Solubilization and *in Vitro* Spermicidal Assessment of Nonoxynol-9 and Selected Fractions Using Rabbit Spermatozoa

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Selected oligomers representing the high, medium, and low molecular weight fractions of the spermicidal agent Nonoxynol-9 (N-9) were separated by HPLC. Nonoxynol-9 and the isolated fractions were formulated with polyvinylpyrrolidone (PVP) in order to increase their water solubility, particularly that of the insoluble low molecular weight fraction. The *in vitro* spermicidal activity of three molecular weight fractions were compared to that of N-9, using rabbit spermatozoa, at equimolar concentrations. Nonoxynol-9/PVP was far more effective in immobilizing the sperm than either N-9 alone or the separate fractions. The relative spermicidal activity of the oligomers was of the order middle molecular weight > high molecular weight > low molecular weight.

KEY WORDS: Nonoxynol-9; solubilization; spermicide; polyvinylpyrrolidone; rabbit spermatozoa.

INTRODUCTION

Nonoxynol-9 [N-9; nonylphenol(polyethoxy) ethanol, Igepal CO-630] is a nonionic surfactant used as the active ingredient in the majority of the commercially available spermicides. It inhibits the *in vitro* growth of venereal pathogens (1–4) including the herpes simplex viruses (5–11). By nature of its synthesis, Nonoxynol-9 is a polymer consisting of at least 17 oligomers of varying ethylene oxide (EO) chain length. The physical and chemical characteristics of these oligomers change as a function of the varying molecular weight (12). As the length of the EO chain increases the oligomers increase in water solubility. Nonoxynol-1 through -6 are considered oil soluble compounds, whereas the oligomers with a longer EO chain are soluble in water and polar organic solvents (12).

These differences in properties seem to affect their biological behavior both *in vitro* and *in vivo*. For instance, it was noted that the dermal toxicity of nonoxynol decreases as the molecular weight increases (12) and that smaller molecular weight nonoxynol may be more toxic to fibroblasts than the larger ones (13). The *in vitro* spermicidal activity of the surfactant was also related to its molecular weight. Thus N-9 (n = 9) was much more effective in inhibiting the motility of the spermatozoa than the higher molecular weight nonoxynols (n = 30, 50, and 100) (13). The lower molecular weight nonoxynols (n = 1 or 4) could not be studied appropriately because of their poor solubility in the aqueous testing medium (13).

Analogous dependence on molecular weight was observed *in vivo*. Oral absorption studies in the rat indicated that increasing the length of the ethylene oxide chain decreased their intestinal absorption (14). Furthermore, data from these laboratories showed that absorption of N-9 through the vaginal membrane was poor (15) and might reflect the preferential absorption of lypophilic low molecular oligomers.

Evidently, N-9 is a multicomponent mixture that exhibits nonuniform chemical and biological activity among its oligomers. Previous research used the commercially available surfactants of the nonoxynol (or Igepal CO) series which are chemically heterogeneous products. It is therefore necessary to fractionate N-9 and test the biological activity of the purified N-9 oligomers. Consequently, the purpose of this work was to study the effect of molecular weight on the spermicidal activity of N-9. For that purpose, three fractions, representing low, middle, and high molecular weight fragments of N-9, were separated by HPLC, and their spermicidal activities compared in vitro using rabbit spermatozoa. In the process, methods to solubilize the low molecular weight nonoxynols in aqueous media had to be developed to permit their biological evaluation.

MATERIALS AND METHODS

Chemicals and Reagents

Igepal CO-210 (avg. n = 1.5), Igepal CO-430 (avg. n = 4), Igepal CO-530 (avg. n = 6), Igepal CO-630 (avg. n = 9), Igepal CO-730 (avg. n = 15), and polyvinylpyrrolidone (K-30) were gifts from GAF Corporation (Wayne, NJ). HPLC solvents and other chemicals were commercially available and were used without further purification.

Zorbax-NH_2 HPLC columns (250 × 4.6-mm I.D.) and (250 × 21.2-mm I.D.) were purchased from DuPont Instruments Products (Wilmington, DE).

Separation of Oligomers of N-9 by Preparative Normal Phase HPLC

Solutions of N-9 (250 mg/ml) in tetrahydrofuran were separated by normal phase preparative HPLC using the conditions reported previously (16).

Three molecular weight fractions of N-9 were isolated: low MW fraction (LMW), 13–27 min, EO 1–EO 4; middle MW fraction (MMW), 32–47.2 min, EO 6–EO 8; and high MW fraction (HMW), 57.6–70.8 min, EO 11–EO 13. The solvent was evaporated under vacuum and the residue was weighed. Stock solutions of the various fractions were made by adding 2.0 ml of methanol. The purity of each fraction

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was assessed by analytical HPLC (Zorbax-NH_2 column, 250 × 4.6-mm I.D.) using the same assay described for [^{14}C]N-9 (15).

The molar concentration of the N-9 fractions was calculated based on the weighted average molecular weight (MW). The latter was obtained using the following equation:

$$MW_L = \frac{(MW_i)(OA_i) + (MW_j)(OA_j) + (MW_k)(OA_k) + \ldots}{OA_i + OA_j + OA_k + \ldots}$$

where MW_L is the weighted average MW of LMW; MW_i, MW_j, and MW_k are the molecular weights of the various oligomers in LMW; and OA_i, OA_j, and OA_k are the relative abundance of the various oligomers in LMW.

Thus the values of 414, 575, and 785 g/mol were calculated for the weighted average molecular weight of MW_L, MW_M, and MW_H, respectively.

Solubilization of Low MW Oligomers of N-9 Using Igepal CO-210 as a Model Compound

Standard Curve

Stock solutions of Igepal CO-210 (n = 1.5) of 1.5, 2.25, 3.75, and 4.5 mg/ml were prepared in methanol. A 0.1-ml aliquot from each stock solution was diluted with 2.9 ml methanol and the UV absorbance was measured at 280 nm, in duplicate readings. The average value of the absorbance was plotted against the concentration. Values were linearly related (r = 0.9999) over the concentration range from 55.3 to 169.53 μg/ml.

Preparation of PVP Coprecipitate

Stock solutions of 1, 2, and 5% (w/v) polyvinylpyrrolidinone (PVP K-30) were made in methanol. A 0.1-ml aliquot was withdrawn from Igepal CO-210 stock solution (4.5 mg/ml) and added to 2.9 ml of the various PVP solutions. The samples were mixed on a vortex mixer for 30 sec and placed in a water bath (100°C) to evaporate the solvent (1 hr). The residual Igepal CO-210/PVP coprecipitates were redissolved in 3.0 ml of distilled water and their absorbance was determined at 280 nm. UV scans (400–200 nm) of PVP-Igepal complexes were obtained and no shift in the wavelength was noted.

In Vitro Spermicidal Tests

Preparation of TEST Buffer

TEST buffer was prepared according to the method of Zavos (17). To a stirred solution of sterile water (700 ml) was added TES (48.3 g), Tris amino (11.6 g), and D-glucose (2.0 g). The volume was brought up to 1 liter, and osmotic pressure was adjusted to 320–325 mOsm using D-glucose, pH 7.2–7.4. The solution was pipetted into 12-ml test tubes and stored at −20°C until needed.

Preparation of N-9 and N-9 Fractions (LMW, MMW, HMW)

Stock solutions of N-9 (7.5 mg/ml), LMW (17.3 mg/ml), MMW (80.6 mg/ml), and HMW (75.5 mg/ml) were prepared in methanol. An aliquot (0.01–0.1 ml) was removed and added to 3 ml of 1% (w/v) PVP K-30 in methanol. The samples were mixed on a vortex mixer for 30 sec and placed in a water bath (100°C) to evaporate the solvent (1 hr). The resulting coprecipitates were redissolved in 3.0 ml of TEST buffer.

Serial dilutions of the stock solutions in TEST buffer were made to achieve N-9/PVP concentrations ranging from 62.5 to 250 μg/ml and LMW/PVP, MMW/PVP, and HMW/PVP concentrations of 40.9–109.2, 56.8–151.4, and 77.5–206.6 μg/ml, respectively.

Solutions of N-9 in TEST buffer were prepared as described above for N-9/PVP but without the addition of PVP.

Source of Spermatozoa

Ejaculates from healthy male New Zealand rabbits (n = 4) were collected using an artificial vagina and a teaser doe. The collected semen was transferred into prewarmed (37°C) containers. Aliquots from each ejaculate were initially evaluated under the microscope for concentration, motility, and progressive motility of the sperm cells. The ejaculates with the highest sperm motility were pooled and used further. The sperm cells were diluted to 40 × 10^6 cells/ml with TEST buffer and maintained in a water bath at 37°C until used.

Evaluation of Motility and Progressive Motility

The sperm motility was estimated via the use of a closed-circuit television system as described by Graham (18). Progressive motility was assessed as described by Zavos and Cohen (19). The sperm samples were independently evaluated in a blind method by two trained technicians and data were averaged for each sample. A microscope stage warmer was used during the study to maintain the sperm samples at physiological temperature (37°C).

In a typical experiment, a solution of the spermicide (1 ml) in TEST buffer was slowly added to an equal volume of sperm cells in TEST buffer. The semen was evaluated for motility and progressive motility immediately following the addition of N-9 (t = 0) and at 15-min intervals for the total duration of 60 min.

The motility was expressed as the percentage of motile sperm cells in five independent fields of view. The progressive motility was determined by the linear progression and drive of the sperm (based on a scale 0–4). The quality of the progressive motility was graded as follows: grade 1, oscillating movement but stationary; grade 2, slow movement with no fixed direction; grade 3 slow progressive movement; and grade 4, fast progressive movement (19).

In one study, the spermicidal effects of N-9 and N-9/PVP coprecipitate on spermatozoa were compared at three different final concentrations of 31.2, 62.5, and 83.3 μg/ml.

In another study, the spermicidal effects of equimolar solutions of N-9/PVP, LMW/PVP, MMW/PVP, and HMW/PVP on spermatozoa were compared at 1.32 × 10^{-7}, 9.87 × 10^{-8}, and 4.94 × 10^{-8} mol/ml.

In all studies, experimental controls were carried out simultaneously, whereby the spermatozoa were evaluated in the presence of TEST buffer only and/or TEST buffer containing 1% PVP only.

RESULTS AND DISCUSSION

The *in vitro* effectiveness of surfactants as spermicides, typified by N-9, has been established since their discovery in the 1940s (20). N-9 is a polymer consisting of at least 17 oligomers of varying hydrophilicity and molecular weight. The objective of this study was to separate these fractions and to evaluate their *in vitro* spermicidal qualities using rabbit spermatozoa. However, the evaluation of each N-9 oligomer would be tedious. Instead, the study was carried out using three N-9 fractions representing the low MW oligomers (LMW, EO 1–EO 4), the middle MW oligomers (MMW, EO 6–EO 8), and the high MW oligomers (HMW, EO 11–EO 13). These fractions were separated using a normal phase gradient elution HPLC assay and the oligomers identified by mass spectrometry as described in the following paper (16). Chromatograms of N-9 and the individual isolated N-9 fractions are shown in Figs. 1 and 2.

Solubility Studies

Low MW polymers of the nonylphenol polyethoxylate class of surfactants are insoluble in aqueous media (12,13). As a result, various methods to increase the water solubility of LMW fraction were investigated using Igepal CO-210 as a model compound. The ideal solubilizing agent should (i) dissolve the compound at the desired concentration, (ii) exhibit no inherent spermicidal activity, (iii) be chemically inert, and (iv) not interfere with/or impair the effectiveness of the spermicide. The use of 5–10% (w/v) cosolvents (e.g., glycerin, PEG, PPG, 95% ethanol, dimethylformamide, tetrahydrofuran, dioxane, and acetone) solutions in water did not solubilize the Igepal CO-210 at the desired concentration (150 μg/ml). Addition of other solubilizing agents was also attempted. Tween-20 surfactants solubilized Igepal CO-210 at the desired concentration (150 μg/ml); however, their inherent sperm toxicity, even at concentrations as low as 0.025% (w/v), prevented their use.

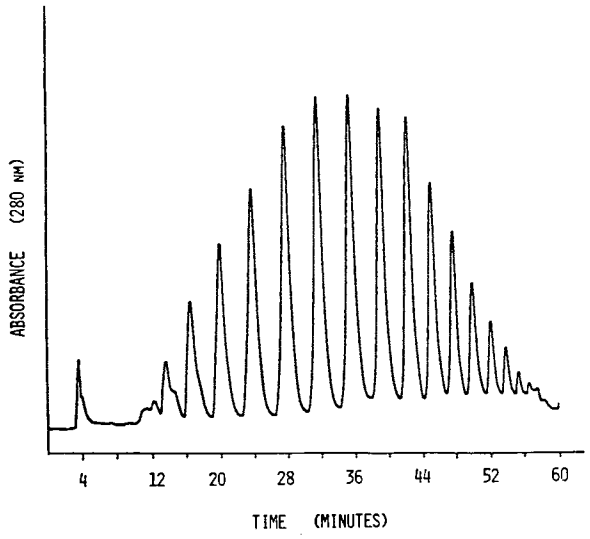

Fig. 1. Analytical separation of Nonoxynol using a normal phase gradient elution assay (15). Zorbax-NH_2 column; mobile phase, 60-min linear gradient from 98%A–2%B to 50%A–50%B, where A = tetrahydrofuran–hexane (20:80, v/v) and B = water–2-propanol (10:90, v/v); flow rate, 1.0 ml/min.

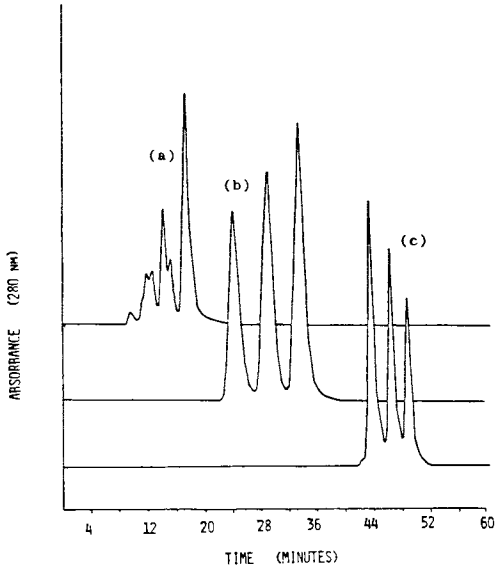

Fig. 2. Chromatograms of separated N-9 fractions: (a) LMW, (b) MMW, and (c) HMW. see the legend to Fig. 1 for analytical HPLC conditions.

The solubility of Igepal CO-210 was not enhanced by the presence of 5–10% (w/v) PVP in water. However, when Igepal CO-210 was coprecipitated with PVP, the resulting material dissolved readily in water, indicating the formation of a strong complex (21). Additionally, in control experiments discussed below, PVP alone in TEST buffer exhibited no toxicity to the sperm. Consequently, N-9 and the isolated fractions were all formulated with PVP for the subsequent *in vitro* studies.

Evaluation of Motility and Progressive Motility

The action of N-9 on human and bovine sperm has been evaluated previously employing electron microscopy techniques (22–24). These studies showed that N-9 lysed all sperm membranes, except the postacrosomal head region and the tail portion. The destruction of the membranes of the acrosome, neck, and midpiece tends to immobilize the sperm, thereby preventing sperm motility, capacitation, and subsequent fertilization of the ovum (23). Therefore, the *in vitro* evaluation of the various N-9 fractions was based on their effect on motility and progressive motility of the sperm.

Initially, a range finding study was conducted to determine the spermicidal concentration of N-9 adequate for the assay. Nonoxynol-9 immobilized sperm on contact at 125 μg/ml, with complete immobilization occurring too fast to allow for assessment of the relative potency of the various spermicides. At concentrations lower than 30 μg/ml, N-9 was ineffective. Hence, concentrations between 30 and 83 μg/ml were utilized in this study.

The spermicidal activity of N-9, N-9/PVP, and N-9 fractions/PVP (HMW, MMW, and LMW) were compared at equimolar concentrations (based on N-9) equivalent to 31.2, 62.5, and 83.3 μg/ml (Tables I and II), as a function of time. At the lowest concentration (31.2 μg/ml; n = 4), no differ-

Table I. Motility[a] of Rabbit Spermatozoa After Exposure to N-9 and Equal Molar Concentrations of N-9 Fractions (LMW, MMW, HMW): The Final Concentration of Spermatozoa Was 20 × 10^6/ml

Time (min)[c]	1% PVP control	N-9 fraction[b]			N-9	N-9 (PVP)
		LMW[d]	MMW[e]	HMW[f]		
			At 31.2 μg/ml			
0	79.4 ± 1.3	75.6 ± 2.4	73.8 ± 1.4	73.8 ± 3.2	74.4 ± 3.2	70.8 ± 8.8
15	80.1 ± 2.0	74.4 ± 1.3	73.1 ± 2.4	72.5 ± 0	73.8 ± 1.4	70 ± 10
30	79.4 ± 2.4	74.4 ± 1.3	71.9 ± 2.4	73.1 ± 1.3	73.8 ± 1.4	70 ± 9
45	78.8 ± 2.5	73.8 ± 1.4	70.6 ± 1.3	73.1 ± 2.4	71.9 ± 1.3	70 ± 7.5
60	78.1 ± 2.4	73.8 ± 2.5	73.1 ± 1.3	72.5 ± 2.0	70.6 ± 1.3	65 ± 8.7
			At 62.5 μg/ml			
0	79.1 ± 4.0	75.0 ± 3.3	70.3 ± 1.9	70.0 ± 1.9	70.3 ± 3.4	50.8 ± 25
15	78.8 ± 2.3	70.3 ± 2.1	22.5 ± 13.6	49.4 ± 16.5	40.6 ± 20.5	20 ± 34.6
30	79.3 ± 3.5	65.0 ± 10.7	4.7 ± 10.3	30.0 ± 25.8	11.6 ± 8.0	1.7 ± 2.9
45	78.8 ± 4.2	60.0 ± 13.1	1.9 ± 4.4	8.5 ± 11.3	2.2 ± 4.3	0
60	77.8 ± 2.5	50.3 ± 22.8	0.3 ± 0.9	1.5 ± 2.8	0	0
			At 83.3 μg/ml			
0	80.0 ± 6.5	69.4 ± 7.5	56.9 ± 17.7	31.3 ± 21.2	61.9 ± 18.2	5 ± 5
15	79.4 ± 6.3	58.8 ± 13.0	1.3 ± 1.4	0	0	0
30	79.4 ± 6.3	47.5 ± 11.4	0	0	0	0
45	76.3 ± 2.5	36.9 ± 13.3	0	0	0	0
60	75.8 ± 5.2	13.1 ± 18.4	0	0	0	0

[a] The motility was expressed as the percentage of motile sperm cells (scale 0–100). Values are the average of four determinations ± SD.
[b] All N-9 fractions (LMW, MMW, HMW) were formulated as PVP coprecipitates.
[c] Time after addition of the spermicide.
[d] Low molecular weight N-9 fraction isolated by HPLC (n = 1–4).
[e] Middle molecular weight N-9 fraction isolated by HPLC (n = 6–8).
[f] High molecular weight N-9 fraction isolated by HPLC (n = 11–13).

ences were noted between the various fractions and N-9 on either the motility or the progressive motility of the sperm. In all instances, the spermicidal activity seemed to be slow and most of the sperm remained viable up to 60 min of incubation.

As expected, the spermicidal activity of the various polymers was enhanced at higher concentrations. At 62.5 μg/ml (n = 8), the order of efficacy in immobilizing the spermatozoa was N-9/PVP = MMW > N-9 > HMW > LMW, with 75% of the motile sperm being immobilized by MMW and N-9/PVP by 15 min, about 50% for N-9 and HMW, and 11% only for LMW. On the other hand, at the highest concentration tested (83.3 μg/ml; n = 4), the order was N-9/PVP > HMW > MMW > N-9 > LMW. Immediately upon addition, N-9/PVP and HMW caused the immobilization of about 95 and 40% of the motile sperm, respectively, while N-9 and MMW immobilized only 25%. Except for the LMW and control groups, all sperm were completely immobilized within 15 minutes of incubation.

Interestingly, N-9, N-9/PVP, HMW, and MMW had comparable activity on the progressive motility of the evaluated sperm. At both active concentrations, the addition of the spermicide seemed to interfere quickly with the linear progression of the sperm and/or immobilizing the sperm and eventually killing the sperm. In contrast, the LMW did not alter the linearity of the progressive motility but, rather, reduced it. Similarly, the activity of the spermicide was faster at the higher concentration.

It is important to note that PVP (1%, w/v, in buffer) did not affect the motility or progressive motility of the sperm. Spermatozoa kept in buffer only (absolute control) or in buffer containing PVP remained viable and quite motile throughout the duration of the study.

CONCLUSION

In conclusion, the *in vitro* spermicidal activity of various molecular N-9 fractions was compared to that of N-9, with rabbit spermatozoa. Nonoxynol-9 and the N-9 fractions were formulated with PVP and evaluated at various concentrations. The data showed that the MMW fraction was at least as spermicidal as the parent N-9 mixture. Both seem to have similar effects on the progressive motility, the MMW fraction being more efficient in decreasing the motility of the rabbit spermatozoa. The HMW fraction had similar effects on the progressive motility yet was not as effective as N-9 in decreasing the sperm motility. The LMW fraction was the least effective spermicide at all the concentrations tested.

When N-9 was coprecipitated with PVP, its spermicidal activity was enhanced. The motility and progressive motility of sperm cells decreased more rapidly when exposed to N-9/PVP than to N-9 alone at all concentrations tested. While PVP had no inherent sperm toxicity, the formation of a N-9/PVP complex seemed to produce a synergistic response which caused a more rapid damage to the sperm than any of the two materials alone.

Table II. Progressive Motility[a] of Rabbit Spermatozoa After Exposure to N-9 and Equal Molar Concentrations of N-9 Fractions (LMW, MMW, HMW): The Final Concentration of Spermatozoa Was 20×10^6/ml

Time (min)[c]	1% PVP control	N-9 fraction[b]			N-9	NVP (PVP)
		LMW[d]	MMW[e]	HMW[f]		
			At 31.25 μg/ml			
0	3.8 ± 0.2	3.4 ± 0.1	2.3 ± 0.2	1.9 ± 0.3	2.1 ± 0.1	2.5 ± 0.0
15	3.9 ± 0.1	3.4 ± 0.3	2.1 ± 0.3	2.0 ± 0.0	2.1 ± 0.3	2.6 ± 0.3
30	3.7 ± 0.2	3.2 ± 0.2	2.3 ± 0.2	2.1 ± 0.1	2.1 ± 0.1	3.0 ± 0.5
45	3.8 ± 0.2	3.1 ± 0.4	2.3 ± 0.2	2.3 ± 0.2	2.3 ± 0.1	3.2 ± 0.6
60	3.7 ± 0.2	3.1 ± 0.1	2.4 ± 0.3	2.3 ± 0.4	2.4 ± 0.1	3.2 ± 0.6
			At 62.5 μg/ml			
0	3.7 ± 0.1	3.0 ± 0.4	1.6 ± 0.3	1.3 ± 0.3	1.3 ± 0.4	1.5 ± 0.7
15	3.6 ± 0.2	3.0 ± 0.4	1.0 ± 0.5	1.3 ± 0.5	0.1 ± 0.2	0.5 ± 0.9
30	3.7 ± 0.0	2.9 ± 0.2	0.5 ± 0.5	1.0 ± 0.0	0.9 ± 0.4	0.3 ± 0.6
45	3.7 ± 0.1	2.7 ± 0.4	0.3 ± 0.5	0.5 ± 0.5	0.4 ± 0.5	0
60	3.7 ± 0.1	2.4 ± 0.6	0.1 ± 0.4	0.1 ± 0.4	0	0
			At 83.3 μg/ml			
0	3.7 ± 0.1	3.1 ± 0.1	1.3 ± 0.3	0.8 ± 0.6	1.1 ± 0.3	0.7 ± 0.6
15	3.5 ± 0.0	2.7 ± 0.4	0.5 ± 0.6	0	0	0
30	3.6 ± 0.1	2.2 ± 0.6	0	0	0	0
45	3.6 ± 0.1	2.3 ± 0.3	0	0	0	0
60	3.4 ± 0.1	1.5 ± 1.1	0	0	0	0

[a] The progressive motility was determined by the linear progression and drive of the sperm (scale 0–4). Values are the average of four determinations ± SD.
[b] All N-9 fractions (LMW, MMW, HMW) were formulated in PVP coprecipitates.
[c] Time after addition of spermicide to sperm cells.
[d] Low molecular weight N-9 fraction isolated by HPLC (n = 1–4).
[e] Middle molecular weight N-9 fraction isolated by HPLC (n = 6–8).
[f] High molecular weight N-9 fraction isolated by HPLC (n = 11–13).

Evidently, the low molecular weight nonoxynols do not contribute significantly to the pharmacological activity of the surfactant N-9. Hence, in view of their suspected toxicity, their removal from the spermicide (either by purification or by controlled synthesis) may lead to a safer product. Alternatively, N-9 could be formulated with PVP to give a more potent spermicidal agent than N-9. The N-9/PVP complex would be more hydrophilic, thus reducing even further its vaginal absorption into the systemic circulation.

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