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DRUG STANDARDS

Quantitative Separation of Progestins and Estrogens from Anovulatory Formulations

ANGEL ALVAREZ FERNÁNDEZ and VICTORIANO TORRE NOCEDA

Abstract □ The quantitative separation of progestins and estrogens, from orally administered anovulatory formulations, by gel filtration on synthetic polysaccharide (SephadexLH -20), and their direct determination by UV spectrometry is described.

Keyphrases □ Progestins, estrogens in dosage forms—quantitative determination □ Column chromatography—separation □ UV spectrophotometry—analysis □ GLC—analysis

A general method is described for the separation of progestins and estrogens from orally administered anovulatory formulations, on synthetic polysaccharide[1] with methanol–water (17:3) as eluant.

The procedure permits the separation of progestins (norethindrone, norethynodrel, megestrol acetate, norgestrel, chlormadinone acetate, and ethynodiol diacetate) from estrogens (ethynyl estradiol, mestranol, estradiol, and estradiol benzoate) in the variable proportions which are usually encountered in commercial anovulatories.

Excipients such as polyvinylpyrrolidone, magnesium stearate, lactose, starch, and talc do not inhibit the separation.

[1] Sephadex LH-20, Pharmacia Fine Chemicals Inc., New Market, N. J.

Methyltestosterone and prednisolone acetate may also be separated by this method from the estrogens cited.

In a formulation containing lynestrenol, mestranol, and α-tocopherol acetate, the progestin-estrogen separation was incomplete; however, the technique quantitatively separates α-tocopherol acetate from mestranol and each could be determined by UV spectrophotometry.

The orally administered anovulatory agents, which have been widely accepted in increasing numbers during these past years, are generally composed of estrogen-progestin mixtures of variable proportions and the quantitative determination of the estrogen is rendered difficult due to its low content and the interference of the progestins. The estrogenic agents principally used are ethynyl estradiol and its methyl ether (mestranol).

The progestational agents found in these formulations correspond to two principal groups. The members of one group are characterized by the absence of the methyl group on C_{19} and are designated as nor-compounds.

The second group consists of substances containing the basic progesterone nucleus with different types of substitutions.

In 1965 Schulz (1) described a method for the determination of mestranol by GLC and he compared the

results with those obtained by TLC. Excipients, such as polyvinylpyrrolidone and stearic acid, interfere with the GLC and must first be eliminated.

Bastow (2), in 1967, spectrophotometrically determined mestranol in the presence of norethynodrel after the reduction of ketosteroid with potassium borohydride, by establishing a mathematical correction for the base line which was valid while the absorption of the reduction products remained linear within the 270–290-mμ interval.

Keay (3) (1967) described the separation and identification of progestins and estrogens by TLC (silica gel with cyclohexane–ethyl acetate, 1:1) employing UV spectrophotometry for the quantitative determination.

Shroff and Grodsky (4) determined mestranol in the presence of norethindrone by GLC, using a column impregnated with semi-inorganic polymer (Silicone XE-60) and experimental conditions which do not give rise to the partial decomposition of mestranol to estrone methyl ether which the authors encountered employing the procedure of Schulz. They compared their results with those obtained by direct UV spectrophotometry using a mathematical correction to determine the absorption of norethindrone.

Heusser (5) separated mestranol from chlormadinone acetate by TLC on silica gel using ether–cyclohexane (4:1) as solvent.

Comer *et al.* (6) (1968) described the determination of mestranol in the presence of chlormadinone acetate, colorimetrically and fluorometrically using sulfuric acid–methanol (70:30) and working at temperatures below 5° in order to inhibit the interference of chlormadinone acetate.

Beyer (7) (1968) described an automatic procedure for the determination of mestranol and ethynyl estradiol in mixtures with nonestrogenic steroids by measuring the red color formed when a chloroformic solution is agitated with sulfuric acid and utilizing solutions containing the same concentration of nonestrogenic steroids in order to correct for their influence in the determination.

Shroff and Huettemann (8) determined mestranol in the presence of norethindrone with 4% phenol in 50% aqueous sulfuric acid.

Recent unpublished work in these laboratories, on the separation of steroids by gel filtration, led the authors to realize the possibilities of finding a general procedure for the separation of progestins and estrogens, employed in anovulatory formulations, by gel filtration on synthetic polysaccharide, and their direct quantitative determination by UV spectrophotometry. The direct spectrophotometric determination of the column eluate requires the use of solvents lacking absorption in the 230–300-mμ range. On the other hand, the small proportion of the estrogen found in these formulations and its relatively low absorptivity (*a*) compared to that of the progestins forces the authors to employ relatively large samples for direct spectrophotometry.

It is evident that, after the separation, both progestins and estrogens may be determined quantitatively by colorimetric procedures, while the separation by gel filtration still remains uniquely efficient.

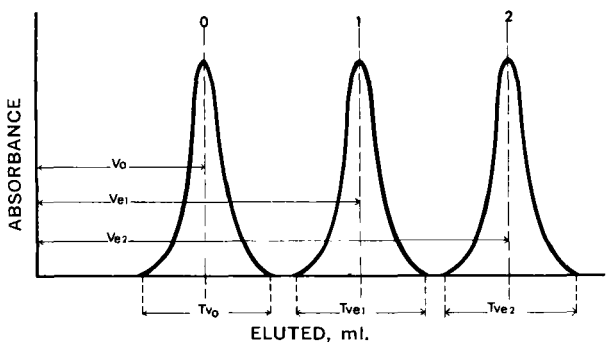


Figure 1—*Parameters used to characterize solute behavior independently of column dimensions. See text for parameter definitions.*

EXPERIMENTAL

Equipment—Chromatographic column SR-25/45[2]; fraction collector (LKB ultrarac 7000); recording spectrophotometer, (Hitachi-Perkin Elmer, model 124).

Materials—Synthetic polysaccharide; eluant: methanol–water (17:3). Methanol and chloroform were of reagent grade.

Procedure—*Preparation of the Column*—The synthetic polysaccharide (45 g.) and methanol (300 ml.) are allowed to stand 12 hr. The gel is then poured into the column allowing the methanol to flow out until the meniscus is approximately 1 cm. above the synthetic polysaccharide. The column is washed with the eluant until the eluate does not absorb in the 230–300-mμ range (about 400 ml. eluant needed).

Definitions—In order to characterize the behavior of a solute, independently of the dimensions of the column, the following parameters were used (see Fig. 1).

V_t = total volume of gel packed, calculated from the inside diameter of the column and the height of the packed gel.

V_e = elution volume, the milliliters of the eluate measured from the moment of sample addition until the solute attains a maximum concentration in the eluate.

V_0 = void volume, the volume, V_e, of a substance which, due to its molecular size, is completely excluded from the gel. This volume corresponds to the volume of the interstitial liquid between the grains of the gel packed in the column.

$K_{av.}$ = partition coefficient, the partition coefficient between the liquid and gel phases independent of the degree of packing of the gel. It is calculated from the formula:

$$K_{av.} = (V_e - V_0)/(V_t - V_0)$$

T_v = total eluted volume, the total volume of the eluted component; it is the eluate volume measured from the moment the component begins to appear in the eluate until the component is completely eluted.

f = dilution factor, the relationship between the milliliters of the component eluted and the milliliters of the sample chromatographed.

$$f = \frac{T_v}{n}$$

where n = the total milliliters of sample chromatographed.

Column Standardization—*Determination of V_0, V_e, V_t, and $K_{av.}$*—The total volume of the synthetic polysaccharide gel packed in the column, calculated from the dimensions of the column, is V_t = 169 ml.

In all of the experiments undertaken, 4 ml. of sample was chromatographed at a velocity of 0.65 ml./min., equivalent to 0.13 ml./cm.^{2}/min., recovering fractions of 3.25 ml. each 5 min.

The value of V_0, void volume, is determined by chromatographing 4 ml. of a solution of polyethylene glycol 20,000 in methanol–water (17:3), and determining its concentration in the eluate by oxidizing with sodium dichromate in sulfuric acid according to Bragdon (10).

The maximum concentration of each solute in the corresponding

[2] Manufactured by Pharmacia Fine Chemicals, Sweden.

Table I—Actual Composition of the Mixtures of Steroids Used and the Compositions Obtained by the Quantitative Determinations

Mixture	Components	Composition, mg./ml.	Chromatographed, mg.	Found, mg.	% Recovered of Material Added
1	Norethindrone acetate	20.0	80.0	81.50	101.9
	Mestranol	0.4	1.6	1.57	98.1
2	Norethindrone acetate	20.0	80.0	80.60	100.8
	Ethynyl estradiol	0.4	1.6	1.62	101.2
3	Megestrol acetate	17.5	70.0	70.00	100.0
	Ethynyl estradiol	0.4	1.6	1.57	98.1
4	Megestrol acetate	17.5	70.0	70.40	100.6
	Mestranol	0.4	1.6	1.63	102.0
5	Norgestrel	4.0	16.0	15.84	99.0
	Mestranol	0.4	1.6	1.62	101.2
6	Norgestrel	4.0	16.0	16.28	101.8
	Ethynyl estradiol	0.4	1.6	1.62	101.2
7	Methyltestosterone	20.0	80.0	79.2	99.0
	Ethynyl estradiol	0.4	1.6	1.57	98.1
8	Methyltestosterone	20.0	80.0	80.80	101.0
	Estradiol	0.4	1.6	1.64	102.7
9	Chlormadinone acetate	3.0	12.0	11.97	99.8
	Estradiol benzoate	0.06	0.24	0.233	97.0
10	Chlormadinone acetate[a]	10.0	40.0	39.88	99.7
	Mestranol	0.4	1.6	1.61	100.6
11	Chlormadinone acetate	10.0	40.0	39.00	99.0
	Ethynyl estradiol	0.4	1.6	1.60	100.0
12	Norethynodrel	20.0	80.0	81.00	101.3
	Mestranol	0.4	1.6	1.65	103.0

[a] Average of six determinations, with a *SD* of 1.45% for chlormadinone acetate and 1.1% for mestranol.

eluates are determined spectrophotometrically at the wavelengths corresponding to maximum absorption for each solute.

The norethynodrel, which has no maximum in the UV, was converted into norethisterone by heating with *N* hydrochloric acid. Lynestrenol and ethynodiol diacetate were determined by GLC on a column of diatomite aggregate[3] impregnated with silicone oil FS-1265. The values obtained are shown in Table II.

The dilution factors for all the steroids studied fall within 10 and 11, and for α-tocopherol acetate the value is 7.5.

Preparation of the Samples—The progestins and estrogens, alone or mixed in the proportions indicated in Table I, were dissolved in 1 ml. of chloroform and diluted to 10 ml. with the eluant. The 1 ml. of chloroform is employed in order to enhance the solubility of the progestins, which is limited, in the eluate mixture.

A finely pulverized sample of the anovulatory formulations analyzed, was weighed so that it contained the equivalent of 4 mg. of estrogen. This sample was extracted five times with chloroform (30, 30, 10, 10, and 5 ml., respectively); the combined filtered extracts were evaporated to dryness under a stream of nitrogen on a water bath. The dry residue was extracted with 1 ml. of chloroform and diluted to 10 ml. with the eluant (centrifuged when necessary). From each of the progestin-estrogen mixtures listed in Tables I and III 4 ml. was chromatographed.

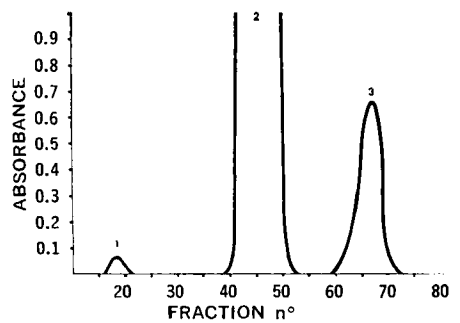


Figure 2—*Elution diagram of polyvinylpyrrolidone–chlormadinone acetate–mestranol (200:100:4) at 278.5 mμ, with methanol–water (17:3). Column SR = 2.5 × 45 cm, fractions of 3.25 ml; sample = 4 ml. Key: 1, polyvinylpyrrolidone; 2, chlormadinone acetate; 3, mestranol.*

[3] AW-DMCS Chromosorb W, Johns-Manville Products Corp., New York, N. Y.

Possible variation in the qualitative and quantitative composition of the excipients used by each manufacturer may necessitate the introduction of modifications in the extraction procedures used on the anovulatory formulations.

Figure 2 shows an elution diagram of a mixture of polyvinylpyrrolidone–chlormadinone acetate–mestranol (240:100:4) (to 278.5 mμ).

Quantitative Determinations—The fractions corresponding to each steroid collected were combined and diluted with the solvent methanol–water (17:3), and the quantitative measurements were obtained by comparison of the magnitude of the UV absorbance, at the characteristic wavelength for each steroid, with that of a standard sample.

Table I gives the composition of the mixture analyzed and the results obtained after analyses. Table II gives the elution constants of the substances utilized, and, finally, Table III gives the results of the quantitative determinations on commercial anovulatories.

RESULTS AND DISCUSSION

The results obtained in the quantitative determinations, which appear in Tables I and III, show conclusively that the separations

Table II—Elution Constants for the Raw Materials Used in This Work

Material	V_e, ml.	V_e/V_t	K_{av}
Polyethylene glycol 20,000	57	—	—
Polyvinylpyrrolidone	60	0.35	—
Methyltestosterone	141	0.83	0.750
Megestrol acetate	144	0.85	0.776
Norethindrone acetate	144	0.85	0.776
Chlormadinone acetate	154	0.91	0.866
Ethynodiol diacetate	155	0.92	0.883
Norgestrel	156	0.92	0.883
Norethynodrel	156	0.92	0.883
Prednisolone acetate	156	0.92	0.883
α-Tocopherol acetate	169	1.00	1.000
Lynestrenol	200	1.18	1.270
Mestranol	214	1.26	1.400
Estradiol	218	1.29	1.430
Ethynyl estradiol	233	1.38	1.570
Estradiol benzoate	237	1.40	1.600

Table III—Results Obtained in the Quantitative Spectrophotometric Determination of Seven Commercial Anovulatory Formulations

Formulation	Components	Labeled, mg.	Found, mg	%
A	Norethynodrel	2.50	2.620	105.0
	Mestranol	0.10	0.104	104.0
B	Norethindrone	2.50	2.470	99.1
	Ethynyl estradiol	0.05	0.0485	97.0
C	Chlormadinone acetate	2.00	1.986	99.3
	Mestranol	0.08	0.0805	100.7
D	Ethynodiol diacetate	1.00	—	—
	Mestranol	0.10	0.097	97.0
E	Lynestrenol	5.00	—	—
	Mestranol	0.15	0.148	98.7
	α-Tocopherol acetate	0.20	0.198	99.2
F	Norgestrel	0.50	0.506	101.3
	Ethynyl estradiol	0.05	0.0503	100.6
G	Norethindrone	2.00	2.036	101.8
	Mestranol	0.08	0.0808	101.0

of the progestin-estrogen mixtures in the combinations studied by the present technique, are applicable to the quantitative determination of anovulatory formulations.

Other 3-ketosteroids such as methyl testosterone and prednisolone acetate may also be separated from estrogens by this procedure.

In only one of the formulations analyzed, shown in Table III, containing lynestrenol–mestranol–α-tocopherol acetate (300:6:8), the separation of progestin–estrogen was incomplete. The separation of the mestranol and α-tocopherol acetate, however, is quantitative, as one may see in Fig. 3, and may be evaluated directly by spectrophotometry due to the lack of absorption of lynestrenol at the wavelength employed.

Knowing the value V_e for a given column, and the total volume of the eluted component, one may work with samples up to one hundred times smaller, evaporating to dryness the total volume of the estrogen eluted, and proceeding to the spectrophotometric determination, with sulfuric acid–methanol (70:30), while the progestins generally may be determined by direct UV spectrophotometry.

It is possible to use smaller columns adjusting the conditions of the separation to the volume of the sample used and the size of the recovered fractions (see Fig. 4).

The technique has shown itself to be precise and reproducible, and may be applied to the analysis of anovulatory formulations, as the results of Table III show, and in which is included the effects due to the variations in weight of the tablets.

Once the elution of the components is complete, the column is ready for use in further determinations.

On the basis of the values given for the constants V_e, V_e/V_t, and K_{av} in Table II, one can predict the possibility of quantitatively separating any of the components listed therein. If $V_{e2} - V_{e1} > (T_{v1} + T_{v2})/2$, the quantitative separation is possible.

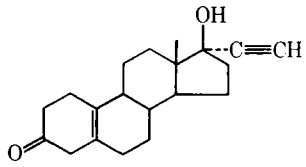
If $V_{e2} - V_{e1} \leq (T_{v1} + T_{v2})/2$ the quantitative separation is impossible. In such a case the possibility of accomplishing the separation in a larger (V_t' larger) column may be calculated, employing the K_{av} found in Table II and determining the new elution volume V_0' corresponding to V_t'; the new elution volume of the solute, V_e', may be obtained from the formula: $V_e' = K_{av}(V_t' - V_0) + V_0'$.

Employing the new values of the V_e' the possibilities for a quantitative separation may be determined as shown above.

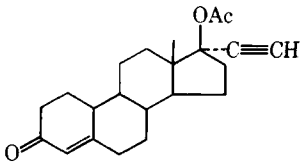
The mixtures studied in the present work contain the following molecules:

Progestins

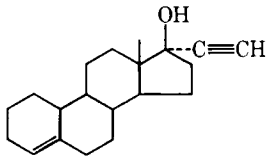
Nor-compounds



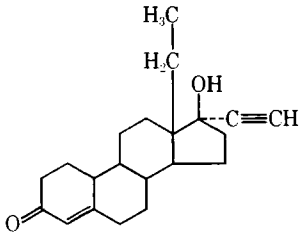
norethynodrel—no maximum absorption in UV in the range 230–350 mμ



norethindrone acetate—absorption maximum 240 mμ (in methanol) $a = 57.0$



lynestrenol—no maximum absorption in UV range 230–350 mμ



norgestrel—absorption maximum 240 mμ (in methanol) $a = 56.0$

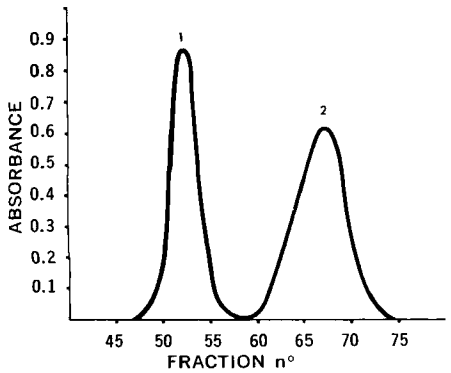


Figure 3—*Elution diagram of α-tocopherol acetate–mestranol (2:1.5) at 278.5 mμ, with methanol–water (17:3). Column SR = 2.5 × 45 cm; fractions of 3.25 ml. sample = 4 ml. Key: 1, α-tocopherol acetate; 2, mestranol.*

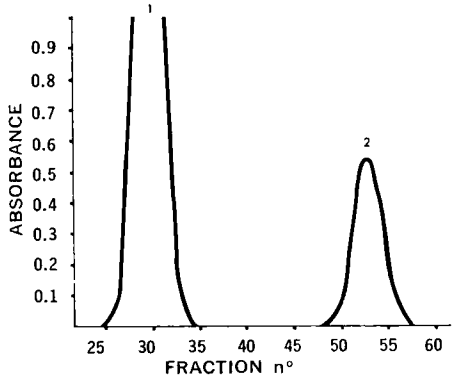
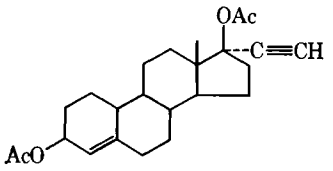
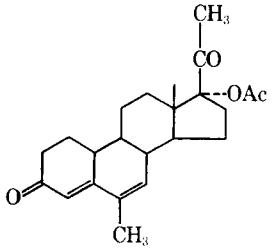


Figure 4—*Elution diagram of chlormadinone acetate–mestranol (1:1) at 278.5 mμ, with methanol–water (17:3). Column = 1.5 × 35 cm. fractions of 1 ml. sample = 1 ml. Key: 1, chlormadinone acetate; 2, mestranol.*

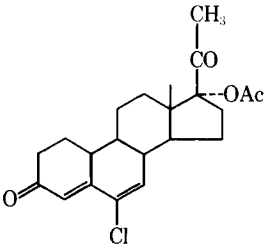


ethynodiol diacetate—no maximum absorption in UV range 230–350 mμ

"substituted" progesterones

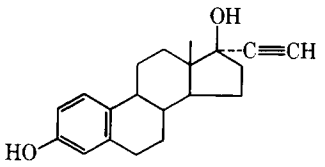


megestrol acetate—absorption maximum 288 mμ (in methanol) a = 630.

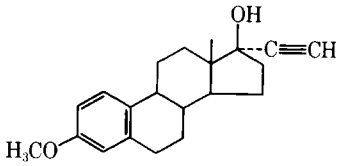


chlormadinone acetate—absorption maximum 284 mμ (in methanol) a = 54.5

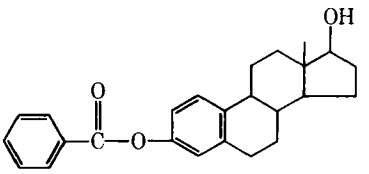
Estrogens



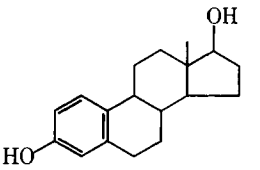
ethynyl estradiol—absorption maximum 281 mμ (in methanol) a = 7.1



mestranol—absorption maximum 278.5 mμ (in methanol) a = 6.3

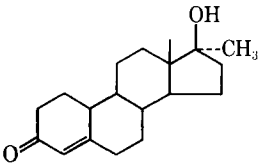


estradiol benzoate—absorption maximum 230 mμ (in methanol) a = 54.5

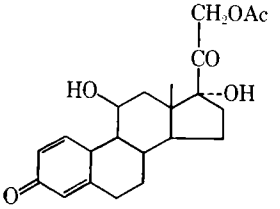


estradiol—absorption maximum 281 mμ (in methanol) a = 7.4

Other steroids



methyl testosterone—absorption maximum 240 mμ (in methanol) a = 53.5



prednisolone acetate—absorption maximum 242 mμ (in methanol) a = 37.3

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