous earth—nitromethane column gives quantitative yields of pure testosterone propionate which is quantitated by its ultraviolet absorbance and identified by its infrared spectrum. The sterols are completely removed by the first 15 ml. of *n*-heptane. A distinct interval of about 30 ml. then follows before the appearance of testosterone propionate in the eluate. The triterpenoids, such as sesamin in sesame oil, are not eluted with *n*-heptane but are retained on the purified siliceous earth–nitromethane column. Testosterone is also retained on this column.

A series of standard samples were prepared to contain 10 to 50 mg. of testosterone propionate per ml. of sesame oil. The recoveries obtained by the column procedure are shown in Table I. Twelve commercial samples with concentrations ranging from 10 to 100 mg./ml. of oil were also analyzed by the column procedure (Table II).

The same commercial samples (Table II) and a number of standard samples (Table III) were also analyzed by the U.S.P. procedure. The results of these U.S.P. analyses were almost always higher than those obtained by the proposed method. Some of these standard samples (samples 1–11, Table III) were prepared by simply adding the weighed testosterone propionate to the measured volume of vehicle in the reaction flask, with no intermediate treatment prior to adding the reagents and completing the analysis. The analyses of these standards at the 50 mg./ml. level by the U.S.P. procedure averaged 3% higher than those by the proposed procedure, while those of the commercial samples averaged 8% higher. In order to resolve this anomaly the effects of the usual procedures used in the manufacturing of commercial samples were investigated.

TABLE II—ANALYSIS OF COMMERCIAL SAMPLES OF TESTOSTERONE PROPIONATE IN OIL

| Labeled mg./ml. of Oil | Vehicle Compn.[a] | Column Procedure Found mg./ml. | Column Procedure Found % Label | U.S.P. Procedure Found mg./ml. | U.S.P. Procedure Found % Label |
|---|---|---|---|---|---|
| 10 | *A* | 9.32 | 93.2 | 10.15 | 101.5 |
| | | 9.24 | 92.4 | 9.28 | 92.8 |
| | | 9.18 | 91.8 | | |
| 25 | *B* | 24.48 | 97.9 | 26.78 | 106.9 |
| | | 24.68 | 98.7 | 29.13 | 116.5 |
| | | 24.63 | 98.5 | | |
| 25 | *C* | 23.62 | 94.5 | 25.10 | 100.4 |
| | | 23.55 | 94.2 | 24.07 | 96.3 |
| | | 24.12 | 96.5 | | |
| 25 | *D* | 22.95 | 91.8 | 24.45 | 97.8 |
| | | 23.10 | 92.4 | 23.75 | 95.0 |
| | | 23.10 | 92.4 | | |
| 25 | *E* | 24.48 | 97.9 | 27.30 | 109.2 |
| | | 24.40 | 97.6 | 26.29 | 105.2 |
| | | 24.15 | 96.6 | | |
| 50 | *B* | 45.80 | 91.6 | 48.30 | 96.6 |
| | | 46.40 | 92.8 | 49.15 | 98.3 |
| | | 46.15 | 92.3 | 49.50 | 99.0 |
| | | | | 51.40 | 102.8 |
| 50 | *C* | 47.65 | 95.3 | 52.40 | 104.8 |
| | | 47.45 | 94.9 | 51.60 | 103.2 |
| | | 48.90 | 97.8 | | |
| 50 | *D* | 47.30 | 94.6 | 52.20 | 104.4 |
| | | 47.00 | 94.0 | 51.50 | 103.2 |
| | | 48.40 | 96.8 | | |
| 50 | *E* | 45.85 | 91.7 | 48.90 | 97.8 |
| | | 45.45 | 90.9 | 50.60 | 101.2 |
| | | 47.20 | 94.4 | | |
| 50 | *F* | 47.45 | 94.9 | 49.00 | 98.0 |
| | | 46.65 | 93.3 | 49.75 | 99.5 |
| | | 46.60 | 93.2 | 49.50 | 99.0 |
| | | 46.50 | 93.0 | 48.80 | 97.6 |
| 100 | *G* | 90.4 | 90.4 | 91.1 | 91.1 |
| | | 91.7 | 91.7 | 92.7 | 92.7 |
| | | 92.5 | 92.5 | | |
| 100 | *H* | 90.2 | 90.2 | 93.1 | 93.1 |
| | | 90.6 | 90.6 | 91.0 | 91.0 |
| | | 92.3 | 92.3 | | |

[a] *A*, Three per cent benzyl alcohol, 0.5% chlorobutanol in sesame oil; *B*, 0.5% chlorobutanol in sesame oil; *C*, 0.08% methylparaben, 0.1% propylparaben in sesame oil; *D*, 1:4000 benzethonium octyloxyacetate in peanut oil; *E*, 0.5% phenol in sesame oil; *F*, 0.1% propylparaben, 3% benzyl alcohol in sesame oil; *G*, 10% benzyl alcohol, 0.08% methylparaben, 0.1% propylparaben in sesame oil; *H*, 25% benzyl benzoate, 0.5% chlorobutanol in sesame oil.

A standard sample (50 mg./ml.) was prepared by dissolving testosterone propionate in sesame oil by warming on a water bath. Portions were sealed in ampuls and subjected to dry heat sterilization. The U.S.P. method gave recoveries of 110 to 112.7% as compared with the average of 102% obtained with the unheated oil. The heating had no effect on recoveries obtained with the proposed procedure.

Blank samples of 1 ml. of sesame oil which were not heated and heated oil treated by the U.S.P. procedure yielded residues which corresponded to 1.4 and 4.1%, respectively, based on a 50-mg./ml. sample of the steroid. Greater amounts of material were contributed by the oil in the presence of testosterone propionate semicarbazone, due to coprecipitation. Weighed amounts of this derivative were dissolved in the reagent prior to the addition of the blank oil. After carrying through the U.S.P. procedure the increase contributed by the heated oil was 2 to 4 times that given by the unheated oil, paralleling the findings of the standard analyses.

The isolated derivatives from the U.S.P. assay were examined by thin-layer chromatography.[5] The material obtained from the blank oils gave a number of spots, several corresponding to the sterol and triterpenoid fractions of the oil. That from the heated oil gave more than that from the unheated oil. These same spots, as well as that of testosterone propionate semicarbazone, were ob-

[5] Samples dissolved in $CHCl_3$ were spotted on silica gel GF 0.25 mm. Solvent system: $CHCl_3$–methanol–$H_2O$, 90:7.5:0.5 (21). Spray the developed chromatogram with 50% $H_2SO_4$. Heat with heat lamp until spots are visible. Examine plate with long wavelength ultraviolet light.

TABLE III—ANALYSIS OF TESTOSTERONE PROPIONATE IN OIL BY U.S.P. XVII ASSAY PROCEDURE

| Concn., mg./ml. of Oil | Vehicle Compn.[a] | Testosterone Propionate mg. Taken | mg. Found | % Recovered |
|---|---|---|---|---|
| 10 | *A* | 51.0 | 67.1 | 131.5 |
| 25 | *A* | 50.2 | 59.6 | 118.7 |
| 25 | *A* | 50.4 | 54.2 | 107.7 |
| 25 | *B* | 50.1 | 55.4 | 110.6 |
| 25 | *B* | 51.8 | 54.9 | 106.0 |
| 50 | *A* | 51.0 | 51.0 | 100.0 |
| 50 | *A* | 51.1 | 52.4 | 102.5 |
| 50 | *B* | 50.0 | 50.1 | 100.2 |
| 50 | *B* | 50.8 | 51.3 | 101.0 |
| 50 | *C* | 55.2 | 57.1 | 103.4 |
| 50 | *C* | 51.9 | 53.4 | 103.0 |
| 50 | *D* | 48.2 | 54.1 | 112.2 |
| 50 | *D* | 48.0 | 54.1 | 112.7 |
| 50 | *E* | 48.2 | 53.0 | 110.0 |

[a] *A*, Three per cent benzyl alcohol in sesame oil; *B*, 3% benzyl alcohol, 0.5% chlorobutanol in sesame oil; *C*, sesame oil; *D*, sesame oil: sample heated on water bath for 5 min. to dissolve testosterone propionate; *E*, sesame oil: sealed in ampul, heated at 160° for 2 hr.

tained from the standards and from the commercial samples. Several of the latter gave in addition a spot corresponding to free testosterone semicarbazone.

In contrast, the testosterone propionate isolated by the proposed procedure gives a single spot by thin-layer chromatography.

## SUMMARY

Spectrophotometrically and chromatographically pure testosterone propionate is recovered quantitatively from its oil dosage forms by a combination of reverse phase and direct phase partition chromatography. The testosterone propionate, together with sterols and triterpenoids as well as any free testosterone, is separated from the bulk of the oil in the reverse phase process; it is then isolated free of these substances by direct phase partition. The procedure is applicable to samples containing such adjuvants as benzyl alcohol, benzyl benzoate, chlorobutanol, phenol, and the parabens.

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# *Technical Articles*

# Effects of Selected Variables on the Microencapsulation of Solids

By L. A. LUZZI* and R. J. GERRAUGHTY

**The hypothesis that solid particles may be protectively encapsulated by coacervation led to the investigation of the extractability of microcapsules, which contained solids, by gastrointestinal fluids. Starting pH, starting temperature, ratio of solid to encapsulating materials, quantity of denaturant, and final pH were varied. The effects of the variables studied on microcapsules were determined by submitting samples to simulated gastrointestinal fluids for periods of up to 2.5 hr. and then comparing the extracts spectrophotometrically to known absorption spectra. Results showed that all variables effected some degree of change in microcapsules.**

THE TERM "coacervation" has recently been used to describe the salting out of a lyophilic solid into liquid droplets rather than solid aggregates (1). The term was introduced into colloidal chemistry by Kruyt and Bungenberg de Jong (2) to describe the flocculation or separation of liquids from solution, where at least one of the liquids contained a colloidal solute.

Coacervation has been subdivided into simple coacervation and complex coacervation. Briefly,

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* Present address: School of Pharmacy, University of Georgia, Athens, GA 30601