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IDENTIFICATION OF FACTORS AFFECTING PRESERVATIVE EFFICACY AND CHEMICAL STABILITY OF LAMIVUDINE ORAL SOLUTION THROUGH STATISTICAL EXPERIMENTAL DESIGN

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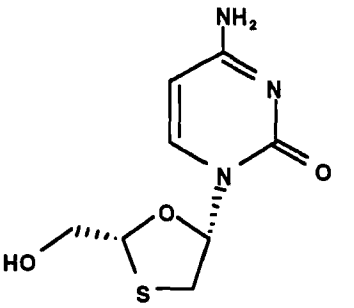
ABSTRACT

To identify factors affecting the chemical stability and preservative efficacy of lamivudine oral liquid formulations, an optimization study using a central composite design was performed. In this design, five factors, each at three levels, were investigated: pH (4.5, 5.5, and 7.5), sucrose (5%, 20%, and 50% w/v), propylene glycol (0%, 2%, and 5% w/v), glycerin (4%, 8%, and 12% w/v), and EDTA (0.100, 0.175, and 0.250 mg/mL). All formulations contained a constant concentration of lamivudine, parabens, and artificial strawberry and banana flavors. All formulations were evaluated for preservative effectiveness against USP and BP standards and for chemical stability at 30°C and 40°C for three months. All formulations were effective against bacteria and yeasts, but indicated reduced preservative effectiveness against the mold Aspergillus niger. Preservative effectiveness improved with increasing pH (4.5 to 7.5) and to a lesser extent with increasing EDTA concentration (0.100 to 0.250 mg/mL). Increasing glycerin concentration (4% to 12% w/v) slightly decreased preservative effectiveness. Over the concentration ranges tested, no change in preservative effectiveness was noted with concentration changes in sucrose or propylene glycol. The pH was the main factor influencing the chemical stability of the drug and preservatives in this study. Lamivudine chemical stability increased with increasing pH from 4.5 to 7.5. Methyl and propylparaben showed extensive degradation at pH 7.5.

INTRODUCTION

Lamivudine, as shown below, is an anti-retroviral agent being evaluated for the treatment of symptomatic and asymptomatic HIV infections. An oral liquid formulation is desired for the pediatric population and for those who cannot swallow more conventional oral dosage forms, such as tablets and capsules.



Lamivudine

The focus of this study was to identify factors affecting the chemical stability and preservative efficacy of the lamivudine oral liquid formulation. Preliminary study showed lamivudine solution to be stable between pH 4.5 and 7.5. A methylparaben /propylpraben combination at pH 5.5 with 20% v/w sucrose was marginally effective in preserving lamivudine formulation. To enhance the lamivudine formulation containing parabens, several ingredients were considered for inclusion in this study. Propylene glycol has been reported to be synergistic in combination with parabens (1,2). Ethylenediaminetetraacetic acid (EDTA), a chelating agent, has been shown to potentiate the activity of many antimicrobial agents by chelating Mg^{2+} and Ca^{2+} ions which are normally responsible for the stability of the cell wall of Gram-negative organisms (3,4) Glycerin was also included in the study as a humectant to prevent possible problems with cap lock. The effect of sucrose level in the formulation on the preservative efficacy was also considered.

An optimization study using a central composite design was performed. In this design, five factors, each with three levels, were investigated: pH (4.5, 5.5, and 7.5), sucrose (5%, 20%, and 50% w/v), propylene glycol (0%, 2%, and 5% w/v), glycerin (4%, 8%, and 12% w/v), and EDTA (0.100, 0.175, and 0.250 mg/mL). All formulations contained 1 mg/mL lamivudine, 1.2 mg/mL methylparaben, 0.15 mg/mL propylparaben, and artificial strawberry (0.8 mg/mL) and banana (0.6 mg/mL) flavors. A full 28-day Antimicrobial Preservative Efficacy (APE) test was performed on all formulations using both USP and BP standards. The chemical stability of these formulations was evaluated at $30^{o}C$ and $40^{o}C$ for 3 months.

EXPERIMENTAL METHODS AND MATERIALS

Preparation of Samples

All ingredients used were compendial or reagent grade except for the flavors (Firmenich, Princeton, NJ) and lamuvidine (Glaxo Research and Development, Ltd., Middlesex, UK), which were used as received from the manufacturers. Samples were prepared by dissolving the parabens in approximately 60% of the final volume of water heated to approximately 80°C. These solutions were then allowed to cool overnight to room temperature. The remaining ingredients were then added to the solution which was brought to final volume with water. Sodium citrate, citric acid, and dibasic sodium phosphate, heptahydrate were used to adjust the solutions to the desired pH. The composition of the formulations tested and their placement in the statistical design space are described in Table 1. The solutions were filled into clear 2 or 5 mL ampules (Kimble-Div. of Owens-Illinois, Chicago Heights, IL). The ampules had been washed with Water for Injection, USP in a Metromatic Products Corp. (Oyster Bay, NY) vial/ampule washer and dried. The ampules were sealed using a propane/oxygen flame with a Cozzoli (Cozzoli Machine Co., Plainfield, NJ) ampule sealer. The sealed ampules were placed into constant temperature ovens (Baxter Scientific Products Model DN-63, Arlington Heights, IL) at 30°C and 40°C. The samples were assayed at initial, 1 and 3 months for lamivudine and parabens according the methods described below. The pH of samples was also measured at each time interval (Orion (Boston, MA) pH meter model 920A with an Orion Ross Combination pH 81-15 electrode). A full 28-day APE test was performed for all formulations .

Quantitative Method for Lamivudine Concentration

The concentration of lamivudine was determined by high pressure liquid chromatography (HPLC). The HPLC system used for the analysis included the following items: Waters (Milford, MA) 600E System Controller, Waters 745 Data Module, Waters 490E Programmable Multiwavelength Detector, Waters 600 Multisolvent Delivery System, Waters 712 WISP Autosampler, Waters autosampler cooler, FIAtron column heater (Oconomowoc, WI), and a Keystone Scientific, Inc. (Bellefonte, PA) BDS Hypersil® C18, 250x4.6 mm, 5 μ particle size, 120 Å pore size, HPLC column. The isocratic assay was performed with 10 μL injection volume and at a flow rate of 1 mL/min using a mobile phase containing 96% (0.025 M ammonium acetate, pH 3.8):4% methanol. Typical retention time was 12-14 minutes with UV detection wavelength of 280 nm and column heater at 30°C. Lamivudine concentration determinations were done in triplicate for each time point.

TABLE 1

Formulations Tested in the Central Composite Design with Face Points Having Five Factors Each at Three Levels.

Formulation Number	Point Type	pH	Sucrose (% w/v)	Propylene Glycol (% w/v)	Glycerin (% w/v)	EDTA (mg/mL)
1	Vertex	4.5	50.0	5.0	12.0	0.100
2	Vertex	7.5	5.0	5.0	4.0	0.250
3	Face	5.5	20.0	2.0	12.0	0.175
4	Face	5.5	20.0	2.0	8.0	0.100
5	Face	5.5	20.0	5.0	8.0	0.175
6	Face	5.5	20.0	2.0	8.0	0.250
7	Vertex	4.5	50.0	0.0	12.0	0.250
8	Face	5.5	20.0	2.0	4.0	0.175
9	Center	5.5	20.0	2.0	8.0	0.175
10	Vertex	7.5	50.0	0.0	12.0	0.100
11	Vertex	7.5	50.0	0.0	4.0	0.250
12	Vertex	4.5	5.0	0.0	12.0	0.100
13	Vertex	7.5	5.0	5.0	12.0	0.100
14	Vertex	7.5	50.0	5.0	12.0	0.250
15	Face	4.5	20.0	2.0	8.0	0.175
16	Vertex	4.5	50.0	0.0	4.0	0.100
17	Face	5.5	5.0	2.0	8.0	0.175
18	Face	5.5	50.0	2.0	8.0	0.175
19	Center	5.5	20.0	2.0	8.0	0.175
20	Vertex	4.5	5.0	5.0	4.0	0.100
21	Vertex	4.5	5.0	5.0	12.0	0.250
22	Face	7.5	20.0	2.0	8.0	0.175
23	Center	5.5	20.0	2.0	8.0	0.175
24	Vertex	7.5	5.0	0.0	4.0	0.100
25	Vertex	7.5	5.0	0.0	12.0	0.250
26	Vertex	7.5	50.0	5.0	4.0	0.100
27	Face	5.5	20.0	0.0	8.0	0.175
28	Vertex	4.5	50.0	5.0	4.0	0.250
29	Vertex	4.5	5.0	0.0	4.0	0.250
30	Center	5.5	20.0	2.0	8.0	0.175

Quantitative Method for Lamivudine Concentration

The concentration of lamivudine was determined by high pressure liquid chromatography (HPLC). The HPLC system used for the analysis included the following items: Waters (Milford, MA) 600E System Controller, Waters 745 Data Module, Waters 490E Programmable Multiwavelength Detector, Waters 600 Multisolvent Delivery System, Waters 712 WISP Autosampler, Waters autosampler cooler, FIAtron column heater (Oconomowoc, WI), and a Keystone Scientific, Inc. (Bellefonte, PA) BDS Hypersil® C18, 250x4.6 mm, 5 μ particle size, 120 Å pore size, HPLC column. The isocratic assay was performed with 10 μL injection volume and at a flow rate of 1 mL/min using a mobile phase containing 96% (0.025 M ammonium acetate, pH 3.8):4% methanol. Typical retention time was 12-14 minutes with UV detection wavelength of 280 nm and column heater at 30°C. Lamivudine concentration determinations were done in triplicate for each time point.

Quantitative Method for Methylparaben and Propylparaben Concentration

The concentrations of methylparaben and propylparaben were determined by HPLC. This HPLC system included the following items: Hewlett Packard 3392A Integrator, Spectraflow 783 Programmable Absorbance Detector (ABI Analytical, Kratos Division, Ramsey, NJ), Waters 712 WISP Autosampler, Waters autosampler cooler, Spectraflow 400 Solvent Delivery System, and Keystone Scientific, Inc. (Bellefonte, PA) Spherisorb® ODS1 C18, 250x4.6 mm, 5 μ particle size, 80 Å pore size, HPLC column. The isocratic assay was performed with 10 μL injection volume and at a flow rate of 1.5 mL/min using a mobile phase containing 50% (0.1 M sodium phosphate):50% acetonitrile. Typical retention times were 2 and 4 minutes for methylparaben and propylparaben, respectively, with UV detection wavelength of 254 nm. Paraben concentration determinations were done in triplicate for each time point.

Antimicrobial Preservative Efficacy (APE) Testing

APE testing was performed according to USP XXII, <51> (5).

RESULTS AND DISCUSSION

Lamivudine Chemical Stability

Results from ANOVA and the quantitative analysis of lamivudine concentration remaining after 3 months at 40°C are given in Table 2. A quadratic model was fit to the three month, 40°C data using RS/1 (BBN Software Products Corp., Cambridge, MA). Higher order terms that did not have a $p<0.05$ were not included in this analysis. Sucrose concentration and pH have significant quadratic effects on the chemical stability of lamivudine, as shown in

TABLE 2
ANOVA Table for the Quadratic Model Fit to the Lamivudine Chemical Stability Data for 3 Months at 40°C.

Source	df†	F-Ratio	Signif.	Coefficient	Std. Error
Constant	1			-0.009278	0.004766
*pH	1	194.40	0.0000	0.044843	0.003072
*Sucrose	1	1.79	0.1942	-0.003091	0.003072
*EDTA	1	1.93	0.1785	-0.004263	0.003072
*pH^2	1	35.03	0.0000	-0.051950	0.008778
*$Sucrose^2$	1	5.92	0.0232	-0.021358	0.008778
*$EDTA^2$	1	5.47	0.0284	0.018090	0.007735
Residual	23				

R^2 = 0.9214, R^2 (adjusted)= 0.9009; †df = degrees of freedom
*indicates factors are transformed to the -1, 1 scale as opposed to their original units.

Table 2. Figure 1 shows the response surface for fraction of lamivudine degradation as a function of pH and sucrose concentration. A higher number in this plot is interpreted as having greater lamivudine chemical stability, i.e., less degradation after three months at 40°C. Although there is little curvature in this plot, it clearly shows that maximum chemical stability of lamivudine is achieved at a sugar concentration of 23-27% w/v and a pH of 6.5-6.7.

Methylparaben Chemical Stability

Results from ANOVA and the quantitative analysis of methylparaben concentration remaining after 3 months at 40°C are given in Table 3. A quadratic model was fit to the three month, 40°C data using RS/1 (BBN Software Products Corp., Cambridge, MA). Higher order terms that did not have a $p<0.05$ were not included in this analysis. Glycerin concentration and pH (quadratic) have highly significant effects on the chemical stability of methylparaben, as shown in Table 3. Figure 2 shows the response surface for fraction of methylparaben degradation as a function of pH and glycerin concentration. A higher number in this plot is interpreted as having greater methylparaben chemical stability, i.e., less degradation after three months at 40°C. This figure shows the dramatic improvement in methylparaben stability by decreasing the pH from 7.5 to 5.8. Little change is noted by decreasing the pH any further. The degradation of methylparaben at higher pH is consistent with its previously reported base catalyzed ester hydrolysis (6). Figure 2 also shows a slight decrease in methylparaben stability with increasing glycerin concentration at lower pH values.

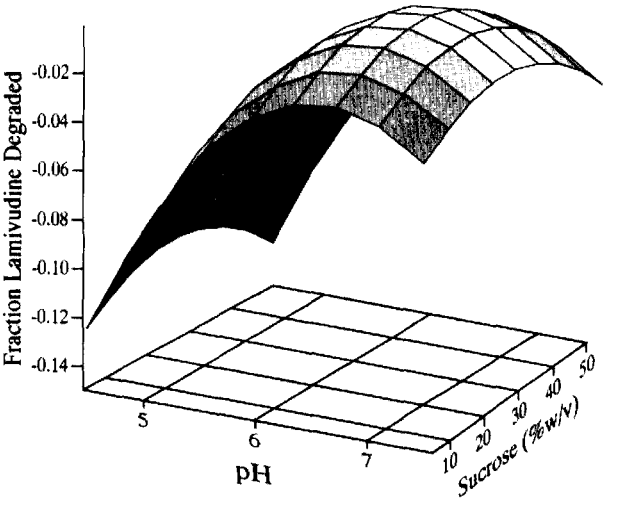


FIGURE 1

Response Surface for Fraction Degradation of Lamivudine as a Function of pH and Sucrose Content After 3 Months at 40°C.

TABLE 3

ANOVA Table for the Quadratic Model Fit to the Methylparaben Chemical Stability Data for 3 Months at 40°C.

Source	df†	F-Ratio	Signif.	Coefficient	Std. Error
Constant	1			-0.037019	0.007080
*pH	1	1882.00	0.0000	-0.188476	0.004789
*Sucrose	1	3.81	0.0643	-0.009850	0.004738
*Propylene Glycol (PG)	1	5.24	0.0325	0.011326	0.004770
*Glycerin	1	21.99	0.0001	-0.022958	0.004788
*pH*Sucrose	1	9.32	0.0061	-0.015292	0.005010
*pH*PG	1	4.67	0.0423	0.010878	0.005032
*pH*Glycerin	1	7.78	0.0110	-0.014067	0.005043
*pH^2	1	241.00	0.0000	-0.137183	0.008837
Residual	21				

R^2 = 0.9905, R^2 (adjusted)= 0.9869; †df = degrees of freedom

*indicates factors are transformed to the -1, 1 scale as opposed to their original units.

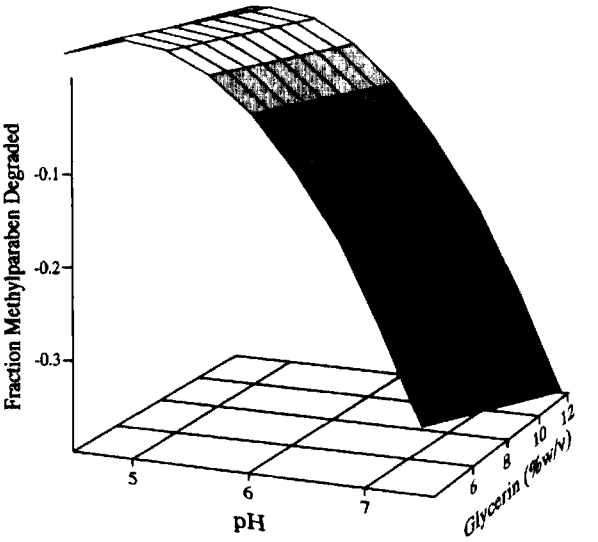


FIGURE 2

Response Surface for Fraction Degradation of Methylparaben as a Function of pH and Glycerin Content After 3 Months at 40°C.

Propylparaben Chemical Stability

Results from ANOVA and the quantitative analysis of propylpraben concentration remaining after 3 months at 40°C are given in Table 4. A quadratic model was fit to the three month, 40°C data using RS/1 (BBN Software Products Corp., Cambridge, MA). Higher order terms that did not have a $p<0.05$ were not included in this analysis. The pH clearly has the highest F-ratio or effect on the chemical stability of propylparaben, as shown in Table 4. Figure 3 shows the response surface for fraction of propylparaben degradation as a function of pH and propylene glycol concentration. A higher number in this plot is interpreted as having greater propylparaben chemical stability, i.e., less degradation after three months at 40°C. Similar to methylparaben stability, propylparaben shows improved stability as the pH is decreased from 7.5 to 6.3 (Figure 3). The degradation of propylparaben at higher pH is consistent with its previously reported base catalyzed ester hydrolysis (7). Figure 3 also shows a slight decrease in the stability of propylparaben at the higher concentrations of propylene glycol.

TABLE 4

ANOVA Table for the Quadratic Model Fit to the Propylparaben Chemical Stability Data for 3 Months at 40°C.

Source	df†	F-Ratio	Signif.	Coefficient	Std. Error
Constant	1			0.022417	0.004979
*pH	1	366.60	0.0000	-0.058557	0.003445
*Propylene Glycol (PG)	1	4.64	0.0406	0.007395	0.003432
*pH^2	1	71.81	0.0000	-0.053278	0.006287
Residual	26				

R^2 = 0.9442, R^2 (adjusted)= 0.9377; †df = degrees of freedom

*indicates factors are transformed to apply to the -1, 1 scale as opposed to their original units.

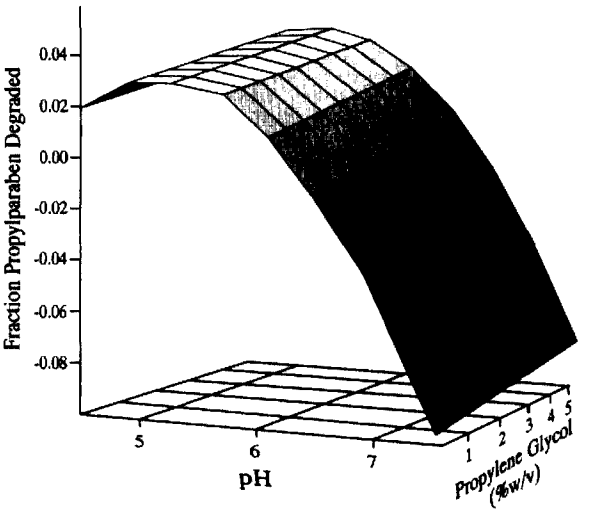


FIGURE 3

Response Surface for Fraction Degradation of Propylparaben as a Function of pH and Propylene Glycol Content After 3 Months at 40°C.

TABLE 5

ANOVA Table for the Quadratic Model Fit to the 25°C Preservative Efficacy Data.

Source	df†	Full Model F-Ratio	Full Model Signif.	Simplified Model Coefficient	Simplified Model Std. Error
Constant	1			2.889312	0.166781
*pH	1	353.40	0.0000	-1.391926	0.115498
*Sucrose	1	1.94	0.1812		
*Propylene Glycol (PG)	1	1.80	0.1968		
*Glycerin	1	13.30	0.0020	0.273121	0.115586
*EDTA	1	6.46	0.0211		
*pH*Sucrose	1	5.27	0.0347		
*pH*Glyceri n	1	7.97	0.0117	-0.229803	0.121755
*pH*EDTA	1	6.30	0.0225		
Glycerin EDTA	1	7.92	0.0119		
*pH^2	1	15.32	0.0011	-0.794512	0.300173
*$Glycerin^2$	1	8.27	0.0105	-0.496443	0.264028
Residual	17				

Full Model: R^2 = 0.9699, R^2 (adjusted)= 0.9486; Simplified Model: R^2 = 0.9094, R^2 (adjusted)= 0.8851; †df = degrees of freedom

*indicates factors are transformed to apply to the -1, 1 scale as opposed to their original units.

Preservative Efficacy Analysis

Since all formulations were effective against bacteria and yeasts, preservative efficacy analysis was based on the data from the mold, Aspergillus niger. The logarithm of the number of organisms per gram plus 1 at 14, 21, and 28 days was taken and then averaged. This value (L) was used to compare the formulations, with a smaller number indicating better preservative efficacy, i.e., fewer organisms remaining at the end of testing. Adding one to the number of organisms before taking the logarithm allowed L values for zero counts to be calculated. Results from ANOVA and the quantitative analysis of preservative efficacy data are given in Table 5. The preservative efficacy data were fit to a quadratic model using RS/1. The pH, glycerin, and EDTA were the factors havinghaving the largest influence on preservative effectiveness, with pH being

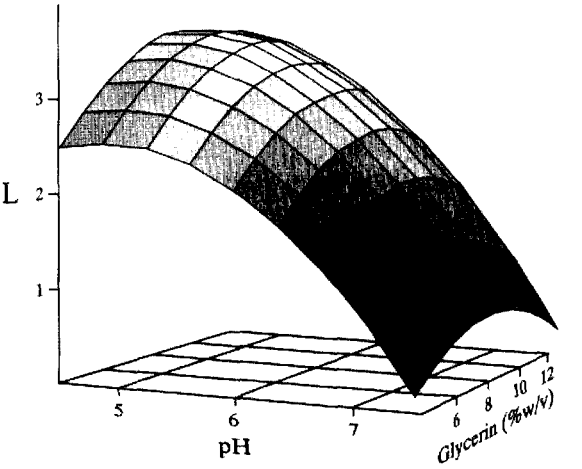


FIGURE 4

Response Surface for the Quadratic Fit to the 25°C Preservative Efficacy Data as a Function of pH and Glycerin Concentration .

the most significant factor. Figure 4 shows the response surface for L as a function of pH and glycerin concentration. Preservative effectiveness improved with increasing pH from 4.5 to 7.5. Increasing the concentration of EDTA slightly improved preservative efficacy, while increasing the concentration of glycerin reduced the preservative efficacy.

CONCLUSIONS

All formulations were effective against bacteria and yeasts, but indicated reduced preservative effectiveness against the mold Aspergillus niger. Preservative effectiveness improved with increasing pH (4.5 to 7.5) and to a lesser extent with increasing EDTA concentration (0.100 to 0.250 mg/mL). Increasing glycerin concentration (4% to 12% w/v) slightly decreased preservative effectiveness. No change in preservative effectiveness was noted with sucrose or propylene glycol in the concentration ranges tested.

The pH was the main factor on the chemical stability of the drug and parabens in this study. While lamivudine chemical stability increased with increasing pH from 4.5 to 7.5, methyl and propylparaben showed extensive degradation with increasing pH. Unfortunately, preservative efficacy was also best at pH 7.5. Further formulation development will be focused in a narrow

range of pH (5.0 to 7.0) to avoid both chemical degradation of lamivudine and parabens while maintaining adequate preservative efficacy.

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