High-Performance Liquid Chromatographic Analysis of Neomycin in Petrolatum-Based Ointments and in Veterinary Formulations

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Abstract □ A high-performance liquid chromatographic (HPLC) method has been developed for the assay of neomycin in petrolatum-based ophthalmic and topical ointments and in veterinary formulations. Neomycin assay interferences from drugs, such as bacitracin and polymyxin B and inactive components, *e.g.* wax, were eliminated by a methanol wash and/or a partitioning method. The extracted neomycin was derivatized with 2,4-dinitrofluorobenzene followed by normal-phase HPLC with detection at 254 nm. The average recovery of neomycin from spiked samples was ~100% with a relative standard deviation of <1%.

Keyphrases □ Neomycin—high-performance liquid chromatography petrolatum-based ointments, veterinary formulations □ Ointments—petrolatum-based, high-performance liquid chromatographic assay for neomycin, veterinary formulations □ Formulations, veterinary—high-performance liquid chromatographic assay for neomycin, petrolatum-based ointments

Neomycin has been formulated into many topical and ophthalmic ointments and veterinary products because of its broad spectrum of antimicrobial activity. The ointments are generally petrolatum based; some contain other antibiotics, such as bacitracin and polymyxin B, or steroidal anti-inflammatory agents, such as hydrocortisone acetate or methylprednisolone acetate.

The antimicrobial potency ratio of neomycins B and C varies with the species of microorganism, presence of divalent cations in the diffusion media, and culture conditions used in the microbiological assay (1). Thus, acceptable precision of the microbiological assay can only be realized if the composition of the unknown preparation is similar to that of the standard preparation. The agar diffusion cylinder cup assay method for neomycin in ointments (2) is often troublesome, giving low recovery with a relative standard deviation larger than desired.

The GC assay method for neomycin in ointments requires prior silylation of the samples, uses a column temperature of ~300°C (3), and is plagued with low recoveries, short column life, instability of derivatized samples, and tedious sample preparation. The high-performance liquid chromatographic (HPLC) method presented here is accurate, precise, and reliable.

EXPERIMENTAL

Apparatus—A modular liquid chromatograph equipped with a 254-nm UV monitor[1], a high-pressure pump[2], and a 40-μL automatic sample injector[3] was used. A normal-phase LiChrosorb column[4], 4.6-mm i.d. × 25 cm, was used to separate neomycins B and C. Peak area was determined using an electronic integrator[5].

Reagents—All the solvents used were distilled-in-glass and UV grade[6]. The 2,4-dinitrofluorobenzene[7] and anhydrous sodium borate[8] used were analytical reagent grade.

For the mobile phase, tetrahydrofuran–chloroform–water–glacial acetic acid (392:598:8:2) was used. The derivatization reagent was freshly prepared by diluting 4 mL of 2,4-dinitrofluorobenzene with 200 mL of methanol to give an ~0.15 M solution. The 0.02 M borate buffer solution (pH 9.0) was prepared from anhydrous sodium borate and water. The mobile phase was pumped through the column at a flow rate of ~1.0 mL/min.

Reference Standard Solution—Neomycin sulfate reference standard USP (Issue K) was dried (3 h, 60°C, <5 mm Hg) and then allowed to cool in a desiccator. A 2.0-g sample was rapidly and accurately weighed, placed in a 1000-mL volumetric flask, dissolved, and diluted to volume with the borate buffer. Aliquots of the neomycin sulfate reference standard solution were placed in tightly capped serum bottles and kept at −20°C until used.

For the determination of neomycin in ointments, 25.0 mL of thawed neomycin stock reference solution was transferred into a 100-mL volumetric flask, 60 mL of 20% methanol was added, and the solution was diluted to volume with the borate buffer. For the determination of neomycin in veterinary products, the 100-mL volumetric flask containing 25 mL of the thawed reference standard solution was diluted to volume with the borate buffer only. A 10-mL quantity of the diluted neomycin reference standard solution thus prepared was transferred into a 250-mL volumetric flask for derivatization.

Sample Preparation—*Ointments Containing Bacitracin and Polymyxin B*—Approximately 5 g of pooled and well-mixed ointment was accurately weighed into a 50-mL round-bottom centrifuge tube followed by 3 mL of methanol. The sample was heated in a 55°C water bath for 5 min with vortexing twice for 20 s, followed by centrifugation at 2000 × *g* for 2 min. The supernatant was removed by aspiration. The methanol wash was repeated twice.

Thirty milliliters of chloroform was then added, and the sample was placed in a 55°C water bath to melt the ointment, vortexing for 15 s. Ten milliliters of 20% methanol was added, vigorously shaken for 20 min on a reciprocating platform shaker, and the sample was centrifuged at 1000 × *g* for ~3 min. The upper aqueous layer was transferred to a 50-mL volumetric flask using a Pasteur pipet with care taken not to withdraw the emulsion interface layer. The methanol–water extraction step was repeated twice using 10 mL of 20% methanol, shaking the centrifuge tube for 10 min each time. The aqueous layer was pooled in a 50-mL volumetric flask, and the sample was diluted to volume with the borate buffer. A 10-mL portion of the diluted sample was accurately transferred into a 250-mL volumetric flask for derivatization.

Ointments Containing Steroids—Approximately 5 g of the ointment was accurately weighted into a 50-mL round-bottom centrifuge tube. The procedure described above for ointments containing bacitracin and polymyxin B was followed, omitting the initial methanolic wash.

Veterinary Formulations—Two types of veterinary formulations, a soluble dry powder containing sucrose and a solution containing salts, preservatives, and a stabilizer (reducing agent), were examined. A sample containing ~50 mg of neomycin sulfate was accurately transferred into a 100-mL volumetric flask and diluted to volume with the borate buffer. The sample was mixed and centrifuged if required. A 10-mL quantity of the sample was quantitatively transferred into a 200-mL volumetric flask for derivatization.

[1] Model 1203 UV monitor III; Laboratory Data Control, Riviera Beach, Fla.
[2] Model M19-60066-022 high-pressure mini-pump; Laboratory Data Control.
[3] Model 710B WISP; Waters Associates, Milford, Mass.
[4] SI-100, LiChrosorb Silica 5-μm particle size, Brownlee Labs, Santa Clara, Calif.
[5] Chromatopac-E1A; Shimadzu Seisakusha, Ltd., Kyoto, Japan.
[6] Burdick and Jackson Labs, Muskegon, Mich.
[7] Aldrich Chemical, Milwaukee, Mis.
[8] Mallinckrodt, St. Louis, Mo.

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Table I—Recovery of Neomycin From a Topical Ointment Containing Hydrocortisone Acetate

Drug Level, %	Neomycin Sulfate, mg/g Added	Recovered	Recovery, %
75	20.62	20.50	99.4
90	24.85	24.64	99.2
100	27.63	27.53	99.6
110	30.19	30.08	99.6
125	34.33	34.04	99.2
Mean			99.4
RSD			0.2
r			0.9999

Derivatization—A 15-mL quantity of the derivatization reagent was added to the 250-mL volumetric flask containing standard solution or sample, and the opening of the flask was covered with aluminum foil. The flask was then placed in a 100°C silicone oil bath for 45 min to form dinitrobenzene-neomycin. The amount of 2,4-dinitrofluorobenzene present was more than a 30-fold excess (4). The flask was then cooled, and mobile phase was added until the lower, yellow, organic phase reached the 250-mL mark. The top (aqueous) layer was removed by aspiration, and the yellow organic phase was chromatographed using the conditions described above. The derivatized neomycin is stable for over 1 week at room temperature when stored in the dark.

Calculations—The concentrations of neomycins B and C and the bioequivalent potency (5) were calculated by using the following equations:

$$\text{Neomycin B } (\mu g/g) = B_{smp}/B_{std} \times W_{std}/W_{smp} \times F1 \times F2 \quad \text{(Eq. 1)}$$

$$\text{Neomycin C } (\mu g/g) = C_{smp}/B_{std} \times W_{std}/W_{smp} \times F1 \times F2 \quad \text{(Eq. 2)}$$

$$\text{Bioequivalent Potency } (\mu g/g) = (\text{Neomycin B}) \times 1/2(\text{Neomycin C}) \quad \text{(Eq. 3)}$$

where B_{std} and B_{smp} are the peak areas of neomycin B in the reference standard and sample, respectively; C_{smp} is the peak area of neomycin C in samples; W_{std} and W_{smp} are the weight of the reference standard powder and samples, respectively; F1 is the dilution factor; and F2 is the assigned potency of the neomycin sulfate reference standard USP (765 μg/mg for Issue K).

RESULTS AND DISCUSSION

Derivatization—Recently, Helboe and Kryger noted formation of insoluble lumps on derivatization (6) which were eliminated when neomycin was derivatized at a lower temperature [60°C instead of 100°C as used by Tsuji *et al.* (4)]. To examine the problem, two lots each of the 2,4-dinitrofluorobenzene reagent obtained from two suppliers[7,9] were employed using the method of Tsuji *et al.* (4). Only one of the four lots of the reagent examined formed yellowish deposits which adhered to the wall of the flask. When derivatized at 60°C the peak areas of neomycins B and C were 94% and 45% of those derivatized at 100°C, respectively. Incomplete derivatization of neomycin in 60°C samples was evident from numerous peaks which eluted near the solvent front. Thus, the formation of insoluble lumps on derivatization may be due to impurities occasionally present in some lots of the derivatization reagent. The use of lower derivatization temperature may result in a serious underderivatization of neomycin.

The use of methanol in the partitioning extraction of neomycin occasionally increased the efficiency of derivatization, resulting in increased peak area. This potential difficulty was eliminated by inclusion of methanol in the reference standard solution prior to derivatization.

Extraction—Differential centrifugation with chloroform to dissolve excipients and to precipitate neomycin, as developed for the GC assay (3), was examined. Precipitated neomycin was dissolved in the borate buffer prior to derivatization. Centrifugation over an extended time (2000 × *g* for 30 min) failed to increase recovery of neomycin from spiked placebo above 98% and 93–95% for ophthalmic and topical ointments, respectively. Various solvents were added to chloroform to lower its specific gravity in an effort to facilitate precipitation of neomycin; no improvement in the recovery was noted.

[9] Eastman Organic Chemicals, Rochester, N.Y.

Table II—Analysis of Neomycin in Ophthalmic and Topical Ointments Containing Hydrocortisone Acetate[a]

Lot	Neomycin Base, mg/g HPLC	GC	Microbiological Assay
	Ophthalmic Ointment		
A	3.86	3.79	3.73
B	3.86	3.74	3.69
C	4.14	3.81	4.04
D	3.87	3.70	3.57
E	3.97	3.70	3.80
	Topical Ointment		
F	3.95	3.60	3.79
G	3.87	3.80	3.54
H	3.77	3.60	3.59
I	3.89	3.52	3.92
J	3.88	3.52	4.04

[a] All lots from The Upjohn Co.

It was noted that the recovery of neomycin from ophthalmic ointments was always higher than that from topical ointments. Topical ointments differ from ophthalmic ointments by inclusion of microcrystalline wax. It is possible that neomycin sulfate is encapsulated by the microcrystalline wax during the manufacturing process. This wax dissolves poorly in chloroform; heat and extended sonication are needed, and the resulting suspension is still turbid. A sample of neomycin was spiked in melted wax and dispersed by vortexing. The sample was then assayed by use of the differential centrifugation method. The recovery was 92%, suggesting the wax encapsulation or adsorption hypothesis. Efforts to remove the wax interference by extended heating and sonication failed to improve the recovery of neomycin.

Aqueous Extraction—Partitioning neomycin for extraction in a methanolic water–chloroform system was then examined. A 20% methanolic water solution was necessary to reduce emulsion formation at the water–chloroform interface.

Ointments Containing Steroids—Recovery of neomycin from both ophthalmic and topical ointments by the partitioning extraction method was examined. Neomycin was spiked in a steroid-containing ointment

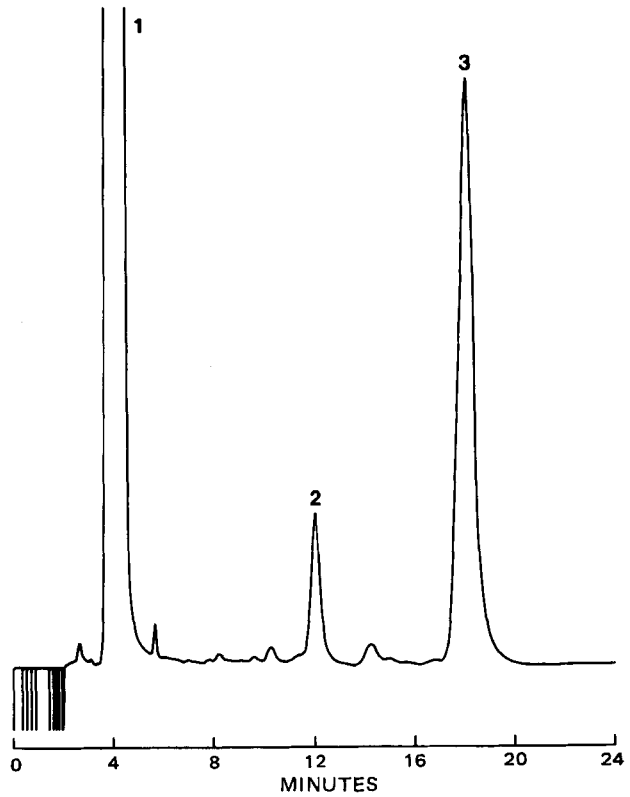

Figure 1—*Typical chromatogram of neomycin in a topical ointment containing hydrocortisone acetate. Key: (1) neomycin C; (2) neomycin B; (3) hydrocortisone acetate.*

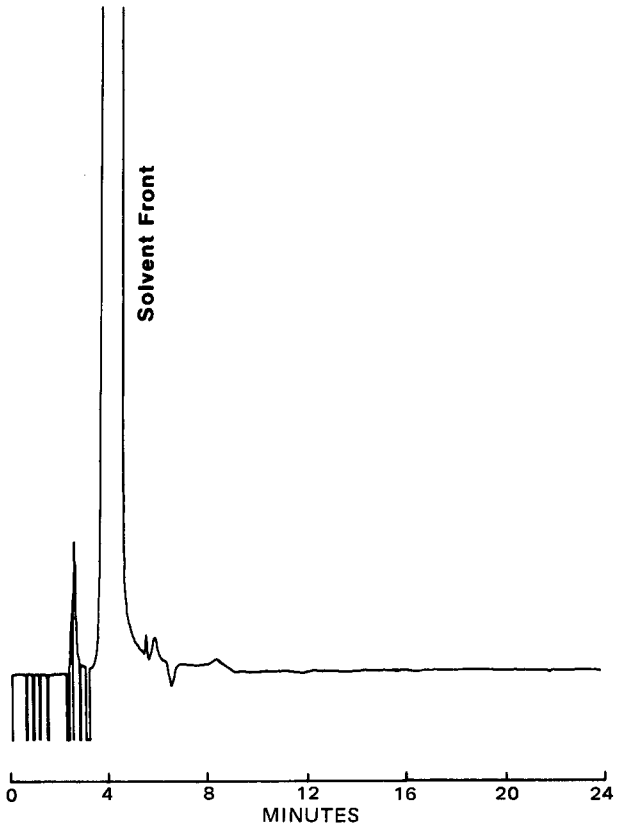

Figure 2—*Chromatogram of placebo of topical ointment containing hydrocortisone acetate, indicating no interfering peaks.*

base at 75–125% of the target. An example of the neomycin recovery study using a topical ointment containing hydrocortisone acetate is shown in Table I. The recovery of neomycin averaged 99.4%, with a relative standard deviation of 0.2%. The correlation coefficient (r) for the recovery study was 0.9999. Similar recoveries and precision were obtained for ophthalmic and topical ointments containing various steroids. Thus, the partitioning method for extraction of neomycin from ointment completely eliminated the interference of microcrystalline wax. Steroids, *e.g.*, hydrocortisone acetate, cortisone acetate, fluorometholone, methylprednisolone, and prednisolone acetate, showed no interference in the HPLC analysis of neomycin in ointments.

Precision of the HPLC method was also examined. Seven individually weighed and extracted samples were examined using the partitioning method. The relative standard deviation of the assay was 0.5%; similar precision was obtained for ophthalmic and topical ointments containing steroids. The HPLC method was then used to assay the ointments, and the values were compared with those of the GC and the microbiological assays. Table II shows an example of such comparison made using both ophthalmic and topical ointments containing hydrocortisone acetate. In general, the values obtained by the HPLC method were the closest to the target and higher than those of the GC or the microbiological methods. However, the difference between HPLC, GC, and microbiological

Table III—Recovery of Neomycin from Topical Ointments Containing Bacitracin and Polymyxin B

Drug Level, %	Day 1 Neomycin Sulfate, mg/g Added	Day 1 Neomycin Sulfate, mg/g Recovered	Day 1 Recovery, %	Day 2 Neomycin Sulfate, mg/g Added	Day 2 Neomycin Sulfate, mg/g Recovered	Day 2 Recovery, %
75	22.45	22.67	101.0	22.47	22.26	99.0
90	27.19	27.24	100.2	26.98	27.12	100.5
100	29.91	29.59	99.0	30.10	29.64	98.5
110	32.77	32.28	98.5	33.04	32.64	98.8
125	37.57	37.22	99.1	37.46	37.46	100.0
Mean			99.6%			99.4%
RSD			1.0%			0.9%
r			0.9996			0.9990

Table IV—Assay of Neomycin in Topical Ointments Containing Bacitracin and Polymyxin B[a]

Lot	Neomycin, mg/g[b] HPLC	Neomycin, mg/g[b] GC
A	4.03	3.75
B	4.25	3.88
C	4.26	3.80
D	4.29	3.88
E	4.33	3.76
F	4.26	3.82
G	4.25	3.84
H	4.24	3.80
I	4.27	3.74
J	4.34	3.90
K	4.25	3.91
L	4.34	3.81
Mean	4.26	3.82

[a] All lots from The Upjohn Co. [b] Manufacturing target: 4.2 mg/g.

Table V—Assay for Neomycin in a Veterinary Formulation[a]

Lot	Neomycin, mg/mL[b] HPLC	Neomycin, mg/mL[b] Microbiological Assay
A	142.7	149
B	142.8	152
C	142.3	148
D	146.5	155
E	136.5	137
F	136.0	136

[a] All lots from The Upjohn Co. [b] Manufacturing target: 142.8 mg/mL.

methods was not statistically significant. Typical chromatograms of the HPLC assay for neomycin in a topical ointment containing hydrocortisone acetate and its placebo are shown in Figs. 1 and 2.

Ointments Containing Bacitracin and Polymyxin B—Bacitracin and polymyxin B are polypeptide antibiotics, and primary amines present in the molecules compete effectively with neomycin for 2,4-dinitrofluorobenzene. Efforts to compensate for the competition by increasing the amounts of the derivatization reagent were unsuccessful. The recovery of neomycin in the presence of bacitracin and polymyxin B was ~92%.

Since bacitracin is readily soluble in methanol, and polymyxin B to a lesser extent, methanol was added to the ointment to remove the interference. (Neomycin sulfate is practically insoluble in methanol.) Samples of topical ointment placebo containing bacitracin and polymyxin B were spiked with neomycin at 75–125% of the manufacturing target. The results of the spiked recovery study are presented in Table III; the recovery of neomycin averaged 99.5% with a relative standard deviation of 1.0%, demonstrating complete removal of the interference.

Precision of the HPLC method for neomycin was examined by analyzing seven individually weighed and extracted ointment samples; the relative standard deviation of the assay was 0.86%. Twelve lots of topical ointments containing bacitracin and polymyxin B were then assayed (Table IV). Although there was significant difference in the assay values between the HPLC and GC methods, the HPLC data were extremely close to the manufacturing target for the product. The HPLC results averaged 101.4% of the target while the GC results averaged only 91%. Thus, the HPLC method was judged to give accurate results.

Veterinary Formulations—The veterinary formulations examined were all soluble in the borate buffer. Therefore, neomycin in the formulation was quantitated by simply derivatizing a dilute borate buffer mixture. The recovery of neomycin was ~100% with a relative standard deviation of <1%. Six lots of a liquid veterinary formulation were assayed for neomycin by HPLC, and the results were compared with those obtained by the microbiological assay method. As shown in Table V, no statistically significant difference existed between the HPLC and the microbiological assay results.

A petition to amend an antibiotic application for the use of the HPLC method as an alternative assay of neomycin in ointment products has been submitted to Food and Drug Administration for approval.

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Pharmacokinetics of Heparin VII: Effect of Pregnancy on the Relationship Between Concentration and Anticoagulant Action of Heparin in Rats

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Abstract □ The effect of pregnancy on the anticoagulant action of heparin was determined by comparing the slope of the relationship between the natural logarithm of the activated partial thromboplastin time (APTT) and heparin concentration (the heparin slope) in the plasma of pregnant and nonpregnant female inbred Lewis rats. Also determined were the prothrombin time, hematocrit, and the activities of coagulation factors II, VII, VIII, X, XI, and XII. The heparin slope was significantly decreased in pregnant rats at the 20th day of gestation but not in rats at the 10th day of gestation, indicative of a decreased anticoagulant action of heparin in late pregnancy. The hematocrit and prothrombin time were decreased, and the baseline APTT (*i.e.*, the APTT without added heparin) as well as the activities of factors II, VII, and X were increased in pregnant rats at the 20th day of gestation. Both pregnant and nonpregnant animals showed a significant negative correlation between prothrombin time and factor II activity and a significant positive correlation between the activities of factors II and X. The effects of pregnancy in rats on heparin slope, prothrombin time, hematocrit, and factors VII, VIII, X, and XII are qualitatively the same as those in pregnant women in the third trimester. The increases in factor II activity and baseline APTT found in the rats were not observed in humans. Pregnant rats, like pregnant women, are relatively resistant to the anticoagulant action of heparin.

Keyphrases □ Heparin—anticoagulant action, effect of pregnancy, rats □ Anticoagulants—heparin, effect of pregnancy on the anticoagulant action, rats □ Pregnancy—effect on the anticoagulant action of heparin in rats

Rats and humans exhibit grossly similar characteristics with respect to the pharmacokinetics and pharmacodynamics of heparin: both species eliminate heparin by dose-dependent kinetics that are not of the Michaelis–Menten type and both show an essentially linear relationship between an index of the anticoagulant response, the logarithm of the activated partial thromboplastin time of plasma (APTT), and the concentration of heparin added to plasma (1). Human pregnancy is associated with a state of progressive blood hypercoagulability due, in part, to increased concentrations or activities of certain components of the coagulation system (2). The heparin dose requirements for treatment or prevention of thromboembolic disorders are increased in human pregnancy (3–6). Recent studies in this laboratory have shown this to be due, at least in part, to a decreased anticoagulant effect of heparin (7). Changes in the distribution (7) and clearance (3) of heparin may also occur, but this is presently uncertain.

A previous investigation of blood coagulation characteristics and response to heparin in rats (8) has revealed a number of important differences relative to humans, despite the gross similarity in the pharmacokinetics and pharmacodynamics of heparin in the two species (1). To further explore the relative characteristics of the two species with respect to heparin, we have determined the effect of pregnancy on the anticoagulant action of heparin and on a number of related physiological variables in rats.

EXPERIMENTAL

Groups of two female inbred Lewis rats[1], 200–250 g, were placed in plastic cages with sawdust bedding taken from cages previously occupied by adult male rats. They had free access to food[2] and water at all times. After 2 d an adult male Sprague–Dawley rat was placed in each cage with the two females for an overnight period. The male rat was removed in the morning, and that day was designated as the first day of gestation. The female rats were then housed in groups of four per cage for the duration of the study; nonimpregnated females from the same colony were kept similarly. This procedure was repeated on three occasions. For the first study, groups of eight pregnant and eight control rats each were tested on the 10th and 20th days of gestation; for the second study, nine pregnant and nine control rats were tested on the 20th day of gestation; for the third study, seven pregnant and seven control rats were tested on the 20th day of gestation.

Blood samples were obtained from the abdominal aorta while the animals were under light ether anesthesia. The hematocrit was first determined from a small sample of blood (<0.1 mL) taken from the tail vein. Sufficient acid citrate anticoagulant (9) was then added to a 5-mL plastic syringe such as to yield a 6:1 plasma–citrate solution volume ratio when 4.5 mL of blood was drawn from the abdominal aorta into the syringe. The citrated blood was transferred to plastic tubes which were centrifuged to separate the plasma as previously described (8). The plasma samples were stored at −80°C for no longer than 3 d. Plasma from two of the control rats (10th day) in the first study contained small clots on thawing. To prevent this in subsequent experiments, the plasma–citrate solution volume ratio was changed from 6:1 to 5:1 in the second and third studies. All animals in the pregnant groups were dissected after blood sampling to confirm pregnancy.

The APTT was determined with a coagulation timer[3] after a 15-min plasma incubation time (unless stated otherwise), using automated APTT[4] as the principal reagent. Heparin of beef lung origin[5] was added to the plasma to yield concentrations of 0.1–0.7 U/mL in six steps; an equivalent volume of heparin-free solvent was added for baseline APTT determination. Details of these procedures have been described previ-

[1] Charles River Farms, Wilmington, Mass.
[2] Charles River Formula RMH 1000, Syracuse, N.Y.
[3] Fibrometer, Baltimore Biological Laboratories, Cockeysville, Md.
[4] General Diagnostics, Morris Plains, N.J.
[5] The Upjohn Co., Kalamazoo, Mich., (lot no. 955FW).

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