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Research Article

Accelerated Stability Modeling for Peptides: a Case Study with Bacitracin

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Abstract. The Accelerated Stability Assessment Program (ASAP) was applied for the first time to a peptide, the antibiotic active pharmaceutical ingredient bacitracin. Bacitracin and its complex with zinc were exposed to temperature and relative humidity conditions from 50 to 80°C and from 0 to 63% for up to 21 days. High-performance liquid chromatography was used to analyze the stressed samples for both degradant formation and loss of the active (bacitracin A) and two inactive isoforms, with identities confirmed by mass spectrometry. These data were then analyzed using a humidity-corrected Arrhenius equation and isoconversion approach to create a shelf-life predicting model for typical storage conditions. Model fitting was found to be good with low residuals in both temperature and relative humidity axes for all parameters examined. The generated model's predictions for both the native and zinc complex of bacitracin for both formation of the major degradation product (F) and loss of the active isoform (A) were consistent with longer-term measured values at 30°C/53%RH and 40°C/75%RH, validating this approach for accelerating the determination of long-term stability of a peptide.

KEY WORDS: accelerated stability; peptide stability; solid-state stability; stability modeling.

INTRODUCTION

In order to establish a drug product shelf-life, it is important to ensure drug safety and efficacy throughout the storage period at the desired storage conditions (temperature, T, and relative humidity, RH). Drug products generally undergo a minimum of 6 months of accelerated stability testing for tentative shelf-life determination and 12 months of long-term testing for confirmation (1). Quality specifications are determined by limits on known and unknown degradants (typically <1% of the active pharmaceutical ingredient, API) and by the loss of the API (typically to 90% of label claim). For practical product development, methods of accurately determining drug product shelf-life in much shorter time periods are highly desirable (2). The use of simple Arrhenius-based predictions has often been erroneous due to the fact that degradation for many APIs and drug products cannot be considered to follow simple kinetics. In addition, most solid-state APIs and drug products show RH sensitivity, which is not accounted for in the classic Arrhenius equation. The Accelerated Stability Assessment Program (ASAP), which uses measured isoconversion values (times to the threshold of the specification limits at each condition, t_{iso}) and a humidity-corrected Arrhenius equation (Eq. 1), is a highly accelerated methodology that has found widespread use for many APIs and drug products (3–7).

$$\ln\frac{1}{t_{iso}} = lnA - \frac{E_a}{RT} + B(RH) \quad (1)$$

In this modified Arrhenius equation, A is the collision frequency, E_a is the activation energy, R is the gas constant, T is the absolute temperature, and B is the humidity sensitivity factor. While ASAP studies are conducted with open exposure to the RH of the designed conditions, in long-term storage, products are typically protected from the environment to varying extents depending on the particular packaging configuration for the product. Modeling the RH as a function of time in packaging can be done explicitly provided that the moisture sorption isotherm of the dosage forms (and any desiccants) and the permeability of the packaging are known. Combining RH as a function of time with the RH sensitivity from ASAP modeling (Eq. 1) has enabled the accurate prediction of chemical stability from short-term data for small molecules (4).

Biological drug products have become an increasingly important category for providing therapies to patients. A variety of stability-indicating methods are generally employed with proteins, including methodologies similar to those used for small molecules (e.g., HPLC, MS) as well as bioassays and techniques only applicable to large molecules (e.g., electrophoresis, size exclusion chromatography). The greater complexity and size of

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biologics force the consideration of higher level structural factors such as thermal transitions, formation of aggregates, and denaturation (8). Nevertheless, most of the degradation processes that result in drug instability are simple chemical reactions (e.g., oxidations, hydrolyses, deamidations). There are reports of the use of elevated temperatures for predicting the stability of proteins (9–17); however, these examples do not model the impact of RH for solid-state proteins and do not report the long-term accuracy of shelf-life projections. While ASAP has been employed to accurately model the shelf-life of numerous small molecule APIs and corresponding drug products, there have been no published reports on the use of ASAP to similarly model the stability of biological molecules. The present study is therefore the first attempt to determine if the ASAP approach for small molecule APIs can also be applied to at least some biological APIs, recognizing that this could greatly accelerate drug development, both of new biologics and of biosimilars. In order to test the humidity-corrected Arrhenius equation, solid biological APIs were of particular interest.

In order to establish general methodologies for accelerating the stability assessment of biological drugs, a simple peptide was selected as a first step. Peptides are composed of amino acids with primary chemical bonds matching those of proteins; however, their small sizes (less than about 40 amino acids) mean that they generally have less complex higher-order structures. It is hoped that if a methodology can be successfully employed for peptides, it is possible that at least the chemical processes involved in the aging of more complex proteins can be similarly modeled.

Bacitracin (Bc) is a cyclic polypeptide produced by *Bacillus licheniformis* and *Bacillus subtilis* which acts mainly against gram-positive bacteria. Commercial Bc is a mixture of at least nine isoforms, with bacitracin A (BcA) as both the main component and the one with the greatest antibiotic activity (18). BcA (Fig. 1) consists of a ring of seven amino acids and a side chain of five amino acids that terminate with a thiazoline ring. The USP monographs (19) specify that bacitracin must contain at least 40% BcA, at least a combined 70% BcA, Bacitracin B1 (BcB1), B2 (BcB2), and B3 (BcB3), and not over 6% of the inactive Bacitracin F (BcF). Complexation by divalent metal cations with bacitracin is known to significantly increase its stability and activity. Commercially, the most common complex is formed as 1:1 (mole/mole) Bc/zinc(II) (20,21), (BcZn), generally provided as an ointment with white petrolatum.

BcF, the predominant degradant product of BcA, is formed through oxidative deamination of the amino-thiazoline ring to a keto-thiazoline ring (Fig. 1) (22). Published reports on Bc and BcZn degradation rates are limited to the solution state (22).

In the present study, Bc and BcZn powders were exposed to a range of temperature and RH conditions over a period of 21 days. Both loss of the original API (BcA) and formation of the primary degradation product (BcF) were then analyzed to determine isoconversion times at each condition. Although specification limits are not well-established for this product, we assigned values in each case to enable modeling. The short-term, high-temperature models for each condition were used to predict the longer term, lower temperature behavior. The native bacitracin is so unstable under many open (i.e., higher RH) conditions that it is difficult to validate against the modeling predictions. With the zinc complex, loss of parent and formation of primary degradant were more readily compared to long-term results.

EXPERIMENTAL

Materials

Bacitracin powder, bacitracin zinc salt, bacitracin A, and ethylenediaminetetraacetic acid (EDTA) (certified reference grade) were purchased from Sigma-Aldrich (St. Louis, MO). Bacitracin F was purchased from Toku-E, EvoPure® (Tokyo, Japan). All bacitracin products were stored at 5°C until use. HPLC-grade water (H_2O), methanol (MeOH), acetonitrile (ACN), hydrochloric acid (HCl), potassium phosphate monobasic, potassium phosphate dibasic, and sodium hydroxide (NaOH) were purchased from Thermo Fisher Scientific (Waltham, MA).

ASAP Studies

Approximately 25 mg of API were weighed into an uncapped glass vial. All vials were placed in Ball® jars with saturated salt solutions placed in separate Gore-Tex®-covered vials to control the RH, in the appropriate constant temperature ovens. After specified time periods, samples were removed from the ovens, reweighed, and stored at 5°C until analysis.

An HPLC system (Agilent 1100 with diode array detection, Agilent Technologies, Inc., Santa Clara, CA) was used to analyze the stressed and long-term samples using a previously reported isocratic method (23). The mobile phase consisted of 0.111 M potassium phosphate dibasic, 0.111 M potassium phosphate monobasic, 1.4 μM EDTA in 37:58.7:4.3 (*v*/*v*/*v*) H_2O/MeOH/ACN. Samples of stressed and BcA and BcF references samples were prepared at 2 mg/mL 0.1 N HCl, while BcZn samples were prepared using a diluent solution of 40 g/L EDTA adjusted to pH 7.0 using 8 N NaOH. The column (Thermo Scientific ODS Hypersil) was set at 30°C and the autosampler at 5°C, using system thermostats. The injection volume was 50 μL, flow rate 1.0 mL/min, run time of 35 min, and UV detection at 254 nm. All samples were analyzed as a group, with controls and standards bracketing every five samples. In order to account for inconsistencies in the system and slight observed degradation of samples in solution, area percent values were corrected against the averages of the standards from the day in which they were run.

The active BcA and known degradant BcF were identified using the corresponding reference standards and confirmed by liquid chromatography-mass spectrometry (AB/Sciex QStar Elite LC-MS instrument, Applied Biosystems/MDS Sciex Instruments, Framingham, MA; Analyst QS 2.0 data collection; 0.34 mL/min flow rate replacing phosphate with ammonium acetate buffer). The amounts of BcA and BcF present in the stressed samples were normalized based on the reference standards. For isoforms B1 and B2, peak identifications were confirmed by LC-MS. Since standards were not available, values were normalized against initial values.

The sorption isotherm of bacitracin was measured using a DVS Intrinsic instrument (Surface Measurement Systems, LTD; London, UK), with a Troemner (Thorofare, NJ) class 7 stainless steel 1.0 g weight for calibration. Analysis of stability data was carried out using ASAP*prime*® Version 5.0 (FreeThink Technologies, Inc., Branford, CT).

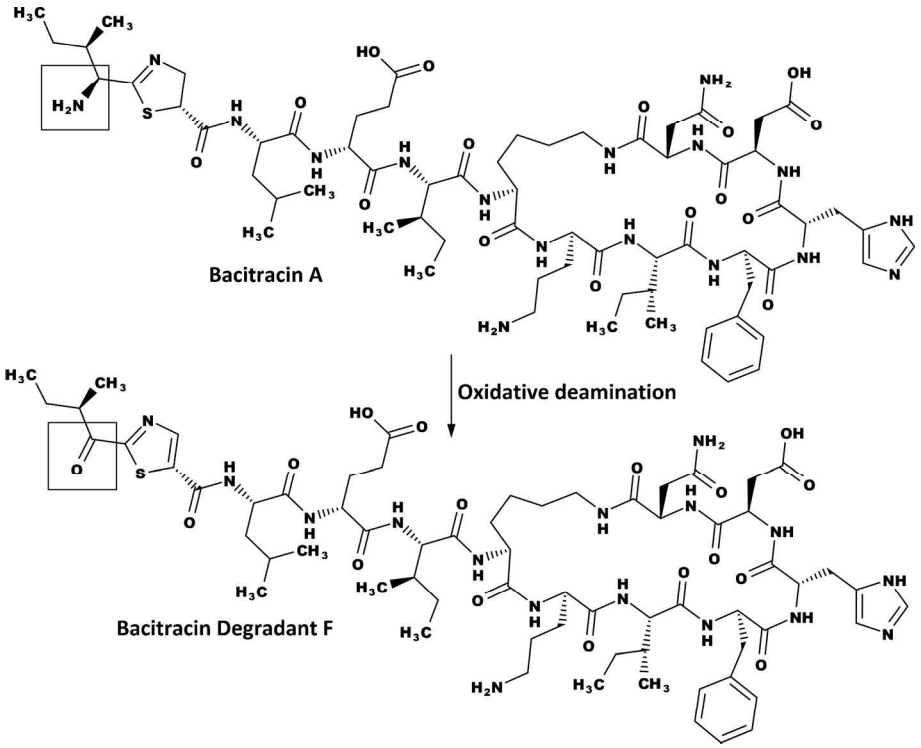

Fig. 1. Bacitracin-A (reactant) reacting by oxidative deamination to give the major degradation product, bacitracin-F

RESULTS AND DISCUSSION

ASAP Experimental Design

Designing an ASAP protocol is dictated by finding a balance between conditions that do not induce a phase change for the material (e.g., melt, deliquescence), provide an adequate range of temperature and RH exposure, ideally hit the specification limit at each condition, and are completed in the desired amount of time. With little known about Bc and BcZn other than that the latter is stored successfully for more than 2 years at room temperature, we first examined the moisture sorption properties of Bc to determine if there was any indication of a critical relative humidity (CRH), which would limit the range of exposure conditions. The sorption isotherm gave no indication of a CRH (water uptake at 80%RH was about 16% *w*/*w*), with hysteresis between sorption and desorption indicating that there may be some molecular rearrangement occurring at high RH conditions.

A general screening protocol for both API forms (Bc and BcZn) was employed involving nine total conditions. ASAP modeling using Eq. 1 requires a minimum of three independent conditions to fit the three parameters. With nine conditions, there are six degrees of freedom, which should provide adequate modeling capability and the ability to assess whether indeed the model fits the data acceptably. The protocol and time points used are shown in Table I. Both native and zinc complexes of bacitracin were exposed as powders to this range of temperature and RH conditions. Whereas most small molecule APIs involve decomposition of a single drug to one or more degradation products, with biological drugs such as bacitracin, the API is not a single pure material. This adds to the complexity both in terms of the analytical determinations and modeling of stability. Determining the stability-indicating changes for such biological APIs, whether they be formation of products or loss of an active, can therefore be challenging in and of itself. For the purposes of the present study, we examined two potentially stability-indicating changes in bacitracin to determine whether both could be reasonably modeled using the ASAP approach: loss of the main active (BcA) and formation of the known degradation product F. In addition, two other isoforms (BcB1 and BcB2) had sufficient signal and change over the ASAP conditions to be modeled.

Loss of Active A

Loss of the active bacitracin form A was examined for both Bc and BcZn, as shown in Table I. For the ASAP data fitting, the isoconversion times at each condition were determined assuming first order loss of active. These isoconversion times were used to fit the modified Arrhenius equation (Eq. 1), resulting in the fitting data shown in Table II. The overall fitting can be compared to the model using dual-axes residual plots (Fig. 2). In these plots, the logarithmic values for the reciprocal isoconversion times are plotted against the best-fit models. In other words, if all data fit the model perfectly, all the points would lie on the zero residual lines for both temperature and RH. As can be seen, the residuals are well within the error bars for determination of the isoconversion times (based on propagating the errors from the measurements estimated to have a 3% standard deviation) and support that the ASAP approach can indeed be used to model the loss of the active peptide. The error bars for the zinc complex are higher since the amount of degradation under the ASAP conditions was less and required extrapolation to determine the isoconversion time in many cases. The appropriateness of the model fitting is

Table I. Results of Designed Aging Using a Range of Highly Accelerated Temperature and Relative Humidity Conditions for Bacitracin (Bc) and Bacitracin Zinc (BcZn)

Time (days)	RH	T (°C)	Active BcA	Active BcZnA	Degradant BcF	Degradant BcZnF	Inactive BcB1	Inactive BcB2
7	27	50	91.76	99.40	1.30	0.31	88.66	94.78
14			88.79	99.21	1.73	0.37	87.2	92.28
21			86.53	98.63	2.10	0.57	88.13	91.71
8	51	50	87.91	99.46	1.52	0.37	91.04	95.75
21			80.46	99.07	3.17	0.63	82.48	70.81
7	11	60	92.18	98.29	1.89	0.53	98.5	100.2
14			82.53	98.61	3.02	0.55	84.29	87.96
21			80.77	95.14	3.44	1.07	83.64	68.59
5	21	60	89.75	98.52	1.72	0.45	92.08	97.26
21			76.65	97.71	4.41	0.93	81.92	68.71
2	63	60	89.53	99.66	1.47	0.37	83.79	97.17
14			51.81	96.78	8.92	1.04	55.22	48.83
2	11	70	88.54	98.28	2.09	1.02	87.45	72.19
19			59.23	94.99	7.22	1.78	71.04	49.85
1	50	70	82.38	98.62	2.34	0.64	80.1	67.32
6			49.91	97.00	8.44	1.25	57.12	42.92
1	0	80	88.34	98.14	1.93	0.97	94.11	92.38
8			64.59	93.28	5.99	1.78	77.46	54.79
1	51	80	68.03	97.43	4.50	0.88	64.77	55.9
2			50.76	96.36	7.00	1.37	54.14	44.12

All data are corrected area percents

further indicated by the high values of R^2 and Q^2 (Table II) for loss of BcA, with or without the zinc complexation. Q^2, the cross-validated R^2, is particularly sensitive to how well the data fit the model since it indicates how close each measured point is to the predicted value when the predictions are made in each case with all the other points except that one (24,25).

The fitted parameters (Table II) indicate that the loss of BcA without the zinc present is relatively rapid, but with an activation energy and B-factor (RH sensitivity) similar to most small molecules. The presence of the zinc greatly increases the stability and lowers the RH sensitivity. Interestingly, the increase in stability caused by the presence of zinc is predominantly due to a combination of a decrease in the collision frequency (A-factor) and sensitivity to RH (B-factor) rather than increasing the activation energy (E_a) for the reaction. This suggests that the zinc is responsible for decreasing mobility of the bacitracin rather than making the barrier to the degradation greater. Whatever the case, the difference in estimated shelf-life is dramatic (mean predicted time to hit the specification limit of 6 months *vs* 10.4 years at 25°C/60%RH, without packaging, for BcA and BcZnA, respectively).

Loss of Bacitracin Isoforms

A similar analysis was performed on BcB1 and BcB2 (data shown in Table I; results in Table II), two isoforms of Bc (not seen to degrade significantly with BcZn). Again, the applicability of the modified Arrhenius equation (Eq. 1) to the loss of activity for these two isoforms is demonstrated by the high R^2 and Q^2 values. Each isoform is predicted to show rapid loss of activity at room temperature; however, since these are not the active API form, their loss of activity would not be expected to limit the shelf-life of the product. The fact

Table II. ASAP Analysis of Data from Using ASAPprime® for Designed Aging Using a Range of Highly Accelerated Temperature and Relative Humidity Conditions for Bacitracin and Bacitracin Zinc

Peptide form	ln A	E_a (kcal/mol)	B	R^2	Q^2	Mean predicted (open) shelf-life at 25°C/60%RH (years)
Active BcA	38.6 ± 5.9	25.7 ± 4.0	0.031 ± 0.009	0.984	0.966	0.5
Active BcZnA	34.3 ± 7.6	24.2 ± 5.2	0.010 ± 0.010	0.938	0.868	10.4
Degradant BcF	44.6 ± 4.9	29.2 ± 3.3	0.021 ± 0.007	0.950	0.838	0.1
Degradant BcZnF	45.8 ± 2.2	32.0 ± 1.5	0.008 ± 0.003	0.981	0.955	6.6
Inactive BcB1	38.3 ± 7.0	25.8 ± 4.7	0.044 ± 0.011	0.957	0.893	0.4
Inactive BcB2	46.2 ± 7.4	30.9 ± 5.0	0.036 ± 0.009	0.970	0.846	1.1

For loss of an active (BcA, BcZnA, BcB1, BcB2), the specification limit was assumed to be 90%. For formation of the main degradation product (BcF, BcZnF), the specification limit was assumed to be 1.0% using a diffusional model to estimate the isoconversion time at each condition

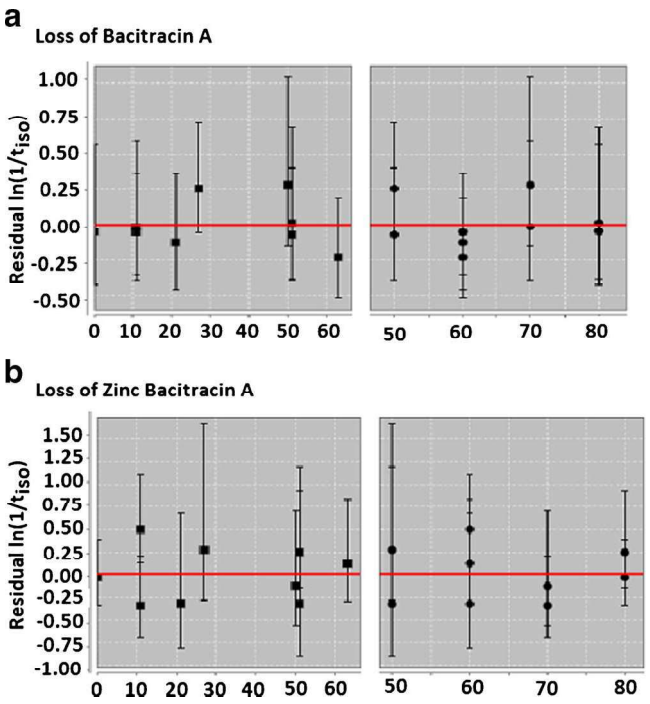

Fig. 2. Residual graphs showing the ASAP model fitting to the moisture-modified Arrhenius equation for loss of the active form of bacitracin in the solid state. The *top graph* (**a**) is for native bacitracin A; the *bottom graph* (**b**) is for the zinc complex. In each case, fitting was done based on estimating the time to an assumed specification limit of 90% of initial value (isoconversion time)

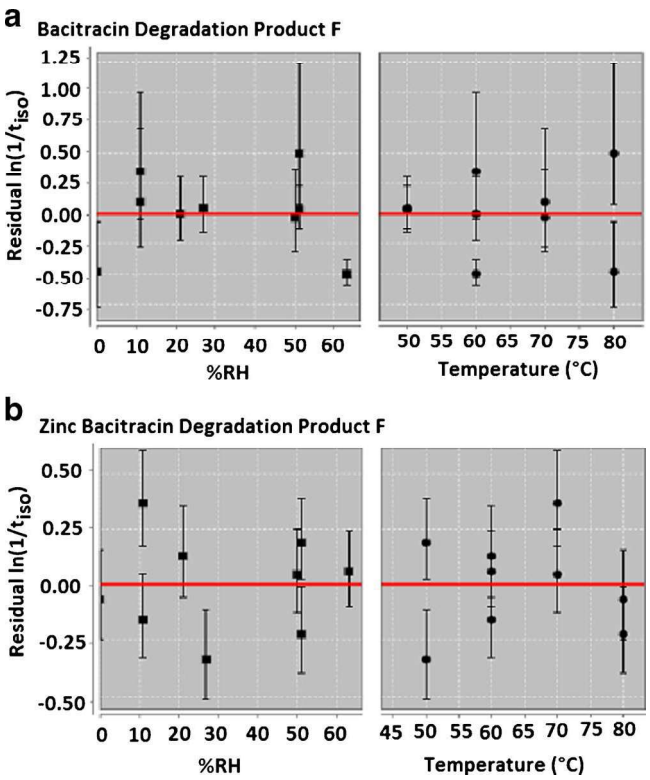

Fig. 3. Residual graphs showing the ASAP model fitting to the moisture-modified Arrhenius equation for formation of the F degradation product of bacitracin in the solid state. The top graph (**a**) is for the native form of bacitracin while the bottom graph (**b**) is for the zinc complex. In each case, fitting was done based on a diffusional model (square root of time) to estimate the time to an assumed specification limit of 1.0% (isoconversion time)

that a particular change occurs rapidly yet does not influence the drug product shelf-life is one of the factors that distinguishes small molecules from biologics, even the relatively small peptide used in the present study.

Formation of Degradant F

Oxidative deamination of both BcA and BcZnA was observed to the corresponding BcF and BcZnF (Table I). While there were a number of other degradation products observed (hence loss of active and formation of F do not directly track), for both bacitracin forms, in both cases, F was the predominant degradant observed. As with loss of the parent APIs, ASAP modeling could be carried out effectively here (assuming a 1.0% specification limit), as shown in Table II. In this case, the growth of the F degradant as a function of time at each condition was found to follow a square root of time dependence, consistent with diffusion being a critical process in the rate-determining step for this reaction in the solid state. That the degradant formation is non-linear is an important reason for applying the isoconversion approach to accelerated stability. If one assumed linearity in the growth of degradant *versus* time (as is commonly assumed in stability studies), the apparent rate constant would depend on the amount of conversion at each condition, resulting in apparent non-Arrhenius behavior. With the isoconversion approach, even without recognizing the curvature in the degradant growth as a function of time, a reasonable fit to the Arrhenius equation can be achieved. An error bar for the data was assumed to be 10% of the value (10%RSD), with a minimum error bar of 0.02%. Using the diffusion model (square root of time) to estimate the isoconversion time at each condition, the fitting to the ASAP model again appears quite good as shown in the residual graphs (Fig. 3) and R^2 and Q^2 values (Table II).

Comparison to Long-Term Results

Ultimately, the best indicator of a predictive model's appropriateness is determined by how well it estimates the long-term behavior. Both the native and zinc complex of bacitracin were stored at 30°C/53%RH (open) for 12 months and 40°C/75%RH (open) for 6 months, with analyses at the 3-, 6- and 12-month time points for both loss of A and formation of F. In Fig. 4, both the ASAP model-predicted and real-time observed formation of F (BcF, BcZnF) at 30°C/53%RH and 40°C/75%RH are shown for both bacitracin forms. In this case, the formation of F for the native form of bacitracin is omitted at 40°C/75%RH due to nearly complete decomposition at that condition by the first time point. Figure 5 shows the same comparison for loss of the parent bacitracin A (BcA, BcZnA). The 40°C/75%RH condition for loss of BcA was again omitted due to extreme degradation at 3 months. As can be seen, with both storage conditions and bacitracin forms, both formation of the major degradant and loss of the parent modeled well using the ASAP process.

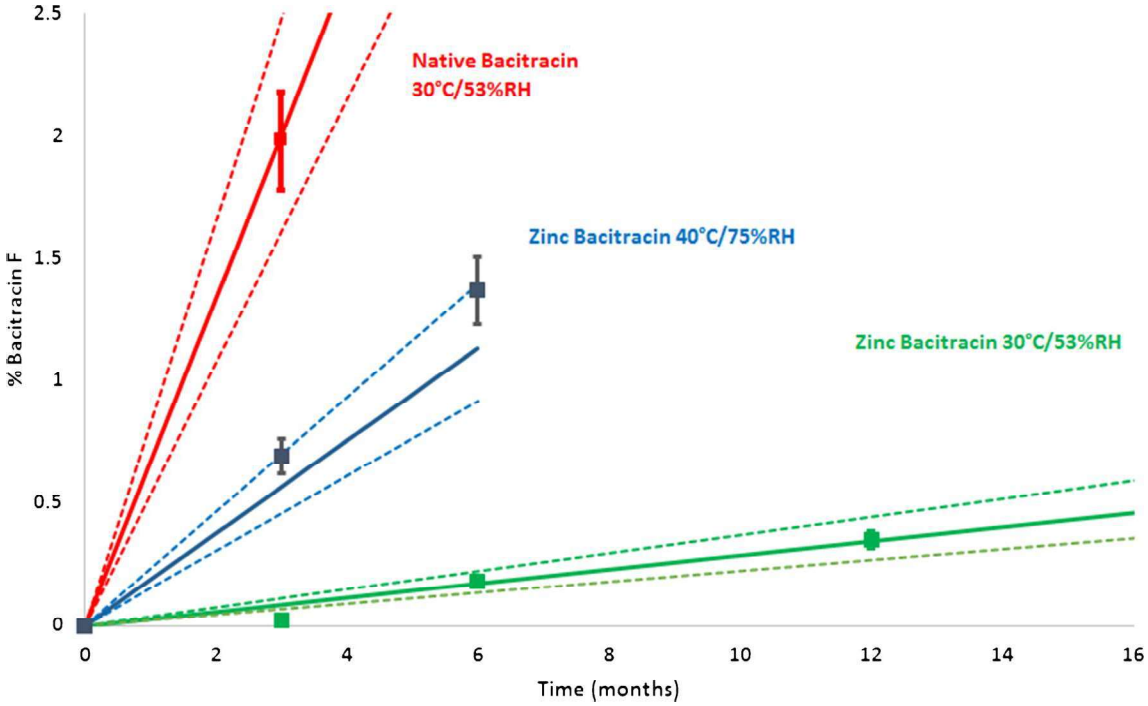

Fig. 4. Comparison between the accelerated stability-model predicted and real-time observed formation of bacitracin degradant F for storage at 30°C/53%RH (*open*) of both the native and zinc complex of bacitracin and 40°C/75%RH for the zinc complex. In each case, the ASAP model predicted mean behavior is shown as a *solid line* with *dashed lines* representing one standard deviation slower and faster predicted degradation. The *squares* represent the measured values with *error bars* representing 10% relative standard deviations

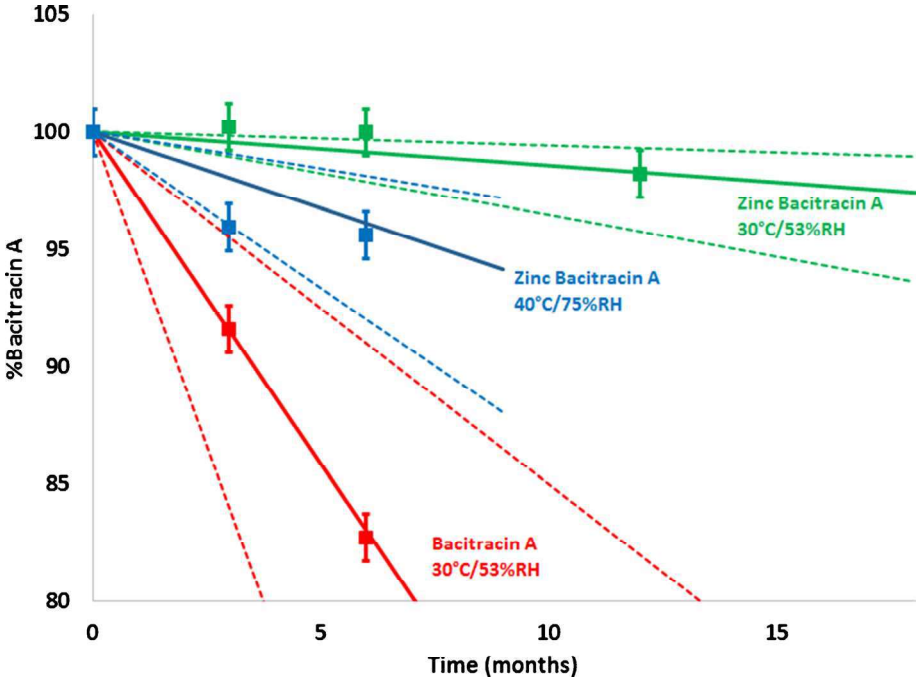

Fig. 5. Comparison between the accelerated stability-model predicted and real-time observed loss of active bacitracin (bacitracin A) of both the native and zinc complex forms. In each case, the ASAP model predicted mean behavior is shown as a *solid line* with *dashed lines* representing one standard deviation slower and faster predicted loss of active. The *squares* represent the measured values with *error bars* representing a 1% standard error

CONCLUSIONS

The ASAP approach was applied for the first time to a peptide, the antibiotic active pharmaceutical ingredient bacitracin. Bacitracin and its complex with zinc were exposed to temperature and relative humidity conditions from 50 to 80°C and from 0 to 63% for up to 21 days. High-performance liquid chromatography was used to analyze the stressed samples for both degradant formation (bacitracin F) and loss of the active (bacitracin A) and two inactive isoforms, with identities confirmed by mass spectrometry. These data were then analyzed using a humidity-corrected Arrhenius equation and isoconversion approach to create a shelf-life predicting model for typical storage conditions. Model fitting was found to be good with low residuals in both temperature and relative humidity axes for all parameters examined. The generated model's predictions for both the native and zinc complex of bacitracin for both formation of the major degradation product (F) and loss of the active isoform (A) were consistent with longer term measured values at 30°C/53%RH and 40°C/75%RH, validating this approach for accelerating the determination of long-term stability of a peptide.

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