Acetonitrile–Diatomaceous Earth Column for Separation of Steroids and Other Compounds

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Abstract □ The retention characteristics of 53 compounds on an acetonitrile–diatomaceous earth column are given for elution with *n*-heptane followed by stripping with chloroform. Nineteen of the compounds were eluted completely, seven were eluted partially, and 27 (including the 12 corticosteroids tested) were not eluted at all by 150 ml. of *n*-heptane. Partially eluted compounds could be completely eluted by larger volumes of *n*-heptane. Elution characteristics in a group of similar compounds are affected greatly by relatively minor changes in structure. The recommended procedure furnishes a simple and rapid method for the separation of mixtures and for the detection of impurities or decomposition in standards. Several pharmaceutical preparations and known mixtures were analyzed as examples of the application of the column procedure to various products.

Keyphrases □ Steroids—separation using acetonitrile–diatomaceous earth column, retention characteristics of 53 compounds, detection of impurities □ Column chromatography—separation of steroids on acetonitrile–diatomaceous earth column, retention characteristics of 53 compounds, detection of impurities □ Acetonitrile–diatomaceous earth column—separation of steroids □ Diatomaceous earth–acetonitrile column—separation of steroids □ Chromatography, column—separation of steroids using acetonitrile–diatomaceous earth column, retention characteristics of 53 compounds, detection of impurities

The separation of mixtures of steroids and of steroids from other compounds in pharmaceuticals can be difficult if the preparation is other than the official one in USP XVIII (1) or NF XIII (2). Both aqueous and nonaqueous immobile phase systems are available for use with diatomaceous earth, but aqueous systems are the most popular for the separation of steroids (3). Examples of nonaqueous systems include the use of kieselguhr treated with dichlorodimethylsilane vapors to retain the less polar component of many solvent systems used in the separation of C_{12}–C_{18} fatty acids (4). This procedure is known as reverse phase chromatography and was used (5) for the analysis of progesterone in oil injectables. An acetonitrile immobile phase was suggested (6) for the separation of corticosteroids from decomposition products and interfering substances in the analysis of pharmaceuticals containing corticosteroids; this column was used also for the separation of estradiol valerate and estradiol isovalerate from estradiol[1].

This paper reports the elution characteristics of 53 compounds from the acetonitrile immobile phase column and the application of such a column to the separation of steroids of similar structure and to the analysis of pharmaceuticals containing either more than one steroid or interfering substances. The method is simple, flexible, and rapid; three determinations can be completed in approximately 3 hr., and the column can be modified for the removal of acidic, basic, and other water-soluble impurities by using trap layers (6).

[1] T. James and B. Radar, Food and Drug Administration, Los Angeles, Calif., personal communication.

EXPERIMENTAL

Equipment—The following were used: UV-visible recording spectrophotometer[2] with 1-cm. quartz cells, glass chromatographic columns for partition chromatography (2.2 × 25 cm., constricted at one end to 0.4 × 5 cm.) and an aluminum tamping rod to fit the column, gas–liquid chromatograph[3] with a flame-ionization detector and a 4-mm. × 0.91-m. (3-ft.) column of 3% SE-33 on 100–120-mesh Gas Chrom Q (column at 252°, detector at 280°, injector port at 258°, and nitrogen carrier gas flow rate at 40 ml./min.), IR spectrophotometer[4], electrobalance[5], and TLC plates coated with silica gel G with fluorescent indicator[6].

Reagents—*Solvents*—All solvents were spectro, certified, analytical reagent, USP, or distilled-in-glass grade. Acetonitrile, *n*-pentane, *n*-hexane, *n*-heptane, 2,2,4-trimethylpentane, cyclohexane, chloroform, methanol, alcohol USP, and absolute ethanol were used. Solvents used in the UV method should be checked photometrically to ensure the absence of aromatic hydrocarbons.

Acetonitrile–n-Heptane (Mutually Saturated)—Mix 25 ml. of acetonitrile and 300 ml. of *n*-heptane (sufficient for two determinations) in a separator, agitate vigorously for 2 min., and allow both layers to clarify. These mutually saturated solutions must be used whenever *n*-heptane or acetonitrile is called for in these directions. Mutually saturated acetonitrile and other hydrocarbons were prepared in the same way for studies without *n*-heptane.

Chloroform (Water Saturated)—Add 50 ml. of water to 250 ml. of chloroform in a separator, agitate for 2 min., and allow both layers to clarify. The water-saturated reagent is required when aqueous trap layers are inserted but should not be used otherwise.

Diatomaceous Earth[7]—Acid washed was used.

Standards—All standard solutions were prepared to contain 1.00, 0.040, or 0.0100 mg./ml. In some studies, individually weighed samples of the solid standards were used.

Alcohol Sulfuric Acid Reagent—Carefully and slowly add 80 ml. of cold, concentrated sulfuric acid (97% w/w) to 20 ml. of cold, absolute ethanol with constant agitation. Cool to room temperature before use. Prepare fresh monthly. Other reagents used in the determinative steps were prepared as directed in the method reference.

Proposed Procedure—*Sample Preparation*—

Tablets: Weigh 20 tablets, grind to pass a 60-mesh sieve, mix thoroughly, and accurately weigh a sample containing approximately 1 mg. of steroid into a beaker. Add 1.5 ml. of acetonitrile, cover the beaker, and swirl occasionally for 10 min. Add 1.5 ml. of *n*-heptane, and swirl until the sample is completely dissolved. As indicated in the tables, the same volumes of absolute methanol and water should be substituted for the acetonitrile and *n*-heptane for some compounds. Continue as directed under *Sample layer*.

Capsules: Weigh the contents of 20 capsules and mix thoroughly. Accurately weigh a sample containing approximately 1 mg. of the steroid into a beaker and proceed as directed under *Tablets* beginning with: "Add 1.5 ml. of acetonitrile, cover the beaker, ..."

Creams: Make a composite by mixing thoroughly the contents of several tubes or jars. Accurately weigh a sample containing approximately 1 mg. of the steroid into a beaker. Proceed as directed under *Tablets* beginning with: "Add 1.5 ml. of acetonitrile, cover the beaker, ..."

[2] Cary model 15.
[3] Barber Colman model 5000.
[4] Perkin-Elmer model 337.
[5] Cahn model G-2.
[6] Eastman No. 6060.
[7] Celite 545, Johns Manville Product Corp., New York, NY 10016

Standards: Accurately weigh approximately 2 mg. of the standard into a beaker. Proceed as directed under *Tablets* beginning with: "Add 1.5 ml. of acetonitrile, cover the beaker, ..."

Column Preparation—

Acetonitrile layer: Insert a glass wool plug in the bottom of the chromatographic column. Thoroughly mix 4.0 g. of diatomaceous earth with 4.0 ml. of acetonitrile, transfer to the column, and pack firmly.

Trap layers: Prepare and insert neutral, acidic, or basic aqueous trap layers as needed according to Graham *et al.* (6).

Sample layer: Thoroughly mix the dissolved sample, prepared as directed under *Sample Preparation*, with 3.0 g. of diatomaceous earth. Transfer to the column above the acetonitrile or trap layer and pack firmly. Dry wash the beaker consecutively with 1.0 g. of diatomaceous earth and glass wool and transfer to the top of the column. Retain the sample beaker for washing with the *n*-heptane and chloroform to be added to the column later.

Column Elution—Wash the sample beaker successively with from four to 12 (depending on the steroid being determined) 25-ml. portions of *n*-heptane and pour onto the column. Adjust the flow rate to 5–7 ml./min., and maintain the liquid head between 8 and 12 cm. above the column bed with a separator attached to the top of the column. Allow the last wash to drain completely. Wash the tip with alcohol USP and save the effluent for further treatment. Rinse the sample beaker with from five to seven (depending on the steroid being determined) 25-ml. portions of chloroform and pour each through the column, maintaining the liquid head as close to the top of the column as possible. Allow the last portion to drain completely from the column. Rinse the tip with alcohol USP and save the effluent for further treatment. Carefully evaporate either or both effluents to dryness on a steam bath under a hood with a current of air to ensure complete removal of the acetonitrile.

Determinative Procedures—*Sample Preparation*—Dissolve the residues from the *n*-heptane and/or chloroform effluents in alcohol USP or absolute methanol and dilute accurately to a volume containing approximately 10 mcg./ml. For GLC, dilute to a volume which contains approximately 1 mg./ml. Use a proper size aliquot of these solutions for quantitation by one of the following determinative methods.

Phenylhydrazine Method—Use the Silber–Porter (7) procedure without modification.

Isoniazid Method—Follow the Umberger (8) procedure using twice the recommended concentration of hydrochloric acid to increase the sensitivity.

UV Method—Scan the alcohol USP or the methanol solution of the sample residue in the region of absorbance using the same solvent as reference. Calculate by comparison with a standard of approximately the same concentration run concurrently.

Alcohol–Sulfuric Acid Method—Dissolve the sample residue in 0.50 ml. of absolute methanol and add 4.00 ml. of the alcohol–sulfuric acid reagent. Scan from 680 to 480 nm. and calculate by comparison with a standard of approximately the same concentration run concurrently.

GLC—Inject a proper size aliquot and calculate by comparison to a standard run concurrently using the area method.

Elution Studies—Each compound studied was put through the proposed procedure using 150 ml. of *n*-heptane and 125 ml. of chloroform. The amount of compound in each of the two eluates was determined by one determinative method to determine if the compound was retained or completely or partially eluted by the *n*-heptane.

Elution curves for those compounds partially or completely eluted by the 150 ml. of *n*-heptane were obtained using 300 ml. of *n*-heptane and collecting 15-ml. aliquots for analysis.

Separation Studies—Several mixtures prepared from standards were analyzed by the proposed method, using 300 ml. of *n*-heptane and 125 ml. of chloroform.

Decomposition Studies—During some elution studies using standards determined by the UV method, it was noted that shoulders appeared in the spectra in some aliquots and that two or more elution bands occurred in some cases. Further study to identify the products of suspected decomposition was performed for androst-4-ene-3,17-dione, dimethoxystilbestrol, norethandrolone, and testosterone enanthate, all of which had been purchased for use as standard substances.

Sample Studies—Several samples of steroid and other types of pharmaceutical preparations were run by the proposed method.

Table I—Effect of Various Hydrocarbons on Elution

Compound	Hydrocarbon	Recovery[a], %: Hydrocarbon Fraction	Recovery[a], %: Chloroform Fraction	Recovery[a], %: Total
Androst-4-ene-3,17-dione[b]	Isooctane	23.3	78.6	101.8
	n-Pentane	52.4	47.0	99.4
	n-Heptane	57.8	42.0	99.8
	n-Hexane	70.8	25.0	95.8
	Cyclohexane	97.7	2.8	100.5
Methyltestosterone[c]	*n*-Pentane	90.0	9.3	99.3
	Isooctane	93.3	5.1	98.3
	n-Heptane	97.1	1.6	98.7
	n-Hexane	98.7	1.1	99.8
	Cyclohexane	99.6	0.5	100.1
Testosterone[d]	Isooctane	5.5	94.7	100.2
	n-Heptane	8.8	92.7	101.5
	n-Hexane	48.9	51.8	100.7
	n-Pentane	75.4	24.3	99.7
	Cyclohexane	102.2	0.6	102.8

[a] Based on the use of 150 ml. of hydrocarbon followed by 125 ml. of chloroform. [b] Aldrich Chemical Co., Milwaukee, Wis. [c] NF Reference Standard. [d] Nutritional Biochemical Corp., Cleveland, Ohio.

METHOD DEVELOPMENT

Length of Acetonitrile Trap Layer—Columns were prepared using methyltestosterone and diatomaceous earth–acetonitrile ratios of 2 g. plus 2 ml., 4 g. plus 4 ml., 6 g. plus 6 ml., and 8 g. plus 8 ml. Twenty 15-ml. aliquots of *n*-heptane were collected and analyzed by the UV method. The primary effect was the increase in the breakthrough volume of *n*-heptane required as the length of the acetonitrile layer increased. Since the breakthrough volume for the 2 g. plus 2 ml. column was less than desirable for many steroids, and the breakthrough volume for the 6 g. plus 6 ml. was larger than necessary, the 4 g. plus 4 ml. ratio was selected as the optimum.

Hydrocarbon Solvent—Various hydrocarbon solvents were studied using testosterone, methyltestosterone, and androst-4-ene-3,17-dione. In each case, the recommended procedure was followed using 150 ml. of the hydrocarbon and 125 ml. of chloroform and then analyzing the residues by the UV method. These three steroids were selected since they appear in both the hydrocarbon and chloroform effluents of the proposed method but in significantly different ratios. There are differences in the elution characteristics of the various solvents as shown in Table I. The hydrocarbon of choice for these three compounds is clearly cyclohexane, but elution data on other steroids prompted the selection of *n*-heptane.

Flow Rate of Hydrocarbon—Testosterone was run by the proposed method, except that the flow rate was adjusted to 1 ml./min. and the 300 ml. of *n*-heptane used was collected in 15-ml. aliquots for UV analysis. The experiment was repeated using a flow rate of 6 ml./min., and both rates were repeated using methandrostanolone. The main effects were a slight broadening of the peaks and the slightly larger volumes (less than 5%) required to attain the peak maxima at the more rapid flow rates. Therefore, the flow rate is not critical and the great saving in time offsets the slight broadening caused by the faster rate.

RESULTS AND DISCUSSION

Fifty-three different compounds were checked by the proposed method, using 150 ml. of *n*-heptane and 125 ml. of chloroform to determine if they were retained on the acetonitrile layer or were partially or completely eluted by the *n*-heptane (Table II). Since the chloroform completely strips the acetonitrile and dissolved substances from the column, any portion of the compound not eluted by the *n*-heptane appears in the chloroform effluent. All 12 corticosteroids listed in Table II [plus nine that were reported previously. (6)] were retained by acetonitrile and were not eluted by the hydrocarbon solvent, even though some were esterified in the C_{17} or C_{21} position. Of the remaining compounds, 19 were completely eluted by the *n*-heptane, seven were partially eluted by the

n-heptane, and 15 were retained by acetonitrile and determined in the chloroform effluent.

The elution curves for compounds that were partially or completely eluted by the *n*-heptane were determined using 15-ml. portions for a total of 300 ml. of the *n*-heptane (last column of Table II); typical elution curves are shown in Fig. 1. The elution curve for the removal of the acetonitrile has been reported (6).

Information concerning the effect of slight changes in structure upon the retention or elution of a compound is found in Table II. As an illustration, consider the first three compounds listed. Androstanolone is completely eluted by the 150 ml. of *n*-heptane, androst-4-ene-3,17-dione is partially eluted, and androst-4-ene-3,11,17-trione is retained completely by the acetonitrile layer. In the conversion of androstanolone to androst-4-ene-3,17-dione, the hydroxyl group at C_{17} is converted to a carbonyl and a single bond is converted to a double bond between C_4 and C_5. The further addition of a carbonyl at C_{11} to form androst-4-ene-3,11,17-trione completely reverses the elution characteristics of the androstanolone.

If the single bond between C_3 and C_5 in androstanolone is converted to a double bond, the resulting compound is testosterone (Table II), which differs from androst-4-ene-3,17-dione only by having a hydroxyl instead of a carbonyl at C_{17}. However, less than 10% of the testosterone but more than 50% of the androst-4-ene-3,17-dione is eluted by the 150 ml. of *n*-heptane. The addition

Table II—Effects of Structural Changes on Elution

Compound	Source[a]	Sample Solvent Used[b]	Determinative Procedure[c]	Recovery[d], % *n*-Heptane Fraction	Recovery[d], % Chloroform Fraction	Volume[e], ml.
Androstanolone	10	MW	SP	99.5	BDL[f]	—
Androst-4-ene-3,17-dione	1	MW	INH	57.8	42.0	200
Androst-4-ene-3,11,17-trione	6	MW	UV	BDL	100.3	—
Betamethasone-17-benzoate	15	MW	UV	BDL	101.9	—
Betamethasone-21-benzoate	15	MW	UV	BDL	101.8	—
Butabarbital	2	MW	UV	BDL	102.5	—
Chlorotrianisene	9	MW	UV	100.4	BDL	—
Corticosterone	12	MW	UV	BDL	99.1	—
Cortisone	7	MW	UV	BDL	99.7	—
Desoxycorticosterone	14	MW	UV	BDL	101.5	—
Dienestrol	9	AH	UV	BDL	97.7	—
Diethylstilbestrol	14	MW	UV	BDL	96.3	—
Diethylstilbestrol dipropionate	8	MW	UV	103.4	BDL	60
Dimethisterone	14	MW	UV	99.1	BDL	128
Dimethoxystilbestrol	10	AH	UV	100.3	BDL	15
Equilenin	12	AH	UV	BDL	100.9	—
Equilin	3	AH	SP	BDL	96	—
Estradiol	9	AH	UV	BDL	99.8	—
Estradiol benzoate	9	AH	UV	100.6	BDL	130
Estradiol valerate	14	MW	UV	102.7	BDL	70
1,3,5(10)-Estratrien-17α-methyl-3,17β-diol-3-methyl ether	12	AH	UV	101.3	BDL	52
Estrone	9	AH	UV	BDL	101.9	—
Estrone-3-methyl ether	12	AH	UV	100	BDL	45
Ethinyl estradiol	14	AH	UV	BDL	100.5	—
Ethisterone	9	MW	UV	BDL	99.8	—
Ethynodiol diacetate	11	MW	AS	102.2	BDL	—
Fluorometholone	9	MW	UV	BDL	102.4	—
Fluoxymesterone	13	MW	UV	BDL	101.6	—
Fluprednisolone	9	MW	UV	BDL	102.1	—
Hexestrol	10	MW	UV	BDL	100.5	—
Hydrocortisone	14	MW	UV	BDL	100.0	—
Lyndiol	5	MW	GLC	100.3	BDL	102
Medroxyprogesterone acetate	14	AH	UV	67.5	32.1	300
Mestranol	11	AH	UV	98.8	BDL	125
Methandrostenolone	14	MW	UV	9.4	90.8	210
Methylprednisolone	9	MW	UV	BDL	100.8	—
Methyltestosterone	9	MW	UV	97.1	1.6	165
Norbolethone	5	MW	UV	101.4	BDL	55
Norethandrolone	11	MW	UV	98.0	BDL	105
Norethindrone	5	MW	UV	BDL	101.6	—
Norethindrone acetate	5	MW	UV	84.8	15.3	200
Norethynodrel	5	MW	UV	16.0	81.7	200
Paramethasone acetate	11	MW	UV	BDL	99.9	—
Prednisone acetate	12	MW	UV	BDL	102.0	—
4-Pregnen-17α,21-diol-3,20-dione	12	MW	UV	BDL	101.5	—
Progesterone	9	MW	UV	100.7	BDL	75
Spironolactone	14	MW	INH	BDL	100.7	—
Stilbene (*trans*)	4	MW	UV	91.5[g]	BDL	—
Testosterone	10	MW	UV	8.8	92.7	200
Testosterone cypionate	14	MW	UV	97.9	BDL	30
Testosterone enanthate	14	MW	UV	97.8	BDL	—
Testosterone propionate	14	MW	INH	99.8	BDL	40
Triamcinolone	14	MW	UV	BDL	98.4	—

[a] 1, Aldrich Chemical Co., Milwaukee, Wis.; 2, Applied Science Laboratories, State College, Pa.; 3, Ayerst Laboratories, New York, NY 10017; 4, Chemical Service, Media, Pa.; 5, Division of Pharmaceutical Sciences, FDA, Washington, D. C.; 6, Eastman Organic Chemicals, Rochester, N. Y.; 7, K & K Laboratories, Plainview, N. Y.; 8, Matheson, Coleman and Bell, East Rutherford, N. J.; 9, NF Reference Standard; 10, Nutritional Biochemical Corp., Cleveland, Ohio; 11, G. D. Searle and Co., Chicago, Ill.; 12, Schwarz-Mann, Orangeburg, N. Y.; 13, The Upjohn Co., Kalamazoo, Mich.; 14, USP Reference Standard; and 15, Warner Lambert Co., Morris Plains, N. J. [b] MW = methanol-water, and AH = acetonitrile-*n*-heptane. [c] SP = Silber-Porter, INH = isonicotinic acid hydrazide, UV = ultraviolet, AS = alcohol-sulfuric acid, and GLC = gas-liquid chromatography. [d] *n*-Heptane (150 ml.) followed by 125 ml. of chloroform in each case. [e] Volume of *n*-heptane needed for complete elution in *n*-heptane fraction. [f] BDL = below detectable limits. [g] Probable loss due to volatilization.

Table III—Separation of Prepared Standard Mixtures[a]

Components	Volume of *n*-Heptane Used, ml.	Recovery, %—*n*-Heptane Fraction	Recovery, %—Chloroform Fraction
Estradiol benzoate[b]–estradiol[c]	175	99.6	101.6
Estradiol valerate[b]–estradiol[c]	150	102.0	101.0
Mestranol[b]–ethinyl estradiol[c]	175	100.2	99.5
Progesterone[b]–estrone[c]	150	99.3	101.9
Progesterone[b,d]–estrone[c]	150	98.9	102.3
Estrone-3-methyl ether[b]–estrone[c]	150	102.3	101.3
Dimethisterone[b]–ethisterone[c]	150	100.8	100.8
Dimethisterone[b]–furosemide[c]	150	99.2	101.2
Testosterone propionate[b,e]–testosterone[b,e]–norethindrone[c]	205[e]	100.0[e] 100.2[e]	99.5
Diethylstilbestrol dipropionate[b]–diethylstilbestrol[c]	150	100.9	98.6

[a] Each mixture contained 2.00 mg. of each component. [b] Eluted in the *n*-heptane fraction. [c] Eluted in the chloroform fraction. [d] This mixture contained 26.0 mg. of progesterone. [e] *n*-Heptane added in two portions: 55 ml. plus 150 ml. The propionate was eluted completely in the first portion and the testosterone in the second.

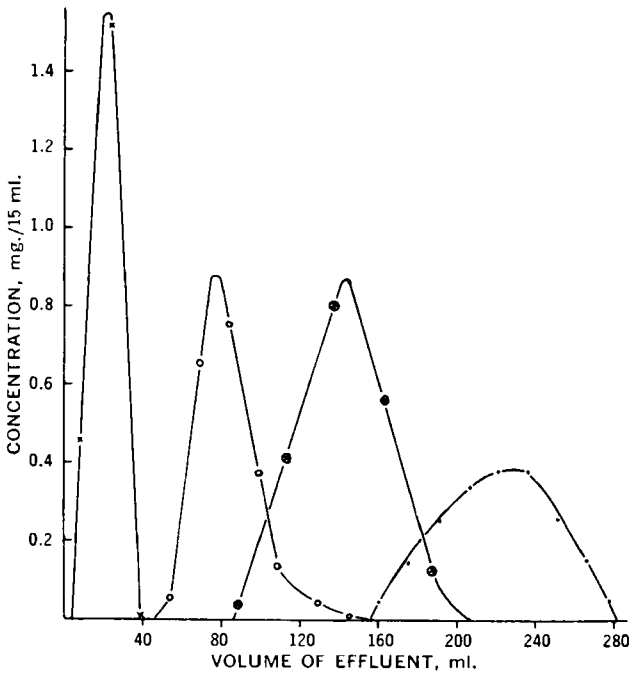

Figure 1—*Typical elution curves for steroids eluted in the* n-*heptane fraction. Key:* ×, *testosterone propionate;* ○, *methyltestosterone;* ⊗, *testosterone; and* ●, *medroxyprogesterone acetate.*

of a methyl group alpha to the C_{17} hydroxyl of testosterone produces methyltestosterone (Table II), which is essentially completely eluted by the *n*-heptane. While the addition of a double bond between C_4 and C_5 greatly decreases the amount of this type of steroid eluted by the *n*-heptane, the difference between a hydroxyl and a carbonyl at C_{17} increases the amount eluted by the *n*-heptane by approximately 50%. However, the effect of the addition of the double bond is offset by the steric hindrance caused by a methyl group alpha to C_{17} since methyltestosterone has essentially the same elution characteristics as androstanolone.

For steroids that are eluted by *n*-heptane, any change in structure that decreases the polarity of the molecule or increases steric hindrance decreases the volume of *n*-heptane necessary for complete elution. Conversely, any change that increases the polarity or decreases steric hindrance increases the volume necessary for complete elution.

Another example using estradiol, estradiol valerate, and estrone (Table II) shows the effect of changes in a single functional group. Estradiol is retained in the acetonitrile layer while estradiol valerate is eluted completely by the 150 ml. of *n*-heptane. Thus, esterification of the hydroxyl group at C_{17} completely reverses the elution characteristics. However, the compound estrone, which is formed by the conversion of the hydroxyl group at C_{17} to a carbonyl, has the same elution characteristics as estradiol. Any change that decreases the polarity of the estradiol molecule, such as esterification at either C_3 or C_{17} or formation of a methyl ether at C_3, apparently produces a molecule that can be eluted by *n*-heptane.

Similarly, diethylstilbestrol is retained by acetonitrile and appears in the chloroform effluent. Conversion to the dipropionate or dimethoxy derivative produces compounds that are eluted completely by *n*-heptane.

Results using the proposed method in the separation and determination of prepared mixtures are shown in Table III. The residues from the evaporation of the *n*-heptane and chloroform effluents were checked by TLC to ensure that separation was complete. For satisfactory separations, one compound should be eluted completely by the *n*-heptane while the other is retained by acetonitrile. The volume of *n*-heptane used depended upon the compounds being separated. The mixture of testosterone propionate, testosterone, and norethindrone is an example of the ability of the proposed method to separate two substances that are completely eluted by the *n*-heptane if there is a sufficient difference in the volumes necessary for complete elution. Information concerning the volumes necessary for complete elution is given in Table II.

The proposed method can also be used to detect decomposition in standard materials by running elution curves, using 15-ml. portions for determination by the UV method. As an example, the UV spectra for the last few portions in the elution of standard androst-4-ene-3,17-dione showed a shoulder at 280 nm. on the expected peak at 240 nm. The fact that the shoulder did not appear until the major portion of the standard had been eluted indicated that the impurity

Table IV—Determination of Pharmaceutical Samples

Type	Components and Declared Amounts	Trap Layer Used	*n*-Heptane Fraction, ml.	Chloroform Fraction, ml.	Found, Percent of Declared—Avg.	Found, Percent of Declared—Range	Number of Determinations
Tablet	Methyltestosterone, 3 mg. Estradiol, 0.2 mg.	Acidic	200	125	109 97	(108–110) (95–98)	3 3
Tablet	Spironolactone, 25 mg.	None	150	125	103	(102–103)	4
Tablet[a]	Methyltestosterone, 10 mg. Esterified estrogens, 1.25 mg.	Acidic	200	125	82 ND[b]	(81–82) ND	3 –
Cream[c]	Iodochlorhydroxyquin, 3% Hydrocortisone, 0.5%	Neutral Aqueous	150	125	99 103	(99) (103–104)	2 2
Tablet[d]	Sodium butabarbital	8% Sodium bicarbonate	150	125	88	(88)	2

[a] Recovery of added methyltestosterone standard was 101.0%. Sample contained stearic acid, which eluted with the *n*-heptane. [b] ND = not determined. [c] Cream also contained allantoin 1%, stearic acid, glycerol monostearate, petrolatum, squalene, propylene glycol, polyoxyethylene stearate, and methylparabens. [d] Tablet also contained stearic acid, which eluted in the *n*-heptane fraction.

was more polar than the standard, but the actual structure was not determined. The impurity in this case was estimated to be approximately 0.2%.

Similarly, the UV spectra for the volumes collected during the elution of standard dimethoxystilbestrol showed two separate components, one contained in the first 15-ml. fraction and the second contained in the fourth through seventh fractions. The first fraction contained 93.6% of the standard and was shown to be the dimethoxystilbestrol. The second compound was shown to be the monomethoxy by its IR spectrum and represented approximately 6.1% of the starting material. The norethandrolone standard was found to contain two impurities, one of which was less polar and the other more polar than norethandrolone, but the actual structures were not determined since they represented only about 0.6% of the original material.

A standard sample of testosterone enanthate, which previous work had indicated might be contaminated, was put through the elution procedure. The undecomposed standard was completely eluted in the first 75 ml. of *n*-heptane and was calculated to represent 64% of the original material. A second compound was eluted between 85 and 200 ml. of *n*-heptane and was shown to be testosterone by its IR spectrum. The complete elution volume of 200 ml. also agrees with the value listed in Table II for testosterone.

The results of several pharmaceutical samples determined by the proposed method are shown in Table IV. The replication and recovery of added standards are satisfactory for these types of materials. The sodium butabarbital tablet is included to illustrate the applicability of the method to samples other than steroids and steroid-like compounds. The butabarbital was present in the chloroform effluent.

The sample must be completely dissolved in the sample solvent before it is mixed with the diatomaceous earth to prepare the sample layer; otherwise, the elution peaks are not symmetrical. Most steroids and other compounds checked will dissolve satisfactorily in either the *n*-heptane–acetonitrile or methanol–water system, but the use of the *n*-heptane–acetonitrile usually produces more symmetrical elution peaks for compounds that are completely soluble in both systems. The volumes required for complete elution, however, are approximately the same for the individual compounds for either system. The *n*-heptane–acetonitrile should be used with all steroids containing an aromatic A ring, since these compounds tail badly when the methanol–water system is used.

In all cases, a white residue, which is insoluble in alcohol USP and methanol, is obtained upon evaporation of the chloroform fraction. The IR spectrum of the residue agrees with the IR spectrum of the diatomaceous earth, and the residue apparently is caused by the fact that some diatomaceous earth is either dissolved or rendered colloidal by the *n*-heptane–acetonitrile–chloroform mixture. The presence of the residue causes an increase in the baseline when the UV method is used for quantitation and must be removed before the measurement is made. The residue can be removed by filtration through a fine frit or can be allowed to settle for 30 or more min. before decantation of the solution. Alternatively, the beaker can be washed several times with small volumes of alcohol USP, methanol, or chloroform (be careful not to remove the residue) followed by evaporation before redissolving in the solvent of choice.

The proposed method can be used for the separation of steroids from steroid esters, for the detection of decomposition in steroid standards, and for the purification of steroid standards, as well as for the analysis of various pharmaceutical preparations. Work is continuing on the application of the method to the separation and determination of components other than steroids in pharmaceutical products.

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