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- (71) Applicant (for all designated States except US): **NEREUS PHARMACEUTICALS, INC.** [US/US]; 10480 Wateridge Circle, San Diego, CA 92121 (US).

CA 92109 (US). **REDDINGER, Natasha** [US/US]; 4933 Naragansett Ave. Apt. 3, San Diego, CA 92107 (US). **BILLSTROM, Cheryl** [US/US]; 6747 Friars Road, Apt. 109, San Diego, CA 92108 (US).

(74) Agent: **HART, Daniel**; Knobbe Martens Olson & Bear, LLP, 2040 Main Street, Fourteenth Floor, Irvine, CA 92614 (US).

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- (72) Inventors; and
- (75) Inventors/Applicants (for US only): **POTTS, Barbara** [US/US]; 2029 Oro Verde Road, Escondido, CA 92029 (US). **SINGH, Ramsharan** [IN/US]; 12292 Blanton Lane, San Diego, CA 92128 (US). **CHU, Jan-Jon** [US/US]; 2621 Valewood Avenue, Carlsbad, CA 92010 (US). **MAI, Bao Viet** [US/US]; 826 Sapphire Street Apt. C, San Diego,

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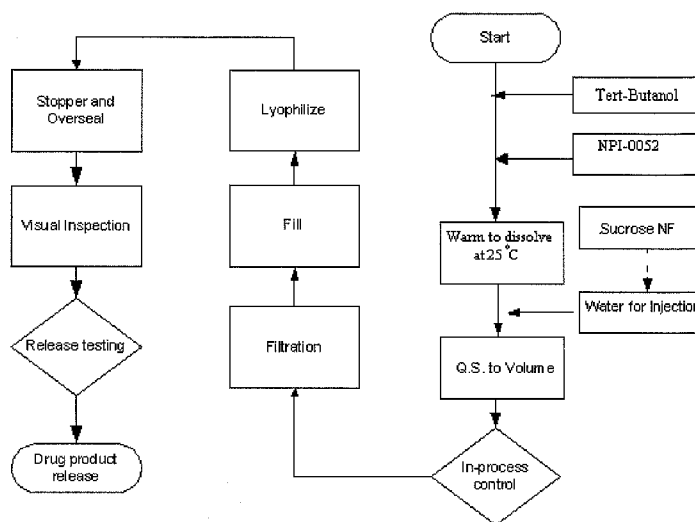


Figure 1

(57) Abstract: Lyophilized formulations comprising Salinosporamide A or analogs thereof are provided. In some aspects, lyophilized formulations comprising Salinosporamide A or analogs thereof and bulking agents are provided. Also provided are methods of lyophilizing Salinosporamide A or analogs thereof. In some aspects, a solvent or co-solvent system is utilized. Also provided are methods of administering Salinosporamide A or analogs thereof to patients.

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LYOPHILIZED FORMULATIONS OF SALINOSPORAMIDE A

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit under 35 U.S.C. § 119(e) of U.S. provisional application Serial No. 60/888,025, filed on February 2, 2007, and U.S. provisional application Serial No. 60/986,891, filed on November 9, 2007, the disclosures of which are hereby expressly incorporated by reference in their entirety and are hereby made a portion of this application.

FIELD OF THE INVENTION

[0002] In some embodiments, the present invention relates to lyophilized formulations of Salinosporamide A.

BACKGROUND OF THE INVENTION

[0003] Cancer is a leading cause of death in the United States. Despite significant efforts to find new approaches for treating cancer, the primary treatment options remain surgery, chemotherapy and radiation therapy, either alone or in combination. Surgery and radiation therapy, however, are generally useful only for fairly defined types of cancer, and are of limited use for treating patients with disseminated disease. Chemotherapy is the method that is generally useful in treating patients with metastatic cancer or diffuse cancers such as leukemias. Although chemotherapy can provide a therapeutic benefit, it often fails to result in cure of the disease due to the patient's cancer cells becoming resistant to the chemotherapeutic agent. Due, in part, to the likelihood of cancer cells becoming resistant to a chemotherapeutic agent, such agents are commonly used in combination to treat patients.

[0004] Similarly, infectious diseases caused, for example, by bacteria, fungi and protozoa are becoming increasingly difficult to treat and cure. For example, more and more bacteria, fungi and protozoa are developing resistance to current antibiotics and chemotherapeutic agents. Examples of such microbes include *Bacillus*, *Leishmania*, *Plasmodium* and *Trypanosoma*.

[0005] Furthermore, a growing number of diseases and medical conditions are classified as inflammatory diseases. Such diseases include conditions such as asthma to cardiovascular diseases. These diseases continue to affect larger and larger numbers of people worldwide despite new therapies and medical advances.

[0006] Therefore, a need exists for additional chemotherapeutics, anti-microbial agents, and anti-inflammatory agents to treat cancer, inflammatory diseases and infectious disease. A continuing effort is being made by individual investigators, academia and companies to identify new, potentially useful chemotherapeutic and anti-microbial agents.

[0007] Marine-derived natural products are a rich source of potential new anti-cancer agents and anti-microbial agents. The oceans are massively complex and house a diverse assemblage of microbes that occur in environments of extreme variations in pressure, salinity, and temperature. Marine microorganisms have therefore developed unique metabolic and physiological capabilities that not only ensure survival in extreme and varied habitats, but also offer the potential to produce metabolites that would not be observed from terrestrial microorganisms (Okami, Y. 1993 *J Mar Biotechnol* 1:59). Representative structural classes of such metabolites include terpenes, peptides, polyketides, and compounds with mixed biosynthetic origins. Many of these molecules have demonstrable anti-tumor, anti-bacterial, anti-fungal, anti-inflammatory or immunosuppressive activities (Bull, A.T. *et al.* 2000 *Microbiol Mol Biol Rev* 64:573; Cragg, G.M. & D.J. Newman 2002 *Trends Pharmacol Sci* 23:404; Kerr, R.G. & S.S. Kerr 1999 *Exp Opin Ther Patents* 9:1207; Moore, B.S 1999 *Nat Prod Rep* 16:653; Faulkner, D.J. 2001 *Nat Prod Rep* 18:1; Mayer, A. M. & V.K. Lehmann 2001 *Anticancer Res* 21:2489), validating the utility of this source for isolating invaluable therapeutic agents. Further, the isolation of novel anti-cancer and anti-microbial agents that represent alternative mechanistic classes to those currently on the market will help to address resistance concerns, including any mechanism-based resistance that may have been engineered into pathogens for bioterrorism purposes.

SUMMARY OF THE INVENTION

[0008] Some embodiments include a lyophilized formulation comprising Salinosporamide A or an analog thereof. One embodiment includes a bulking agent. In one

embodiment, the bulking agent comprises polyvinylpyrrolidone, a sugar, a sugar analog, or mixtures thereof. In one embodiment, the bulking agent comprises mannitol, lactose, trehalose, a cyclodextrin or mixtures thereof. In one embodiment, the bulking agent comprises sucrose. In one embodiment, the cyclodextrin is selected from the group consisting of a hydroxypropyl beta-cyclodextrin, a sulfobutylether beta-cyclodextrin, or mixtures thereof. In one embodiment, the bulking agent comprises KOLLIDON®. In one embodiment, the bulking agent comprises mannitol and sucrose.

[0009] Another embodiment includes a container comprising lyophilized Salinosporamide A or an analog thereof. In one embodiment, a bulking agent is included in the container. In one embodiment, the container comprises from about 0.2 to 4 mg of Salinosporamide A or an analog thereof. In one embodiment, the container is a type 1 flint tubing vial.

[0010] Another embodiment includes a method of lyophilizing compounds comprising β lactone rings, for example, Salinosporamide A or analog thereof, including dissolving the Salinosporamide A or analog thereof and a bulking agent in a solvent or co-solvent system, freezing the resulting solution, and drying the frozen solution. In one embodiment, the solvent comprises an alcohol. In one embodiment, the alcohol is t-butyl alcohol. In one embodiment, the co-solvent system comprises t-butyl alcohol and water. One embodiment includes dissolving the Salinosporamide A or analog thereof in an alcohol, dissolving the bulking agent in water, and mixing the resulting two solutions.

[0011] Another embodiment includes a method of administering Salinosporamide A or an analog thereof, including reconstituting the Salinosporamide A or analog thereof by adding a solvent or co-solvent diluent to a lyophilized formulation of the Salinosporamide A or analog thereof, and injecting the resulting solution into a patient. One embodiment includes storing the solution from about 2°C to about 8°C prior to injection.

[0012] Other embodiments include a method of treating cancer, inflammation, or an infectious disease, comprising administering to a patient or animal a formulation of Salinosporamide A or analog thereof prepared from a lyophilized product described above. In one embodiment, the cancer is selected from one or more of breast cancers; osteosarcomas, angiosarcomas, fibrosarcomas and other sarcomas; leukemias; sinus tumors; ovarian, uretal,

bladder, prostate and other genitourinary cancers; colon, esophageal and stomach cancers and other gastrointestinal cancers; rectal cancers; lung cancers; lymphomas; myelomas; pancreatic cancers; liver cancers; kidney cancers; endocrine cancers; skin cancers; melanomas; angiomas; and brain or central nervous system (CNS; glioma) cancers. In one embodiment, the infectious disease is selected from one or more of infection with *B. anthracis*, *Plasmodium*, *Leishmania*, and *Trypanosoma*. In one embodiment, the administration comprises injecting the formulation into the patient. In one embodiment, the administration comprises administering the formulation intravenously.

BRIEF DESCRIPTION OF THE DRAWINGS

[0013] Figure 1 is a flow chart illustrating methods of manufacturing lyophilized formulations.

[0014] Figure 2 is a graph showing results of a projected shelf life analysis using a 95% confidence interval for a lyophilized Salinosporamide A formulation (Lot# NPI-0052-06-107) stored at -20°C.

[0015] Figure 3 is a graph showing results of a projected shelf life analysis using a 95% confidence interval for a lyophilized Salinosporamide A formulation (Lot# NPI-0052-06-107) stored at 5°C.

[0016] Figure 4 is a graph showing results of a projected shelf life analysis using a 95% confidence interval for a lyophilized Salinosporamide A formulation (Lot# NPI-0052-06-107) stored at 25°C.

[0017] Figure 5 is a graph showing an accelerated stability study for several lyophilized Salinosporamide A formulations stored at various temperatures, where Salinosporamide A purity was measured as a function of time. Two samples containing sucrose were prepared according to the methods of Examples 7 and 10, with 60 mg and 80 mg of sucrose, respectively. Two samples containing Kollidon were prepared according to the method of Examples 3 and 4 with the samples comprising KOLLIDON® 12PF or KOLLIDON® 17PF. At various times, the lyophilized product was reconstituted and the purity determined.

[0018] Figure 6 is a graph showing an accelerated stability study for several lyophilized Salinosporamide A formulations stored at various temperatures, where the amount of the NPI-0049/2083 impurities was measured as a function of time. Two samples containing sucrose were prepared according to the methods of Examples 7 and 10, with 60 mg and 80 mg of sucrose, respectively. Two samples containing Kollidon were prepared according to the method of Examples 3 and 4 with the samples comprising KOLLIDON® 12PF or KOLLIDON® 17PF. At various times, the lyophilized product was reconstituted and the purity determined.

[0019] Figure 7 is a graph showing an accelerated stability study for several lyophilized Salinosporamide A formulations stored at various temperatures, where the amount of the NPI-0050 impurity measured as a function of time. Two samples containing sucrose were prepared according to the methods of Examples 7 and 10, with 60 mg and 80 mg of sucrose, respectively. Two samples containing Kollidon were prepared according to the method of Examples 3 and 4 with the samples comprising KOLLIDON® 12PF or KOLLIDON® 17PF. At various times, the lyophilized product was reconstituted and the purity determined.

[0020] Figure 8 is a graph showing long term stability results of various lyophilized Salinosporamide A formulations at 5°C, where Salinosporamide A purity was measured as a function of time.

[0021] Figure 9 is a graph showing long term stability results of various lyophilized Salinosporamide A formulations at 5°C, where the amount of the NPI-0049/2083 impurities was measured as a function of time.

[0022] Figure 10 is a graph showing a shelf life analysis of a lyophilized Salinosporamide A formulation (Lot # NPI-0052-06-111) stored at 5°C with a confidence interval of 95%.

[0023] Figure 11 is a graph showing results for % potency of dose solutions at 5°C and 25°C. Diluent and dose solutions are indicated in Table 25.

[0024] Figure 12 is a graph showing results for % Potency of various dose solutions at 5°C. Dose solutions are indicated in Table 25.

[0025] Figure 13 is a graph showing a sub-ambient Differential Scanning Calorimetry thermograph for a Salinosporamide A formulation without an annealing step. The study was run at a rate of 10°C/min between 30°C and -60°C with an annealing temperature at -12°C for 2 minutes.

[0026] Figure 14 is a graph showing a sub-ambient Differential Scanning Calorimetry thermograph for a Salinosporamide A formulation with annealing at -12°C. The study was run at a rate of 10°C/min between 30°C and -60°C with an annealing temperature at -12°C for 2 minutes.

[0027] Figure 15 is a graph showing a correlation between the residual tert-butanol of finished drug product per vial and the total lyophile weight per vial.

[0028] Figure 16 is a graph showing Salinosporamide A *in-situ* phase solubility in 10, 20 and 30% H₂O-TBA (v/v) solutions. Salinosporamide A was prepared at 2 mg/mL in TBA as a stock solution. HPLC water was spiked into the Salinosporamide A solution aliquot to make up the equivalent 10, 20 and 30% H₂O-TBA solutions at room temperature.

[0029] Figure 17 is a graph showing sucrose *in-situ* phase solubility in % H₂O-TBA to determine the miscible zone and phase solubility for formulation development. The line represents the estimated sucrose solubility curve.

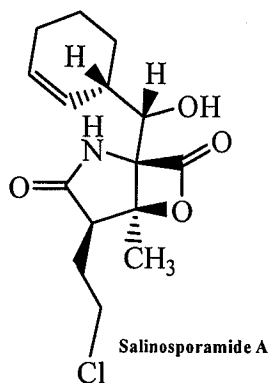
[0030] Figure 18 is a graph showing mannitol *in-situ* phase Solubility in %H₂O-TBA to determine the miscible zone and phase solubility for formulation development. The line represents the estimated mannitol solubility curve.

[0031] Figure 19 is a graph showing Salinosporamide A bulk solution stability as a function of % purity over time at 2-8 °C and 25 °C, for a bulk solution containing 1 mg/mL API with 35 mg/mL sucrose in 14% H₂O-TBA.

[0032] Figure 20 is a graph showing Salinosporamide A bulk solution stability as a function of % degradant, NPI-2054, over time at 2-8 °C and 25 °C, for a bulk solution containing 1 mg/mL API with 35 mg/mL sucrose in 14% H₂O-TBA.

DETAILED DESCRIPTION OF CERTAIN EMBODIMENTS

[0033] Salinosporamide A (NPI-0052) is a potent 20S proteasome inhibitor that is currently in clinical development for the treatment of cancer. The structure of Salinosporamide A is:



[0034] Salinosporamide A and its analogs have various biological activities. For example, the compounds have chemosensitizing activity, anti-microbial, anti-inflammation, radiosensitizing, and anti-cancer activity. Studies have been conducted that show Salinosporamide A and its analogs have proteasome inhibitory activity, effect NF- κ B / I κ B signaling pathway, and have anti-anthrax activity. Salinosporamide A and several analogs, as well as biological activity of the same, are described in U.S. Provisional Patent Applications Nos., 60/480,270, filed June 20, 2003; 60/566,952, filed April 30, 2004; 60/627,461, filed November 12, 2004; 60/633,379, filed December 3, 2004; 60/643,922, filed January 13, 2005; 60/658,884, filed March 4, 2005; 60/676,533, filed April 29, 2005; 60/567,336, filed April 30, 2004; 60/580,838, filed June 18, 2004; 60/591,190, filed July 26, 2004; 60/627,462, filed November 12, 2004; 60/644,132, filed January 13, 2005; 60/659,385, filed March 4, 2005; 60/790,168, filed April 6, 2006; 60/816,968, filed June 27, 2006; 60/836,155, filed August 7, 2006; 60/844,132, filed September 12, 2006; 60/885,379, filed January 17, 2007; 60/791,625, filed April 13, 2006; 60/797,553, filed May 3, 2006 and 60/849,908, filed October 5, 2006; U.S. Patent Applications Nos., 10/871,368, filed June 18, 2004; 11/118,260, filed April 29, 2005; 11/412,476, filed April 27, 2006; 11/453,374, filed June 15, 2006; and 11/517,899, filed September 8, 2006; and International Patent Applications Nos., PCT/US2004/019543, filed June 18, 2004; PCT/US2005/044091, filed December 2, 2005;

PCT/US2005/014846, filed April 29, 2005; and PCT/US2006/016104, filed April 27, 2006; each of which is hereby incorporated by reference in its entirety.

[0035] In order to develop a suitable parenteral formulation and manufacturing process for Salinosporamide A and its analogs, factors such as the potency, solubility and chemical stability of the active pharmaceutical ingredient (API) were considered. The API is stable for at least 12 months when stored at -20°C desiccated but is labile in aqueous solution at neutral and basic pH values. The predominant degradation pathway identified under these conditions involves hydrolysis of the β -lactone ring with subsequent elimination of chlorine.

[0036] Accordingly, in some embodiments, a dry powder formulation of Salinosporamide A is provided that may be stably stored for prolonged periods prior to use. The powder formulation may then be reconstituted using a suitable solvent or co-solvent system prior to injection into a patient (e.g., IV bolus injection or IV infusion). In some embodiments, the reconstituted formulation may be administered to animals, for example, mammals. In some embodiments, the dry powder formulation is a lyophilized formulation. In some embodiments, the lyophilized formulation includes the API and a bulking agent. Non-limiting examples of suitable bulking agents include polyvinylpyrrolidone (povidone), a sugar, a sugar analog, or mixtures thereof. Non-limiting examples of suitable sugars or sugar analogs include sucrose, mannitol, lactose, trehalose, and cyclodextrins. Non-limiting examples of suitable cyclodextrins include hydroxypropyl beta-cyclodextrin and sulfobutylether beta-cyclodextrin. In one embodiment, the bulking agent is KOLLIDON® 12PF or 17PF (BASF, Germany). In one embodiment, the bulking agent is mannitol. In another embodiment, the bulking agent is a combination of mannitol and sucrose. In yet another embodiment, the bulking agent is sucrose.

[0037] In some embodiments, the lyophilized formulations described above are placed in vials in which the formulation can be reconstituted with a suitable solvent or co-solvent. In various embodiments, each vial comprises from about 0.2 mg to about 4 mg, from about 1 mg to about 3 mg, from about 2 mg to about 4 mg, or from about 1.5 mg to about 2.5 mg of API. In one embodiment, each vial comprises about 2 mg of API. In various embodiments, each vial comprises from about 10 mg to about 100 mg, from about 20 mg to

about 90 mg, from about 30 mg to about 80 mg, from about 40 mg to about 80 mg, or from about 60 mg to about 80 mg of bulking agent.

[0038] In some embodiments, a lyophilized formulation as described above may be stored at about 2°C to about 8°C or alternatively below -10°C (e.g., about -20°C) for prolonged periods without significant degradation. In various embodiments, the lyophilized formulation maintains purity in excess of about 95% after about 6 months, 12 months, or 18 months of storage. In vials containing about 2 mg of API and 80 mg bulking agent, the moisture content may be below about 2 mg, 1.5 mg, 1 mg, or 0.5 mg and the residual alcohol content may be below about 6 mg, 4 mg, or 2 mg. Such low solvent amounts may support the stability of the API during storage.

[0039] In some embodiments, the dry powder formulation described above is obtained by lyophilization of a bulk solution of the API and the bulking agent. In one embodiment, the solvent used in the pre-lyophilized solution is non-aqueous. In some embodiments, the non-aqueous solvent is an alcohol. In one embodiment, the alcohol is t-butyl alcohol. In some embodiments, the solvent is a co-solvent system. In some embodiments, the co-solvent system is a mixture of water with an alcohol. In one embodiment, the alcohol is t-butyl alcohol. In various embodiments, the co-solvent system comprises from about 5% to about 30%, from about 10% to about 25%, or from about 15% to about 20% water. In some embodiments, the pH of the solution is adjusted using a suitable acid, base, or buffer. In one embodiment, diluted HCl is used to adjust the pH. In some embodiments comprising a co-solvent mixture, the pH of water is adjusted prior to mixture. In various embodiments, the pH of the water is adjusted to be from about 3.0 to about 5.0.

[0040] Some embodiments include a method of manufacturing the lyophilized formulation described above. For example, figure 1 depicts a flow chart illustrating one suitable manufacturing process. Depending on the particular embodiment, steps may be added to those depicted in the flowchart or some steps may be removed. In addition, the order of steps may be rearranged depending on the application. In the process depicted in figure 1, t-butyl alcohol is added to an appropriate vessel followed by addition of the API (e.g., Salinosporamide A (NPI-0052)). The API is dissolved in the t-butyl alcohol by

warming. In some embodiments, a shear mixer is used. Separately, the bulking agent (e.g., sucrose) is dissolved in water. The water solution is then added to the t-butyl alcohol solution. Additional t-butyl alcohol is added to obtain the desired final volume. In process control is then performed on the resulting solution. In process control may include HPLC assays to determine potency and purity of the API, checking the fill volume (e.g., 2.0 mL +/- 0.05 mL), checking the specific gravity (e.g., 0.840 +/- 0.005), checking the bioburden (e.g., < 10cfu/100 ml), and checking the clarity of the solution.

[0041] If the solution passes the in-process control, it is then filtered such as by sterile filtration using a 2" OPTICAP® DURAPORE® PVDF 0.22µm membrane (Millipore, Billerica, MA) (e.g., for a 12 L batch). Filling prior to lyophilization may be conducted at room temperature or from about 2°C to about 8°C. The solution is then lyophilized. The lyophilization cycle may include annealing at about -15°C (e.g., for about 1-4 hours), freezing at about -40°C to about -50°C (e.g., for from about 1 to about 4 hours), first drying at about -31°C (e.g., for about 24 hours), second drying at about 25°C (e.g., for about 30 hours), and unloading at about 5°C or 20°C. The chamber vacuum used may be about 200 mTorr. The lyophilized product may then be stoppered and oversealed prior to visual inspection, release testing, and final product release. In some embodiments, packaging includes using a SCHOTT® Type 1 USP 20 mm flint tubing vial (Schott North America, Inc., Elmsford NY) with a West FLUROTEC® coated stopper and a West flip-off seal (West Pharmaceutical Services, Lionville, PA).

[0042] The lyophilized API may be reconstituted into a solution using any suitable solvent or co-solvent system. Non-limiting solvent systems are described in more detail in U.S. Provisional Application No. 60/849,908, filed October 5, 2006, which is incorporated herein by reference in its entirety. In one embodiment, the solvent system comprises a mixture of polyethylene glycol (for example, PEG-400, or PEG-300), ethanol, and a citrate buffer. In another embodiment, the solvent system comprises a mixture of propylene glycol, ethanol, and citrate buffer. In one embodiment, about 10 ml or about 20 ml of the solvent system may be added to a vial containing about 2 mg of API to produce a 0.2 or 0.1 mg/ml dosing solution, respectively. In some embodiments, a reconstituted solution of about 0.01 mg/ml, about 0.02, about 0.03 mg/ml, about 0.04 mg/ml, about 0.05 mg/ml, about

0.06 mg/ml, about 0.07 mg/ml, about 0.08 mg/ml, or about 0.09 mg/ml is prepared. The reconstituted solution may then be administered to a patient (e.g., via IV injection or infusion). In some embodiments, the reconstituted solution may be administered to an animal, for example, a mammal, for example, a rodent. In some embodiments, the dosing solution is refrigerated and administered within 3-5 hours to avoid hydrolytic degradation. In another embodiment, the refrigerated dosing solution is administered within 8-24 hours of reconstitution. In some embodiments, the dosing solution is administered at room temperature within 1-3 hours.

[0043] In various embodiments, the formulations, methods, uses, and kits described herein include any compound comprising a β lactone ring, for example, Salinosporamide A. Techniques are provided that allow for the compounding of bulk solutions and subsequent lyophilization of β lactone containing compounds while protecting against hydrolysis, which is an inherent problem with formulating such compounds.

EXAMPLES

Example 1 – HPLC protocols for evaluating purity and potency of lyophilized API

[0044] To evaluate the purity and potency of lyophilized Salinosporamide A, an HPLC protocol was developed allowing detection of Salinosporamide A and its degradants in the presence of the bulking agent(s). For each protocol, the lyophilized formulation was reconstituted in a diluent comprising 0.2% TFA in 45% water/55% acetonitrile. 4.0 mL of the diluent was added to vials containing lyophilized formulations comprising 2 mg of Salinosporamide A. The contents were mixed until all solids were dissolved and a clear solution obtained. After reconstitution, the theoretical concentration of Salinosporamide A is 0.5 mg/mL.

Protocol for sucrose as the bulking agent

[0045] Five calibration solutions of Salinosporamide A were prepared having concentrations of 40 μ g/mL, 200 μ g/mL, 500 μ g/mL, 600 μ g/mL, and 800 μ g/mL, respectively. As a check of system suitability, the 500 μ g/mL standard solution is used as a check standard and is injected six times at the beginning of the sequence after the diluent injection, two injections after every ten sample injections, and two injections at the end of the

run to ensure system suitability throughout the entire run. The % RSD of the area response for the six suitability injections at the beginning of the run plus the check standard injections is checked to be $\leq 2.0\%$. The chromatographic conditions were as indicated in Table 1.

Table 1.

Chromatographic conditions for sucrose containing samples.

Column:	ACE C18, 150 × 4.6 mm, 5 μ m, 100Å		
Flow rate:	1.5 mL/min		
Column temperature:	30°C		
Autosampler temperature:	5°C		
Inject volume:	50 μ L (with needle wash using ACN)		
Run time:	41 min.		
Mobile Phase:	Eluant A: 0.01% TFA in pure water or HPLC water Eluant B: 0.01% TFA in HPLC acetonitrile (ACN)		
Gradient Table:	Time (min)	Eluant A (%)	Eluant B (%)
	0	95	5
	23	55	45
	32	20	80
	36	0	100
	37	95	5
	41	95	5
DAD Detector Setting	Signal:		
	Wavelength (nm)	Band Width	Reference
	210	4	Off
	220	4	Off
	230	4	Off
	240	8	Off
	250	8	Off
	Spectrum: Store all data in the range of 200-600 nm with step size of 2.0 nm. Time: Stop time as pump. Required lamp: UV and Vis.		

	Autobalance: Prerun. Slit: 4 nm. Margin for Negative absorbance: 100 mAU
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[0046] The typical injection sequence was: diluent (2 injections) → 500 µg/mL standard (6 injections) → 40 µg/mL standard → 200 µg/mL standard → 500 µg/mL standard → 600µg/mL standard → 800 µg/mL standard → 500 µg/mL standard (2 injections) → working sample 1 (2 injections) → working sample 2 (2 injections) → working sample 3 (2 injections) → etc. → 500 µg/mL standard (2 injections) → diluent.

[0047] The peak areas from the single injections of the calibration solutions were plotted versus the concentration to determine a linear regression line. The concentration of Salinosporamide A from the test samples are determined based on this calibration curve. Potency is determined as the percent of the measured concentration relative to the theoretical concentration. Peak areas for certain impurities are corrected by multiplication by correction factors based on the Relative Response Factor (RRF). The correction factors listed in Table 2 were used for Salinosporamide A (NPI-0052) and several impurities.

Table 2
HPLC correction factors.

Peak ID	220 nm			240 nm		
	RF	RRF	Correction Factor	RF	RRF	Correction Factor
NPI-0052	8.557	1.000	1.000	3.183	1.000	1.000
NPI-0049	80.699	9.431	0.106	21.844	6.862	0.146
NPI-2083	80.699	9.431	0.106	21.844	6.862	0.146
NPI-0050	12.130	1.418	0.705	27.143	8.527	0.117
NPI-2107	181.430	21.202	0.0472	61.311	19.261	0.0519

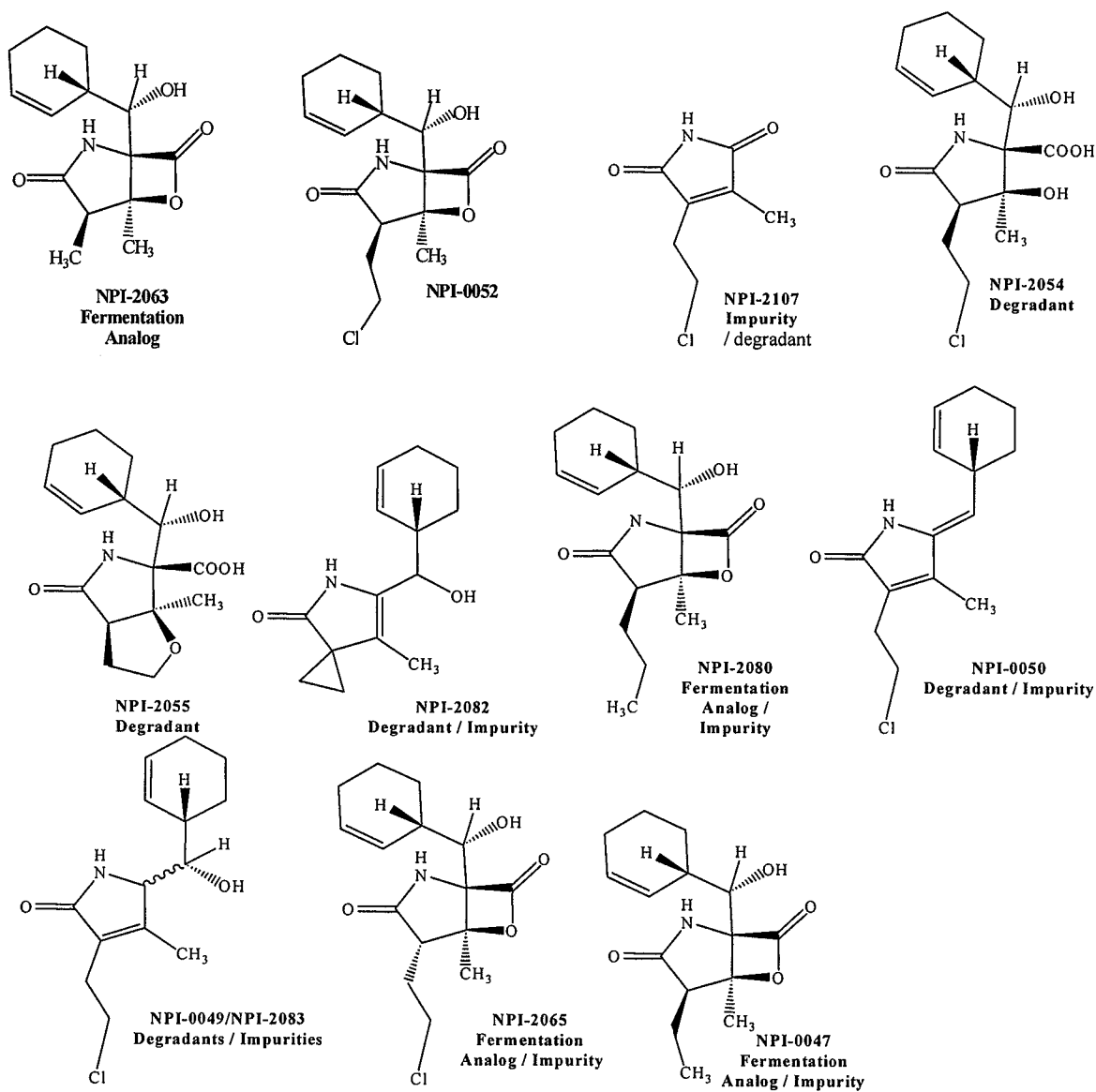
[0048] The relative retention time, molecular weight, and m/z data were determined for Salinosporamide A (NPI-0052) and related compounds based on a chromatogram run on an MSD system using UV detection at 220 nm. The results are indicated in Table 3.

Table 3.

Chromatograph and mass spectrometry data for Salinosporamide A (NPI-0052) and related compounds.

Compound ID	Mol. Weight	Retention Time (min.)	Relative Retention Time (RRT)	<i>m/z</i> [M+H]⁺
NPI-2055	295.3	8.512	0.398	296.1
NPI-2107	173.6	12.717	0.594	174.1
NPI-2054	331.8	13.247	0.617	332.1
NPI-2063	265.3	16.008	0.753	266.1
NPI-2082	233.3	17.666	0.825	234.2
NPI-0049/2083	269.8	18.530	0.866	270.0
NPI-0047	279.3	19.049	0.890	280.1
NPI-2065	313.8	20.734	0.969	314.1
NPI-0052	313.8	21.402	1.000	314.1
NPI-2080	293.4	22.066	1.031	294.1
NPI-0050	251.8	27.583	1.289	252.1
<i>Following data are from individual injections of NPI-0049 and NPI-2083</i>				
Compound ID	Mol. Weight	Retention Time (min.)	Relative Retention Time (RRT)	<i>m/z</i> [M+H]⁺
NPI-0049	269.8	18.517	0.865	270.0
NPI-2083	269.8	18.453	0.862	270.0

[0049] The chemical structures of the above-identified compounds are :



Protocol for povidone as the bulking agent

[0050] Povidone interferes with HPLC analysis of Salinosporamide A and its degradants when UV detection is used, especially at low wavelength. Povidone is a polymeric compound that generally elutes as a broad peak on reverse phase columns, which makes it challenging to separate from analyte peaks. Accordingly, a reverse phase HPLC gradient method that separates povidone from Salinosporamide A and its degradants was developed. Both evaporative light scattering (ELS) and UV detection were evaluated to optimize quantitation and good chromatographic results. Degradants and impurities were identified using mass spectroscopy. This method was tested for linearity, accuracy, precision,

specificity, robustness and quantitation and detection limits. A comparative study using the ELS and UV detection techniques were performed.

[0051] Seven calibration solutions of Salinosporamide A were prepared having concentrations of 50 µg/mL, 100 µg/mL, 240 µg/mL, 500 µg/mL, 600 µg/mL, 720 µg/mL, and 800 µg/mL, respectively. As a check of system suitability, the 500 µg/mL standard solution is used as a check standard and is injected six times at the beginning of the sequence after the diluent injection, two injections after every ten sample injections, and two injections at the end of the run to ensure system suitability throughout the entire run. The %RSD of the area response for the six suitability injections at the beginning of the run plus the check standard injections is checked to be $\leq 2.0\%$. The chromatographic conditions were as indicated in Table 4.

Table 4.

Chromatographic conditions for povidone containing samples.

Column:	Phenomenex Synergi POLAR-RP C18, 4µ, 250×4.6 mm 80 Å		
Flow rate:	1.0 mL/min		
Column temperature:	40°C		
Autosampler temperature:	4°C		
Inject volume:	50 µL (with needle wash using ACN)		
Run time:	56 min.		
Mobile Phase:	Eluant A: 0.01% TFA in pure water or HPLC water Eluant B: 0.01% TFA in HPLC grade tetrahydrofurn (THF) containing 5% HPLC grade acetonitrile (ACN)		
Gradient Table:	Time (min)	Eluant A (%)	Eluant B (%)
	0	100	0
	10	95	5
	12	90	10
	15	90	10
	17	80	20
	30	50	50
	40	50	50

	42	35	65
	46	10	90
	48	10	90
	50	80	20
	52	95	5
	54	100	0
	56	100	0
DAD Detector Setting	Signal:		
	Wavelength (nm)	Band Width	Reference
	210	4	Off
	220	4	Off
	230	4	Off
	240	8	Off
	250	8	Off
	Spectrum: Store all data in the range of 200-600 nm with step size of 2.0 nm. Time: Stop time as pump. Required lamp: UV and Vis. Autobalance: Prerun. Slit: 4 nm. Margin for Negative absorbance: 100 mAU		
ELSD Setting			
Nitrogen Gas Pressure	4.0 Bar		
Gain	7		
Nebulization Temperature	40°C		

[0052] The typical injection sequence was: diluent (2 injections) → 500 µg/mL standard (6 injections) → 50 µg/mL standard → 100 µg/mL standard → 240 µg/mL standard → 500 µg/mL standard → 600 µg/mL standard → 720 µg/mL standard → 800 µg/mL standard → 500 µg/mL standard (2 injections) → working sample 1 (2 injections) → working sample 2 (2 injections) → working sample 3 (2 injections) → etc. → 500 µg/mL standard (2 injections) → diluent.

[0053] The peak areas from the single injections of the calibration solutions were plotted versus the concentration to determine a linear regression line. The concentration of Salinosporamide A from the test samples are determined based on this calibration curve. Potency is determined as the percent of the measured concentration relative to the theoretical concentration. Peak areas for certain impurities are corrected by multiplication of correction factors based on the Relative Response Factor (RRF). The correction factors listed in Table 5 were used.

Table 5.
HPLC correction factors.

Peak ID	240 nm		
	RF	RRF	Correction Factor
NPI-0052	5.501	1.000	1.000
NPI-0050	39.898	7.252	0.138
NPI-0049	23.407	4.255	0.235

[0054] The relative retention time, molecular weight, and m/z data were determined for Salinosporamide A (NPI-0052) and related compounds based on a chromatogram run on an MSD system using UV detection at 240 nm. The results are indicated in Table 6.

Table 6.
Chromatograph and mass spectrometry data for Salinosporamide A (NPI-0052) and related compounds.

Compound ID	Mol. Weight	Retention Time (min.)	Relative Retention Time (RRT)	m/z [M+H] ⁺
NPI-2055	295.3	21.880	0.629	296.1
NPI-2107	173.6	N/D	N/D	174.1
NPI-2054	331.8	N/D	N/D	332.1
NPI-2082	233.3	28.219	0.812	234.2
NPI-2083	269.8	29.280	0.842	270.0
NPI-0049	269.8	29.717	0.855	270.0

NPI-2063	265.3	29.929	0.861	266.1
NPI-0047	279.3	32.059	0.922	280.1
NPI-2080	293.4	33.809	0.973	294.1
NPI-2065	313.8	34.761	1.00	314.1
NPI-0052	313.8	34.761	1.00	314.1
NPI-0050	251.8	35.493	1.02	252.1

N/D: Not determined

Example 2 – Non-aqueous lyophilizing solution

[0055] 2.0 mg Salinosporamide A was added to a 20 ml Type 1 USP flint tubing vial and t-butyl alcohol was added to bring the total volume to 1 ml. A clear solution was obtained. The t-butyl alcohol was essentially removed during lyophilization to produce a lyophilized formulation.

Example 3 – Water/t-butyl alcohol lyophilizing solution (Kollidon 12PF)

[0056] 2.0 mg Salinosporamide A, 30.0 mg of KOLLIDON® (12PF), and t-butyl alcohol were added to a 20 ml Type 1 USP flint tubing vial. 100 mg of water (water pH was adjusted to pH 3.0-5.0 with diluted HCl) and additional t-butyl alcohol were added to bring the total volume to 2 ml. A clear solution was obtained. The t-butyl alcohol and water were essentially removed during lyophilization to produce a lyophilized formulation.

Example 4 – Water/t-butyl alcohol lyophilizing solution (Kollidon 17PF)

[0057] 2.0 mg Salinosporamide A, 30.0 mg of KOLLIDON® (17PF), and t-butyl alcohol were added to a 20 ml Type 1 USP flint tubing vial. 100 mg of water (water pH was adjusted to pH 3.0-5.0 with diluted HCl) and additional t-butyl alcohol were added to bring the total volume to 2 ml. A clear solution was obtained. The t-butyl alcohol and water were essentially removed during lyophilization to produce a lyophilized formulation.

Example 5 – Water/t-butyl alcohol lyophilizing solution (mannitol)

[0058] 2.0 mg Salinosporamide A and t-butyl alcohol were added to a 20 ml Type 1 USP flint tubing vial. 20.0 mg of mannitol was dissolved in 380 mg of water (water pH was adjusted to pH 3.0-5.0 with diluted HCl) and added to the vial. Additional t-butyl alcohol

was added to bring the total volume to 2 ml. A clear solution was obtained. The t-butyl alcohol and water were essentially removed during lyophilization to produce a lyophilized formulation.

Example 6 – Water/t-butyl alcohol lyophilizing solution (mannitol/sucrose)

[0059] 2.0 mg Salinosporamide A and t-butyl alcohol were added to a 20 ml Type 1 USP flint tubing vial. 60.0 mg of sucrose and 20 mg of mannitol were dissolved in 320 mg of water (water pH was adjusted to pH 3.0-5.0 with diluted HCl) and added to the vial. Additional t-butyl alcohol was added to bring the total volume to 2 ml. A clear solution was obtained. The t-butyl alcohol and water were essentially removed during lyophilization to produce a lyophilized formulation.

Example 7 – Water/t-butyl alcohol lyophilizing solution (sucrose)

[0060] 2.0 mg Salinosporamide A and t-butyl alcohol were added to a 20 ml Type 1 USP flint tubing vial. 60.0 mg of sucrose was dissolved in 240 mg of water (water pH was adjusted to pH 3.0-5.0 with diluted HCl) and added to the vial. Additional t-butyl alcohol was added to bring the total volume to 2 ml. A clear solution was obtained. After lyophilization, the water and t-butyl alcohol content were measured. Water content was determined using the Karl Fischer coulometric method and t-butyl alcohol content was determined using a GC headspace method. The water content was 0.24 mg. The t-butyl alcohol content was 1.28 mg.

Example 8 – Water/t-butyl alcohol lyophilizing solution (sucrose)

[0061] 2.0 mg Salinosporamide A and t-butyl alcohol were added to a 20 ml Type 1 USP flint tubing vial. 70.0 mg of sucrose was dissolved in 280 mg of water (water pH was adjusted to pH 3.0-5.0 with diluted HCl) and added to the vial. Additional t-butyl alcohol was added to bring the total volume to 2 ml. A clear solution was obtained. After lyophilization, the water and t-butyl alcohol content were measured. Water content was determined using the Karl Fischer coulometric method and t-butyl alcohol content was determined using a GC

headspace method. The water content was 0.15 mg. The t-butyl alcohol content was 1.72 mg.

Example 9 – Water/t-butyl alcohol lyophilizing solution (sucrose)

[0062] 2.0 mg Salinosporamide A and t-butyl alcohol were added to a 20 ml Type 1 USP flint tubing vial. 80.0 mg of sucrose was dissolved in 320 mg of water (water pH was adjusted to pH 3.0-5.0 with diluted HCl) and added to the vial. Additional t-butyl alcohol was added to bring the total volume to 2 ml. A clear solution was obtained. After lyophilization, the water and t-butyl alcohol content were measured. Water content was determined using the Karl Fischer coulometric method and t-butyl alcohol content was determined using a GC headspace method. The water content was 0.05 mg. The t-butyl alcohol content was 2.01 mg.

Example 10 – Water/t-butyl alcohol lyophilizing solution (sucrose)

[0063] 2.0 mg Salinosporamide A and t-butyl alcohol were added to a 20 ml Type 1 USP flint tubing vial. 80.0 mg of sucrose was dissolved in 320 mg of water (water pH was adjusted to pH 3.0-5.0 with diluted HCl) and added to the vial. Additional t-butyl alcohol was added to bring the total volume to 2 ml. A clear solution was obtained. After lyophilization, the water and t-butyl alcohol content were measured. Water content was determined using the Karl Fischer coulometric method and t-butyl alcohol content was determined using a GC headspace method. The water content was 0.21 mg. The t-butyl alcohol content was 1.83 mg.

Example 11 – Stability studies

[0064] The potency and purity of the lyophilized formulations of Examples 7-10 were studied using the procedures described in Example 1 under various storage conditions and times. The measured potency for the various examples and conditions are indicated in Table 7 and the purity values are given in Table 8.

Table 7.

Potency of lyophilized formulations.

Elapsed Time (days)	2 mg Salinosporamide A / 80 mg Sucrose					2 mg Salinosporamide A / 60 mg Sucrose	
	Ex 9 -20°C (%)	Ex 9 2-8°C (%)	Ex 9 25°C (%)	Ex 10 40°C (%)	Ex 10 50°C (%)	Ex 7 40°C (%)	Ex 7 50°C (%)
0	94.9	94.9	94.9	97.4	97.4	95.5	95.5
7	N/D	N/D	N/D	100.9	98.8	98.8	98.8
15	N/D	N/D	N/D	94.9	96.7	99.3	99.9
28	N/D	N/D	N/D	94.7	89.7	95.3	94.5
35	94.7	88.7	90.7	93.3	87.6	94.2	92.0
56	N/D	N/D	N/D	95.5	N/D	98.4	N/D
69	94.3	94.9	94.8	N/D	N/D	N/D	N/D
96	98.2	100.1	95.9	N/D	N/D	N/D	N/D

N/D: Not determined

Table 8.

Purity of lyophilized formulations.

Elapsed Time (days)	2 mg Salinosporamide A / 80 mg Sucrose					2 mg Salinosporamide A / 60 mg Sucrose	
	Ex 9 -20°C (%)	Ex 9 2-8°C (%)	Ex 9 25°C (%)	Ex 10 40°C (%)	Ex 10 50°C (%)	Ex 7 40°C (%)	Ex 7 50°C (%)
0	98.4	98.4	98.4	98.7	98.7	98.7	98.7
7	N/D	N/D	N/D	98.1	93.4	98.4	94.7
15	N/D	N/D	N/D	97.6	92.8	98.2	94.1
28	N/D	N/D	N/D	97.4	91.1	98.0	93.6
35	98.8	98.1	98.4	97.6	94.7	97.6	92.7
56	N/D	N/D	N/D	96.6	N/D	97.4	N/D
69	98.4	98.4	97.6	N/D	N/D	N/D	N/D
96	98.5	98.5	97.7	N/D	N/D	N/D	N/D

N/D: Not determined

[0065] Based on the measured purity values of the Example 9 lyophilized product, a projected shelf life analysis was conducted using a 95% confidence interval. For various storage conditions, the amount time before purity dropped to 95% was projected. The results are depicted in Figures 4-6.

[0066] Accelerated stability studies were conducted on the lyophilized product of Examples 4, 7, and 10. The product was stored at various temperatures. At various times, the lyophilized product was reconstituted and the purity determined as discussed above. Figure 5 depicts the resulting Salinosporamide A purity measured as a function of time. The two samples containing sucrose were from Examples 7 and 10 (60 mg and 80 mg of sucrose, respectively). The two samples containing Kollidon were prepared as in Example 4 with one being KOLLIDON® 12PF and the other being KOLLIDON® 17PF. Figure 6 depicts the amount of the NPI-0049/2083 impurities measured as a function of time. Figure 7 depicts the amount of the NPI-0050 impurity measured as a function of time. Among these samples, lyophilized product containing sucrose as the bulking agent exhibited the highest stability.

Example 12 – Reconstitution strategies

[0067] Various lyophilized compositions were reconstituted using various amounts of either propylene glycol (PG) or polyethylene glycol (PEG) with ethanol and citrate buffer (CB). The time required to successfully solubilize the lyophilate was measured. Tables 9-13 list the results using PG with 60 mg, 70 mg, 80 mg, 80 mg, and 40 mg sucrose in the lyophilate, respectively. Tables 14-17 list the results using PEG-400 with 60 mg, 70 mg, 80 mg, and 40 mg sucrose in the lyophilate, respectively.

Table 9.

Reconstitution of Salinosporamide A/60 mg sucrose lyophilate using PG/EtOH/CB diluent.

Diluent Composition				
PG (%, v/v)	EtOH (%, v/v)	CB (%, v/v)	Diluent Volume (mL)	Dissolution Time
40	10	50	20	5 min
60	10	30	10	>5 min
50	0	50	10	> 5 min

50	10	40	10	2 min
50	20	30	10	< 2 min
40	10	50	10	> 5 min
40	20	40	10	< 2 min
30	20	50	10	> 5 min

Table 10.

Reconstitution of Salinosporamide A/70 mg sucrose lyophilate using PG/EtOH/CB diluent.

Diluent Composition				
PG (%, v/v)	EtOH (%, v/v)	CB (%, v/v)	Diluent Volume (mL)	Dissolution Time
40	10	50	20	< 5 min
60	10	30	10	4.5 min
50	10	40	10	4 min
50	20	30	10	< 2 min
40	20	40	10	< 2 min

Table 11.

Reconstitution of Salinosporamide A/80 mg sucrose lyophilate using PG/EtOH/CB diluent.

Diluent Composition				
PG (%, v/v)	EtOH (%, v/v)	CB (%, v/v)	Diluent Volume (mL)	Dissolution Time
40	10	50	20	5 min
60	10	30	10	2-5 min
50	0	50	10	> 5 min
50	10	40	10	2 min
50	20	30	10	2 min
40	10	50	10	> 5 min
40	20	40	10	2 min
30	20	50	10	2 min

Table 12.

Reconstitution of Salinosporamide A/80 mg sucrose lyophilate using PG/EtOH/CB diluent.

Diluent Composition				
PG (%, v/v)	EtOH (%, v/v)	CB (%, v/v)	Diluent Volume (mL)	Dissolution Time
40	10	50	20	5 min
40	10	50	10	> 5 min
40	20	40	10	1 min

Table 13.

Reconstitution of Salinosporamide A/40 mg sucrose lyophilate using PG/EtOH/CB diluent.

Diluent Composition				
PG (%, v/v)	EtOH (%, v/v)	CB (%, v/v)	Diluent Volume (mL)	Dissolution Time
40	10	50	20	> 5 min
60	10	30	10	> 5 min
50	0	50	10	> 5 min
50	10	40	10	> 5 min
50	20	30	10	> 5 min
40	10	50	10	> 5 min
40	20	40	10	> 5 min
30	20	50	10	> 5 min

Table 14.

Reconstitution of Salinosporamide A/60 mg sucrose lyophilate using PEG/EtOH/CB diluent.

Diluent Composition				
PEG400 (%, v/v)	EtOH (%, v/v)	CB (%, v/v)	Diluent Volume (mL)	Dissolution Time
50	20	30	10	< 2 min
40	20	40	10	< 2 min

Table 15.

Reconstitution of Salinosporamide A/70 mg sucrose lyophilate using PEG/EtOH/CB diluent.

Diluent Composition				
PEG400 (%, v/v)	EtOH (%, v/v)	CB (%, v/v)	Diluent Volume (mL)	Dissolution Time
50	10	40	10	< 5 min
50	20	30	10	< 2 min
40	20	40	10	< 2 min

Table 16.

Reconstitution of Salinosporamide A/80 mg sucrose lyophilate using PEG/EtOH/CB diluent.

Diluent Composition				
PEG400 (%, v/v)	EtOH (%, v/v)	CB (%, v/v)	Diluent Volume (mL)	Dissolution Time
50	20	30	10	< 2 min
40	20	40	10	< 2 min

Table 17.

Reconstitution of Salinosporamide A/40 mg sucrose lyophilate using PEG/EtOH/CB diluent.

Diluent Composition				
PEG400 (%, v/v)	EtOH (%, v/v)	CB (%, v/v)	Diluent Volume (mL)	Dissolution Time
50	20	30	10	> 5 min
40	20	40	10	> 5 min

Example 13 – Stability of reconstituted solution

[0068] The potency and purity of reconstituted lyophilate was determined using various diluents. The lyophilate included 70 mg of sucrose. Four different diluents were tested. Diluent I included 40% propylene glycol, 20% ethanol, and 40% citrate buffer (10 mM, pH 5.0). Diluent II included 50% propylene glycol, 20% ethanol, and 30% citrate buffer (10 mM, pH 5.0). Diluent III included 40% polyethylene glycol 400, 20% ethanol, and 40% citrate buffer (10 mM, pH 5.0). Diluent IV included 50% polyethylene glycol 400, 20%

ethanol, and 30% citrate buffer (10 mM, pH 5.0). Table 18 lists the results as a function of time after reconstitution when the solution is stored at 5°C. Table 19 lists the results when the solution is stored at 25°C.

Table 18.

Potency and purity of a Salinosporamide A stored at 5°C after reconstitution from a Salinosporamide A/sucrose lyophilate.

Time (h)	Potency (%)	Recovery Based on time t=0	Degradation rate (%/h)	Purity (%)
Diluent I				
0	100.80	100	-	98.25
3	100.37	99.57	0.14	98.05
5	100.06	99.26	0.15	98.09
24	97.65	96.88	0.13	97.15
Diluent II				
0	100.37	100	-	98.00
3	100.23	99.86	0.05	97.88
5	100.01	99.65	0.07	97.90
24	98.71	98.35	0.07	97.24
Diluent III				
0	98.82	100	-	97.82
3	98.67	99.85	0.05	97.77
5	98.45	99.62	0.08	97.62
24	97.19	98.35	0.07	97.25
Diluent IV				
0	101.94	100	-	98.37
3	101.68	99.75	0.08	98.30
5	101.50	99.57	0.09	98.30
45	100.46	98.55	0.06	97.93

Table 19.

Potency and purity of a Salinosporamide A stored at 25°C after reconstitution from a Salinosporamide A/sucrose lyophilate.

Time (h)	Potency (%)	Recovery Based on time $t=0$	Degradation rate (%/h)	Purity (%)
Diluent I				
0	99.06	100	-	97.93
3	96.45	97.36	0.88	96.76
5	94.88	95.78	0.84	96.12
24	80.04	80.79	0.80	88.51
Diluent II				
0	98.64	100	-	98.17
3	96.86	98.20	0.60	97.45
5	94.88	96.19	0.76	96.91
24	85.45	86.63	0.56	90.91
Diluent III				
0	98.83	100	-	98.34
3	97.16	98.30	0.57	97.31
5	95.87	97.00	0.60	96.61
24	85.78	86.79	0.55	92.11
Diluent IV				
0	98.81	100	-	98.44
3	97.32	98.50	0.50	97.72
5	96.26	97.42	0.52	97.14
24	86.15	87.19	0.53	92.44

Example 14 – Stability studies of various lyophilized formulations and dosing solutions of Salinosporamide A

[0069] To determine the stability of various lyophilized formulations of Salinosporamide A and the reconstituted dosing solutions, stability studies were carried out

on various lots of lyophilized formulations prepared using sucrose, Kollidon 12PF or 17PF as excipient, and stored at -20, 5, 25, 40, and 50 °C.

Drug product formulation stability

[0070] Several lots of Salinosporamide A lyophile formulations were diluted with diluent containing various compositions of Propylene Glycol (PG), PEG 400, Citrate Buffer and EtOH. In a typical formulation preparation, Salinosporamide A lyophile was dissolved with an appropriate amount of diluent at 5 and 25°C and mixed to obtain a clear solution of dose formulation. Stability studies of the formulations were conducted at 5 and 25 °C for comparison and formulation selection.

[0071] Results of accelerated stability of Salinosporamide A formulations prepared with Kollidon or Sucrose as excipients are shown in Figures 5 and 6. The stability of Salinosporamide A lyophile was monitored under accelerated conditions (40 and 50°C). The % Purity of NPI-0052 decreases more rapidly with Kollidon as excipient. A main impurity peak, NPI-2083/0049 is formed with Kollidon excipient under accelerated conditions. Formulations prepared with Kollidon are less stable than formulations prepared with Sucrose. Therefore, a sucrose lyophile was selected for further development.

[0072] Long term stability results of Salinosporamide A lyophile drug product at 5°C for up to 12 months are presented in Figures 8-10, and Tables 20-22. Vials were stored at -20, 5, 25 °C for stability analysis. Various lots of drug product with sucrose as excipient and varying compositions were prepared and stability studies were performed at -20, 5 and 25°C. For the formulations prepared with sucrose, the results suggest that within the range of sucrose tested, increasing the amount of sucrose lowers the stability. Based on the stability data, lyophile formulation with 2 mg/vial API and 60 mg/vial sucrose was selected as lead formulation. Stability data with shelf life prediction for the final formulation of Salinosporamide A (Lot # NPI-0052-06-111) stored at 5°C with 95% confidence interval was evaluated. A shelf life of at least 81 months and 167 months at 5°C and -20 °C, respectively, is projected. Long term storage condition of 5°C is proposed based on desired stability results for product shelf life.

Table 20.Compositions of Salinosporamide A for stability analysis.

Salinosporamide A Drug Product Number	Salinosporamide A Composition (per vial)	
	NPI-0052 (mg)	Sucrose (mg)
NPI-0052-06-107	2.0	80
NPI-0052-06-110	2.0	80
NPI-0052-06-111	2.0	60
NPI-0052-06-120	2.0	70
NPI-0052-07-004	2.0	60

Table 21.Stability of Salinosporamide A Lot # NPI-0052-06-111

Time (month)	Purity (%)		
	20±2 °C	5±3 °C	25±2 °C
0	98.69	98.69	98.69
1	98.57	N/D	N/D
3	98.43	98.39	97.87
4	98.57	98.3	97.71
6	98.63	98.52	97.87
9	98.69	98.51	97.67
12	98.56	98.34	97.53

N/D: Not determined

Table 22.Shelf life of Salinosporamide A Lot # NPI-0052-06-111

Lyo DP Lot # NPI-0052-06-111 (2 mg NPI-0052 / 60 mg sucrose)	20±5 °C (month)	5±3 °C (month)	25±2 °C (month)
Projected Shelf Life	167	81	28
Recommended Shelf Life	24	24	24

Dose solution stability results

[0073] Table 23 and Figures 11 and 12 describe the diluents evaluated for dose solution stability. NPI-0052 hydrolyzes in the presence of aqueous diluents. Stability studies were performed in various aqueous co-solvent systems at 5 and 25°C to select dose solutions with sufficient stability to support practical use in the clinical setting. It is clear that the stability of dose solutions is best at 5°C. The final drug product diluent contains 40% PG/ 20% EtOH/ 40% CB. Although several diluents are more stable for up to 24 hours, the final drug product diluent was chosen based on stability, high total water content in the dose formulation as well as reconstitution time of less than 2 minutes.

[0074] Accelerated stability data indicated that formulation with sucrose as excipient is more stable than the formulation prepared using Kollidon as excipient. Further, stability studies suggested that a parenteral formulation of NPI-0052 lyophile reconstituted with 40%PG-40%CB-20%EtOH is stable for up to 24 hours at 5°C.

Table 23.

Diluent selection for dose solution stability

NPI-0052 Drug Product Diluent	Drug Product Diluent Composition (% v/v)		
	Ethanol	PG/ PEG 400	Citrate Buffer (10 mM, pH 5.0)
Diluent 1	20	40 (as PG)	40
Diluent 2	20	50 (as PG)	30
Diluent 3	20	40 (as PEG400)	40
Diluent 4	20	50 (as PEG400)	30
Diluent 5	10	40 (as PG)	50

Example 15 – Lyophilization development of Salinosporamide A in a water/t-butyl alcohol co-solvent Systems

[0075] Process parameters affecting lyophilization and residual solvents, namely, water and t-butyl alcohol, were evaluated to develop a robust lyophilization process for water/tert-butanol co-solvent formulation containing sucrose and Salinosporamide A.

[0076] Salinosporamide A was formulated with 16-40 mg/mL sucrose in 12-16 % water/tert-butanol (v/v) co-solvent systems. The formulation was filled in 20 mL flint tubing vials (Wheaton) and subsequently lyophilized to a dry powder for evaluation. A freeze dryer

system (Virtis Genesis 25 SXL) with sample thief device was utilized for the studies. In-process samples were retrieved during the drying process and the sublimation rate of the lyophilization process was analyzed by percentage weight loss. The desorption rate and residual solvents content were determined by Karl Fischer Coulometric Titration (Mettler Toledo DL32) for moisture and GC headspace method (Hewlett-Packard 5970 MSD GCMS) for tert-butanol, respectively. Thermal characteristics of the drug solutions were analyzed by freeze-thaw cycle studies and sub-ambient Differential Scanning Calorimetry (DSC, TA Instruments Q1000). The morphology of the finished drug product was evaluated using DSC and X-ray powder diffraction (XRPD), Shimadzu XRD-6000.

Freeze-thaw studies

[0077] The water-tert-butanol co-solvent formulations containing sucrose as bulking excipient exhibit a freezing temperature at about -10°C immediately after a super-cooling event at -18°C to about -21°C . The freezing point of tert-butanol is depressed by water and sucrose to sub-zero temperature. This result supports the use of 5°C loading temperature prior to lyophilization. The ice melting event from the thawing cycle is observed to start at about -7°C .

Sub-ambient DSC

[0078] The studies were run at a rate of $10^{\circ}\text{C}/\text{min}$ between 30°C and -60°C and with an annealing temperature at -12°C for 2 minutes. The thermograph shows two on-set eutectic melting temperatures at -7°C and 11°C , and an apparent glass transition temperature (T_g') of sucrose at about -32°C . After annealing step at -12°C , only one significant eutectic temperature at -7°C is observed with no presence of T_g' (see thermographs in Figure 13 and 14).

Sublimation rate

[0079] In-process samples were collected from the primary drying cycle when shelf temperature reached at -34°C and -30°C with vacuum pressure at 200 mtorr. The sublimation rate based on % weight loss was determined to be 0.10-0.12 g/hr or 0.04-0.05 $\text{g}/\text{cm}^2/\text{hr}$. It was observed that a high shelf temperature exceeding -24°C can cause the cake matrix to vibrate and puff; this is because a high vapor pressure of tert-butanol and fast sublimation (mass transfer) can push the partially dry cake matrix upward before the primary

drying cycle is complete. Therefore, the shelf temperature can advantageously be maintained at $-31 \pm 3^\circ\text{C}$ during the primary drying cycle with constant chamber pressure, 200 mtorr, in this case, to avoid overheating of product temperature.

[0080] There is no appreciable change in weight loss for the secondary drying cycle especially after 30 hr. An extended 2nd drying cycle at 25°C or an elevated temperature does not significantly affect the % residual tert-butanol in the final drug product.

[0081] The lyophilization cycle for Salinosporamide A formulation started with pre-freezing at -45°C , an annealing step at -12°C , and 2nd freezing step at -45°C . The shelf temperature was kept at -31°C for 24 hr during primary drying and 25°C for 30 hr during 2nd drying, respectively, while chamber pressure was kept at 200 mtorr. The moisture content and residual tert-butanol of the finished drug products are tabulated in Table 24. Interestingly, the residual tert-butanol content was found to have a good linearly correlation to the theoretical total solid weight in the lyophile (see Figure 15).

Table 24.

% Residual Solvents of Salinosporamide A Lyophilized Products

Solid Content in Lyophile Vial	H ₂ O Content		Tert-Butanol	
	(mg/vial)	(%, w/w)	(mg/vial)	(%, w/w)
2 mg active with 32 mg Sucrose	n/a	n/a	0.61	1.8
2 mg active with 60 mg Sucrose	0.23	0.4	1.28	2.1
2 mg active with 70 mg Sucrose	0.15	0.2	1.72	2.4
2 mg active with 80 mg Sucrose	0.21	0.3	1.83	2.2

[0082] The DSC thermograph of the lyophile shows a T_g at 52°C , in good agreement with amorphous sucrose T_g at a low moisture environment. The lyophile was reconstituted with a co-solvent diluent within 2 minutes.

Example 16 – Bulk solution solubility and stability

[0083] A water-tert-butanol system with a suitable ratio was evaluated to accommodate both the drug and excipient solubility, as well as minimize the rate of hydrolysis for a lyophilization preparation.

Solubility and stability studies

[0084] Salinosporamide A solubility was determined in 100% tert-butanol (TBA) and in TBA containing 3% (w/v) Kollidon 12PF and 3% (w/v) Kollidon 17PF, respectively. Both Kollidons can dissolve in TBA at approximately 4%(w/v). 3% Kollidon can serve as the bulking agent to support a lyophilization preparation. Salinosporamide A was added and saturated in the vehicles at ambient room temperature overnight. Salinosporamide A solutions were filtered and analyzed by HPLC to determine the solubility.

[0085] Salinosporamide A *in-situ* phase solubility and stability were determined in 10, 20 and 30% H₂O-TBA (v/v) solutions. Salinosporamide A was prepared at 2 mg/mL in TBA as a stock solution. HPLC water was spiked into the Salinosporamide A solution aliquot to make up the equivalent 10, 20 and 30% H₂O-TBA solutions at room temperature. Each drug solution was mixed and immediately filtered. The filtrate was collected as time zero sample and analyzed by HPLC at real time to avoid drug degradation. The filtrate was subsequently sampled and filtered at 4, 24 and 48 hr to monitor drug stability at ambient room temperature.

[0086] Salinosporamide A solubility in 100% TBA was determined to be 2mg/mL by HPLC. The drug is stable at ambient room temperature overnight. The drug solubility in 3% Kollidon 12PF-TBA and 3% Kollidon 17PF-TBA was determined to be 4.9 and 4.2mg/mL, respectively. Both drug solutions were stable at room temperature overnight.

[0087] Salinosporamide A *in-situ* phase solubility and stability in %H₂O-TBA are illustrated in Figure 16 and Table 25. The results indicate that a stable bulk solution can be prepared at 1mg/mL Salinosporamide A in the 10-30% H₂O-TBA co-solvent systems at room temperature.

Table 25.

Salinosporamide A (NPI-0052) Stability in H₂O-TBA Co-solvent Systems

Room Temp. Stability (hr)	HPLC Assay (% Purity)		
	10% H ₂ O-TBA	20% H ₂ O-TBA	30% H ₂ O-TBA

0	100.0	100.0	100.0
4	99.5	99.8	99.8
24	99.8	99.8	99.5
48	99.7	99.7	99.3

[0088] Sucrose and mannitol *in-situ* phase solubility was conducted in various %H₂O-TBA co-solvent systems to determine the miscible zone for formulation development. Sucrose and mannitol *in-situ* phase solubility in %H₂O-TBA are illustrated in Figure 17 and 18, respectively. The miscible zones and the phase solubility curves are estimated. When sucrose is used as the bulking excipient, 3% sucrose will be soluble in TBA solution containing at least 14% H₂O in TBA; and 5% sucrose in at least 25% H₂O in TBA. When mannitol is used as the bulking excipient, 1% mannitol will be soluble in TBA solution containing at least 15% H₂O in TBA; and 1.8% mannitol in at least 25% H₂O in TBA, respectively. However, the lyophilized drug product containing mannitol does not yield acceptable reconstitution performance with diluent.

Sucrose formulation stability

[0089] Salinosporamide A was formulated with sucrose (as bulking excipient) in 14% H₂O-TBA as a clear solution prior to lyophilization. The bulk solution stability was monitored at 2-8°C and 25°C. The solution was 2 mL filled in 20 mm 20 cc flint tubing vial and subsequently lyophilized to yield a white powder for stability evaluation.

[0090] As depicted in the graph of Figure 19, Salinosporamide A bulk solution formulated with sucrose in 14% H₂O-TBA was stable at 2-8°C for at least 24 hr and at 25°C for at least 6 hr, respectively. As depicted in the graph of Figure 20, the primary hydrolytic degradant (NPI-2054) can be controlled to less than 0.1% in the solution prior to lyophilization. This stability is sufficient to support product stability during the lyophile manufacturing process. The bulk solution was filled in the vial and lyophilized to yield a stable drug product with suitable reconstitution performance with diluent.

[0091] Thus, Salinosporamide A can be formulated with sucrose as bulking excipient in H₂O-TBA co-solvent system as a clear bulk solution prior to lyophilization. The lyophilization subsequently removes the solvents and produces a stable Salinosporamide A

lyophile. The drug product has demonstrated satisfactory stability at 2-8°C and can be readily reconstituted with a propylene glycol/Ethanol/Citrate buffer diluent to yield a ready-to-use solution for IV administration. Although common bulking agents, such as sucrose or mannitol, have poor solubility in alcohols, the API solubility can be improved with an *in-situ* co-solvent approach prior to lyophilization.

[0092] The above example further demonstrates that any compound comprising a β lactone ring, such as Salinosporamide A, can be protected from hydrolysis during bulk solution compounding and lyophilization by using a lyophilization preparation from a TBA solvent or H₂O-TBA co-solvent system.

[0093] Although the invention has been described with reference to embodiments and examples, it should be understood that numerous and various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims.

WHAT IS CLAIMED IS:

1. A lyophilized formulation comprising Salinosporamide A or an analog thereof.
2. The formulation of claim 1, comprising a bulking agent.
3. The formulation of claim 2, wherein the bulking agent comprises polyvinylpyrrolidone, a sugar, a sugar analog, or mixtures thereof.
4. The formulation of claim 3, wherein the polyvinylpyrrolidone comprises KOLLIDON® 12PF or 17PF.
5. The formulation of claim 2, wherein the bulking agent comprises mannitol, sucrose, lactose, trehalose, a cyclodextrin or mixtures thereof.
6. The formulation of claim 5, wherein the cyclodextrin is selected from the group consisting of a hydroxypropyl beta-cyclodextrin, a sulfobutylether beta-cyclodextrin, or mixtures thereof.
7. The formulation of claim 2, wherein the bulking agent comprises sucrose.
8. The formulation of claim 2, wherein the bulking agent comprises mannitol and sucrose.
9. A container comprising lyophilized Salinosporamide A or an analog thereof.
10. The container of claim 9 comprising a bulking agent.
11. The container of claim 10, wherein the bulking agent is sucrose.
12. The container of claim 9 comprising from about 0.2 mg to about 4 mg of Salinosporamide A or an analog thereof.
13. The container of claim 9, wherein the container is selected from the group consisting of a type 1 flint tubing vial, an amber tubing vial, a clear molded vial, and an amber molded vial.
14. A method of lyophilizing a compound comprising a β lactone ring, comprising:
 - dissolving the compound and a bulking agent in a solvent or co-solvent system;
 - freezing the resulting solution; and
 - drying the frozen solution.

15. The method of claim 14, wherein the compound is Salinosporamide A or analog thereof.
16. The method of claim 14, wherein the solvent comprises an alcohol.
17. The method of claim 16, wherein the alcohol is t-butyl alcohol.
18. The method of claim 14, wherein the co-solvent system comprises t-butyl alcohol and water.
19. The method of claim 14, comprising:
 - dissolving the compound in an alcohol;
 - dissolving the bulking agent in water; and
 - mixing the resulting two solutions.
20. A method of administering Salinosporamide A or an analog thereof, comprising:
 - reconstituting the Salinosporamide A or analog thereof by adding a diluent to a lyophilized formulation of the Salinosporamide A or analog thereof; and
 - administering the resulting solution to a patient or animal.
21. The method of claim 20, comprising storing the solution from about 2°C to about 8°C prior to injection into the patient or animal.
22. The method of claim 20, wherein the diluent comprises a co-solvent system.
23. The method of claim 20, wherein the diluent comprises polyethylene glycol, ethanol, a buffer, or mixtures thereof.
24. The method of claim 23, wherein the buffer is a citrate buffer.
25. The method of claim 20, wherein the diluent comprises propylene glycol, ethanol, a buffer, or mixtures thereof.
26. The method of claim 25, wherein the buffer is a citrate buffer.
27. The method of claim 20, wherein the diluent comprises 40% propylene glycol, 20% ethanol, and 40% citrate buffer (10 mM, pH 5.0).
28. The method of claim 20, wherein administering the solution comprises injecting it into the patient or animal.
29. A method of treating cancer, inflammation, or an infectious disease, comprising administering, according to the method of claim 20, Salinosporamide A or an

analog thereof to a patient or animal inflicted with cancer, inflammation, or an infectious disease.

30. A kit for administering Salinosporamide A or an analog thereof, comprising:
a first container comprising lyophilized Salinosporamide A or an analog thereof; and
a second container comprising a diluent.
31. The kit of claim 30, wherein the first container comprises a bulking agent.
32. The kit of claim 31, wherein the bulking agent comprises from about 60 mg to 80 mg of sucrose.
33. The kit of claim 30, wherein the first container comprises from about 0.2 mg to about 4 mg of Salinosporamide A or an analog thereof.
34. The kit of claim 30, wherein the first container is selected from the group consisting of a type 1 flint tubing vial, an amber tubing vial, a clear molded vial, and an amber molded vial.
35. The kit of claim 30, wherein the diluent comprises a co-solvent system.
36. The kit of claim 30, wherein the diluent comprises polyethylene glycol, ethanol, a buffer, or mixtures thereof.
37. The kit of claim 36, wherein the buffer is a citrate buffer.
38. The kit of claim 30, wherein the diluent comprises propylene glycol, ethanol, a buffer, or mixtures thereof.
39. The kit of claim 38, wherein the buffer is a citrate buffer.
40. The kit of claim 30, wherein the diluent comprises 40% propylene glycol, 20% ethanol, and 40% citrate buffer (10 mM, pH 5.0).

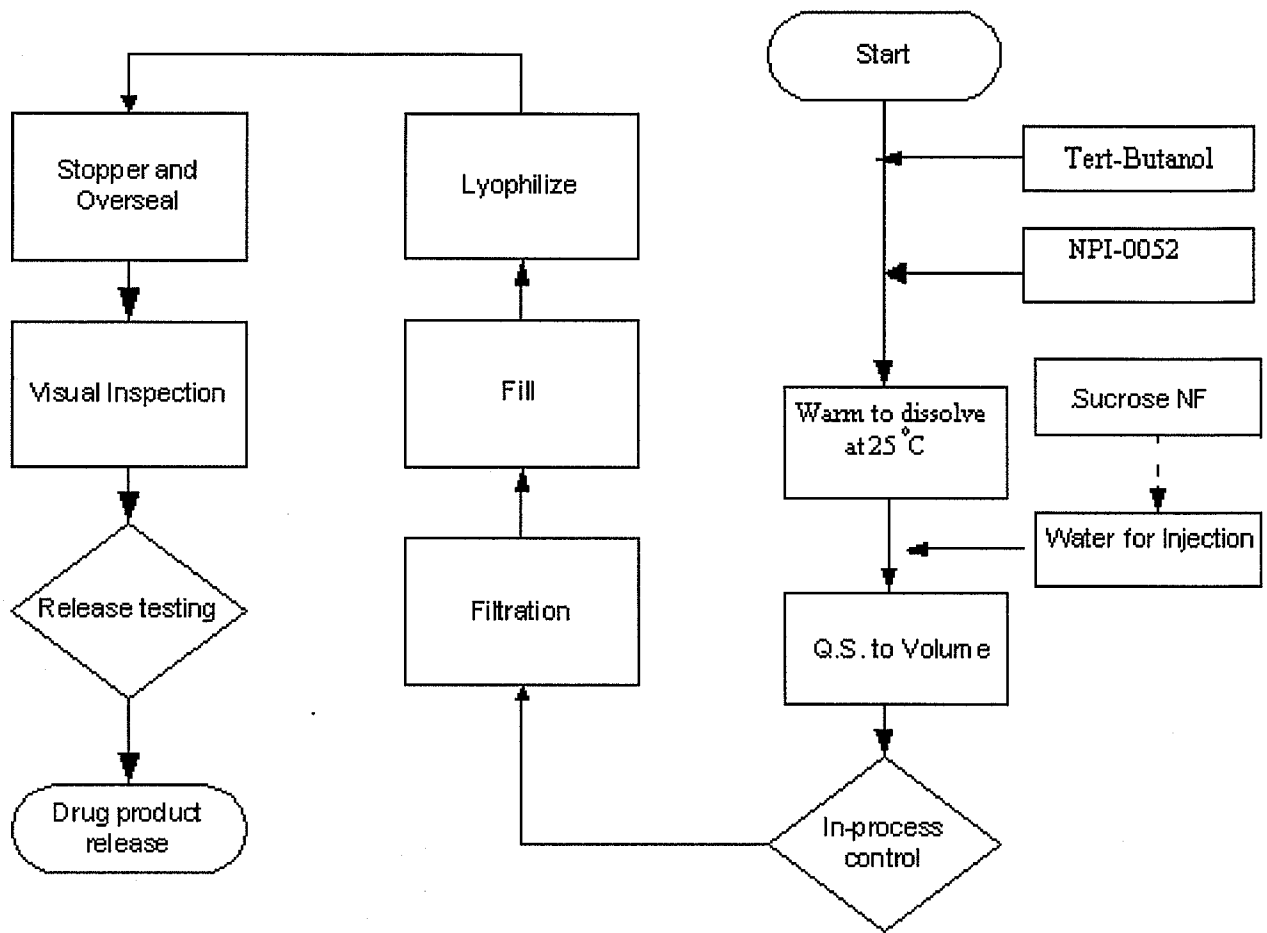


Figure 1

Shelf Life Analysis (Lot# 06-107, -20⁰C, CI = 95%)

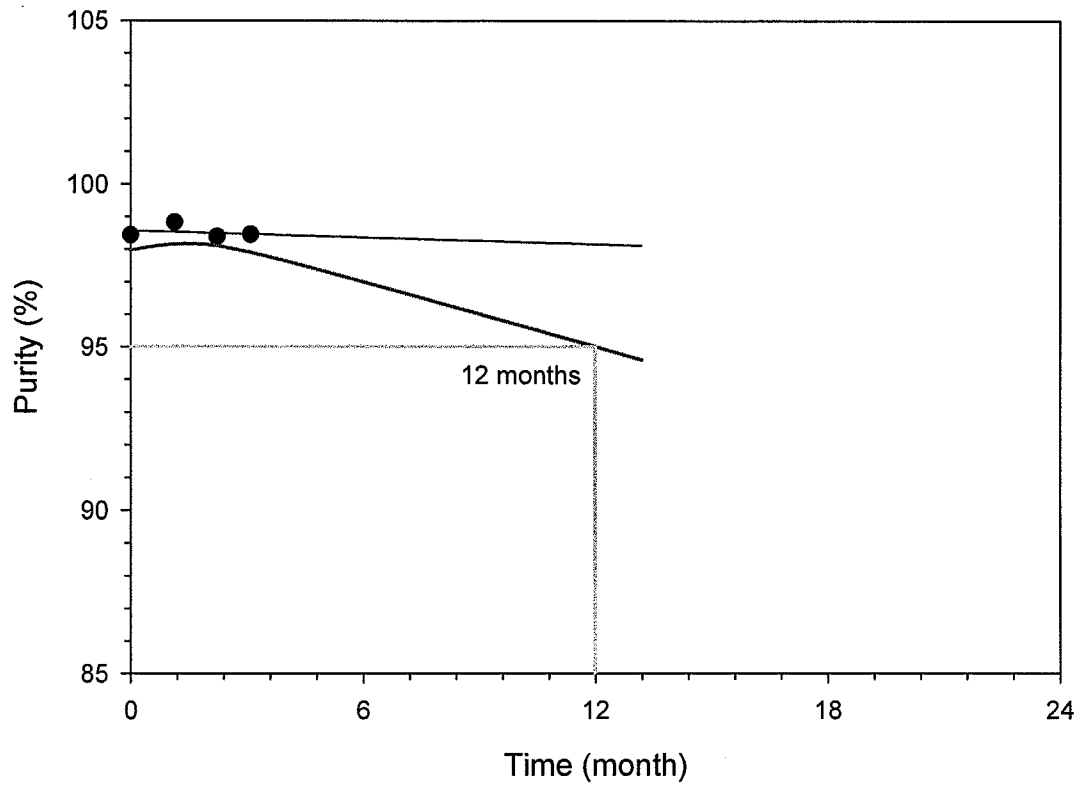


Figure 2

Shelf Life Analysis (Lot# 06-107, 5⁰C, CI = 95%)

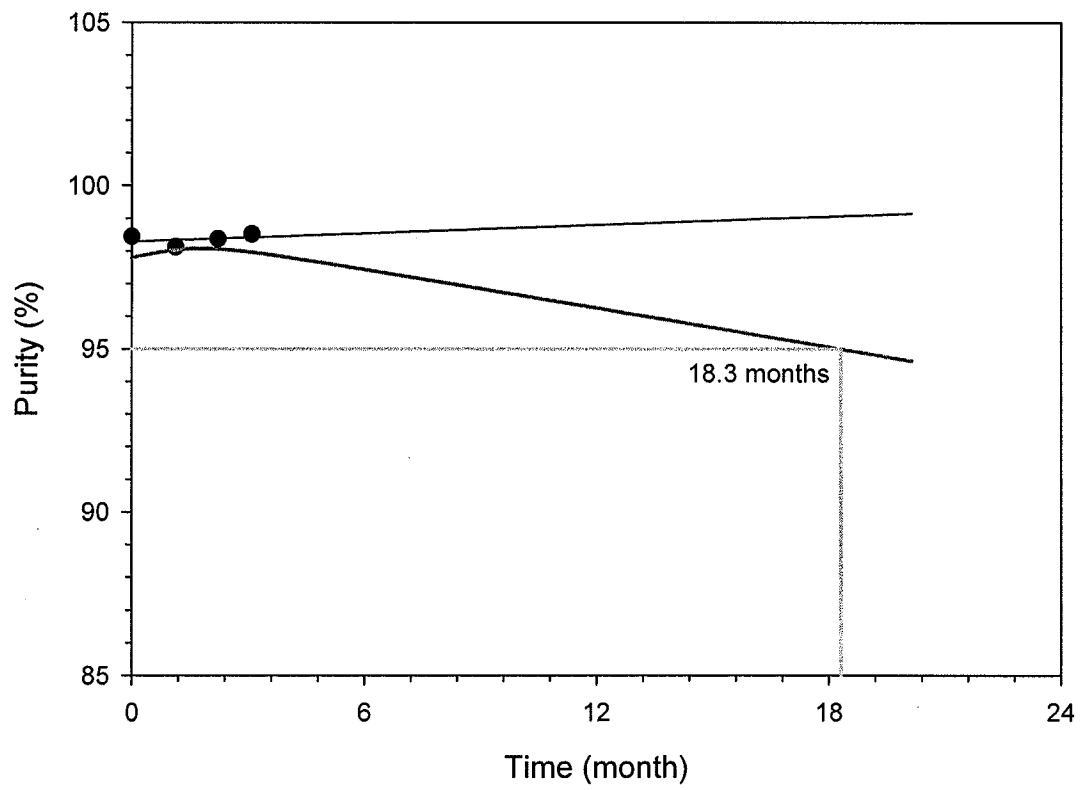


Figure 3

Shelf Life Analysis (Lot# 06-107, 25⁰C, CI = 95%)

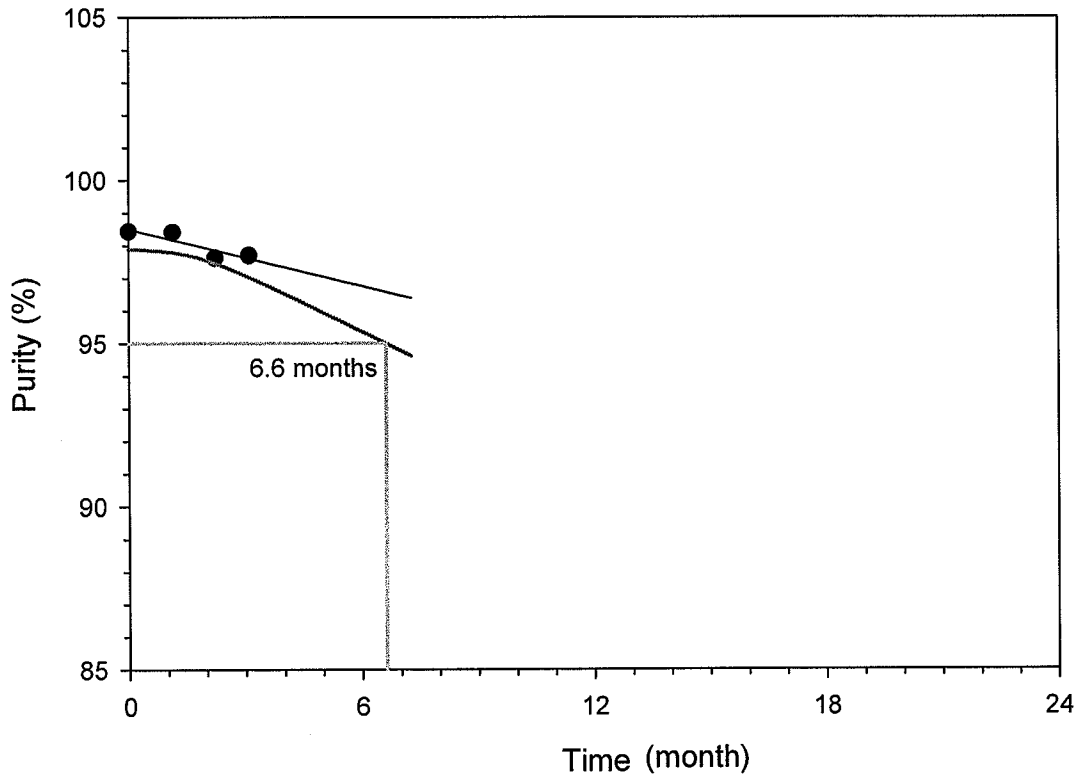


Figure 4

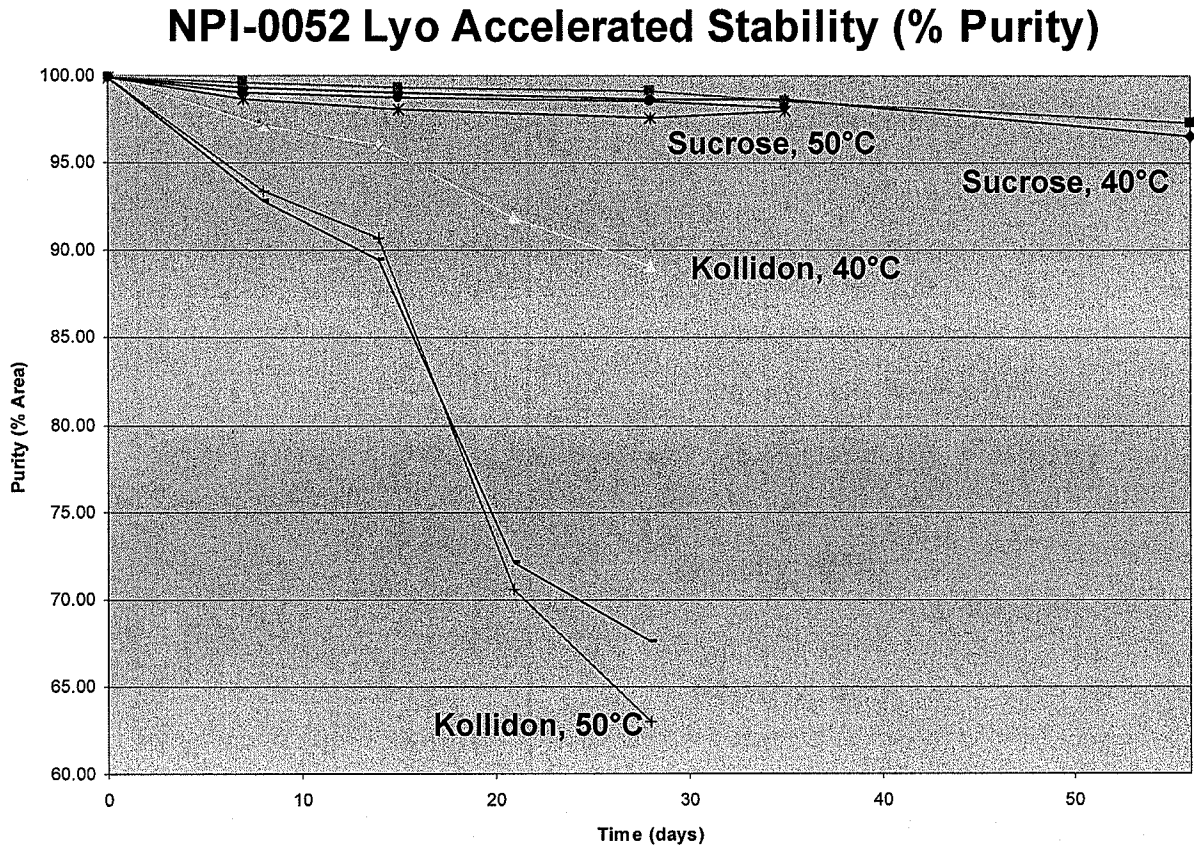


Figure 5

Lyo Accelerated Stability (% NPI-0049/2083)

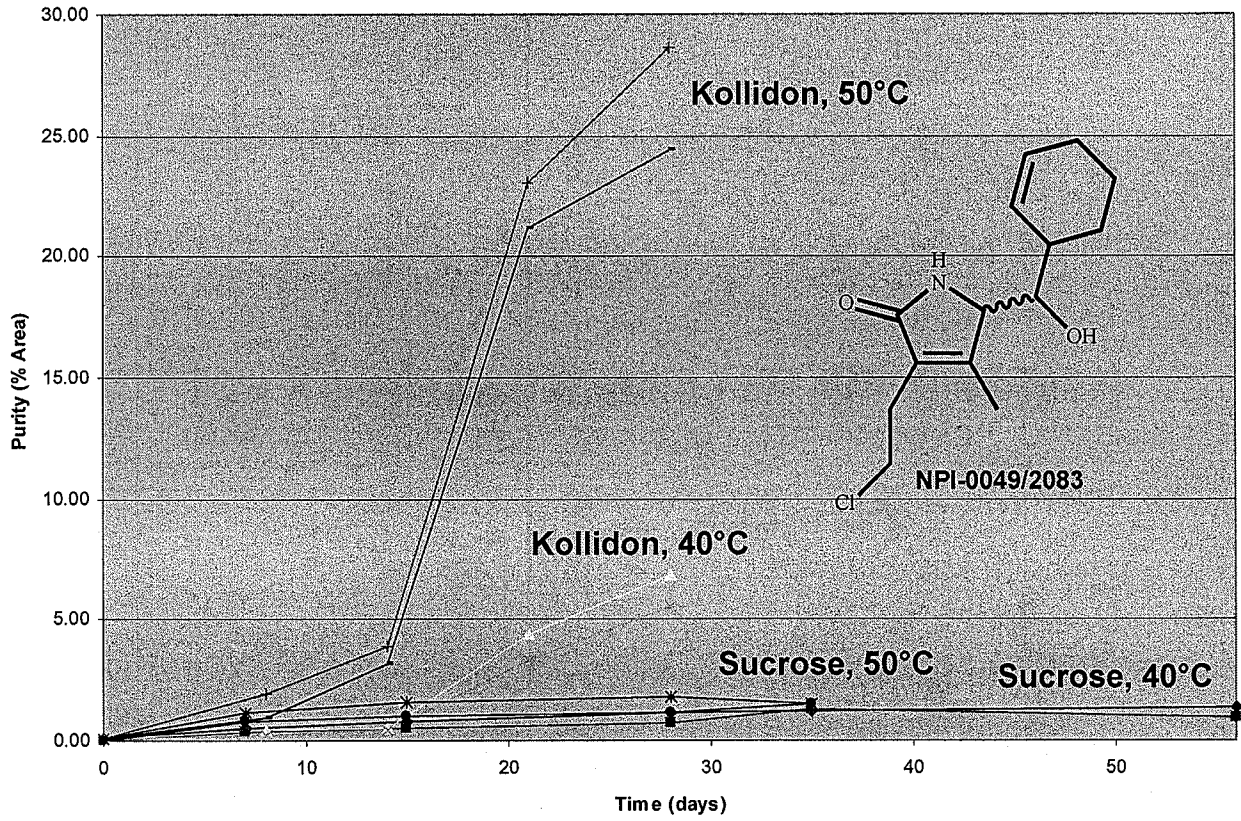


Figure 6

Lyo Accelerated Stability (% NPI-0050)

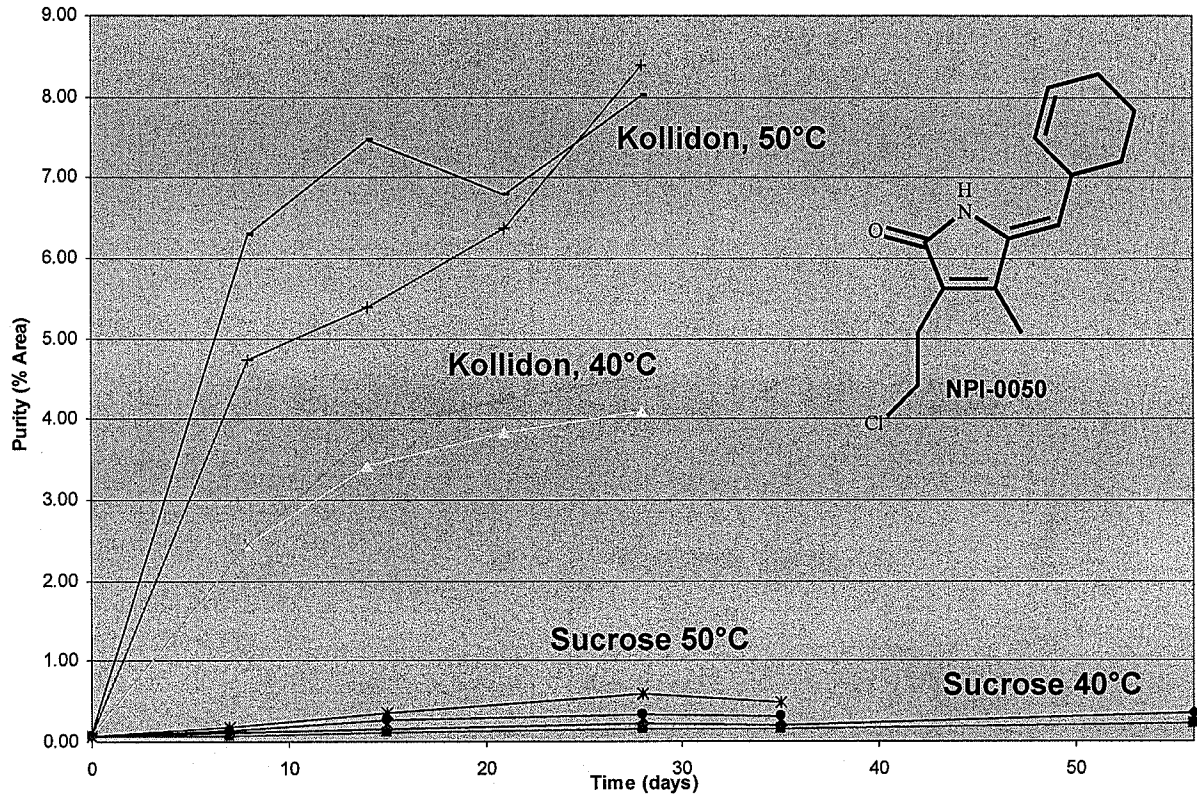


Figure 7

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5°C Stability, % Purity of NPI-0052 Normalized

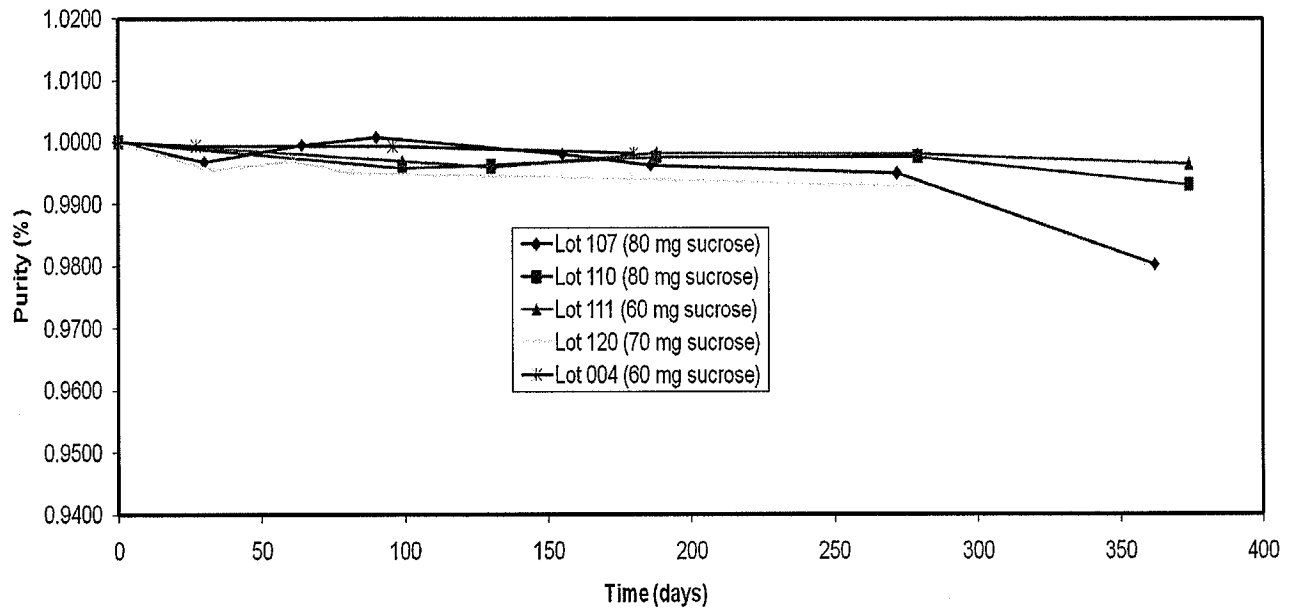


Figure 8

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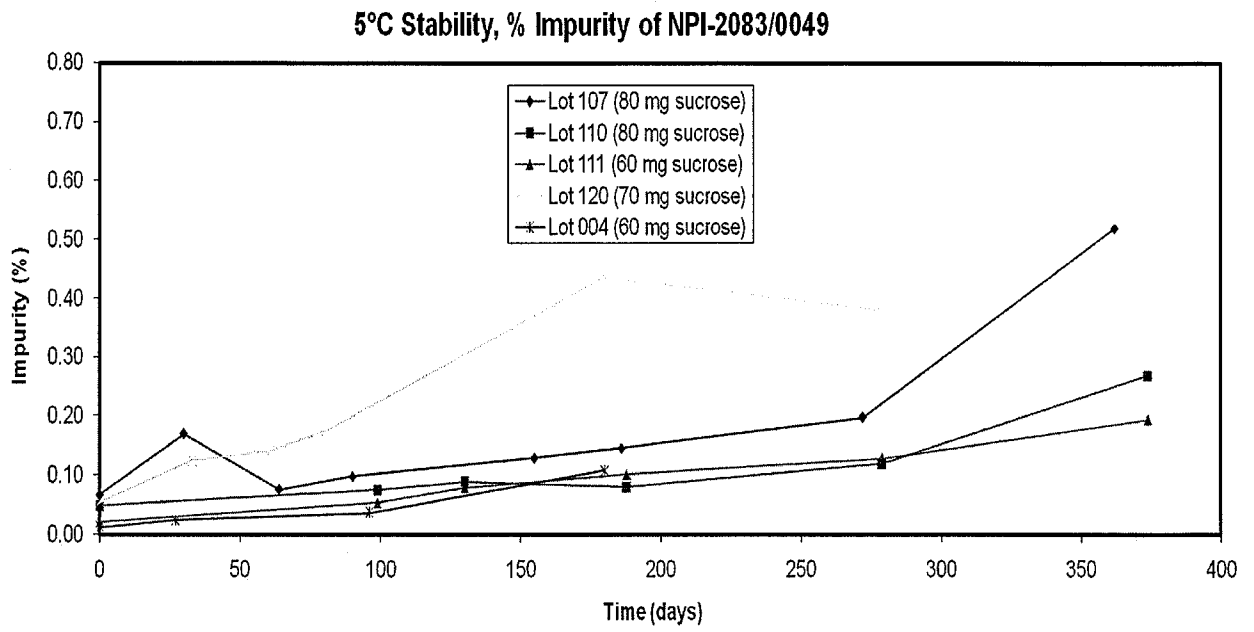


Figure 9

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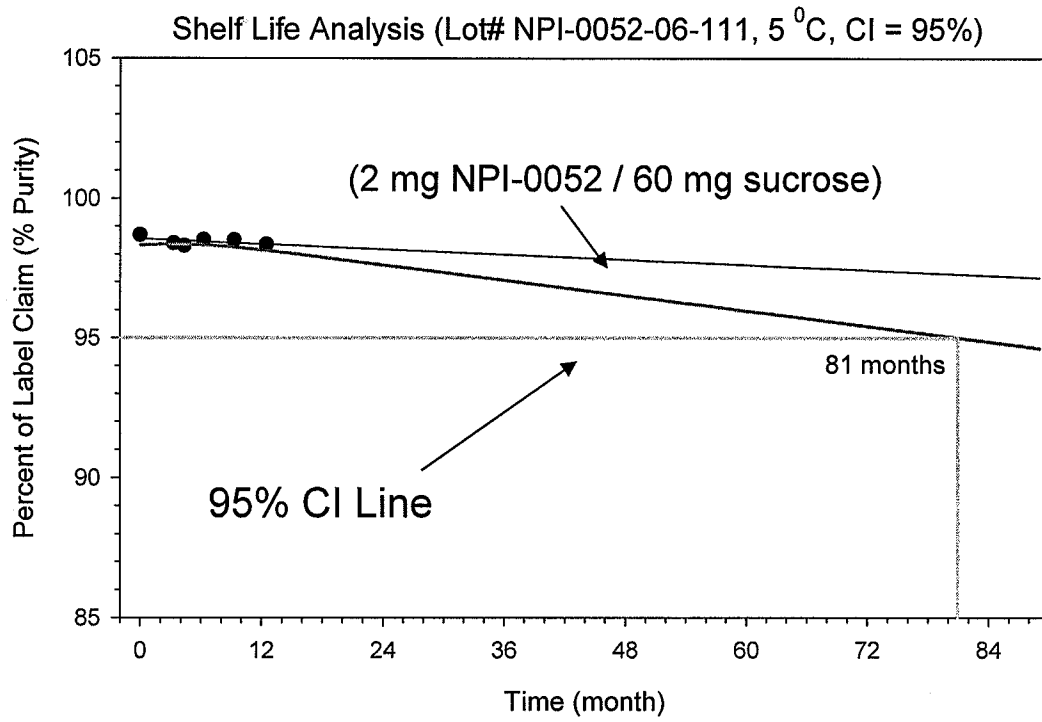


Figure 10

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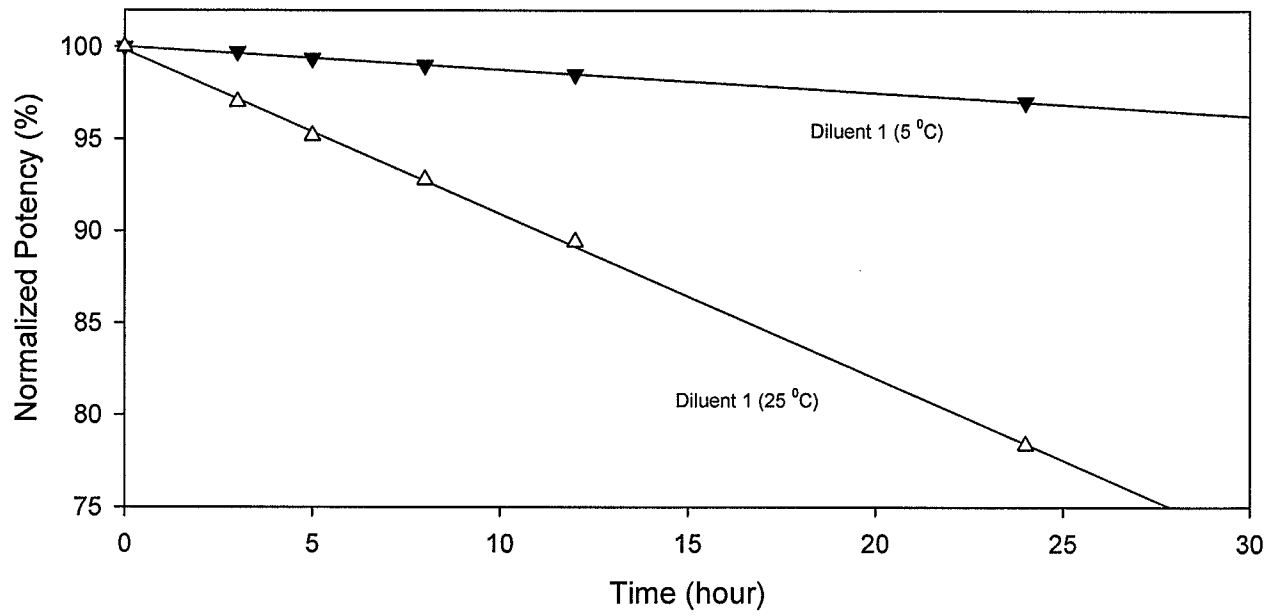


Figure 11

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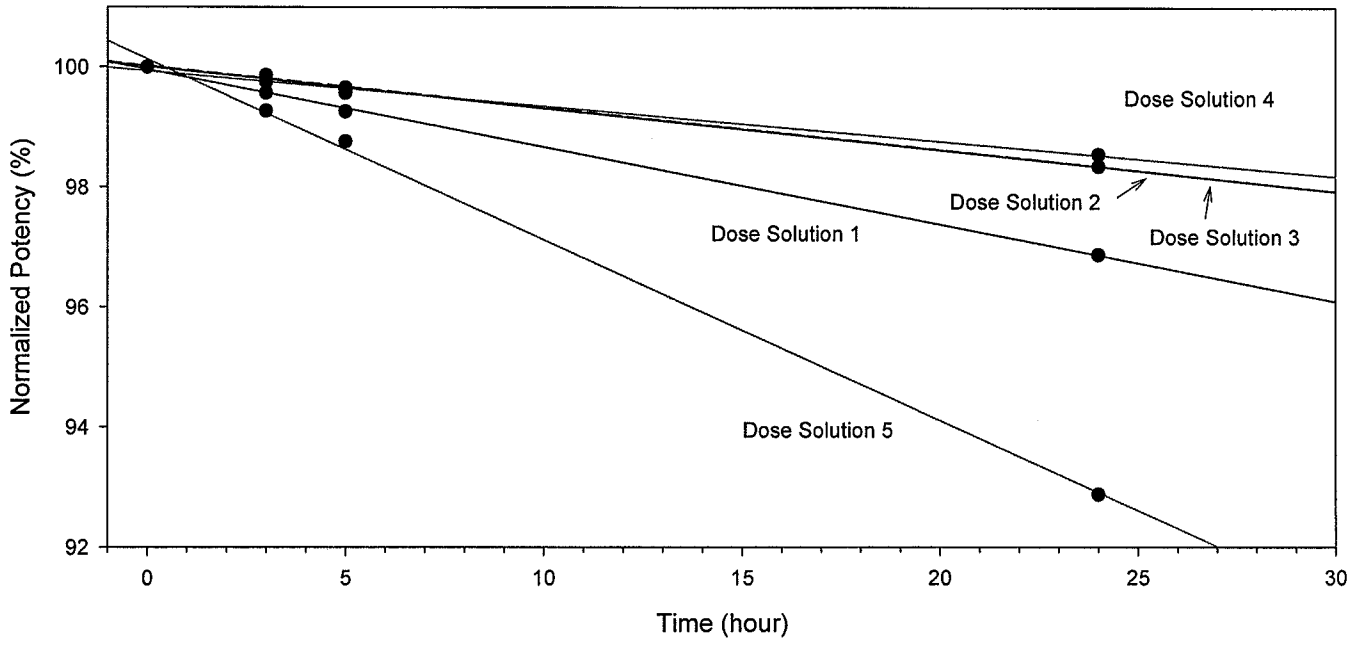


Figure 12

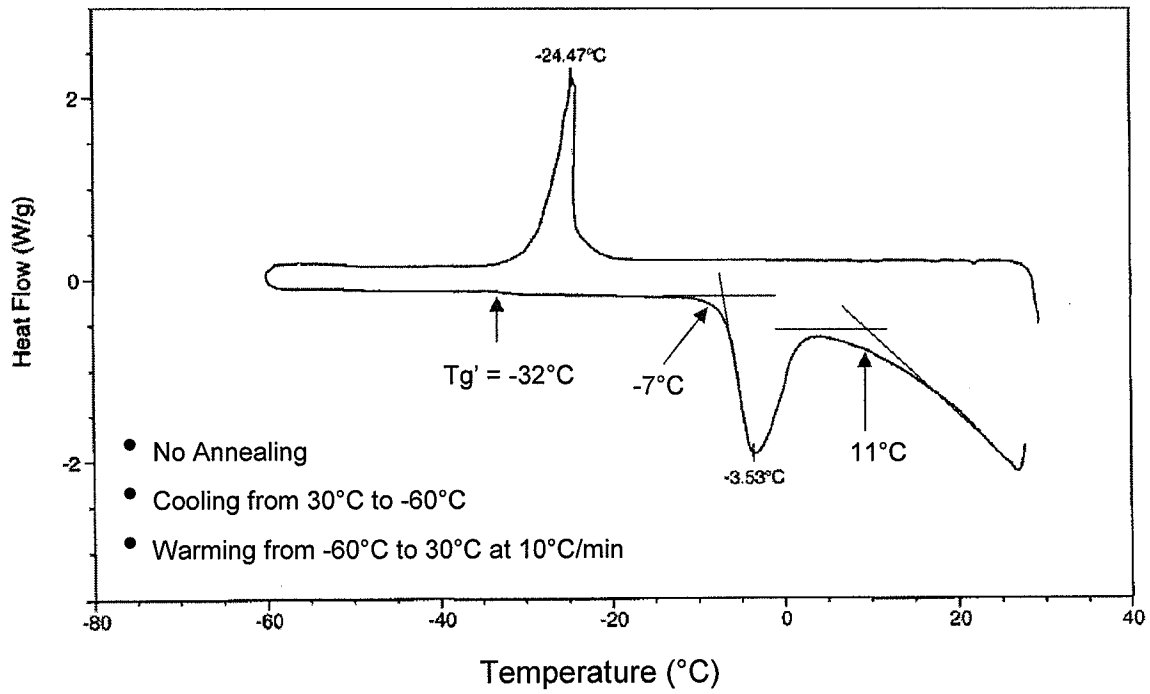
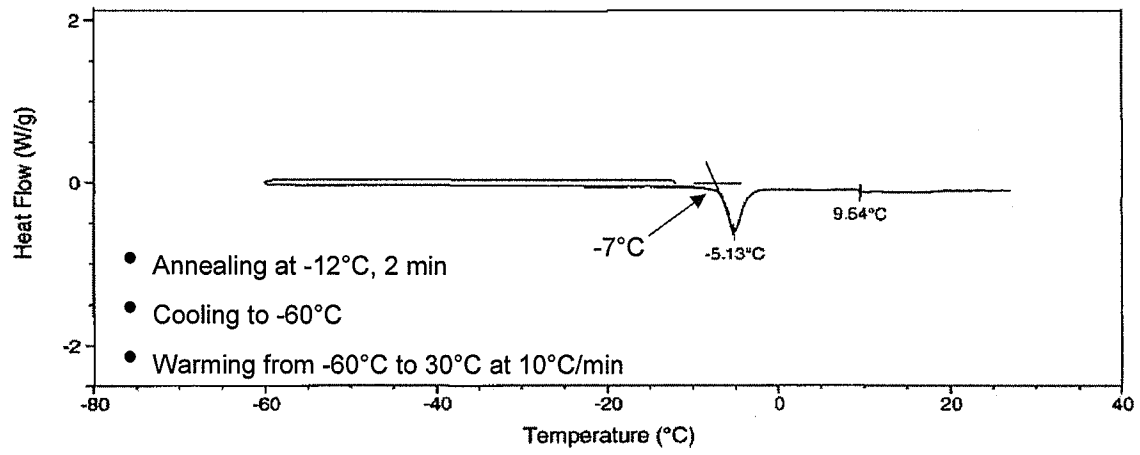
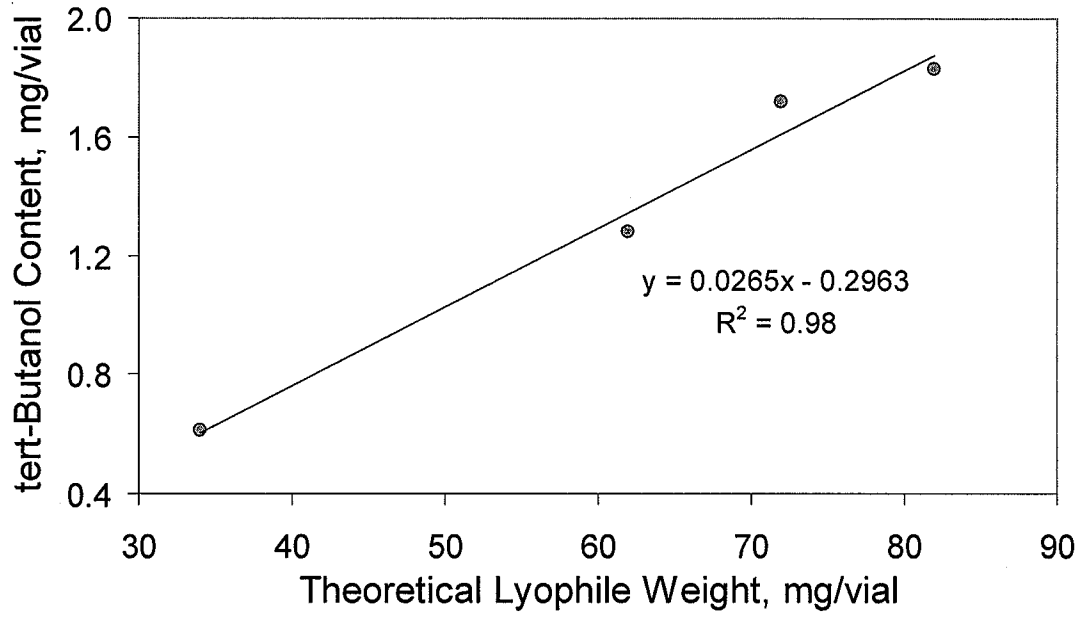


Figure 13

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**Figure 14**

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**Figure 15**

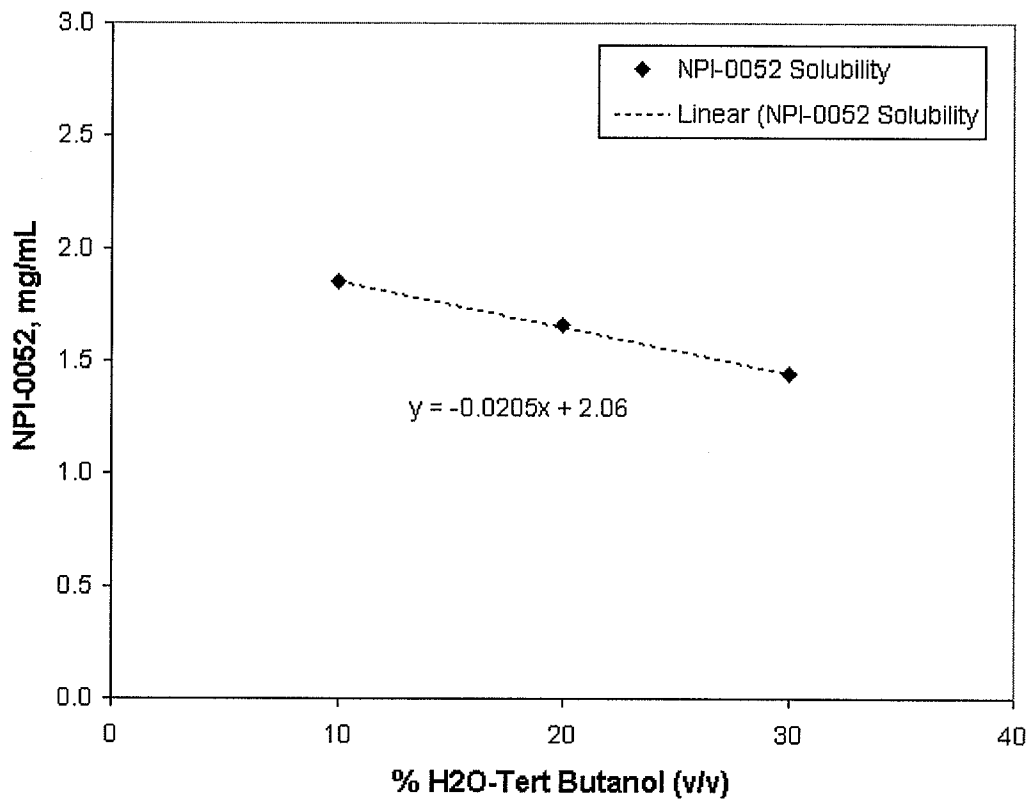


Figure 16

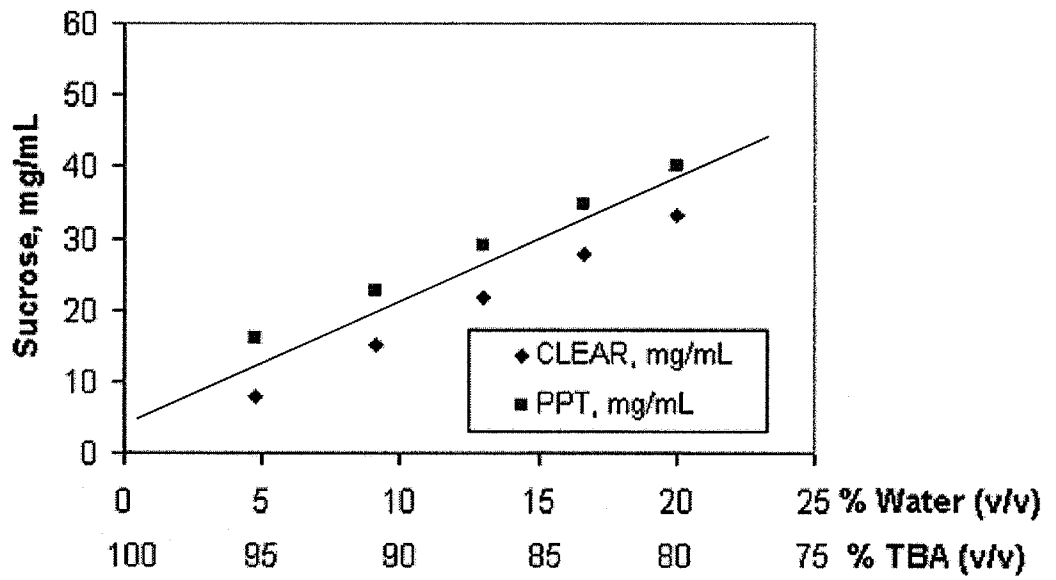


Figure 17

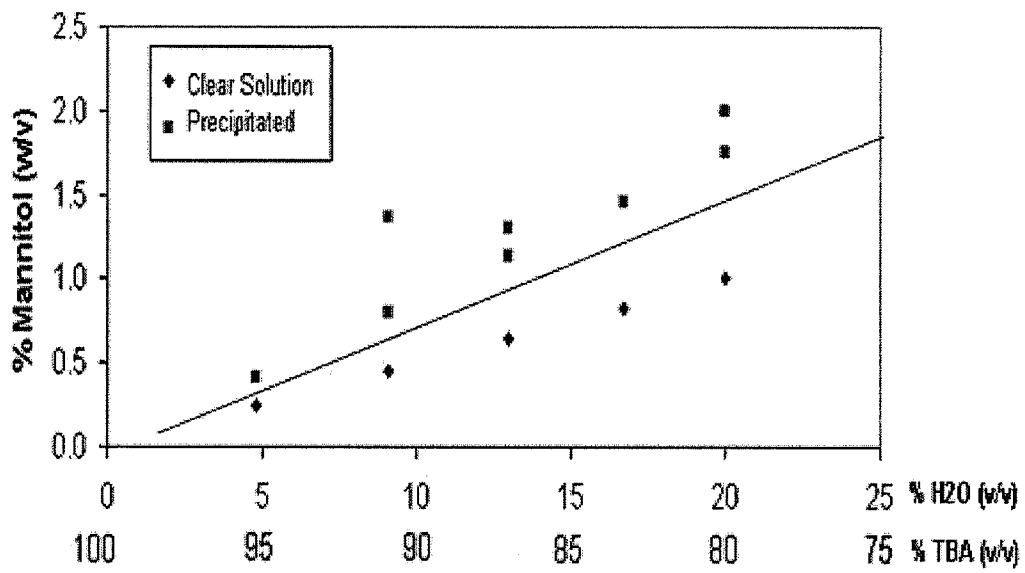


Figure 18

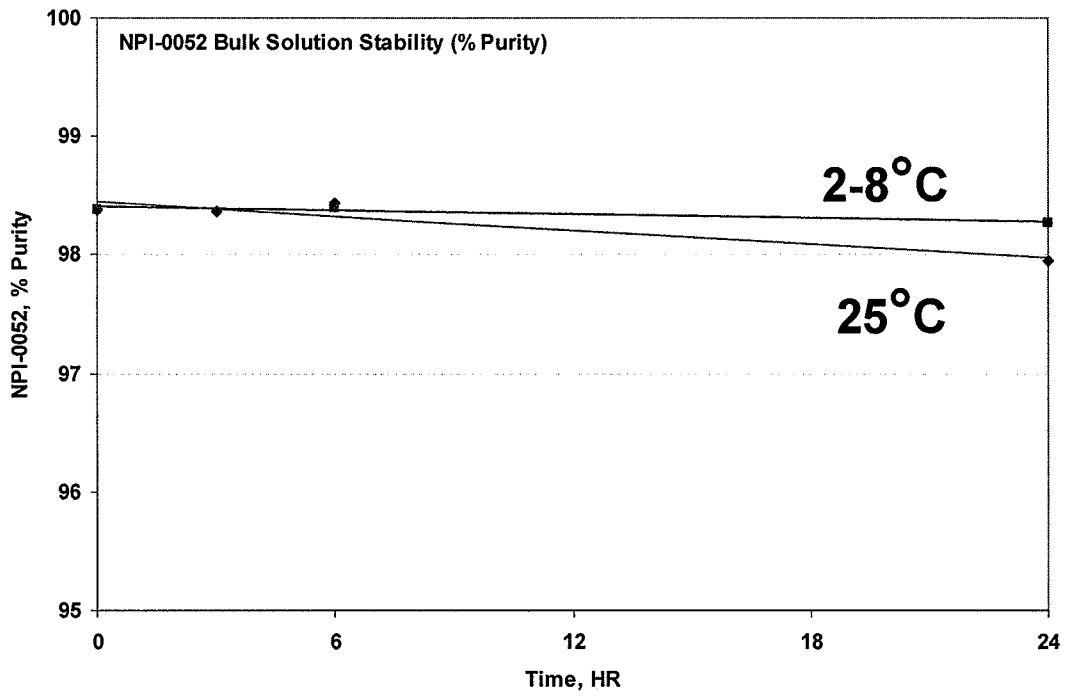


Figure 19

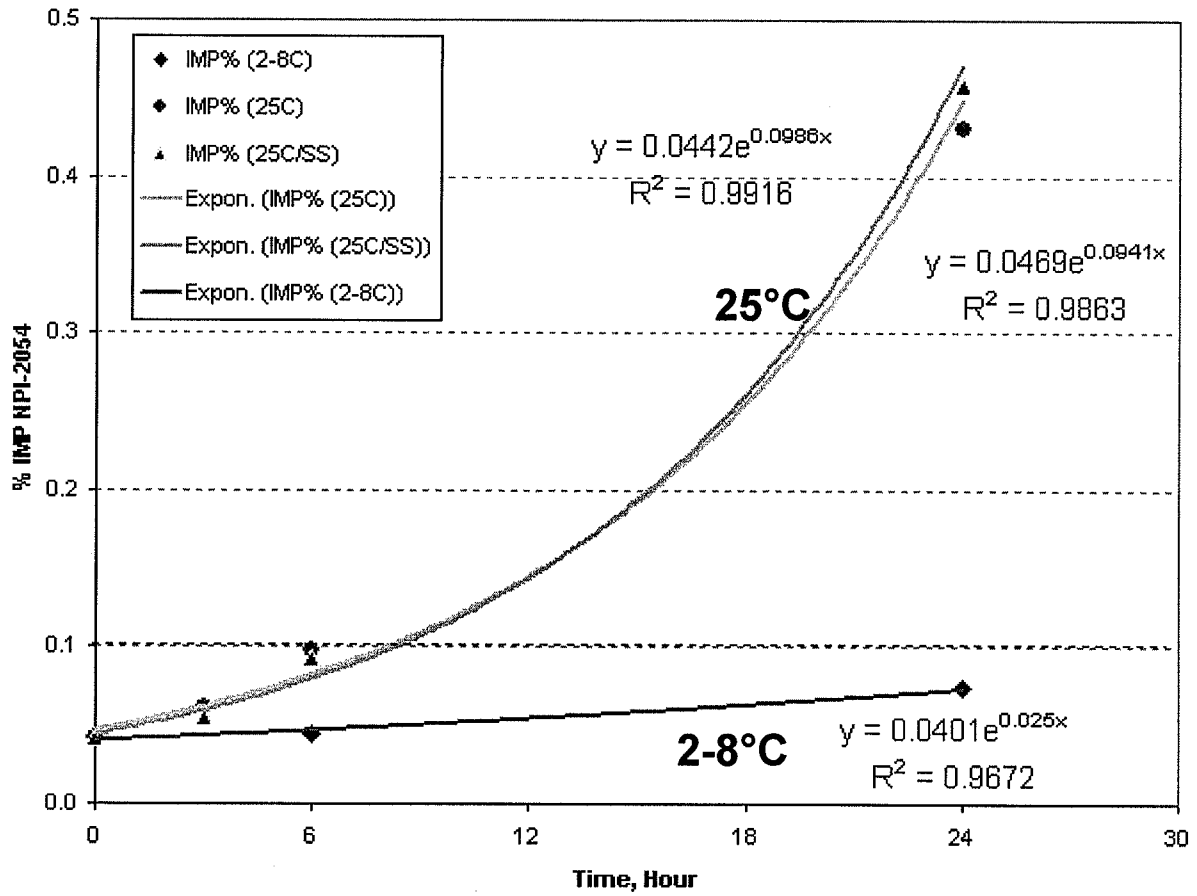


Figure 20