Influence of Long-term Stability Conditions on Microbicidal Nucleoside Prodrug (WHI-07)-loaded Gel-Microemulsion

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ABSTRACT

The objective of this study was to evaluate the long-term stability of the antiretroviral spermicide WHI-07 (5-bromo-6-methoxy-5,6-dihydro-3′-azidothymidine-5′-(p-bromophenyl)-methoxyalaninyl phosphate) in a polymer-based microemulsion. The recovery and stability of WHI-07 in gel-microemulsion was examined by a validated high-performance liquid chromatography (HPLC) method. The stability was examined over a period of 24 weeks at 3 controlled temperatures (4ºC, 25ºC, and 40ºC). The recovery of the prodrug from 0.5% to 2.0% WHI-07-loaded gel-microemulsion was 99.8%. HPLC analysis revealed that a 2% WHI-07-loaded gel-microemulsion stored at room temperature and cold temperatures for 24 weeks retained >90% of the prodrug, whereas those stored at 40ºC maintained 90% of initial WHI-07 for at least 10 weeks. The observed stability of WHI-07 in gel-microemulsion is of great importance for its widespread utility in various climatological conditions.

KEYWORDS: Gel-microemulsion, prodrug, stability, WHI-07.

INTRODUCTION

Newly acquired human immunodeficiency virus type 1 (HIV-1) infections are largely the result of heterosexual transmission.[1,2] Women are vulnerable to heterosexual transmission of HIV-1 owing to substantial mucosal exposure to seminal leukocytes, the predominant HIV-1-infected cell types in semen.[3-6] Consequently, the development of safe, efficacious vaginal microbicides capable preventing the transmission of HIV-1 from infected leukocytes via semen is of paramount importance in the fight against the spread of sexually transmitted AIDS.[7-9]

WHI-07 (5-bromo-6-methoxy-5,6-dihydro-3′-azidothymidine-5′-(p-bromophenyl)-methoxyalaninyl phosphate), a novel aryl phosphate derivative of zidovudine (ZDV) is an anti-HIV-1 prodrug with in vivo microbicidal and contraceptive properties.[10-14] WHI-07 was rationally designed to bypass the kinase activation step, especially in thymidine kinase deficient or lacking monocytes/macrophages, the main carriers of HIV-1 in semen and female genital tract secretions.[5,6,15] WHI-07 displays selective cytotoxicity to leukocytes with a high selectivity index against genital tract epithelial cells.[16-18] Furthermore, the primary metabolite of WHI-07 identified as 3′-azidothymidine-5′-(p-bromophenyl)-methoxyalaninyl phosphate (Ala-Me-ZDV-BPP; Figure 1) exhibits broad-spectrum anti-HIV-1 activity against drug-resistant strains. The selective spermicidal and leukotoxic properties of WHI-07 are useful for its utility as a prophylactic contraceptive to prevent genital transmission of HIV-1.

WHI-07 was formulated in an oil-in-water (o/w) microemulsion-based system as a vaginal drug delivery vehicle. Microemulsions appear to have the ability to deliver larger amounts of topically applied agents into the mucosa than the traditional gels or creams.[19-21] Polymer-based WHI-07 gel-microemulsion offers several benefits for vaginal delivery, including increased solubility, protection from enzymatic hydrolysis, increased bioavailability for prolonged contraceptive and antiretroviral effects, as well as decreased toxicity. In animal toxicity studies performed in mice, rabbits, cats, and/or pigs, intravaginal administration of gel-microemulsion with or without 2.0% WHI-07 was not associated with any mucosal, systemic, developmental, and/or reproductive toxicity.[22-28] Consequently, the shelf-life/stability of WHI-07-loaded gel-microemulsion at 3 controlled temperatures (4ºC, 25ºC, and 40°C) was determined using a validated high-performance liquid chromatography (HPLC) method for up to 24 weeks (6 months).

MATERIALS AND METHODS

Materials

Captex 300 (medium chain triglyceride) was obtained from ABITEC Corp, (Janesville, WI). Cremophor EL (polyethoxylated castor oil) was from BASF Corp (Mount Olive, NJ); polyethylene glycol (PEG-200) (humectant) was from Union Carbide Corp (Danbury, CT); Phospholipon 90G (purified

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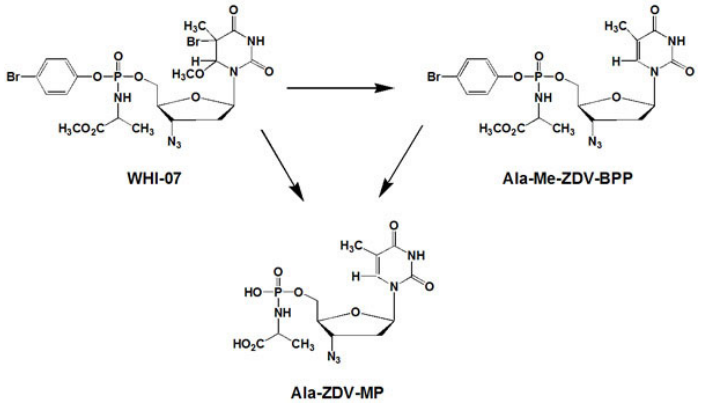

Figure 1. Chemical structure of WHI-07 and its major metabolites identified by analytical HPLC and ^{1}H-NMR spectrum.

soya lecithin) was from American Lecithin Co (Danbury, CT); and propylene glycol was obtained from Spectrum Quality Products Inc (New Brunswick, NJ). SeaSpen PF and Viscarin GP-209 carrageenans (natural polymers) were obtained from FMC Corp (Newark, DE). Rhodigel (xanthan gum) was from Rhodia Food Ingredients (Cranbury, NJ). All other chemicals and solvents (HPLC grade) were used as received. Deionized water used as diluent was purified using Millipore Milli-Q System (Bedford, MA).

Synthesis of WHI-07

Large-scale synthesis of WHI-07 employed a 3-step strategy as outlined in Figure 2. In brief, the reaction was performed at <5ºC with a solution of p-bromophenol, phosphorous oxychloride, and dichloromethane in the presence of triethylamine to yield its phosphorochloridate derivative (1). The reaction product was purified by vacuum distillation and condensed with L-alanine methyl ester HCl at low temperature in methylene chloride to furnish the precursor (2). This precursor was used to functionalize ZDV (3). The resulting aryl phosphoramidate derivative of ZDV (4) obtained in the organic phase was purified by column chromatography using 1:1 ethyl acetate-hexanes as the eluent on a Biotage 75-L silica gel column (Biotage, Charlottesville, VA). The phosphate ester (4) was further modified by treatment with bromine in anhydrous methanol to obtain the target aryl phosphate derivative of ZDV (5) with 5-bromo and 6-methoxy substitutions on the thymine ring (ie, WHI-07 [5-bromo-6-methoxy-5,6-dihydro-3′-azidothymidine-5′-(p-bromophenyl) methoxyalaninyl phosphate]. The prodrug formed was purified by chromatography using ethyl acetate-hexanes (2:1). Purity of WHI-07 was assessed by the proton (^{1}H), carbon (^{13}C), phosphorus (^{31}P) nuclear magnetic resonance (NMR) spectra, liquid chromatography-mass spectrometry (LC-MS) data, and by HPLC.

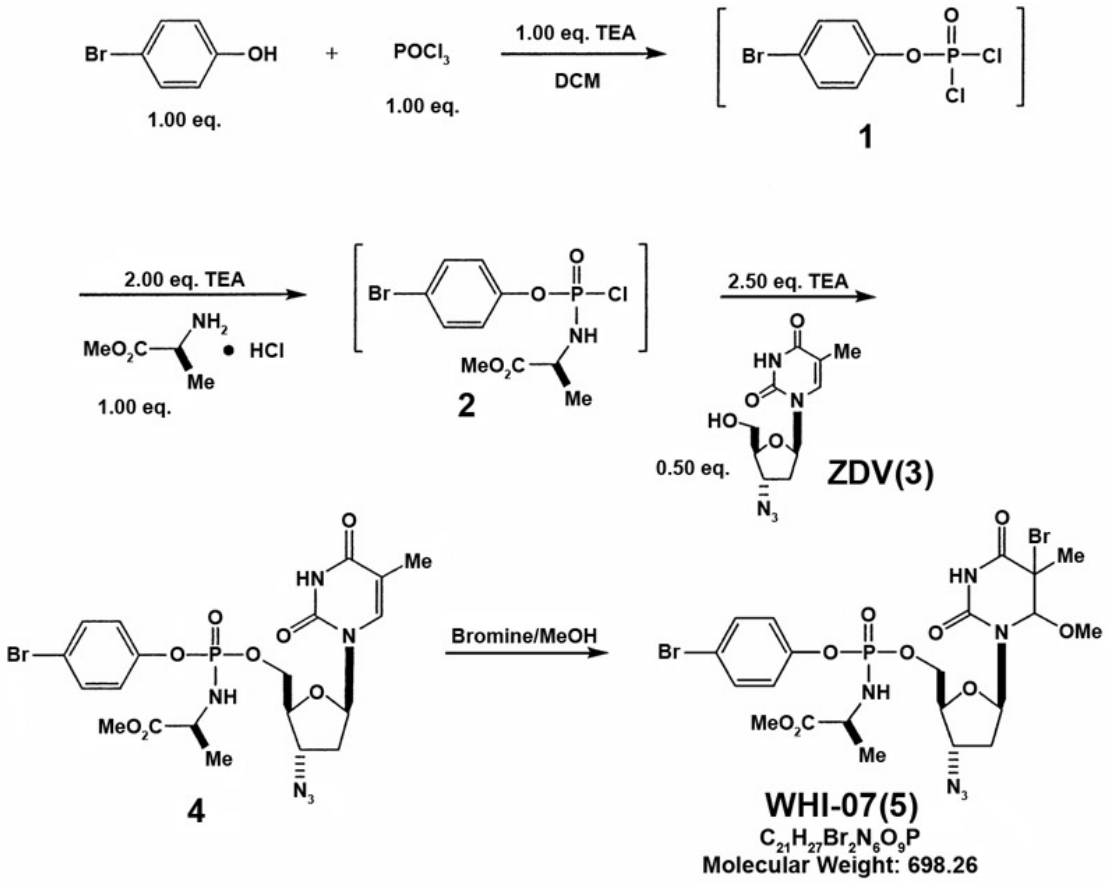

Figure 2. Large-scale synthetic scheme used for WHI-07. TEA indicates triethylamine; DCM, dichloromethane; ZDV, zidovudine.

Partition Coefficient of WHI-07

In brief, quadruplicate samples of WHI-07 were dissolved in 5 mL each of octanol (10-15 mg/mL) and diluted with 5 mL of deionized water; after vigorous mixing for 10 minutes, the mixture was left to stand overnight to allow the compound to equilibrate into the 2 phases. Aliquots from the bottom aqueous layer were carefully removed using an air-filled syringe. Aliquots of the recovered water and octanol fractions were directly injected into the HPLC column for analysis. Peak areas (mAU*s) of WHI-07 in the octanol and water layers were determined, and the peak area ratios were used to represent the partition coefficient (Log K_D) of WHI-07.

HPLC Analysis

For long-term stability studies of WHI-07, a previously validated HPLC method, optimized for single as well as combination microbicides in gel-microemulsion, was used. Sample analysis was performed using an Agilent series 1100 HPLC system equipped with HP1000 series quaternary pump, autosampler, automatic electronic degasser, automatic thermostatic column compartment, diode-array UV detector, and ChemStation software (Agilent Technologies Inc, Pal Alto, CA). The analytical column used was a Lichrospher 100, RP-18 (5 μm; 250 × 4.6 mm) in conjunction with a Lichrospher 100, RP-18 (5 μm) guard column (4 × 4 mm) (Agilent). The mobile phase was composed of acetonitrile and water with 0.1% trifluoroacetic acid and 0.1% triethylamine (pH 2.4) in a ratio of 43:57 (vol/vol). The column was equilibrated and eluted under isocratic conditions with a flow rate of 1 mL/min at 30ºC. The UV wavelength detector for WHI-07 was set at 220 nm (reference: 400 nm; [UV (MeOH) λ_{max} 217, 226, and 270 nm]. Sample injection volume was 30 μL. Method run time was 40 minutes. Peak width, response time, and slit were set at >0.03-minute, 0.5 seconds, and 4 nm, respectively. Under these conditions, the chromatogram of WHI-07 consisted for 4 stereoisomeric peaks. A run time of 40 minutes was used to determine potential late eluting impurities and degradation products in sample solutions.

Solubility Studies of WHI-07 in Microemulsion

A microemulsion-based system was optimized by identifying the suitable excipients through systematic mapping of ternary phase diagrams and lipophilic drug solubilization studies.[29,30] The solubility of WHI-07 in microemulsion components such as Captex 300 and PEG 200 was performed using UV-visible (vis) spectrophotometer at 272 nm. Appropriate amounts of WHI-07, Captex 300, or PEG 300 were mixed in glass vials at ambient temperature for 2 hours, filtered using a 0.45-μm Acrodisc syringe filter (Gilman Sciences, Ann Arbor, MI), and diluted with ethanol for UV-vis measurement. A calibration curve was prepared by preparing 2 stock solutions of WHI-07 (3.85 and 4.2 mg/mL) in ethanol. These stock solutions were diluted in ethanol to prepare the 13 standard solutions with concentrations ranging from 0.06 to 2.1 mg/mL and A_{272} ranging from 0.07 to 1.886. The standard curves were constructed by plotting the absorbance values as well as the HPLC peak area of WHI-07 against the WHI-07 concentration and were used for calculating the drug concentration of the samples.

Preparation of Gel-Microemulsion

For solubility studies, WHI-07 was solubilized in the microemulsion with concentrations ranging from 0.5% to 2.0% (by weight). For long-term stability studies, WHI-07 (2.0%) was solubilized in gel-microemulsion. In brief, a microemulsion was prepared by combining separate mixtures of Captex 300/Phospholipon 90G and Cremophor EL/propylene glycol/PEG 200. Aqueous polymer suspensions were added to the microemulsion to increase the viscosity. Two types of polymers, SeaSpen PF and Viscarin GP-209NF carrageenans, were used to prepare a gel-microemulsion with adequate viscosity.

The optimized gel-microemulsion for WHI-07 consisted of 10.8% Captex 300, 5.1% Phospholipon 90G, 7.6% Cremophor EL, 4.2% propylene glycol, 4.2% PEG 200, 0.9% each of SeaSpen PF and Viscarin GP-209NF carrageenans, 0.2% sodium benzoate, and water (66.1%).

The viscosities of gel-microemulsions were determined using a Brookfield DV-E viscometer (Brookfield Engineering Laboratories, Stoughton, MA) with spindle No. 3 (speed: 10 rpm). The particle diameter of the microemulsion in the absence of polymers was determined by laser light scattering using Nicomp 380 submicron particle sizer (Particle Sizing Systems Inc, Santa Barbara, CA). For particle size measurement, WHI-07-containing microemulsion concentrate was diluted with water to bring the intensity between 300 and 500 kHz.

Analytical Studies of WHI-07 in Gel-Microemulsion

The HPLC analytical method for WHI-07 in gel-microemulsion was validated with respect to precision, linearity, and reproducibility. Quantitation of WHI-07 in gel-microemulsion was performed by extracting the samples in organic solvents (ethanol, methanol, or acetonitrile) and analyzed by HPLC after appropriate dilution. For the optimized gel-microemulsion with carrageenan polymers, methanol was preferred as the solvent.

For the calibration curve, 101 mg of WHI-07 was dissolved in 1.5 mL of microemulsion with composition of 36%

Cremophor EL, 24% Phospholipon 90G, 20% PEG-200, 20% propylene glycol, and 3.5 mL of the polymer suspension. Gel-microemulsion without WHI-07 served as control. Four standard solutions of WHI-07 were prepared with concentrations ranging from 0.05, 0.10, 0.20, to 0.30 mg/mL. The diluted formulation was mixed, and the polymers in the gel were precipitated out in the presence of acetonitrile, clarified by centrifugation (2000 rpm for 10 minutes), and 0.5 mL of the recovered supernatant was used for HPLC analysis. Standard curve was constructed by plotting WHI-07 concentration versus peak area.

To test the precision of the method, 10 WHI-07-loaded gel-microemulsion samples were prepared and analyzed by HPLC as follows: 101 mg of WHI-07 was solubilized in a glass vial with 1.5 mL of the microemulsion and 3.5 mL of the polymer suspension (1.3% SeaSpen PF carrageenan, 0.7% Xantural [xanthan gum]). A control gel was prepared by mixing 1.2 mL of the microemulsion with 2.8 mL of the polymer suspension. To each of the 10 test vials, 0.2 mL of the WHI-07 gel, 0.3 mL of the control gel, and 4 mL of acetonitrile were added. The contents were vortexed, centrifuged, and 0.5 mL of the supernatant was subjected to HPLC analysis. The peak areas of WHI-07 were obtained to determine the precision of the method.

Stability of WHI-07 in Gel-Microemulsion

8-Day Stability Studies

Gel-microemulsion samples with 0.5% and 2.0% WHI-07 were prepared, stored at room temperature (25ºC ± 3ºC/60% relative humidity [RH]; Caron 6010 environmental test chamber, Marietta, OH), and analyzed for WHI-07 concentrations on day 1, 4, and 8 following extraction for HPLC. The percentage change was based on the observed concentrations of WHI-07 on day 1 and day 8, which were calculated from the HPLC peak areas as described above in Analytical Studies of WHI-07 in Gel-Microemulsion.

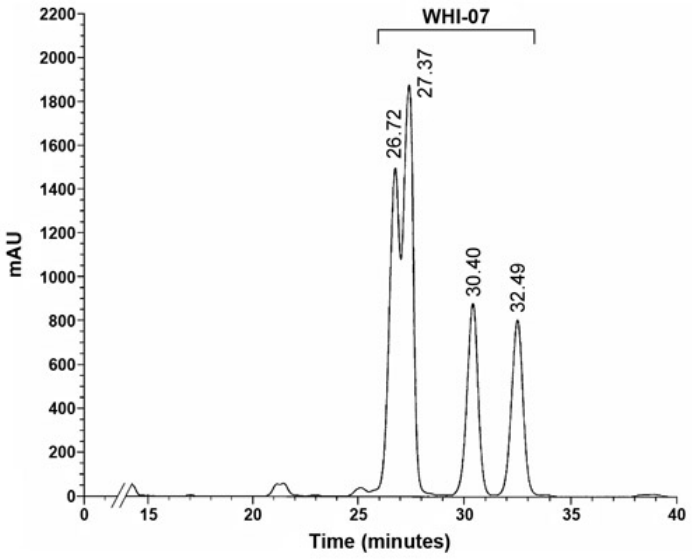

Figure 3. A representative HPLC chromatogram of WHI-07 depicting 4 diastereoisomeric mixtures (2:2:1:1).

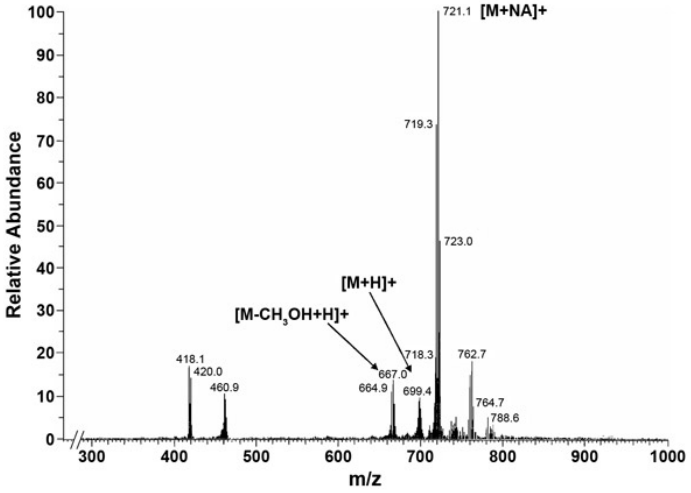

Figure 4. Mass spectrum of WHI-07.

24-Week/6-Month Stability Studies

The long-term stability of WHI-07 in gel-microemulsion was monitored at 3 controlled temperatures over a period of 24 weeks. Thirty glass vials, each containing 1 mL of 2% WHI-07-loaded gel-microemulsion, were stored at room temperature (25ºC ± 3ºC/60% RH; Caron 600 chamber) and at 4ºC ± 2ºC (refrigerated). Fifteen glass vials were stored at (40ºC ± 3ºC/75% RH; Caron 6010 chamber). On week 1, 2, 3, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, and 24, one vial each was taken, and the gel-microemulsion with and without 2% WHI-07 was diluted in 1 mL of 60% methanol, mixed for 30-minutes, and clarified by centrifugation; then the supernatant was filtered through a 0.2-μm Acrodisc syringe filter and 0.1-mL aliquots were assayed by HPLC immediately after preparation. The areas of the 4 peaks of WHI-07 were used to quantify the fraction remaining at each time interval as described above.

Table 1. Octanol:Water Partition Coefficient for WHI-07*

	Peak Area (mAU*s)		
Sample No.	Octanol	Water	Ratio
1	5324.3	46.74	113.9
2	6159.2	55.32	111.3
3	5712.4	49.32	115.8
4	7347.4	67.25	109.2

*Average octanol:water partition coefficient: log K_D = 2.05.

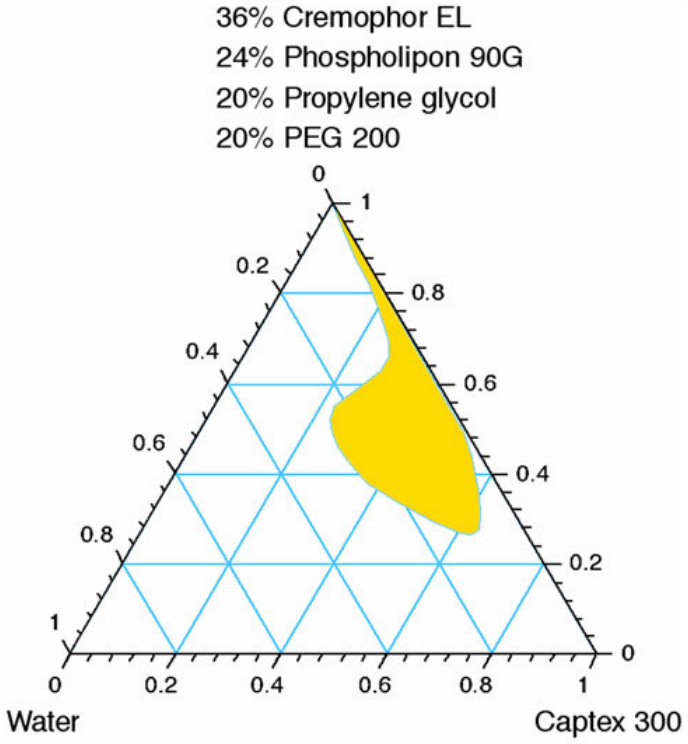

Figure 5. Ternary phase diagram for mixtures of oil/water/surfactant systems. The single-phase o/w microemulsion region formed is shown in orange.

Statistical Analysis

The results are reported as mean or means ± SD. A least square regression analysis was applied using GraphPad Prism Version 4.0a software (GraphPad, San Diego, CA) to determine coefficient of correlation (R^2) between different data sets and statistical significance of correlations. Differences were considered significant at $P < .05$.

Results and Discussion

Characteristics of WHI-07

Owing to the stereochemistry of the phosphorous chiral center, synthesis of WHI-07 with 100% purity resulted in diastereoisomeric mixture (5R, 6R and 5S, 6S) of 4 isomers with HPLC retention times of 26.7 minutes, 27.3 minutes, 30.4 minutes, and 32.5 minutes, respectively (Figure 3). The average percentages of 4 peak areas were 28.92%, 35.37%, 18.16%, and 17.56%, respectively. The 2 major peaks comprised 64.3% of WHI-07. Mass spectral analysis was consistent with the calculated molecular weight of WHI-07 (699 *m/z* $[M + H]^+$ and 721 *m/z* $[M + Na]^+$; see Figure 4).

WHI-07 is practically insoluble in water (<0.003 wt%) but quite soluble in Captex 300 (4.1% ± 0.4 wt%) and the hydrophilic cosolvent PEG 200 (13.1% ± 0.5 wt%) as well as in several organic solvents including acetonitrile, chloroform, ethanol, methanol, and dimethyl sulfoxide. The calculated average octanol-water partition coefficient (Log K_D) value for WHI-07 was 2.05 (Table 1).

Gel-Microemulsion Formulation

Owing to the lipophilic nature of WHI-07, a submicrometer (30-80 nm) particle size microemulsion was developed for its topical delivery. Suitable microemulsions were identified through systematic mapping of ternary phase diagrams. Figure 5 shows the ternary phase diagram of the o/w microemulsion system. The shaded nongrid area represents the region of single-phase transparent microemulsion used for WHI-07 solubilization. The ingredients selected for the o/w microemulsion included drug solubilizers and stabilizers (Captex 300, Cremophor EL, Phospholipon 90G, PEG 200,

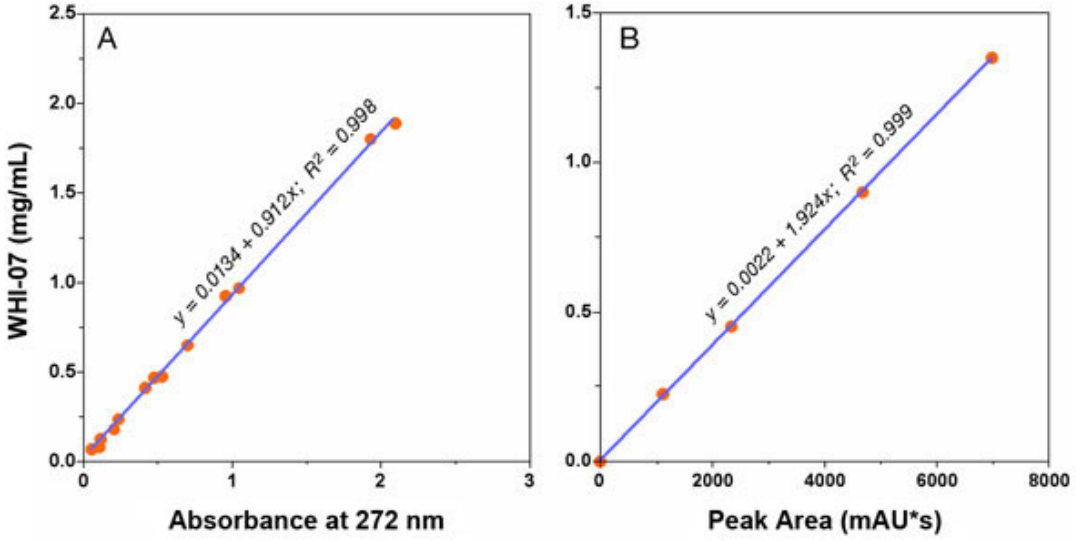

Figure 6. Correlation between WHI-07 concentrations in ethanol versus absorbance at 272 nm (A) or HPLC peak area (B).

Table 2. Precision of the HPLC Analytical Method for WHI-07 in Gel-Microemulsion*

Vial No.	Peak Area (mAU*s)	Deviation	RSD
1	2312.8	18.04	
2	2285.2	−7.56	
3	2272.0	−22.76	
4	2279.4	−15.36	
5	2296.0	1.24	
6	2275.6	−19.16	
7	2299.4	4.64	
8	2291.2	−3.56	
9	2332.8	38.04	
10	2303.2	8.44	
Mean	2294.76	14.08	0.61%

*RSD indicates relative standard deviation.

and propylene glycol) and a preservative (sodium benzoate). Natural gel-polymer suspensions were selected as additives to the microemulsion-based system to obtain a gel of desirable viscosity (300-1200 cP) with high thickening capability and compatibility with the microemulsion for intravaginal use. The resulting translucent gel-microemulsion had a pH of 7.2. The desired viscosity was obtained by increasing the polymeric content of the gel-microemulsion.

The calibration curve for WHI-07 solubilized in ethanol was linear in the concentration range of 0.06 to 2.3 mg/mL with a correlation coefficient of 0.998 (Figure 6A). Similarly, the relationship between WHI-07 concentration in the gel-microemulsion solutions and peak area (mAU*s) on the chromatograms was linear from 0 to 1.3 mg/mL with a linear correlation coefficient of 0.999 (Figure 6B). The precision of the HPLC analytical method for WHI-07 determined by the HPLC peak area of 10 WHI-07-containing gel-microemulsion samples revealed a relative standard deviation (RSD) of 0.61% (mean peak area: 2294 ± 14; n = 10; Table 2). Thus, the HPLC method developed had a good precision for the analysis of WHI-07 content in gel-microemulsion. WHI-07 remained stable after dilution to 0.5%, 1.0%, and 2.0% in gel-microemulsion. Following extraction in acetonitrile, the average drug concentrations

Table 3. Solubility of WHI-07 in Gel-Microemulsion as Determined by HPLC*

Vial No.	Initial Concentration	Observed Concentration	Average	RSD
1	0.5%	0.52%		
2	0.5%	0.49%	0.50%	0
3	1.0%	1.02%		
4	1.0%	0.92%	0.97%	3.0%
5	2.0%	1.88%		
6	2.0%	1.99%	1.94%	3.0%

*HPLC indicates high-performance liquid chromatography; RSD relative standard deviation.

Table 4. Eight-day Stability of WHI-07 in Gel-Microemulsion as Determined by HPLC*

	WHI-07 Concentration	
Storage Time (days)	0.5%	2.0%
1	0.50	2.0
4	0.49	2.0
8	0.51	1.97
% Change on day 8	2%	1.5%

*HPLC indicates high-performance liquid chromatography.

were found to be 0.5%, 0.97%, and 1.94%, respectively. Thus, the recovery rate was 99.8%, and all samples tested met pharmaceutical requirement (RSD <5%) (Table 3).

Stability of WHI-07 in Gel-Microemulsion

Stability was defined as the retention of at least 90% of the initial concentration. WHI-07 was stable in gel-microemulsion at room temperature when stored at 0.5% and 2.0% concentrations on day 1, 4, and 8 during the 8-day initial observation period (Table 4). At both concentrations, WHI-07 was recovered as compared with the initial concentration analyzed by HPLC; the percentage change on day 8 was minimal (≤2%).

For the 6-month stability study, a 2.0% WHI-07-loaded gel-microemulsion was stored at 3 controlled temperatures (4ºC, 25ºC, and 40ºC), and at each time interval, the samples were extracted in methanol and WHI-07 concentrations were determined by HPLC. Results of the HPLC analysis

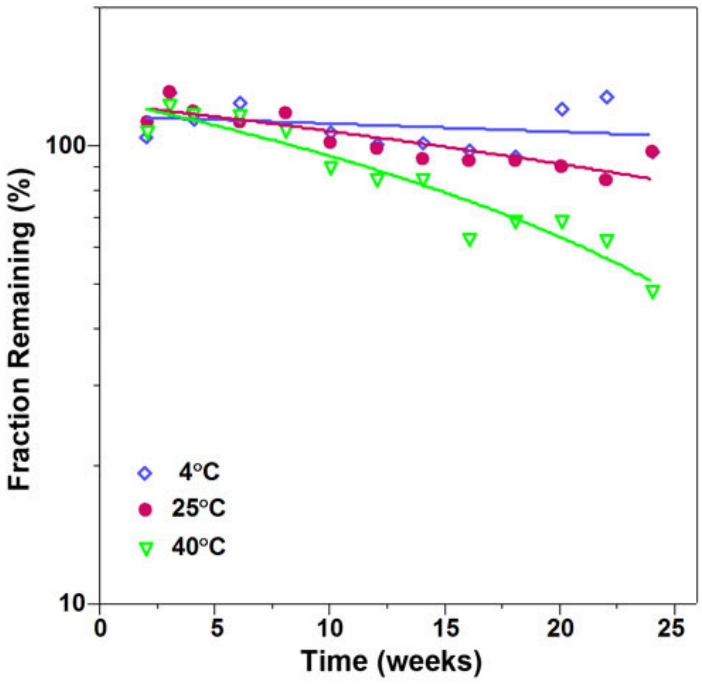

Figure 7. Stability of 2% WHI-07 in o/w gel-microemulsion stored at 3 controlled temperatures for 24 weeks.

showed that 2% WHI-07 gel-microemulsion stored at 4ºC and 25ºC for 24 weeks maintained at least 90% of the initial concentration (Figure 7). No significant differences were observed between the stability of WHI-07 in gel-microemulsion at 4ºC and 25ºC. At 40ºC, WHI-07 was stable in gel-microemulsion for at least 10 weeks, after which a steady decline in concentration was seen. After 24 weeks, more than 40% of the initial concentration was lost at 40ºC. When WHI-07 concentrations in gel-microemulsions stored at 4ºC, 25ºC, and 40ºC were plotted according to second-order equations, a decline in concentration was apparent only at 40ºC during the 24-week observation period (Figure 8). This decrease in WHI-07 content on storage at 40ºC was not associated with the appearance of degradation products or additional peaks (Figure 9). The observed decline in WHI-07 content in gel-microemulsion stored at 40ºC is most likely owing to intrinsic changes in the gel-microemulsion when stored at higher temperatures. Physical changes that may occur on long-term storage of gel-microemulsion include creaming, discoloration, flocculation, settling, and/or phase separation. The neat gel-microemulsion has been found to be stable when stored at room temperature for 5 years (O.J. D'Cruz, unpublished observations, September 2004). In the present study, throughout the 24-week observation period, no change in color, flocculation, phase separation, or precipitation was apparent in gel-microemulsions stored at 4ºC and 25ºC. Therefore, for the expected concentration range of 0.5% to 2.0% in clinical trials, no problems for WHI-07 stability in gel-microemulsion are anticipated.

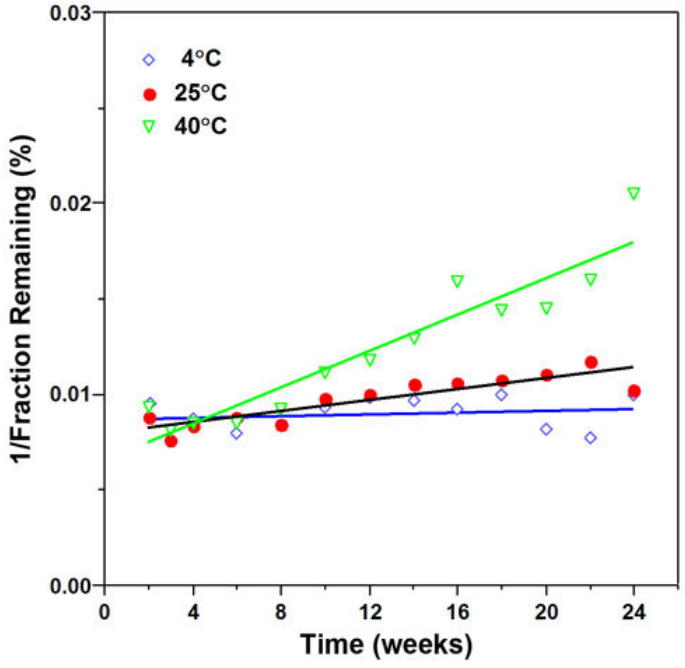

Figure 8. Second-order rate plot for the stability of 2% WHI-07 in o/w gel-microemulsion stored at 4°C, 25°C, and 40°C for 24 weeks.

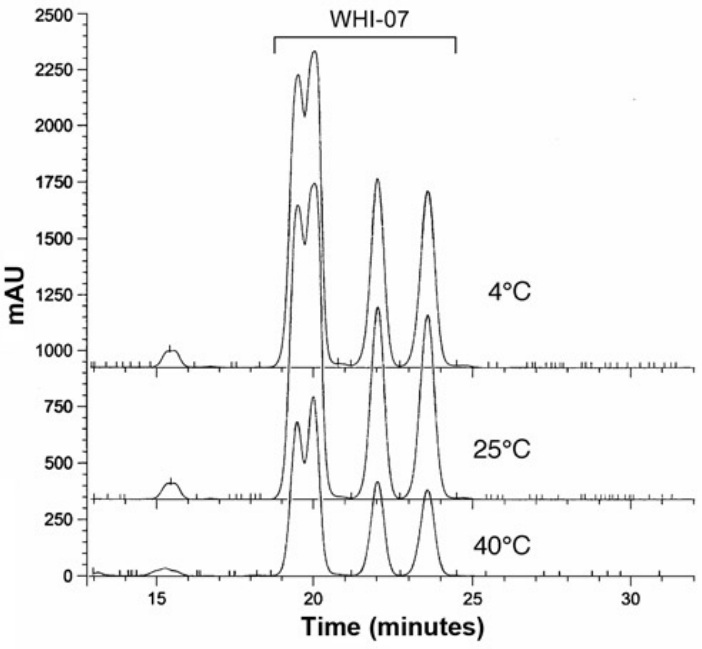

Figure 9. A representative HPLC chromatogram of WHI-07 in gel-microemulsion stored at 4°C, 25°C, and 40°C for 24 weeks.

The observed stability of WHI-07 in gel-microemulsion is of great importance for its widespread utility in various climatological conditions. WHI-07 was shown to be stable for at least 6 months at room temperature when formulated at 2% in gel-microemulsion. Therefore, WHI-07 gel-microemulsion, because of its documented in vivo contraceptive and antiretroviral activities[13,14] and its lack of inflammatory/toxic effects,[10,11,16,17] and because of its stability may be useful as a dual-function prophylactic vaginal contraceptive for sexually active women.

Conclusion

To be a microbicide, the nucleoside analog prodrug WHI-07 must have adequate chemical and physical stability in vaginal drug delivery systems. WHI-07 was formulated in an o/w microemulsion-based system composed of water-insoluble lipids (Captex 300, Phospholipon 90G), water-soluble organic solvents (PEG-200, propylene glycol), and nonionic surfactants (Cremophor EL). This study showed that WHI-07 is stable in natural polymer-based microemulsion for its potential utility as an anti-HIV spermicide for sexually active women.

Acknowledgments

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