Table III—Recovery of Aminophylline and Amobarbital Mixed Standard in 50% Ethanol in 4% (v/v) Acetic Acid[a]

Mixture	Aminophylline		Amobarbital	
	Amount Taken, mg	Recovery, %	Amount Taken, mg	Recovery, %
A	350.0	100.7	67.0	97.4
B	350.0	98.9	67.0	98.6
C	360.0	99.3	69.0	99.1
D	340.0	97.5	65.0	99.4
E	300.0	97.9	57.5	101.3
F	350.0	98.6	67.0	100.6
G	360.0	99.1	69.0	99.8
H	350.0	97.7	67.0	97.8
I	350.0	98.6	67.0	98.9
J	340.0	98.9	65.0	99.4
Average, %	—	98.7	—	99.2
SD, %		±0.92		±1.67

[a] Synthetic mixes were prepared by dissolving an accurately weighed amount of both compounds in 50% ethanol in 4% acetic acid in a 50-ml volumetric flask. Each mixture was treated and diluted as the sample, excluding the chromatographic step.

between 320 and 220 nm, the absorption curves showed that the maximum at 285.5 nm remained unchanged and that the maximum at 254 nm was absent. The absorption at 240 nm increased significantly in intensity with respect to that of aminophylline determined individually at the same concentration.

The cause of this deviation at pH 13 may have been that the keto–enolic equilibrium of the doubly ionized form of amobarbital with the absorption maximum at 254 nm was probably shifted by a much larger concentration of the enolized aminophylline to exhibit an absorption maximum at 240 nm (singly ionized form). To eliminate such a possible keto–enolic equilibrium effect, a new approach was followed. The differential absorption curves at pH 10, previously plotted for the separated compounds, showed that aminophylline and amobarbital exhibited fairly strong maxima at about 240 nm and that there was a wide variation between the intensities of absorption of both components at 285.5 nm. Figure 2 shows the differential absorption spectra at pH 10 of aminophylline and amobarbital. These conditions seemed to be theoretically adequate for the quantitative determination of mixtures of aminophylline and amobarbital (8).

This hypothesis was verified experimentally when mixtures of the two substances, analyzed at pH 10, gave a positive response. The results in Table III show that the procedure is both accurate and precise. Compared to the NF procedure, the proposed method significantly shortens the time required for the analysis of aminophylline, amobarbital, and ephedrine hydrochloride, thus making possible the quantitative determination of more samples per day.

REFERENCES

(1) "British Pharmacopoeia," Pharmaceutical Press, London, England, 1973, p. 32.

(2) "The National Formulary," 13th ed., Mack Publishing Co., Easton, Pa., 1970, pp. 266, 267.

(3) R. J. Hyatt, *J. Assoc. Offic. Agr. Chem.*, **38**, 624 (1955).

(4) *Ibid.*, **36**, 673 (1954).

(5) C. G. Cunninghan and S. Barkan, *J. Assoc. Offic. Anal. Chem.*, **58**, 525 (1975).

(6) G. B. Pleat, J. H. Harley, and S. E. Wiberley, *J. Am. Pharm. Assoc., Sci. Ed.*, **40**, 107 (1951).

(7) "The National Formulary," 13th ed., Mack Publishing Co., Easton, Pa., 1970, pp. 695, 696.

(8) A. E. Gillam, "Electronic Absorption Spectroscopy," 2nd ed., Edward Arnold, London, England, 1962, p. 214.

ACKNOWLEDGMENTS AND ADDRESSES

Received October 14, 1975, from *Saphar Laboratories (PTY) Ltd., P.O. Box 2368, Industria West, Johannesburg, South Africa.*

Accepted for publication August 5, 1976.

Liquid Chromatography in Pharmaceutical Analysis VIII: Determination of Isoniazid and Acetyl Derivative in Plasma and Urine Samples

S. J. SAXENA, J. T. STEWART, I. L. HONIGBERG ˣ, J. G. WASHINGTON, and G. R. KEENE

Abstract □ Parameters are described for the qualitative and quantitative analysis of a mixture of isoniazid and its acetyl derivative. The compounds are chromatographed on an octadecylsilane column, using absolute methanol–distilled water (60:40) at pH 2.5 containing 0.01 *M* dioctyl sodium sulfosuccinate. The flow rate was 2.0 ml/min (2500 psig). The separation and quantification are applicable to plasma and urine samples. The determination in each biological fluid can be achieved in approximately 90 min with percentage accuracies for isoniazid of 5.25 and 7.45 and for the acetyl derivative of 4.47 and 1.56 in plasma and urine, respectively.

Keyphrases □ Isoniazid and acetyl derivative—high-pressure liquid chromatographic analyses, plasma and urine □ High-pressure liquid chromatography—analyses, isoniazid and acetyl derivative, plasma and urine □ Tuberculostatic antibacterials—isoniazid and acetyl derivative, high-pressure liquid chromatographic analyses, plasma and urine

Interest in high-pressure liquid chromatography (HPLC) for the determination of drugs and their metabolites in biological media led to the analysis of isoniazid (I) and its major metabolite, the acetyl derivative (II). Presently, I is very effective as an antituberculosis drug, and the determination of I and II is important in studies related to I disposition and metabolism. A review of analytical methodology concerning I and its metabolites is available (1).

This paper reports the HPLC separation of I and II and the application of the procedure to the quantification of these compounds in plasma and urine. The HPLC separation is effected utilizing ion-pair formation with dioctyl sodium sulfosuccinate on an octadecylsilane column. Generalities of ion-pair formation were reviewed extensively (2, 3). Some investigators (4–6) used ion-pair formation as a tool for effecting chromatographic analysis of selected organic ions. The mechanism for such a separation apparently depends upon the reversible formation of ion-pairs in the chromatographic system and the separa-

Table I—Calibration Data for Standard Drug Solutions

Compound	Initial Concentration, μg/ml[a]	D/IS Ratio[b]	Slope	Intercept	$r \pm s_{yx}$[c]
For plasma levels:					
I	10.0	0.2046 ± 0.0298[d]	0.0055	−0.0395	0.9873
	15.0	0.4248 ± 0.2268			±0.0326
	20.0	0.4858 ± 0.1166			
	30.0	0.7945 ± 0.0926			
II	13.3	0.2291 ± 0.0043	0.0041	0.0214	0.9999
	20.0	0.3332 ± 0.0096			±0.0019
	26.6	0.4321 ± 0.0096			
	40.0	0.6510 ± 0.0149			
For urine levels:					
I	60.0	0.2893 ± 0.0955	0.0009	0.0181	0.9970
	80.0	0.3841 ± 0.0530			±0.0112
	120.0	0.5748 ± 0.0585			
	140.0	0.6470 ± 0.1095			
II	186.6	0.7388 ± 0.0122	0.00089	0.1294	0.9977
	266.6	1.0425 ± 0.0000			±0.0157
	320.0	1.2086 ± 0.0342			
	373.3	1.3629 ± 0.0285			

[a] Based on a typical 3-ml biological sample. [b] Data represent three replicate injections of calibration solutions; D/IS is the ratio of the integrated area of the drug at some concentration divided by the integrated area of the internal standard. [c] The r is the correlation coefficient determined from linear regression analysis, and s_{yx} is the standard error of the estimated concentration. [d] Confidence limits at $p = 0.05$.

tion of the constituents on the basis of differences in the lipophilicity of the ion-pairs formed.

The analysis of an isoniazid–pyridoxine hydrochloride dosage form, using ion-pair formation with dioctyl sodium sulfosuccinate, was recently reported (7). The chromatographic separation described herein takes approximately 10 min. The overall analysis time is approximately 90 min, which includes extraction of the drug and metabolite from the biological fluid (8) followed by HPLC separation and quantification.

EXPERIMENTAL[1]

Reagents and Chemicals—Powdered samples of I[2] and II (9) were used in the analytical procedure, and dioctyl sodium sulfosuccinate[3] was used as a component of the mobile phase. All other chemicals and solvents were commercially available and were utilized as received.

Mobile Phase—The mobile phase, consisting of absolute methanol–distilled water (60:40), was prepared fresh daily. It was made 0.01 *M* with respect to dioctyl sodium sulfosuccinate (4.4 g/liter), and its pH was adjusted to 2.5 by dropwise addition of 2 *M* sulfuric acid (<1 ml).

Internal Standard Solution—The stock internal standard solution (8 mg/10 ml) was prepared by dissolving 1-benzoyl-2-isonicotinoylhydrazine in distilled water. The substituted hydrazine was originally synthesized by Kerr *et al.* (10). It was recrystallized from water (mp 232–234°) before use.

Standard Solutions for Calibration Curves—Stock solutions of I (6 mg/10 ml) and II (8 mg/10 ml) were prepared by dissolving weighed amounts of each powder in distilled water.

Determination of Calibration Curve—*For Plasma Levels*—Into individual 60-ml separators were placed accurately pipetted volumes[4] of 0.050, 0.075, 0.100, and 0.150 ml of both I and II standard solutions. A 0.200-ml volume of the internal standard solution was added to each separator, and the volume of each solution was adjusted to 6 ml with distilled water. Then 1 ml of 0.1 *N* sodium hydroxide solution and solid ammonium sulfate, about 1 g/ml of aqueous solution, were added. The latter was added in small portions to each solution followed by gentle shaking for 5 min. Each solution was then shaken for 5 min with 40 ml of water-saturated 1-butanol–chloroform (30:70).

The layers were allowed to separate, and 30 ml of the organic layer (bottom) from each separator was transferred to clean 45-ml stoppered conical centrifuge tubes. Four milliliters of 0.1 *N* sulfuric acid was added to each tube, and the contents were shaken for 5 min. The layers were allowed to separate, with centrifugation if necessary, and a 40-μl aliquot of the acidic layer (top) was injected into the liquid chromatograph. The concentrations of both I and II were subjected to a linear regression analysis, and the slope and intercept for each component were calculated (Table I).

For Urine Levels—Into individual 60-ml separators were placed the following accurately pipetted volumes of I and II standard solutions, respectively: 0.300 and 0.700 ml, 0.400 and 1.000 ml, 0.600 and 1.20 ml, and 0.700 and 1.40 ml. A 0.800-ml quantity of the internal standard solution was added to each separator, and the volume of each mixture was adjusted to 6 ml with distilled water. The solutions were then treated in the same manner as described for plasma levels.

Determination of I and II in Human Plasma and Urine—To 3.0 ml of human plasma in a 60-ml separator were added accurately pipetted quantities of 0.100 ml of both I and II standard solutions and 0.200 ml of the internal standard solution. To 3.0 ml of human urine in another separator were added 0.400 and 0.900 ml of I and II solutions, respectively, along with 0.800 ml of the internal standard solution. Each plasma and urine sample was then treated in the same manner as described for the standard solutions. Control blanks for each biological fluid were also included. Replicate 40-μl injections were made for each sample.

Chromatographic Separation and Quantification—The degassed mobile phase was pumped through an octadecylsilane column[5] at a flow rate of 2.0 ml/min (2500 psig) at room temperature until a stable baseline was obtained. Replicate 40-μl injections of sample and standard solutions were made using a 100-μl syringe[6]. The wavelength of the UV detector was set at 266 nm. The chart recorder provided a record of drug elution from the column as peaks on a chromatogram. In all cases, the solute was measured by digital integration of the peak area[1].

RESULTS AND DISCUSSION

The analysis of the I–II mixture and the application to plasma and urine extracts necessitated the examination of three separate problems: (*a*) the development of a set of HPLC operating parameters that would separate the two compounds without peak overlap, (*b*) the detection and quantification of each compound at the level found in biological fluids, and (*c*) the contribution of extracted plasma and urine blanks to the chromatograms.

Initially, the separation was achieved on a cation-exchange column using aqueous 0.01 *M* ammonium acetate (pH 3.5) as the mobile phase. However, this procedure was rejected due to the lack of reproducible

[1] A Waters Associates liquid chromatograph (model ALC/GPC 201), equipped with an M-6000 pump, a Perkin-Elmer model LC-55 variable wavelength UV–visible detector, an Infotronics integrator (model CRS-204) with digital printout, and a Waters packed column, 4 mm i.d. × 30 cm, was used.

[2] Matheson, Coleman and Bell, East Rutherford, N.J.

[3] Sigma Chemical Co., St. Louis, Mo.

[4] Volumes of standard drug solutions of ≤1.0 ml added to plasma and urine samples were pipetted using Eppendorf pipets (Brinkmann Instruments, Westbury, N.Y.). Quantities >1 ml were delivered using Class A graduated volumetric pipets.

[5] Micro-Bondapak/C_{18}, <10 μm, Waters Associates, Milford, Mass.

[6] Model B-110, Precision Sampling Corp., Baton Rouge, La.

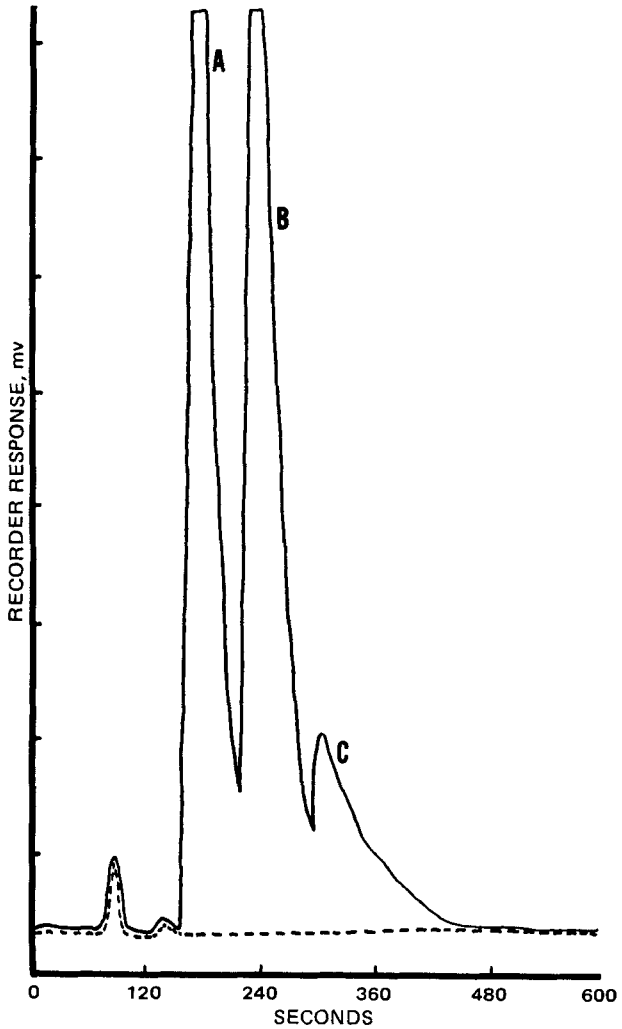

Figure 1—*Liquid chromatogram of I and II in plasma extract on octadecylsilane column using absolute methanol–distilled water (60:40) at pH 2.5 with 0.01* M *dioctyl sodium sulfosuccinate. Key: - - -, blank plasma extract; —, plasma extract containing drug and metabolite; A, II; B, 1-benzoyl-2-isonicotinoylhydrazine; and C, I.*

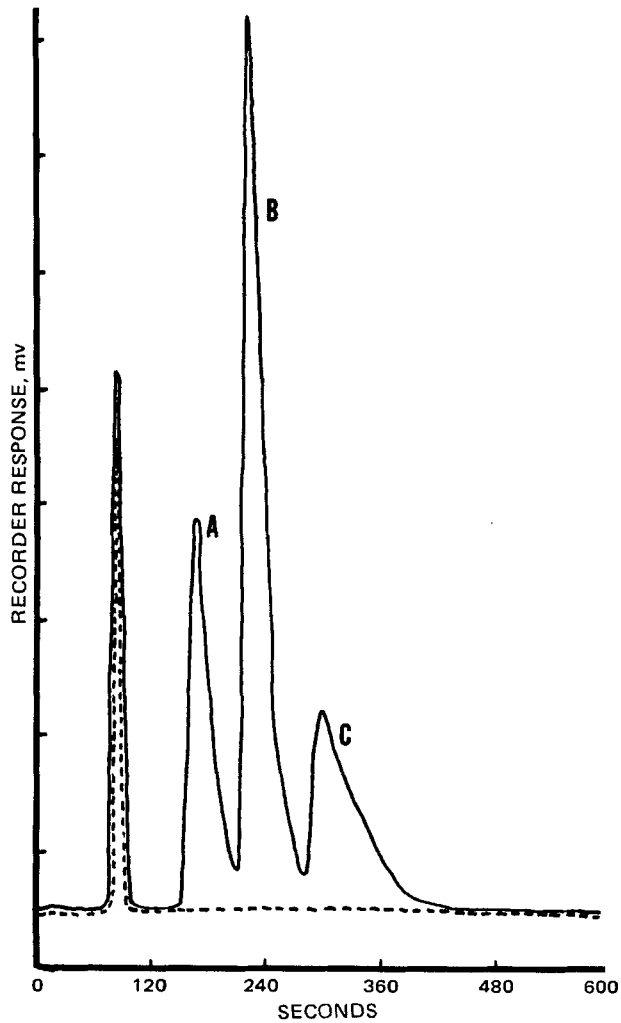

Figure 2—*Liquid chromatogram of I and II in urine extract on octadecylsilane column using absolute methanol–distilled water (60:40) at pH 2.5 with 0.01* M *dioctyl sodium sulfosuccinate. Key: - - -, blank urine extract; —, urine extract containing drug and metabolite; A, II; B, 1-benzoyl-2-isonicotinoylhydrazine; and C, I.*

resolution of the compounds from different batches of resin from the same supplier and due to a loss in resolution of I and II from plasma and urine extracts.

Attempts to separate I and II using conventional reversed-phase chromatography were also unsuccessful. Studies showed that the compounds were most propitiously separated on an octadecylsilane column using absolute methanol–distilled water (60:40) at pH 2.5 with 0.01 *M* dioctyl sodium sulfosuccinate. The mobile phase had been successfully employed for the determination of a I–pyridoxine hydrochloride mixture (7) and was readily adaptable to the I–II problem. Adequate separation was achieved between I and II in this solvent mixture as evidenced by an approximate resolution (R_s) value of 2.0 (11). Furthermore, use of this mobile phase allowed 1-benzoyl-2-isonicotinoylhydrazine to be used as a suitable internal standard.

A flow rate of 2.0 ml/min (2500 psig at room temperature) was the most satisfactory, since separations could be obtained in less than 10 min. The void volume of the column was 2.8 ml. UV detection at 266 nm was employed, since both I and II give maximum absorption at this wavelength and the detector would therefore provide maximum sensitivity for the low concentration levels present in biological fluids.

The solutions used in the determination of the standard curves as well as the plasma and urine samples were extracted according to a literature procedure for I (8). To make the sample compatible with the mobile phase, the extraction method was slightly modified with the use of 0.1 *N* sulfuric acid in the reextraction step. The internal standard was added to the original samples prior to extraction to improve precision. The drug concentrations used in the preparation of the standard curves and biological samples were in the range of those reported for plasma and urine levels (1, 12). Minimum detectability of I and II utilizing the chromatographic procedure described herein was 200 and 50 ng, respectively.

Calibration curves for both plasma and urine levels of I and II were obtained as follows. Digital integration of the area under the curve for each peak on the chromatograms was performed. The ratios of I and II peak areas to the area of the internal standard were calculated for each chromatogram. A linear regression analysis of these data at the four concentrations of each component gave the slope, intercept, and correlation coefficient for the plasma and urine calibration curves (Table I).

The liquid chromatograms of I–II from the spiked plasma and urine samples are shown in Figs. 1 and 2. No interferences from blank plasma and urine samples were noted. The ratios of drug peak areas/internal standard peak areas (D/IS) were calculated for I and II. The constants (slope and intercept) shown in Table I for the linear regression equations were used to solve for drug concentration in each biological sample: [D/IS

Table II—Analysis of I and II in Plasma and Urine

Biological Fluid	Compound	Initial Concentration Added, μg/ml	Amount Found[a], μg/ml	Accuracy, %
Plasma	I	20.0	18.9 ± 2.51[b]	5.25
	II	26.6	25.41 ± 0.36	4.47
Urine	I	80.0	74.04 ± 1.65	7.45
	II	240.0	243.74 ± 2.15	1.56

[a] Based on three replicate determinations of each solution. [b] Confidence limits at $p = 0.05$.

= (slope × concentration) + intercept]. Calculations were performed on a programmable calculator[7].

The data in Table II demonstrate the utility of HPLC in the analysis of I and II in biological fluids at plasma and urine concentration levels.

REFERENCES

(1) H. G. Boxenbaum and S. Riegelman, *J. Pharm. Sci.*, **63**, 1191 (1974).

(2) R. Modin, *Acta Pharm. Suec.*, **9**, 157 (1972).

(3) B. A. Perrson, *ibid.*, **8**, 217 (1971).

(4) B. L. Karger and B. A. Perrson, *J. Chromatogr. Sci.*, **12**, 521 (1974).

(5) B. L. Karger, S. C. Su, S. Marchese, and B. A. Perrson, *ibid.*, **12**, 678 (1974).

(6) D. P. Wittmer, N. O. Nuessle, and W. G. Haney, *Anal. Chem.*, **47**, 1422 (1975).

(7) J. T. Stewart, I. L. Honigberg, J. P. Brant, W. A. Murray, J. L. Webb, and J. B. Smith, *J. Pharm. Sci.*, **65**, 1536 (1976).

(8) "Manual of Analytical Toxicology," I. Sunshine, Ed., Chemical Rubber Co., Cleveland, Ohio, 1971, pp. 191, 192.

(9) H. H. Fox and J. T. Gibas, *J. Org. Chem.*, **18**, 1375 (1953).

(10) V. N. Kerr, D. G. Ott, and F. N. Hayes, *J. Am. Chem. Soc.*, **82**, 186 (1960).

(11) B. L. Karger, L. R. Snyder, and C. Horvath, "An Introduction to Separation Science," Wiley, New York, N.Y., 1973, pp. 146–150.

(12) J. H. Peters, *Am. Rev. Resp. Dis.*, **81**, 485 (1960).

[7] Olivetti-Underwood Programma 101.

ACKNOWLEDGMENTS AND ADDRESSES

Received October 9, 1975, from the *Department of Medicinal Chemistry, School of Pharmacy, University of Georgia, Athens, GA 30602.*

Accepted for publication August 5, 1976.

Presented at the Pharmaceutical Control and Analysis Section, APhA Academy of Pharmaceutical Sciences, Atlanta meeting, November 1975.

[x] To whom inquiries should be directed.

In Vitro and *In Vivo* Considerations of a Novel Matrix-Controlled Bovine Progesterone-Releasing Intravaginal Device

V. W. WINKLER [x], S. BORODKIN, S. K. WEBEL, and J. T. MANNEBACH

Abstract □ An *in vitro* system measuring progesterone release from silicone matrixes into serum is described. Drug release followed a matrix-controlled diffusion model in which the cumulative quantity released was linear with the square root of time. A bovine intravaginal device, consisting of a steel coil coated on both sides with a progesterone silicone matrix, was used as an *in vivo* drug delivery system. *In vivo* drug release also was matrix controlled, with rates comparable to those obtained *in vitro*. However, lag times of 1.7 and 4.2 days before reaching the steady-state rate were obtained for the outer and inner surfaces, respectively. The combination of effects resulted in a pseudo-zero-order drug-releasing device in which cumulative drug released was linear with time for at least 16 days.

Keyphrases □ Progesterone—release from silicone matrixes, *in vitro* system compared to bovine intravaginal device *in vivo* □ Silicone matrixes—release of progesterone, *in vitro* system compared to bovine intravaginal device *in vivo* □ Drug delivery—progesterone from silicone matrixes, *in vitro* system compared to bovine intravaginal device *in vivo* □ Vaginal devices, bovine—progesterone in silicone matrix, compared to *in vitro* system □ Hormones—progesterone, release from silicone matrixes, *in vitro* system compared to bovine intravaginal device *in vivo*

Intravaginal devices coated with a progesterone silicone matrix were used successfully (1–3) to control the bovine estrus cycle and to synchronize estrus by regulating pituitary release of gonadotropic hormones. Estrus occurs at a predictable time upon removal of the device developed in these laboratories 9–21 days after intravaginal implantation. The drug release rate profile of this device is defined in this report through correlation of *in vitro* and *in vivo* studies.

EXPERIMENTAL

Matrix Preparation—Progesterone silicone matrixes at various drug concentrations were prepared by first mixing the required amount of micronized drug into silicone fluid 360[1] and then adding Silastic elastomer 382[1]. The mixture was blended for 30 min to ensure random distribution of drug before adding the polymerizing catalyst, stannous octanoate[1].

***In Vitro* Study**—Matrixes used in the *in vitro* studies were prepared by streaking the silicone drug mixture containing catalyst onto fiber glass cloth and allowing it to cure. A template was used to obtain a uniform thickness of 1.5 mm. The cured matrixes were cut accurately into 2 × 2-cm pieces, mounted onto stainless steel plates with medical adhesive[1], and allowed to dry for 48 hr.

Two types of *in vitro* experiments were performed. The first followed the release of progesterone as a function of time from a silicone matrix containing 6.75% drug. The second concerned the release rate as a function of drug concentration using 5, 7.5, and 10% progesterone in silicone matrixes. The 6.75% matrix was prepared from a different lot of silicone liquid and elastomer, restricting direct comparison of the two *in vitro* experiments.

In both experiments, a specified number of matrixes mounted on stainless steel were individually placed in 25 × 150-mm glass tubes. To each tube, 15 ml of sheep serum[2] and 2 drops of toluene were added to retard microbial growth. Each tube was capped with a rubber stopper, placed on a shaker, and incubated at 37°. Serum changes at the end of Days 1, 2, 3, 4, 7, 11, 15, and 18 for one set of matrixes were matched with serum changes at the end of Days 0.1, 0.2, 0.3, 0.7, 1, 1.3, 2, 2.3, 3, 4, 5, 8, 12, 15, 18, and 20 for a duplicate set of matrixes.

The progesterone released in selected mounted matrixes was determined at various scheduled times by analyzing the amount of drug re-

[1] Dow Corning Corp., Midland, MI 48640.

[2] International Scientific Industries, Cary, IL 60013.