A Simple, Sensitive Assay for the Spermicide Nonoxynol-9 in Biological Fluids by High-Performance Liquid Chromatography

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Abstract □ A new high-performance liquid chromatographic technique has been developed to quantitate nonoxynol-9 in serum, urine, and vaginal fluid. The method is rapid, involves minimal sample preparation, and can be used to analyze a large number of biological fluid samples. The assay elutes a single nonoxynol-9 peak with no interfering components. This was accomplished using a 10-μm pelicular packed amine column, a normal-phase solvent system, and fluorescence detection. Nonoxynol-9 levels as low as 0.23 μg/mL in urine can be detected.

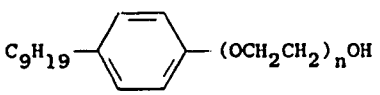
Nonoxynol-9 (N-9), a nonionic surfactant, has been the spermicide of choice in vaginal contraceptives for over 20 years. While N-9 is generally considered to be safe and effective, absorption through mucous membranes has never been directly quantitated.[1,2] To investigate potential absorption of N-9 from a vaginal polyurethane sponge, a method was needed to quantitate microgram concentrations of N-9 per milliliter of fluid.

Nonoxynol-9 is an alkylphenolethoxylate with the general formula $C_9H_{19}C_6H_4(OCH_2CH_2)_nOH$ (see structure). The "n" denotes the average number of ethylene oxide units (n = 9). No practical method to quantitate N-9 in biological fluids has been available. The best methods published to date employ HPLC to separate each ethoxymer, and then summation of the peak areas.[3-6] These methods are time consuming (30 to 60 min per sample) and they are not very sensitive. Methods using reversed-phase solvent systems to obtain a single peak result in erratic recoveries as high as ±20%, even in solutions containing high levels of N-9. To successfully quantitate a large number of biological samples, a method must be relatively simple and highly sensitive. To achieve this goal, a method was developed using normal-phase HPLC and fluorescence detection for analysis of N-9 in serum, urine, and vaginal fluid.

Experimental Section

Materials and Reagents—Nonoxynol-9 (Igepal CO-630) was purchased from GAF (Linden, NJ). Water was deionized and distilled in the laboratory. All solvents were HPLC grade.

Biological Fluid Samples—Blood was collected from volunteers in 10-mL Vaccutainer tubes (Catalog #6430; Becton and Dickinson, Rutherford, NJ), allowed to coagulate, and was refrigerated until assayed. The tubes did not contain anticoagulants or other modifiers. Urine samples were collected and refrigerated. Swabs for collection of vaginal fluids were Puritan brand cotton-tipped, with three-inch wood shafts. Vaginal fluid was collected by swabbing the walls of the vagina with pre-weighed swabs.



Nonoxynol-9

High-Performance Liquid Chromatography Apparatus—The HPLC system employed consisted of a gradient controller (model 680; Waters Associates, Cambridge, MA), two high-pressure pumps (model 510; Waters), an automated sampler (model WISP 710B; Waters), a fluorescence detector (Shimadzu, model RF530; Cole Scientific, Calabasas, CA), and an integrator (model 3390A; Hewlett-Packard, Avondale, PA). The fluorescence detector settings for excitation and emission wavelengths were 275 and 575 nm, respectively. The integrator received a 1-V full-scale signal from the detector; integrator attenuation was set at 2^2.

Column and Mobile Phase—The HPLC column was a R-SIL-amine (NH_2) column with 10-μm irregularly shaped packing material (Catalog #8586; Alltech Associates, Deerfield, IL). Column dimensions were 4.6 mm × 25.0 cm length. An HPLC prefilter (Catalog #28689; Alltech), with a 2-μm frit was fitted to the column inlet. The flow rate of the mobile phase, 95% tetrahydrofuran (THF) and 5% acetonitrile, was set at 1.0 mL/min. The sample injection volume was 20 μL.

Calibration Standards—Standards were diluted from a 1000-μg/mL N-9 standard in THF. The calibration standard concentration was 5.00 μg/mL. Since the fluorescence signal intensity and peak symmetry is influenced by different solvents, solutions to dilute the 1000-μg/mL N-9 standard were designed to reflect as nearly as possible their respective sample solutions based on sample preparation. The diluent was 100% distilled deionized water for the urine standard, 75% THF:25% distilled deionized water for the serum standard, and 100% acetonitrile for the vaginal fluid standard.

Procedure—Prior to use, the urine samples were brought to ambient temperature and mixed well. Four different amounts of N-9 were spiked into known volumes of each urine sample and mixed. Five-milliliter aliquots were transferred into centrifuge tubes and spun at 4000 rpm for 5 min.

Blood samples were removed from the refrigerator and allowed to reach room temperature. They were then mixed and centrifuged at 4000 rpm for 5 min. The serum was separated and an aliquot was spiked with a known amount of N-9. One to two serial dilutions of the spiked serum were performed using the same unspiked serum sample. Four concentrations of N-9 in serum were generated from the serial dilutions for each sample. The aliquots of serum were transferred to a centrifuge tube and combined with an equal volume of THF. This sample mixture was centrifuged at 4000 rpm for 5 min. An aliquot of the supernate was transferred to a second centrifuge tube, mixed with an equal volume of THF, and centrifuged as before. A volume of the final supernate was used for analysis.

Swabs used to collect vaginal fluids were placed in centrifuge tubes and weighed. The swabs were then spiked with 100-μL aliquots of different concentrations of N-9 in distilled deionized water. Samples were processed by adding 1.0 mL of acetonitrile to each tube, vortexing, and allowing to stand at ambient temperature for 15 min. Each tube was weighed, then vortexed once more. The swabs were removed and the tube was centrifuged at 4000 rpm for 5 min. An aliquot of the supernate was used for analysis.

Results and Discussion

The use of a fluorescence detection system eliminated interfering components and allowed simple sample preparation and short assay times. Initially, attempts were made to monitor N-9 in biological samples with UV-vis spectropho-

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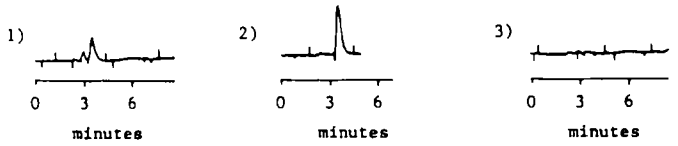

Figure 1—Typical chromatograms in blood serum: (1) sample spiked with N-9 (10.00 μg/mL); (2) N-9 standard (5.00 μg/mL); (3) sample blank.

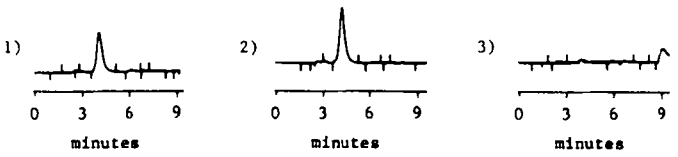

Figure 2—Typical chromatograms in urine: (1) sample spiked with N-9 (4.76 μg/mL); (2) N-9 standard (6.56 μg/mL); (3) sample blank.

tometry. The most common λ_{max} used for the detection of N-9 is 276 nm, which is also a common wavelength for many biological compounds. Nonoxyl-9 could not be separated from these interfering compounds at this wavelength. Extensive sample clean up would have been necessary, and this would sacrifice both the simplicity of the method and sensitivity, due to reduced recoveries. Due to the simple sample preparation required, not all particulates were eliminated during sample preparation; this led to frequent column inlet clogging. Attempts to filter samples through several types of filter papers led to adsorption of N-9 and reduced recoveries. Ultimately, the problem was solved by installing an HPLC column inlet prefilter containing an easily replaceable 2-μm frit. The frit needed to be changed approximately every 40 h of run time.

Using fluorescence detection, a linear regression analysis of a series of N-9 standards in water containing 0, 0.66, 1.31, 2.62, 6.56, 15.74, and 65.60 μg/mL against integrator area

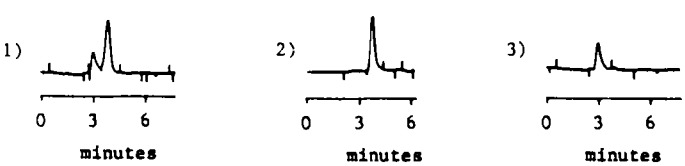

Figure 3—Typical chromatograms in vaginal fluid: (1) vaginal swab spiked with N-9 (5.57 μg/g); (2) N-9 standard (4.80 μg/g); (3) sample blank.

counts results in a correlation coefficient of 0.9996 (n = 13, y intercept = −3678, slope = 31 149). Chromatograms of serum, urine, and vaginal fluid spiked with N-9 are shown in Figures 1, 2, and 3, respectively. Also included here are chromatograms of respective N-9 standards and control samples without added N-9. An extraneous peak appears intermittently in chromatograms, eluting just prior to N-9. The peak did not interfere with quantitation under any circumstances and was not identified.

Column-to-column reproducibility was tested over the course of methods development. Several Alltech R-SIL-amine columns were used. All produced quantifiable results and endured >100 sample injections. Two columns required minor adjustments of the tetrahydrofuran concentration in the mobile phase to maintain N-9 retention time between 3.5 and 5 min.

The results for biological fluid samples containing a known amount of N-9 are presented in Table I. The percent mean recovery of N-9 in serum was 106%. Similarly, urine and vaginal fluid recoveries were 95 and 102%, respectively. The relative standard deviation for recoveries was 6.9 for serum, 7.5 for urine, and 8.1 for vaginal fluids. These results indicate that this method is sufficiently quantitative to determine the concentration of N-9 in biological samples at very low levels.

The detection limits were established for each sample type. The limits are based on the equation $3S_m/m$, where S_m is the

Table I—Recovery Data for Nonoxynol-9 in Biological Fluids[a]

Blood Serum				Urine				Vaginal Fluid			
Actual Concentration of Nonoxynol-9, μg/mL	Percent Recovery	Mean	±SD	Actual Concentration of Nonoxynol-9, μg/mL	Percent Recovery	Mean	±SD	Actual Concentration of Nonoxynol-9, μg/mL	Percent Recovery	Mean	±SD
0	ND			0	ND			0	ND		
0	ND			0	ND			0	ND		
0	ND			0	ND			0	ND		
0	ND	—	—	0	ND	—	—	0	ND	—	—
2.50	118			2.44	105			1.17	118		
2.50	97			2.44	100			1.21	123		
2.50	91			2.38	102			1.20	101		
		102	14	2.38	102	102	2			114	2
5.00	108			4.88	84			5.59	99		
5.00	106			4.88	79			5.60	107		
5.00	115			4.76	86			5.57	99		
5.00	106	109	4	4.76	91	85	5	5.49	98	101	4
10.00	106			9.76	102			12.43	95		
10.00	100			9.76	102			12.33	98		
10.00	114			9.52	100			12.47	96		
10.00	109	107	6	9.52	98	100	2	12.05	95	96	1
20.00	109			12.20	92			24.64	101		
20.00	102			12.20	94			24.45	101		
20.00	106			11.90	96			24.38	101		
20.00	105	106	3	11.90	91	93	2	23.90	101	101	0

[a] ND = None detected; detection limits: blood serum, <1.01 μg/mL; urine, <0.23 μg/mL; vaginal fluid, <0.46 μg.

standard deviation of the lowest spiked concentrations, and m is the slope of the spiked calibration curve.[7] The detection limits were 1.01 μg/mL for serum, 0.23 μg/mL for urine, and 0.46 μg for vaginal fluid. (The detection limit in vaginal fluids was reported in micrograms since the amount of fluid absorbed on a swab generally ranges from 2 to 55 mg; this value can easily be converted to micrograms per gram of vaginal fluid as needed.)

Over 400 N-9 assays were conducted on blood serum, urine, and vaginal fluid samples as an auxiliary study during clinical trials for a novel intravaginal drug delivery product. No interfering components were observed. Furthermore, there are no known N-9 degradation products.

In summary, a simple, sensitive, single-peak quantitative method has been developed for the analysis of N-9 in biological fluids to a level as low as 0.23 μg/mL. The method may also be readily adapted for other applications in the pharmaceutical and cosmetic industries.

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