Intravaginal Controlled Administration of Flurogestone Acetate I: Development of a Stability-Indicating Liquid Chromatographic Method and Stability Kinetics of Flurogestone Acetate

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Abstract □ Flurogestone acetate is a synthetic progestin useful for estrus synchronization in sheep. The estrus synchronization is achieved by inserting the drug-impregnated vaginal sponge in the sheep for up to 15 d, during which time flurogestone acetate is administered continuously by intravaginal absorption to suppress estrus and ovulation. The sponge is then withdrawn to regain estrus within 2–4 d. A review of the literature suggests that the analytical methods currently available are not specific, sensitive, and rapid enough. A stability-indicating high-performance liquid chromatographic method was developed, which is capable of characterizing the stability kinetics of flurogestone acetate under various environmental conditions and quantifying the release and absorption profiles of the drug from the vaginal sponges.

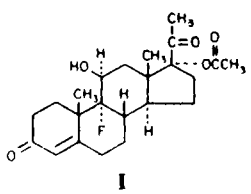
Keyphrases □ Flurogestone acetate—stability-indicating HPLC method, stability kinetics □ HPLC—stability-indicating method, flurogestone acetate □ Stability kinetics—flurogestone acetate

Flurogestone acetate (I) is a synthetic progestin used in sheep for estrus synchronization (1). The objective for developing an effective treatment for estrus synchronization is to establish a stage at which estrus can be induced to make the females fertile in a desired season. Estrus synchronization is achieved by prolonged continuous administration of flurogestone acetate-releasing vaginal sponges to the sheep for 15 d; 2–4 d after withdrawal of the sponges most sheep regain their estrus.

Over the years, flurogestone acetate has been assayed by GC (2), gravimetric analysis (2), and radioactive tracers (3). However, these methods lack assay specificity, speed, sensitivity, and/or convenience; therefore, a high-performance liquid chromatographic (HPLC) method was developed to provide a rapid, quantitative, and specific assay for this drug in the presence of its degradation products. This assay was used to study several stability characteristics of flurogestone acetate, including the effects of acid, base, and temperature.

EXPERIMENTAL SECTION

Materials—A high-performance liquid chromatograph equipped with a reciprocating-piston pump[1], injector[2], UV detector (254 nm)[3], and a strip-chart recorder[4] was employed in conjunction with a 10-μm (3.9 mm × 30 cm) C_{18} column[5] for separation.



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All chemicals and reagents were analytical grade unless indicated. Flurogestone acetate[6], testosterone[7], anhydrous dibasic sodium phosphate[8], anhydrous citric acid[8], dibasic potassium phosphate[8], monobasic potassium phosphate[8], and glass-distilled methanol[9] were used as received. Deionized water[10] was freshly prepared and used throughout the study.

Analytical Procedure—A fixed quantity of testosterone was added to each sample as an internal standard to assure injection-to-injection reproducibility. Aliquots were injected into the HPLC column and the peak height ratios of flurogestone acetate to testosterone were then determined and compared with the standard curve for calculation of the flurogestone acetate concentration. Methanol–water (50:50) was used as the mobile phase. The column and the solvent were at ambient temperature. At a solvent flow rate of 2 mL/min, the retention times for flurogestone acetate and testosterone were ~21 and ~32 min, respectively.

Standard Calibration Curve—The standard stock solution of flurogestone acetate and testosterone (internal standard) were prepared in methanol and stored at 4°C. The flurogestone acetate stock solution was diluted as necessary. The standard calibration curves were constructed by plotting peak height ratio *versus* flurogestone acetate concentration. A straight-line fit of the data was made by linear regression analysis, and the correlation coefficient was determined.

Stability Studies—*Effect of pH*—Three methanolic stock solutions (which contained 1.00–1.49 mg/mL of flurogestone acetate) were prepared and then diluted to 71.5, 39.8, and 5.03 μg/mL with 0.1 M HCl (pH 1.0), 0.1 M NaOH (pH 13), and simulated vaginal fluid (pH 7.3), respectively. The simulated vaginal fluid was prepared from 870 mL of 0.4 M Na_2HPO_4 and 130 mL of 0.2 M citric acid made up to 2 L with water. The diluted acidic, basic, and simulated vaginal fluid solutions were placed in well-sealed glass containers and stored at 94°C (by reflux), 30°C, and 38°C (shaken in a water bath[8]), respectively, until the completion of the study. Aliquots (1 mL of the acidic solution or 2 mL of either basic or simulated vaginal fluid solutions) were withdrawn periodically from each flask and assayed for flurogestone acetate

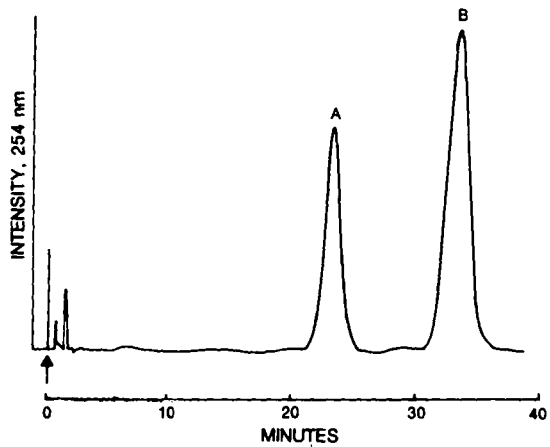

Figure 1—*Representative chromatogram for flurogestone acetate (A) and testosterone (B), as internal standard. Arrow indicates the time of injection.*

[1] Model 6000A; Waters Associates, Milford, Mass.
[2] Model U6K; Waters Associates.
[3] Model 440; Waters Associates.
[4] Omniscribe Recorder; Houston Instruments, Austin, Tex.
[5] μ-Bondapack C_{18}; Waters Associates.
[6] Searle Laboratories, Skokie, Ill.
[7] Sigma Chemical Co., St. Louis, Mo.
[8] Fisher Scientific, Fair Lawn, N.J.
[9] J. T. Baker Chemical Co., Phillipsburg, N.J.
[10] Nanopure; Sybron/Barnstead, Boston, Mass.

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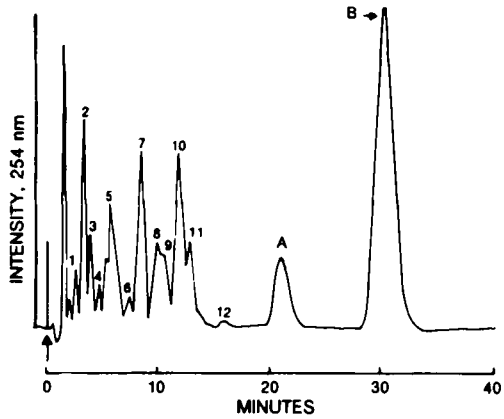

Figure 2—*Chromatogram of flurogestone acetate (A) after 95 h in 0.1 M HCl (pH 1.0) at 94°C. Key: (1–12) degradation products with retention times at 2.02, 3.19, 3.74, 4.71, 5.82, 7.73, 8.81, 9.56, 11.15, 12.61, 13.23, and 15.28 min; (B) testosterone (internal standard).*

and its degradation products. However, the acidic and basic solutions were first neutralized to pH 5.5 and 6.59, respectively, with 1 mL of 0.1 M K_2HPO_4 (pH 9.3) or 2 mL of 0.2 M KH_2PO_4 (pH 4.46); after vortexing, 50 μL of testosterone (0.61 or 0.13 mg/mL) was added to each sample. An aliquot (~100 μL) of each sample was assayed by the HPLC method.

Effect of Temperature—Flurogestone acetate stock solution prepared in simulated vaginal fluid (5.75 μg/mL) was placed in well-sealed glass containers and stored in a shaking water bath at 38°C, 70°C, 80°C, and 90°C. Each experiment was performed in triplicate. An aliquot (2 mL) was withdrawn periodically from each flask and, after cooling, 50 μL of testosterone (0.113 mg/mL) was added. The sample (100 μL) was then assayed by the HPLC method for flurogestone acetate and degradation products.

Treatment of Data—Semilogarithmic plots of the flurogestone acetate concentration as a function of time were constructed to evaluate the kinetics and rates of drug degradation. An Arrhenius plot (4) was then constructed from the kinetic data obtained from the studies with different temperatures. The line of best fit was determined by linear regression analysis.

RESULTS AND DISCUSSION

Chromatographic Data—A typical chromatogram for flurogestone acetate in the presence of testosterone (the internal standard) is shown in Fig. 1. Peaks for flurogestone acetate and testosterone are observed at retention times of 21.2 and 31.5 min, respectively. No interfering peaks were detected in the 40-min run time. The standard curve constructed for flurogestone acetate was linear over the concentration ranges of 0–10 and 10–40 μg/mL ($r > 0.99$) when the peak height ratio of flurogestone acetate to testosterone was used.

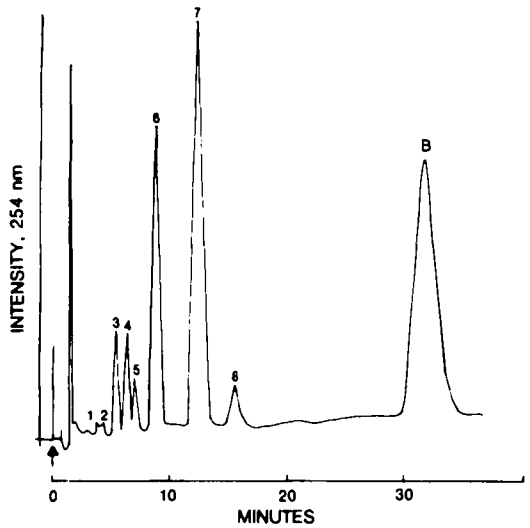

Figure 3—*Chromatogram of flurogestone acetate after 24 h in 0.1 M NaOH (pH 13) at 30°C. Key: (1–8) degradation products with retention times at 3.65, 4.16, 5.08, 6.00, 6.88, 8.34, 11.69, and 14.75 min; (B) testosterone (internal standard).*

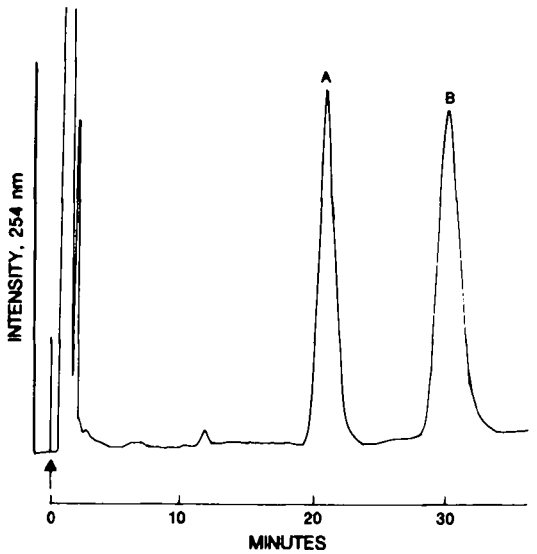

Figure 4—*Chromatogram of flurogestone acetate (A) after 22 d in simulated vaginal fluid (pH 7.3) at 38°C, with testosterone (B) as internal standard. Very little degradation was observed.*

The retention time varied slightly from day to day and with the pH of the injected solution.

The sensitivity of detection for flurogestone acetate at an injection volume of 50 μL was ~ 0.65 μg/mL (at 0.005 AUFS), which was equivalent to the injection of 28 ng of drug into the column. The assay sensitivity can be improved substantially by increasing the injection volume, by using a less-polar mobile phase (by increasing the ratio of methanol to water, *e.g.*, from 50:50 to 70:30), or by changing the detector wavelength from the conventional 254 nm to 236–238 nm, which is the maximum absorbance for flurogestone acetate (ϵ = 17,520) (5).

Solution Stability and Effect of Acid and Base—The HPLC method was used to study the solution stability of flurogestone acetate and to detect the degradation products, if any, under various conditions. Flurogestone acetate solutions in 0.1 M HCl (pH 1), 0.1 M NaOH (pH 13), or simulated vaginal fluid (pH 7.3) were stored at various elevated temperatures for different intervals; samples were taken periodically for chromatographic assay. The samples from 0.1 M HCl solutions at 94°C yielded 12 well-defined peaks of degradation products (Fig. 2); 8 degradation-product peaks formed under alkaline conditions at 30°C (Fig. 3). Under the chromatographic conditions all the degradation peaks appear before 18 min and none interfered with the primary peaks of flurogestone acetate (at 21.2 min) or testosterone (at 31.5 min). The samples from the simulated vaginal fluid (pH 7.3) at 38°C (the vaginal temperature of the sheep) yielded only a very small degradation peak after 22 d (Fig. 4).

A semilogarithmic plot of flurogestone acetate concentrations as a function

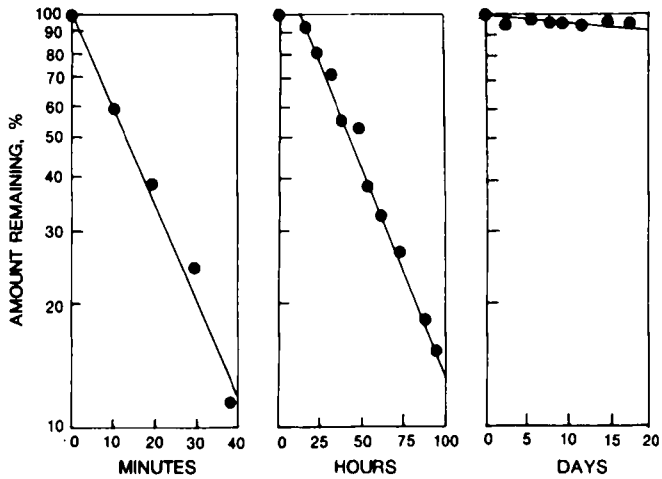

Figure 5—*Degradation profiles of flurogestone acetate in basic (a), acidic (b), and simulated vaginal fluid (c) solutions. Key: (a) pH 13 (30°C), k = 0.053 min^{-1}; (b) pH 1 (94°C), k = 0.023 h^{-1}; (c) pH 7.3 (38°C), k = 0.0018 d^{-1}.*

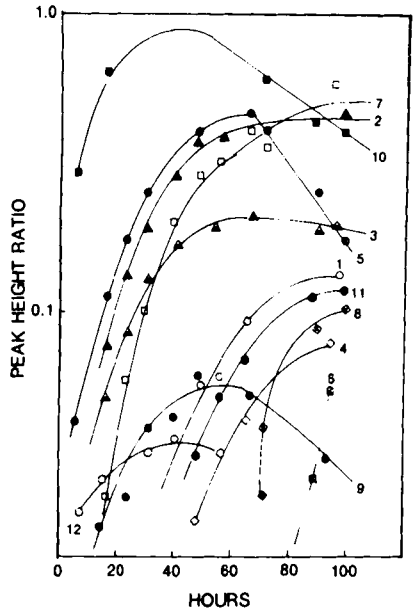

Figure 6—*Time course for the appearance of various degradation products of flurogestone acetate in 0.1 M HCl (pH 1) at 94°C. Key: (1-12) degradation product peaks in Fig. 2.*

of time yields a linear relationship, indicating that the degradation could follow first-order kinetics (Fig. 5). The data in Fig. 5 indicate that the degradation of flurogestone acetate is most rapid at pH 13, intermediate at pH 1, and slowest at pH 7.3. The apparent first-order half-life determined from these first-order kinetic plots is 13 min at pH 13, 30.1 h at pH 1, and ~370 d at pH 7.3, respectively.

Flurogestone acetate degradation, subject to both acid and base catalysis, is minimized at pH 7.3 (the vaginal pH of the sheep). The drug degradation profiles (Figs. 6 and 7) illustrate the time course for the appearance and disappearance of degradation products in acidic (pH 1) and alkaline (pH 13) solutions.

Thermal Stability in Simulated Vaginal Fluid—The thermal stability of flurogestone acetate in simulated vaginal fluid was studied at 70°C, 80°C, and 90°C. The semilogarithmic plot of drug concentration as a function of time was linear at various temperatures (Fig. 8), indicating first-order degradation kinetics. The apparent first-order degradation kinetic constants determined from the slope of these linear plots are 0.0490, 0.1303, and 0.3941 d^{-1} at 70°C, 80°C, and 90°C, respectively. The results suggest that the

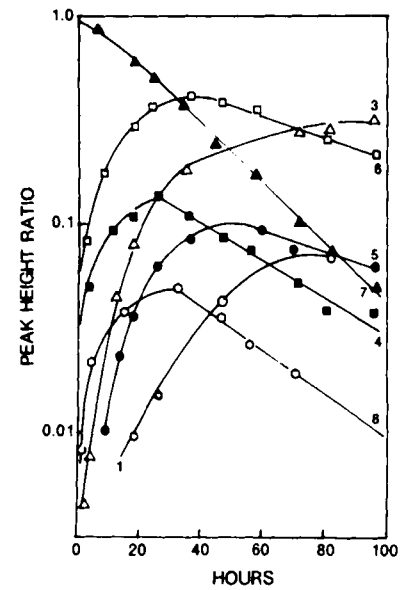

Figure 7—*Time course for the appearance of various degradation products of flurogestone acetate in 0.1 M NaOH (pH 13) at 30°C. Key: (1-8) degradation product peaks in chromatogram in Fig. 3.*

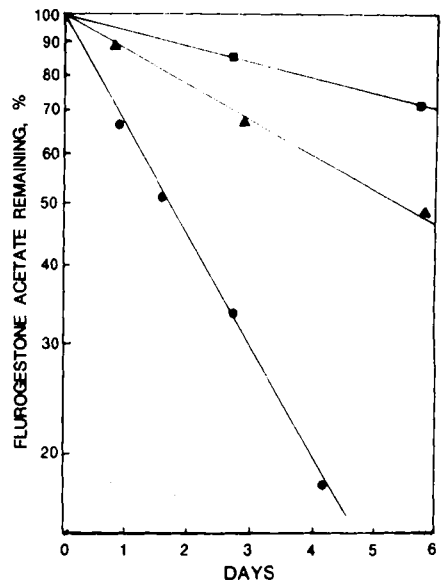

Figure 8—*Thermal stability of flurogestone acetate in the simulated vaginal fluid (pH 7.3) at 70°C (■), 80°C (▲), and 90°C (●). The first-order degradation constants were calculated to be 0.0490 d^{-1} (70°C), 0.1303 d^{-1} (80°C), and 0.3941 d^{-1} (90°C).*

thermal degradation of flurogestone acetate could follow the Arrhenius relationship (4) of:

$$\log k = \log A - \frac{E_a}{2.303R} \cdot \frac{1}{T}$$

where k is the apparent rate constant of degradation obtained from the kinetic plots in Fig. 8, R is the gas constant (1.98 cal/mol·K), T is the absolute temperature (K), E_a is the energy of activation, and A is the frequency factor. The exponential relationship between the apparent first-order rate constants (k) obtained and the reciprocal of the absolute temperature is shown in Fig. 9. Extrapolation of the data obtained at elevated temperatures to the vaginal temperature (38°C) results in a predicted first-order rate of 0.0010 d^{-1}, which compares favorably (95% confidence limit) with the rate constant of 0.0018 d^{-1} obtained experimentally at 38°C. E_a and A values, derived from the slope and intercept, are 25.55 kcal/mol and 9.27×10^{14} d^{-1}, respectively.

The potential utility of the temperature-dependent relationship is determined by the controlling mechanism of degradation (6). Since the E_a value

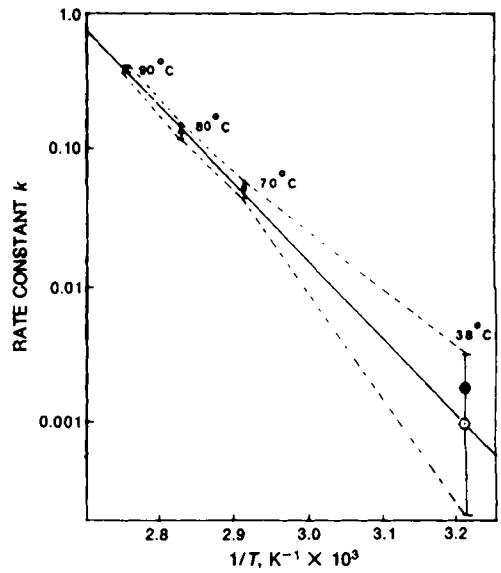

Figure 9—*Arrhenius plot (—) of the thermal degradation rate constants of flurogestone acetate in the simulated vaginal fluid (pH 7.3) with 95% confidence limits (- - -) (7, 8). The filled data points are actual data obtained experimentally; the open data point is the calculated value extrapolated from the Arrhenius plot of the data at elevated temperatures; E_a = 25.55 kcal/mol.*

(25.55 kcal/mol) is rather high, the degradation of flurogestone acetate (in vaginal fluid) could be the result of a solvolytic process, which generally has an energy of activation in the range of 10–30 kcal/mol (6). The higher the value of E_a, the greater the thermal stability of the drug.

CONCLUSIONS

The HPLC method developed is specific and allows a good separation of flurogestone acetate from its degradation products. The degradation is both acid- and base-catalyzed, but is much slower in the simulated vaginal fluid (pH 7.3). Thermodynamic studies indicate that the degradation in simulated vaginal fluid is very slow, as it requires a fairly high energy of activation (25.55 kcal/mol).

This assay should be suitable for determination of the release kinetics of flurogestone acetate from the vaginal sponges and its absorption rate through the vaginal wall. Since it is also a stability-indicating method, the metabolism of flurogestone acetate, if any, in the simulated vaginal fluid or vaginal mucosa could be detected (9).

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Intravaginal Controlled Administration of Flurogestone Acetate II: Development of an *In Vitro* System for Studying the Intravaginal Release and Permeation of Flurogestone Acetate

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Abstract □ A relatively simple and easily constructed *in vitro* system was developed for studying the mechanism of release and intravaginal absorption of flurogestone acetate from vaginal sponges. The stability-indicating high-performance liquid chromatographic method developed earlier was used to provide a rapid, reproducible, and sensitive assay. The *in vitro* intravaginal release/permeation system developed was capable of determining the rate of flurogestone acetate release from vaginal sponges and, simultaneously, the rate of absorption through the vaginal wall. The design, calibration, and applicability of the system, and the release and absorption profiles of the drug from vaginal sponges in this system are discussed.

Keyphrases □ Drug release—flurogestone acetate, intravaginal, sheep □ Flurogestone acetate—intravaginal release and permeation measurement *in vitro* □ Intravaginal administration—flurogestone acetate, sheep

In 1975, the use of flurogestone acetate-releasing vaginal sponges (Fig. 1) to replace the conventional daily injection of progesterone for estrus synchronization in sheep was introduced (1). On intravaginal administration, the polyurethane sponges release the drug continuously for 15 d, this suppresses estrus and ovulation. Two to four days after removal of the sponges, the sheep reenter estrus and ovulate. During this period, artificial insemination can be performed at a predetermined schedule to promote a specific breeding program (2).

Knowledge of the pattern and rate of drug release is essential for an understanding of the action of flurogestone acetate-impregnated sponges in the vagina (3). Development of a suitable *in vitro* drug release/permeation system is essential to understand the mechanisms of drug release and permeation under *in vivo* conditions (4). Currently, the release profiles are determined by extracting the residual drug content in the vaginal sponges and then assaying it by GC (2) or by radioactivity count (5). However, these *in situ* experiments require the use of large numbers of animals over an extended period of time. Also, the lack of assay sensitivity can jeopardize the

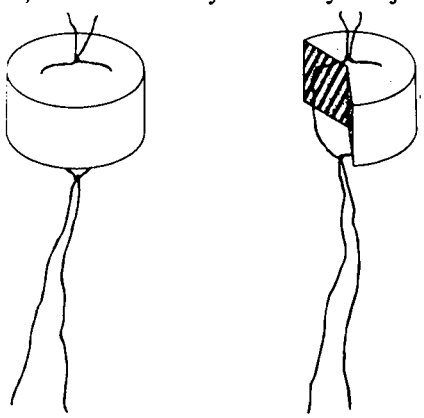

Figure 1—*Diagrammatic illustration of the flurogestone acetate-releasing vaginal sponges*[1].

[1] Synchro-Mate Pessary; G. D. Searle and Co., Chicago, Ill.

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