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Stability of low concentrations of guanine-based antivirals in sucrose or maltitol solutions

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Abstract

Three guanine-based antiviral drugs, entecavir, lobucavir, and acyclovir showed degradation in presence of sucrose in ready-to-use solutions held at 50 °C, with more degradation at pH 4 than at pH 6 or 7. LC/MS analysis of the solutions showed isomeric adducts of the drugs and reducing sugars. Sucrose, a disaccharide and a non-reducing sugar, was the source of monosaccharides, the reducing sugars. Sucrose showed pH-dependent hydrolysis at 50 °C into two monosaccharides, fructose and glucose, with more sucrose hydrolyzing at pH 4 than pH 6 or 7. Additionally, the three drugs showed pH-dependent degradation at 50 °C in fructose and glucose solutions with the following rank order: pH 7 > pH 6 > pH 4. This indicated that the increased degradation of the drugs in sucrose solutions at pH 4 was mainly due to more hydrolysis of sucrose into fructose and glucose compared to pH 6 or 7, and subsequent reactions of the fructose and glucose with the drugs. Based on structures of the major degradants, it is proposed that the main cause of the degradation was nucleophilic addition of the primary amine group of the drugs to the carbonyl group of the fructose and glucose. This reaction was facilitated as the solution pH increased from 4 to 7. All the drugs showed satisfactory stability regardless of the storage temperature or solution pH in maltitol, an alternate sweetener. The free aldehyde or ketone group in maltitol precursors is reduced to a hydroxyl group after the hydrogenation process making maltitol less susceptible to nucleophilic addition.

Keywords: Stability; Nucleophilic addition; Sucrose; Maltitol; Entecavir; Lobucavir; Acyclovir; pH effect

1. Introduction

During the formulation development of a low-strength entecavir oral solution, it was observed that entecavir degraded in the presence of sucrose, a commonly used sweetener for oral solutions. This was unexpected since entecavir exhibited satisfactory stability in aqueous solutions of pH 2–9.4. The observed degradation was of concern since a sweetener was needed to mask the bitter taste of entecavir. To understand the degradation mechanism of entecavir in the presence of sucrose, stability of two other guanine-based and structurally similar antiviral compounds, lobucavir and acyclovir (Fig. 1), was also studied in presence of sucrose. Additionally, the solution stability of these antivirals in the presence of fructose, glucose, and maltitol at different pH values and temperatures was studied. Results of the stability study and possible mechanisms of degradation of the antivirals in presence of sweeteners are reported.

2. Materials and methods

The stability of 0.2 mg/mL solutions of entecavir, lobucavir, and acyclovir was studied in 50% (w/v) sucrose, 26% (w/v) fructose, 26% (w/v) glucose, or 50% (w/v) maltitol. The stability of each drug was evaluated in 10 mM citrate buffer at pH 4, 6, and 7 for sucrose, fructose, and glucose solutions, and pH 4, 5, and 6 for maltitol solutions. The solutions were stored in glass vials with rubber stoppers and crimped with aluminum caps to prevent evaporation of the liquid. The vials were placed at 5 °C (control) and 50 °C for 13 weeks. The solutions were analyzed by HPLC for loss in potency of the drugs and formation of degradants.

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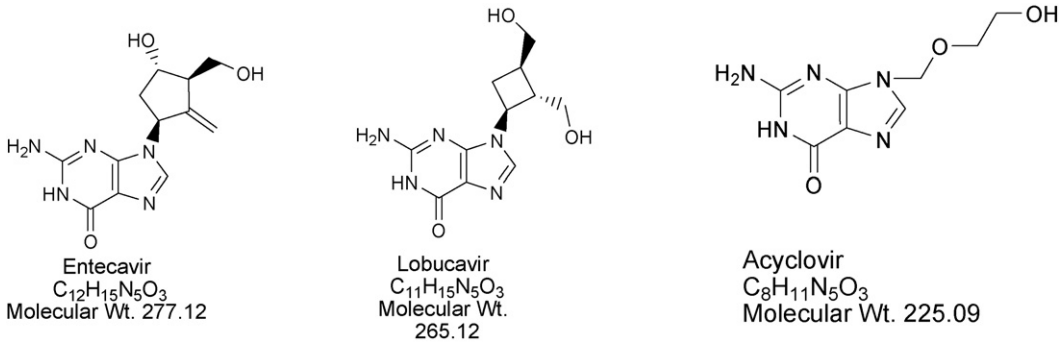


Fig. 1. Structures of guanine-based drugs. Both entecavir and lobucavir have pK_a values 2.9 and 9.7 (BMS unpublished data). The pK_a values of acyclovir are 2.3 and 9.3 (Package insert—Zovirax® suspension).

2.1. Materials

Entecavir and lobucavir were manufactured by Bristol-Myers Squibb Company. The remaining compounds were obtained commercially and were used as received. Acyclovir was obtained from Toronto Research Chemicals Inc (North York, Ontario, Canada). Sucrose (NF) was obtained from Domino Specialty Ingredients (Arabi, LA). Fructose (USP crystals) and glucose (dextrose anhydrous, USP/EP/BP) were obtained from Fisher Scientific (Fair Lawn, NJ). Maltitol NF (Lycasin 80/55®) was purchased from Roquette America, Inc (Keokuk, Iowa).

2.2. Methods

2.2.1. Preparation of solutions

The formulation compositions for antiviral drug solutions are shown in Table 1. The drug, sweeteners, citric acid, and sodium citrate were added to water and continuously stirred at room temperature for at least one hour until all the components were dissolved. The solutions were then adjusted to various pH values using IN hydrochloric acid or IN sodium hydroxide solution. Additional water was added to make up the final batch volume. The batch volume for entecavir and lobucavir solutions was 250 and 100 mL for acyclovir. The corresponding blank solutions were prepared by repeating the same procedures except that the drug was not added. The blank solutions were used in the stability study for identifying excipient-related degradant peaks.

2.2.2. Stability studies

The solutions were stored in 60-cc glass vials at 50-mL fill volume for entecavir and lobucavir solutions or in 30-cc glass vials at 25-mL fill volume for acyclovir solution, with rubber stoppers and aluminum caps to prevent evaporation of the liquid. The vials were placed in 50 °C controlled stability chamber (Model 317502, HOTPACK, PA) in a vertical position during the stability studies. Control samples were placed in a refrigerator (Bally Engineered Structures, Inc., Bally, PA) at 2–8 °C.

At each time point, approximately 1.5 mL samples were withdrawn for HPLC analysis. At the 2.5 week time point, approximately 10 mL samples were withdrawn into a 20 mL glass vial for pH determination. The solutions were equilibrated at room temperature before measuring the pH. After sampling for HPLC and pH determination, the glass vials were resealed and returned to the storage conditions.

2.3. Analytical methods

The pH of the samples was measured using a glass electrode (Model 511053, Beckman Coulter Inc., CA) and Ø350 pH meter (Beckman Coulter Inc., CA) at room temperature. The

Table 1
Composition of antiviral drug solution, 0.2 mg/mL, in 10 mM citrate buffer

Ingredient	Concentration (mg/mL)			
	Sucrose 50%	Maltitol 50%	Fructose 26%	Glucose 26%
Sucrose	500	–	–	–
Maltitol solution[a]	–	650	–	–
Fructose	–	–	262	–
Glucose	–	–	–	262
Drug			0.2	
Citric acid anhydrous			0.37	
Sodium citrate dihydrate			2.40	
Water			qs to 1 mL	
Sodium hydroxide			qs as needed for pH adjustment[b]	
Hydrochloric acid			qs as needed for pH adjustment[b]	
pH adjusted to	4, 5, 6, or 7	4, 5, or 6	4, 5, or 6	4, 6, or 7

[a] Contains 75% solid maltitol.
[b] 1N sodium hydroxide solution and/or IN hydrochloric acid solution may be used to adjust the final solution pH.

Table 2
Stability of entecavir in 50% sucrose solutions

Time (weeks)	Storage condition	In 50% sucrose solution					
		pH 4		pH 6		pH 7	
		Potency (% initial)	pH	Potency (% initial)	pH	Potency (% initial)	pH
Initial	RT	100.0	4.0	100.0	6.0	100.0	7.0
2.5	5 °C	100.0		100.0		100.0	
	50 °C	93.5		98.6		99.2	
4.5	5 °C	90.3		96.2		96.6	
	50 °C	73.9		92.3		95.2	
8	5 °C	87.2	4.0	91.1	5.7	93.5	6.5
	50 °C	48.4	3.6	83.8	5.7	82.2	6.9
13	5 °C	88.4	4.0	89.4	5.7	95.7	6.5
	50 °C	31.9	3.3	74.5	5.2	77.4	6.8

electrode was calibrated routinely prior to analysis of the samples. The solutions were analyzed by an isocratic method for loss in potency and a gradient method for degradant formation using a Waters Alliance 2690 HPLC with Waters 486 UV detector. The software used was Millennium version 4.0. A YMC ODS-AQ column (S-3 μm, 12 nm, 150 × 4.6 mm) with mobile phases A (25 mM potassium phosphate buffer, pH 3.3) and B (analytical grade acetonitrile) was used. The column temperature was ambient.

The injection volume was 5 μL with UV detection wavelength of 254 nm for both methods. The isocratic assay for potency was performed using a mobile phase of 93% A and 7% B for entecavir and lobucavir and 95% A and 5% B for acyclovir at a flow rate of 1 mL/min. The run time was 8 min. The typical retention times were 5.9, 5.8, 4.0 min for entecavir, lobucavir, and acyclovir, respectively, in the isocratic assay.

The gradient assay for degradant was performed at a flow rate of 1 mL/min with 96% A and 4% B for 12 min, followed by a 14-min linear gradient change to 40% A and 60% B and held for 3 min, and finished with 6-min hold of 96% A and 4% B. The total run time was 35 min. The typical retention times were 13.5, 12, and 4.4 min for entecavir, lobucavir, and acyclovir, respectively, in the gradient assay.

Degradants were characterized by LC/MS with exact mass measurements (±5 ppm) and product ion LC/MS using a Waters Q-ToF-2 quadrupole time-of-flight hybrid instrument. The chromatographic conditions used were similar to those used for the gradient degradant assay with the exception of the use of 0.1% formic acid as the mobile phase modifier in place of potassium phosphate.

The assay for monosaccharide was performed using a Phenomenex Luna Amino column (S-3 μm, 12 nm, 150 mm × 4.6 mm) eluted at 1 mL/min with mobile phase containing 80% acetonitrile and 20% water. The column temperature was 30 °C. The injection volume was 10 μL. Detection was achieved using a Waters Quattro Micro quadrupole mass spectrometer operated in the negative ion electrospray mode and monitoring the deprotonated ion at *m/z* 181.

3. Results and discussion

The stability of 0.2 mg/mL entecavir, lobucavir, and acyclovir in 50% (w/v) sucrose solution was studied at different pH values and temperatures as shown in Tables 2–4. All three drugs showed degradation in the presence of sucrose and the degradation trend was similar. For any given time point, as the storage temperature

Table 3
Stability of lobucavir in 50% sucrose solutions

Time (weeks)	Condition	In 50% sucrose solution					
		pH 4		pH 6		pH 7	
		Potency (% initial)	pH	Potency (% initial)	pH	Potency (% initial)	pH
Initial	RT	100.0	4.0	100.0	6.0	100.0	7.0
2.5	5 °C	100.0		100.0		100.0	
	50 °C	97.2		98.8		99.0	
4.5	5 °C	96.9		99.5		99.0	
	50 °C	68.4		93.8		96.4	
8	5 °C	100.6	4.0	99.1	6.2	100.3	7.0
	50 °C	54.9	3.7	89.4	5.7	93.9	7.0
13	5 °C	99.5	4.0	100.5	6.0	99.0	7.0
	50 °C	41.1	3.4	74.7	5.4	87.6	6.7

Table 4
Stability of acyclovir in 50% sucrose solutions

Time (weeks)	Condition	In 50% sucrose solution					
		pH 4		pH 6		pH 7	
		Potency (% initial)	pH	Potency (% initial)	pH	Potency (% initial)	pH
Initial	RT	100.0	4.0	100.0	6.0	100.0	7.0
2.5	5 °C	100.0		100.0		100.0	
	50 °C	96.7		100.0		100.3	
4.5	5 °C	102.5		102.8		103.9	
	50 °C	89.2		100.0		101.9	
8	5 °C	102.9	4.3	102.8	5.6	102.4	6.5
	50 °C	65.7	3.5	93.0	5.8	97.6	7.2
13	5 °C	98.5	4.3	100.8	5.7	97.6	6.7
	50 °C	40.2	3.2	78.0	5.2	88.4	6.9

increased from 5 to 50 °C, drug degradation increased and the solution pH decreased slightly (Tables 2–4). The lowering of pH in sucrose solutions in un-buffered and buffered solutions has been reported (L'Homme et al., 2003). This has been attributed to generation of acid degradation products of sucrose such as formic and acetic acid. In fact, even the blank formulations showed a decrease in pH (data not shown).

Moreover, as the pH of the drug solution increased from 4 to 6, the drug degradation decreased (Tables 2–4). The initial appearance of all solutions was clear and colorless. However, at the 50 °C storage condition, they turned slightly yellow after 8 weeks storage and light orange after 13 weeks storage, especially the pH 4 solutions. The rate of drug degradation does not appear to be first-order (Fig. 2). The shape of the degradation profile suggests that the mechanism could be sequential or autocatalytic.

In contrast to the above observations with sucrose, all three drugs showed satisfactory stability in maltitol, an alternate sweetener. Solution concentrations were usually within ±2% of initial, with the greatest loss equal to <5% after 13 weeks at 50 °C. There was no major effect of temperature or pH on the stability. The appearance (clear and colorless solution) and pH of the solutions remained unchanged throughout the stability study.

LC/MS characterization of the degraded samples in sucrose showed a series of products corresponding to the drug conjugated with dehydrated monosaccharide. The number of dehydration ranged from one to three and multiple isomeric forms of each dehydration product were detected.

Sucrose is a disaccharide, a non-reducing sugar, known to undergo pH-dependent non-enzymatic hydrolysis to fructose and dextrose (glucose), both monosaccharide, reducing sugars. Based on these observations, the reaction sequence in Scheme 1 is proposed.

To better understand the kinetics and pH-dependent degradation behavior in sucrose solutions, experiments were conducted to a) determine the rate of sucrose degradation into fructose and glucose and b) determine the rate of degradation for lobucavir, acyclovir, and entecavir in the presence of individual monosaccharides, i.e. fructose and glucose.

Sucrose can degrade into fructose and glucose as shown by the stability study of 50% (w/w) sucrose solution at different pH values and at 50 °C storage temperature (Table 5). As shown in Table 5, at pH 4, the sucrose solution showed more hydrolysis than at pH 6 or 7. The hydrolysis of sucrose was negligible at pH 7. The appearance of the pH 4 solution changed from clear and colorless to light yellow to orange after storage at 50 °C for 8 weeks. As the hydrolysis proceeded, a decrease in pH was observed in solutions initially at pH 4 and 6.

Table 6 shows the degradation of lobucavir, acyclovir, and entecavir in 26.5% fructose or glucose solutions at 50 °C. As the pH of the solution increased from 4 to 7, the extent of degradation increased for all the drugs. Interestingly, the degradation was more pronounced in the fructose solutions compared to glucose solutions. The solutions were clear and colorless initially. After storage at 50 °C for 2.5 weeks, the fructose solutions turned light orange. The fructose solutions stored at 5 °C and all glucose solutions remained clear and colorless.

3.1. Effect of pH

Tables 5 and 6 show the effect of pH on sucrose degradation into fructose and glucose and their reactivity with the drugs, respectively. The overall effect of pH on the drug degradation in sucrose solutions is affected by the following factors:

1. degradation of sucrose to fructose and glucose;
2. ring-opening of fructose and glucose;
3. rate of amine reaction with the aldoses.

Non-enzymatic inversion of sucrose is an acid catalyzed reaction (L'Homme et al., 2003). As the pH is increased from 4 to 6, the amount of fructose and glucose generated should be lowered. At any given pH, both glucose and fructose are in a rapid equilibrium with the reactive acyclic "aldose" moieties. Determination of an equilibrium constant for the ring opening of sugars is not trivial (Kannane and Labuza, 1989; Labuza, 1981) and very few reports are available even on simple sugars like glucose and fructose at physiologically relevant pH. Cantor and Penniston

Table 5
Hydrolysis of 50% (w/v) sucrose solutions at 50 °C

Time (weeks)	pH 4			pH 6			pH 7		
	Amount glucose (% w/v)	Amount fructose (% w/v)	pH	Amount glucose (% w/v)	Amount fructose (% w/v)	pH	Amount glucose (% w/v)	Amount fructose (% w/v)	pH
Initial	0	0	4.0	0	0	6.0	0	0	7.0
2.5	13.5	10.4		0.01	0.19		0.02	0.01	
4.5	19.4	12.8		0.16	0.18		0.02	0	
8	24.5	15.5	3.5	1.04	0.47	5.6	0.02	0	7.2
13	30.4	15.7		1.02	1.05	5.5	0.02	0.01	6.9

(Cantor and Pennington, 1940) reported that the % acyclic glucose increased from 0.012 at pH 6.5–0.040 at pH 7.5. Bunn and Higgins (Bunn and Higgins, 1981) also proposed that fructose appears to equilibrate with the larger fraction of acyclic form. Furthermore, the pH-dependent interaction of hemoglobin with various sugars was also correlated with the relative fractions of acyclic forms of reducing sugars (Bunn and Higgins, 1981). As pointed out by Kannane and Labuza (1989), the reaction rate increases with the amount of acyclic form in the solution but the proportionality is unknown. It is noteworthy that the amount of acyclic form of fructose and glucose is likely to be a small fraction of the total monosaccharide concentration but comparable to the drug concentration in the systems studied here.

The mechanism of Schiff base formation has been well-studied (Cordes and Jencks, 1962). The pH-rate profile for amine-aldehyde reaction has been reported to be a bell-shaped curve and the rate determining step transitions from acid-catalyzed amine attack on the carbonyl to dehydration of carbinolamine addition product at neutral pH (Scheme 2). However, for the more relevant reactions between sugars and amines, the amine degradation rate has been shown to increase with pH in the range of 3–8 (Lee and Nagy, 1988; Kannane and Labuza, 1989). As the pH is increased, the fraction of free amine necessary for nucleophilic addition also increases. It is noteworthy that based on the pK_a of the guanine-based drugs (pK_a values between 2.5 and 3) and the pH range studied, a majority of the drug is expected to be in the un-protonated form (95–99%).

A kinetic model was derived to model the loss of entecavir in the presence of sucrose using the following assumptions: (a) sucrose hydrolysis is a first order reaction, (b) the glycosamine formation is the main pathway for the loss of drug as well as the monosaccharides i.e., fructose and glucose and (c) the individual loss of reducing sugar and amine is individually a first-order reaction and overall a second-order reaction (Boekel (2001) and Baisier and Labuza (1992).

$$\frac{-\mathrm{dSu}}{\mathrm{d}t} = k_s \times [\mathrm{Su}] \quad (1)$$

$$\frac{\mathrm{d}[F_T]}{\mathrm{d}t} = k_s \times [\mathrm{Su}] - k_F \times D_T \times [F_T] \quad (2)$$

$$\frac{\mathrm{d}[G_T]}{\mathrm{d}t} = k_s \times [\mathrm{Su}] - k_G \times D_T \times [G_T] \quad (3)$$

$$\frac{-\mathrm{d}[D_T]}{\mathrm{d}t} = k_F \times D_T \times [F_T] + k_G \times [G_T] \quad (4)$$

where Su, F_T, G_T and D_T are total sucrose, fructose, glucose and drug concentration, respectively.

In order to solve the above equations, the rate constants k_s, k_F and k_G need to be determined independently. The sucrose inversion constant, k_s is determined at pH 4.0 by following the glucose levels in sucrose solution at 50 °C. As expected, the hydrolysis of sucrose at pH 4 was found to be first-order and the rate constant k_s was estimated to be $0.068 \pm 0.008\ \mathrm{week}^{-1}$. Values of k_F and k_G were determined by following drug hydrolysis in 1.46 M solution of fructose or glucose at pH 4 at 50 °C. In spite of the amine drug-reducing sugar reaction being a bimolecular one, the rate of drug degradation in the presence of fructose or glucose at pH 4 can be fitted to a first-order rate profile (Fig. 3). While following Maillard kinetics of model amino acid-glucose systems, others have found that the loss of amino acid is first-order although the overall reaction is of second-order (Baisier and Labuza, 1992; Boekel, 2001). In the

Table 6
Stability of lobucavir, acyclovir, and entecavir in fructose and glucose solutions stored at 5 °C and 50 °C for 2.5 weeks

Drug	Storage condition	In 26% (w/v) fructose solution						In 26% (w/v) glucose solution					
		pH 4		pH 6		pH 7		pH 4		pH 6		pH 7	
		Potency (% initial)	pH	Potency (% initial)	pH	Potency (% initial)	pH	Potency (% initial)	pH	Potency (% initial)	pH	Potency (% initial)	pH
Lobucavir	5 °C	100.0	4.0	100.0	5.9	100.0	6.9	100.0	4.0	100.0	5.9	100.0	6.9
	50 °C	78.7	3.8	58.6	5.7	56.8	6.5	91.9	4.0	87.1	5.7	78.1	6.6
Acyclovir	5 °C	100.0	4.0	100.0	6.0	100.0	7.0	100.0	4.0	100.0	5.9	100.0	6.8
	50 °C	80.5	3.8	67.6	5.5	57.1	6.4	90.6	4.0	85.3	5.8	71.0	6.6
Entecavir	5 °C	100.0	4.0	100.0	5.9	100.0	6.9	100.0	4.0	100.0	5.9	100.0	6.9
	50 °C	83.2	4.0	61.4	5.8	49.3	6.6	92.8	4.0	87.8	5.7	80.9	6.6

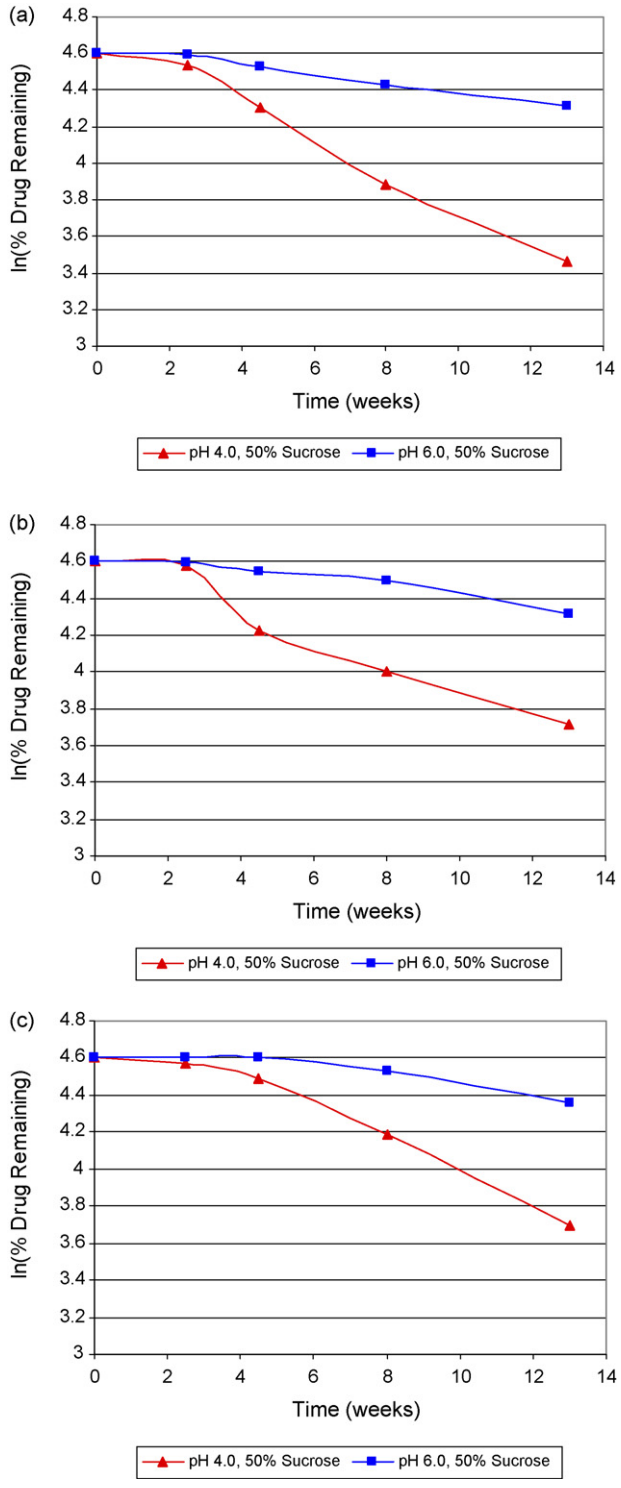
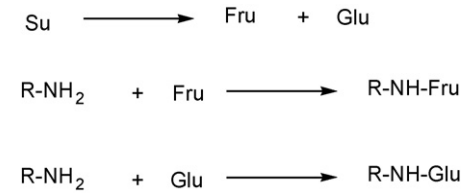


Fig. 2. Stability of guanine-based drugs in 50%w/w sucrose solutions at 50 °C at pH 4 and pH 6. (a)–(c) Correspond to entecavir, lobucavir and acyclovir, respectively.

current study, we observe a pseudo-first order loss of entecavir because of the large molar excess of fructose or glucose (1.46 M) relative to the drug (0.008 M). The pseudo-first order rate constants obtained for entecavir degradation in fructose and glucose are 0.221 ± 0.02 and 0.048 ± 0.004 week^{-1}, respectively. These pseudo-first order rate constants are dependent on the fructose or glucose concentrations. The concentration-independent second order rate constants (k_F and k_G) are obtained by simply dividing the pseudo-first order rate constant by the molar concentration of the fructose or glucose (1.46 M). The degradation was greater in the presence of fructose relative to glucose i.e. k_F and k_G were found to be 0.151 and 0.033 week^{-1} M^{-1}, respectively.



Scheme 1. Reaction scheme for sucrose hydrolysis and drug degradation at a fixed pH.

The reaction Scheme 3 was tested at pH 4, 50 °C by predicting the drug degradation in 50% sucrose solutions at pH 4. The rate expressions given by Eqs. (1)–(4) were simultaneously solved using the differential equation solver SCIENTIST (Micromath Inc.). At initial conditions ($t = 0$), D_T, Su, F_T, G_T were fixed as 0.008 M, 1.46 (or 0.58), 0 and 0 M. The rate constants k_S, k_F and k_G were fixed at 0.068 week^{-1}, 0.151 week^{-1} M^{-1} and 0.033 week^{-1} M^{-1}, respectively. Fig. 4 shows the prediction of drug degradation in the presence of 50% sucrose. Overall, a good agreement between the predicted and experimental values is observed thus validating the model.

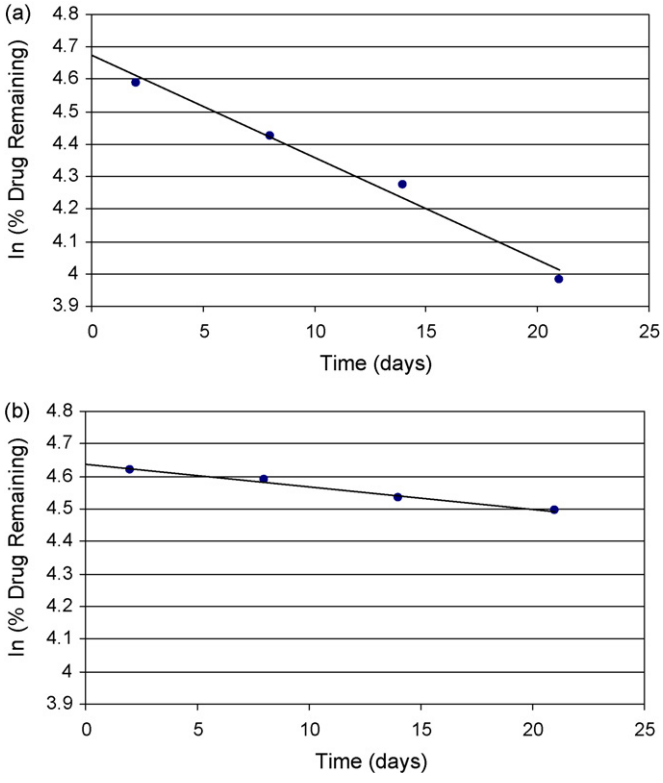
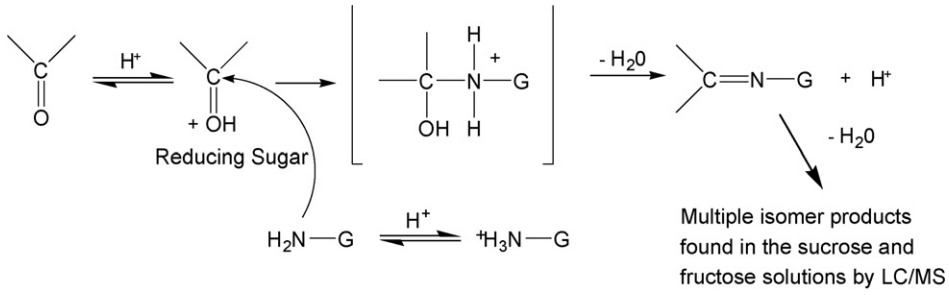
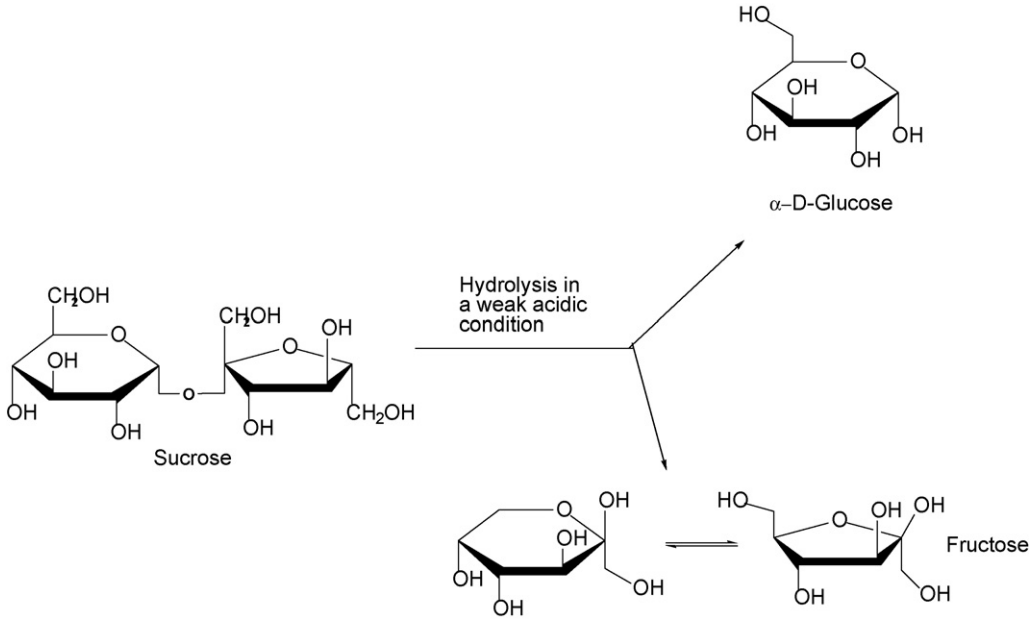


Fig. 3. Stability of entecavir in 26% (w/w) fructose and 26% (w/w) glucose solution at pH 4.0 and 50 °C. (a) and (b) Correspond to fructose and glucose, respectively. The points represent experimental data and the line represents the best fit to the data.



Scheme 2. Reaction mechanism of a primary amine drug with a reducing sugar.



Scheme 3. Hydrolysis of sucrose into glucose and fructose.

The effect of pH on the drug degradation in the presence of sucrose can be predicted if pH-dependencies of the individual rate constants used in Eqs. (1)–(4) are known. The acid hydrolysis of sucrose has been studied extensively and the pH-dependence is known. The pH effect on the reaction rate constants used in Eqs. (1)–(4) is complicated because of the lack of information on the pH-dependence of equilibrium between open and closed form of reducing sugars and the exact proportionality of this ratio on the rate constant.

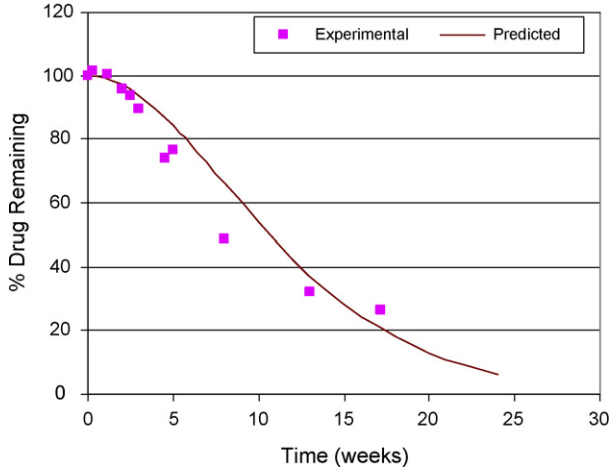


Fig. 4. Stability of entecavir in 50% (w/w) sucrose solutions at pH 4.0 and 50 °C. The points represent experimental data and the line represents the predicted values based on the reaction scheme represented by Eqs. (1)–(4).

The alternative sweetener, maltitol, is made from the hydrogenation of partially hydrolyzed starch. During the hydrogenation process, the free aldehyde or ketone group is reduced into a hydroxyl group, which is generally less reactive. For this reason, all three drugs demonstrated satisfactory stability with maltitol as a sweetener. These results indicate that maltitol, a hydrogenated sweetener, is most suitable for use in formulating a guanine based antiviral drug in an aqueous solution.

4. Conclusions

The instability of three antiviral drugs in ready-to-use solutions containing sucrose was due to hydrolysis of sucrose at pH 4 into fructose and glucose, the reducing sugars. The primary amine-containing drugs underwent nucleophilic addition with the ketone group of the acyclic form of fructose and glucose, the hydrolytic products of sucrose. Such reaction was not feasible with the alternate sweetener maltitol where the free

aldehyde or ketone group is reduced to a hydroxyl group after the hydrogenation process. Based on these results, it was concluded that maltitol would be the preferred sweetener to prepare ready-to-use solutions of entecavir, lobucavir, and acyclovir.

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