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Journal of Chromatography B, 677 (1996) 204–208

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Short communication

Determination of the spermicide nonoxynol-9 in vaginal lavage by high-performance liquid chromatography

Jason L. McPherson[a,b], James H. Nichols[a,c], Patricia Barditch-Crovo[a,b], Fayez M. Hamzeh[a,b,*]

[a]*The Division of Clinical Pharmacology, The Johns Hopkins University School of Medicine, Baltimore, MD 21287-5554, USA*
[b]*Department of Medicine, The Johns Hopkins University School of Medicine, Baltimore, MD 21287-5554, USA*
[c]*Department of Pathology, The Johns Hopkins University School of Medicine, Baltimore, MD 21287-5554, USA*

Received 28 April 1995; revised 14 September 1995; accepted 25 September 1995

Abstract

A sensitive normal-phase high-performance liquid chromatographic method using a bonded-phase aminosilica column has been developed for the measurement of the spermicide nonoxynol-9 in vaginal lavage fluid. The mean multiple correlation coefficient (r^2) for nonoxynol-9 was 0.999 over the calibration range 3.125–50 μg/ml for the standards. Quality control samples measured at two different concentration levels gave intra-day precision values (coefficient of variation, C.V.) in the range of 0.61 to 1.63% and the intra-day accuracy values (mean relative error, M.R.E.) in the range of 0.13–0.62%. Inter-day precision and accuracy values from five different calibration standard concentration values ranged from 2.25 to 5.09% C.V. and 4.02 to 7.56% M.R.E. Nonoxynol-9 samples examined for peak area stability at room temperature over a 24-h time period had a M.R.E. of 14.9%. Quality control samples stored at −70°C, and tested after one month by comparison to baseline samples, had a M.R.E. of −10% and −7.53% for the low and high quality control samples, respectively. The method is sensitive and simple, with short runtimes, to enable the processing of numerous samples from a clinical trial.

Keywords: Nonoxynol-9

1. Introduction

Nonoxynol-9 (N-9) is a surfactant which was approved by the FDA in 1980 as a safe and effective over-the-counter contraceptive agent [1]. N-9 (Fig. 1) is a nonionic polyethoxylated alkylphenol liquid mixture corresponding to the general formula: $C_9H_{19}C_6H_4(OCH_2CH_2)_nOH$, in which the average number of n is about 9. The mixture contains not less than 90.0% and not more than 110% of N-9. In vitro, N-9 has been shown to contain both antibacterial and

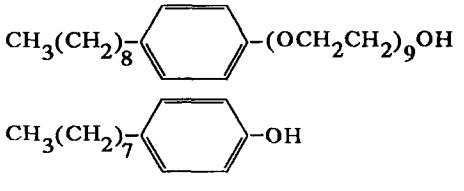

Fig. 1. Representative molecular structures of nonoxynol-9 (top) and the I.S., 4-octylphenol (bottom).

*Corresponding author. Address for correspondence: The Johns Hopkins Hospital, Osler 527, Division of Clinical Pharmacology, 600 North Wolfe St., Baltimore, MD 21287-5554, USA.

0378-4347/96/$15.00
SSDI 0378-4347(95)00413-0

antiviral activity, including HIV [2–6]. It has also been reported that N-9, when used in conjunction with physical barrier methods, may reduce the transmission of HIV in vivo [7]. However, questions remain unanswered concerning the effectiveness of N-9 in the prevention of sexually transmitted disease transmission, especially as related to dose dosage regimen and adequate drug exposure [3].

There have been several published methods to quantitate the family of polyethoxylated alkylphenols [8–10]. These methods used gradient elution to separate components of a polyethoxylated alkylphenol liquid mixture (i.e. $n=1-17$). Although these methods achieve good separation total quantitation of all components requires summation of individual peak areas which multiplies analytical imprecisions. Furthermore, these methods have long runtimes, ranging from 40 to 100 min [9,10]. These assays are thus unsuitable for processing the large number of samples that might be generated from clinical trial of N-9.

This paper describes the development and validation of a N-9 assay when N-9 is contained in vaginal lavage fluid. The assay is logistically simple so that a large number of samples could be processed with minimal sample preparation. This method, unlike previous methods [8–13], employs an internal standard, which enhances the assay versatility, precision and accuracy.

2. Experimental

2.1. Chemicals

N-9 was a gift from Rhône-Poulenc (Cranbury, NJ, USA) and 4-octylphenol, 99% purity (internal standard) was obtained from Aldrich (Milwaukee, WI, USA). HPLC grade water, acetonitrile (ACN), and tetrahydrofuran (THF) were obtained from Baxter Scientific Products (Columbia, MA, USA).

2.2. HPLC conditions

The HPLC system consists of an automated sampler (Hitachi 655A-40 Autosampler), a high pressure pump (Hitachi L-6200 Intelligent pump), and a fluorescence spectrophotometer (Hitachi Model F-1050). Separation was achieved on an aminosilica-based column with 10-μm irregularly shaped packing material (Alltech R-SIL-Amine (NH_2) column, 25 cm×4.6 mm I.D.) (Deerfield, IL, USA). An HPLC inline 2-μm prefilter (Anspec SS Frit, Ann Arbor, MI, USA) was placed prior to the analytical column and changed before each analytical run. Data integration was performed on a Waters Millipore Millennium Chromatography Manager, software version 2.00.

Mobile phase consisted of THF–ACN (98:2 v/v) at a flow-rate of 1.0 ml/min. The mobile phase was filtered through a 0.22-μm Nylon-66 membrane filter (Micron Separations from Supelco, Bellefonte, PA, USA, Catalog No. E02W04700) and vacuum degassed for 2 min.

Separate fluorescence scans of N-9 and the I.S. were evaluated in the sample matrix and in mobile phase to find the optimum excitation and emission wavelengths for the assay. Excitation and emission wavelengths chosen for the fluorescence detection were 227 and 612 nm respectively.

2.3. Calibration, quality control samples, and internal standard

Solutions used to dilute the N-9 calibration and quality control samples were chosen to represent the vaginal lavage fluid, which was 100% deionized (DI) water. QC and calibrators were prepared in lavage fluid and DI water and injected for comparison. There were no differences seen when QC and calibrators were prepared in lavage fluid or DI water. Therefore the matrix effect was null and the assay was validated with the QC and calibrators prepared in DI water.

The N-9 calibrators and quality control (QC) samples were prepared separately by dilution from a 1000 μg/ml N-9 solution in DI water. Calibration concentrations for construction of a calibration curve were 3.125, 6.25, 12.5, 25, and 50 μg/ml. The low and high quality control sample concentrations were 12.54 and 25.4 μg/ml in deionized water. Excess aliquots of the calibrators and QC samples were stored in polyethylene tubes at −70°C until usage. The QC samples for validation purposes were from one batch that was frozen (at −70°C) and used

throughout the validation period. Calibration samples represent several separate weighings.

Internal standard working solutions were prepared in a similar fashion to the N-9 calibrators and QC samples. A 100 μg/ml internal standard stock solution was weighed daily in mobile phase. Working solutions of the internal standard (25 μg/ml) were diluted from stock standard in mobile phase.

2.4. Sample preparation

Prior to analysis, the calibrators, QC samples, and subject specimens were brought to room temperature (25°C) and mixed well. A 0.5-ml aliquot of each sample was added to a disposable 12×75 mm borosilicate glass tube. A 0.5-ml volume of internal standard working solution was added, the tubes were vortexed briefly and 20 μl injected onto the column.

2.5. Quantitation

Linear least squares fit calibration curves for N-9 in DI water were obtained by plotting the peak-area ratios (N-9/I.S.) after analysis versus their corresponding added concentrations.

2.6. Validation design

Specificity

The specificity of the assay was demonstrated by showing the resolution of N-9, and 4-octylphenol (internal standard) from endogenous peaks.

Linearity

Linearity was measured by analyzing calibration curves run on five different days. Calibration curves consisted of five different calibration concentrations, a zero calibrator, and a blank, all run in duplicate. Separate weighings of analytical standards for calibration curves and quality control samples were performed. The linearity (r^2 correlation coefficient) of the calibration curve was determined by the linear least squares regression analysis of the calibration standards curve for both the front and back curves. Acceptance criteria of multiple correlation coefficient of each calibration curve was ≥0.980.

Intra-day and inter-day precision and accuracy

The Intra-day (within day) precision and accuracy was measured by analyzing one set of QC samples in the same run (with $n=4$ low and 4 high). The inter-day precision and accuracy (different days) values were determined from analysis of low and high QC samples and a replicate calibration curve over five days. To calculate the inter- and intra-day precision and accuracy of the method the following formulae were used: precision was defined as the coefficient of variation (C.V. %) which is the standard deviation divided by the mean multiplied by 100; accuracy (or bias %) is defined as the difference between the mean concentration and the spiked concentration divided by the spiked concentration.

Limit of detection and quantification

The limit of detection for N-9 was determined statistically from the zero calibrator over five runs. The limit of quantification was the lowest calibration standard with a C.V.% and M.R.E.% ≤10%.

Benchtop stability of processed analyte

Samples were examined for stability at room temperature over a 24 -time period. Stability of N-9 and internal standard were evaluated separately. Peak areas of the zero and 24-h-old specimen injections were compared to the same calibration curve.

Freezer storage stability

Freezer stability was validated using six sets of four low and four high QC samples. Samples were stored at −70°C and tested at 0 h, 24 h, 48 h, and 1 week. Analyte concentrations at each time point after freezing were compared to the 0-h samples. Quadruplicate QC samples were analyzed at each time point after freezing. Long term stability was preformed on two sets of 4 QC samples at intervals of 1 and 4 months.

3. Results and discussion

3.1. Chromatography

Fig. 2, shows representative chromatogram of a blank vaginal lavage sample, calibration standard (25.0 μg/ml I.S. and 12.50 μg/ml N-9), and a study

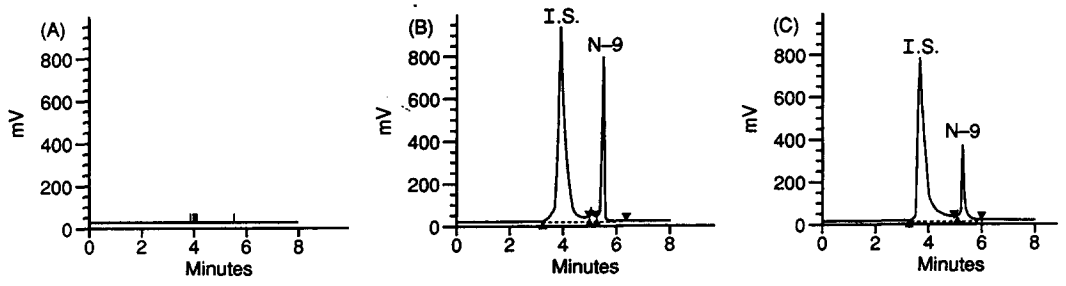

Fig. 2. Representative chromatograms of (A) blank vaginal lavage (DI water) sample; (B) D.I. water spiked with internal standard (I.S.) and 12.50 μg/ml of nonoxyonol-9 (N-9); (C) subject sample containing 7.93 μg/ml of N-9. The I.S. elutes at 4.0 min and N-9 elutes at 5.2 min.

subject sample (25.0 μg/ml I.S. and 7.93 μg/ml N-9). Retention times for the I.S. 4-octylphenol and N-9 were 4.0 and 5.7 min, respectively. The retention times of the analytes were very consistent with drift less then ±0.05 min over the 5-day validation period. The excitation and emission wavelengths were selected to optimize peak intensity between the two analytes.

3.2. Validation

Specificity

Chromatogram A of Fig. 2, shows that there are no interferences found in the retention time windows of N-9 or the I.S.. Therefore, our chromatographic method is capable of resolving N-9 and the I.S. from any interferences.

Linearity

The calibration curve was linear at the concentrations ranging between 3.125 to 50 μg/ml. A typical equation of the line $y=0.00962x+0.01528$ (with y=response and x=concentration). Linearity was assessed from the multiple correlation coefficient (r^2) of a nonweighted linear least squares regression analysis. The mean of the correlation coefficient between days is 0.999 with $n=5$. The average slope value was 0.00962±0.0004 ($n=5$).

Intra-day and inter-day precision and accuracy

The intra-day and inter-day precision and accuracy values of the method are summarized in Table 1. The intra-day precision values for the low and high QC sample had C.V.% of 0.61% and 1.63%, respectively. The intra-day accuracy values for the low and high QC samples had M.R.E.% of 0.13% and 0.62%, respectively. Inter-day precision values for the low and high QC sample ($n=5$) ranged from 5.44% to 5.54% (C.V.%) and the inter-day accuracy values had a M.R.E.% range of 0.07% to 0.04%.

The inter-day precision and accuracy values for replicate standards over the 3.125–50 μg/ml range are summarized in Table 2. These values were determined by back-calculation of concentrations from respective calibration curves ($n=5$). The inter-day precision values range from 4.02% to 7.56% (C.V.%) with inter-day accuracy M.R.E.% values between 2.35% to −5.09%.

Table 1
Intra-day and inter-day precision and accuracy data for N-9 quality control samples

	Concentration (μg/ml)	
	15.4 μg/ml	25.41 μg/ml
Intra-day (n=4)		
Measured (mean±S.D.)	15.42±1.12	25.57±0.37
Precision (C.V., %)	0.61	1.63
Accuracy (M.R.E., %)	0.13	0.62
Inter-day		
1	14.01	23.78
2	16.57	23.82
3	15.41	26.92
4	15.91	26.96
5	15.42	25.57
Mean±S.D.	15.5±0.84	5.41±1.41
Precision (C.V., %)	5.44	5.54
Accuracy (M.R.E., %)	0.07	0.04

Table 2
Inter-day precision and accuracy data of calibration curves

Standard concentration (μg/ml)	Measured concentration mean (μg/ml)	Accuracy (C.V., %)	Precision (M.R.E., %)
3.125	3.07 ± 0.14	4.50	−1.65
6.250	6.30 ± 0.48	7.56	0.87
12.50	13.14 ± 0.54	4.08	5.09
25.00	25.50 ± 1.36	5.33	2.01
50.00	48.87 ± 1.97	4.02	−2.25

Note: These values were determined by back-calculation of concentrations from respective calibration curves (n=5).

Limit of detection and quantitation

The limit of detection was 0.48 μg/ml, where the N-9 peak is three times higher than the noise level. The limit of quantitation was 3.125 μg/ml. At this concentration level, the precision (C.V.%) and M.R.E.%, values were 4.5%, and −1.65%, respectively.

Benchtop stability of the analyte

Over a 24-h period (at 25°C) the peak area of N-9 (in mobile phase) had a M.R.E.% of 14.9% when compared to the 0-h samples. The peak area of the I.S. over the same period had a M.R.E.% of −0.6%. Assays were, therefore, not run longer than 12 h to ensure analytical accuracy. With the short run times of this method, this should not become a problem.

Sample storage stability

Aliquots of quality control samples were stored at −70°C for the duration of the validation (1 month) without significant breakdown. The M.R.E.% of the samples stored for 1 month at −70°C were −10% and −7.53% for the low and high QC respectively.

4. Conclusions

The described method was developed and validated to quantitate N-9 in vaginal lavage fluid. Comparable methods that quantitate N-9 in vaginal fluids lack the simplicity, precision and accuracy, of this method [12]. This new assay is logistically simple so that a large number of samples can be analyzed in a short time frame. Our assay is performed without an extraction step and, therefore, requires minimal sample preparation. This assay will be useful in determining the relationship of N-9 pharmacokinetics to its clinical uses for the prevention of pregnancy and perhaps, sexually transmitted diseases.

Acknowledgments

This research was supported by the Department of Health and Human Services, Food and Drug Administration contract number 223-94-3002. I would also like to thank Mr. Ebenezer Asafu-Adjaye for his technical help with the numerous fluorescence scans needed for this project.

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