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Pharmaceutical development of a parenteral lyophilized formulation of the antimitastatic ruthenium complex NAMI-A

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Abstract

This paper describes the development of a stable pharmaceutical dosage form for NAMI-A, a novel antimetastatic ruthenium complex, for Phase I testing. NAMI-A drug substance was characterized using several spectrometric and chromatographic techniques. In preformulation studies, it was found that NAMI-A in aqueous solution was not stable enough to allow sterilization by moist heat. The effect of several excipients on the stability of the formulation solution was investigated. None of them provided sufficient stability to allow long-term storage of an aqueous solution of NAMI-A. Therefore, a lyophilized product was developed. Five different formulations were prepared and subjected to thermogravimetric analysis and stability studies at various conditions for one year. Minimal degradation during the production process is achieved with a formulation solution of pH 3-4. Of the acids tested, only hydrochloric acid (0.1 mM) both stabilized the formulation solution and was compatible with the lyophilized product. This product was stable for at least one year when stored at –20°C, 25°C/60% relative humidity (RH) and 40°C/75% RH, and was also photostable.
Introduction

NAMI-A (imidazolium trans-tetrachloro(dimethylsulfoxide)imidazoleruthenium(III), H$_2$im[trans-RuCl$_4$(DMSO)Him], Figure 1 [1] is a novel ruthenium anticancer agent. Preclinical pharmacological and toxicological studies showed selective activity against lung metastases of murine tumors [1-4] and low toxicity in mice and dogs [5-7]. Its action seems to be independent of the origin (type of primary tumor) and stage of growth of the metastases [4,5]. NAMI-A possesses no direct tumor cell cytotoxicity [3-5,8], although an interaction with cell cycle regulation has been observed with a transient accumulation of cells in the G$_2$/M phase. Furthermore, it increases connective tissue disposition around tumor cells and blood vessels, reducing intravasation and thus tumor blood supply, but also preventing tumor cells from invading surrounding tissue and blood or lymphatic vessels [3,8,9]. Based on the promising activity and toxicity profile, NAMI-A was developed as an antimetastatic agent.

Figure 1. Chemical structure of NAMI-A, molecular weight 458.18 g/mol.

The aim of this study was to develop a stable, parenteral pharmaceutical dosage form for use in Phase I clinical trials. The starting dose for these studies was set at a daily times 5 schedule of 2.4 mg/m$^2$. However, because up to 400 mg/m$^2$ had been administered to dogs, the dose in the Phase I trials was expected to increase substantially. Therefore, a dosage unit content of 100 mg was considered most appropriate to cover the expected Phase I dosing range. The development of a suitable parenteral formulation was performed based on the EORTC/CRC/NCI Joint Formulation Working Party guidelines [10].
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Material and methods

Chemicals

NAMI-A drug substance was supplied by SIGEA Srl (Trieste, Italy). NAMI-A lyophilized products were manufactured in-house (Department of Pharmacy & Pharmacology, Slotervaart Hospital, Amsterdam, The Netherlands). Sterile Water for Injections (WfI, Ecotainer®), 0.9% (w/v) NaCl (normal saline) and 5% (w/v) dextrose were obtained from B. Braun (Melsungen, Germany). Methanol (HPLC grade) was obtained from Biosolve Ltd. (Amsterdam, The Netherlands). Trifluoromethanesulfonic acid, citric acid, sodium acetate trihydrate, acetic acid 96% (v/v), sodium dihydrogen phosphate dihydrate, di-sodium tetraborate decahydrate, sodium carbonate (anhydrous), sodium chloride, perchloric acid (70-72% (w/v)), sodium hydroxide pellets, quinine monohydrochloride dihydrate, and dimethyl sulfoxide (dried) were all purchased from Merck (Darmstadt, Germany). Sodium dodecylsulphate was obtained from Fluka Chemica GmbH (Buch, Switzerland), hydroxypropyl-β-cyclodextrin (molar substituttion of 0.8) from Sigma-Aldrich (St. Louis, MO, USA), and mannitol from BUFA BV (Uitgeest, The Netherlands). All reagents were of analytical grade and used without further purification. Excipients and primary packaging used in the manufacture of NAMI-A lyophilized product were of European Pharmacopoeia III (Ph.Eur.III) grade. Excipients were approved after in-house quality control (based on monographs in the Ph.Eur.).

1H nuclear magnetic resonance (NMR) spectroscopy

1H NMR spectra were recorded at room temperature with a Gemini-300 instrument (Varian NMR Instruments, Palo Alto, CA, USA). The sample was dissolved in DMSO-d6 (1-2 mg in 0.65 ml). The central DMSO line at 2.505 ppm was used as the reference line.

Mass spectrometry (MS)

Positive and negative electrospray mass spectra were recorded with a Fisons VG Platform II (Micromass, Manchester, UK) single quadrupole mass spectrometer. The instrument was calibrated with sodium iodide in the range of m/z 100-1000. Nitrogen was used as nebulizer gas and as curtain gas, and the cone voltage was 30V.
Infrared (IR) spectroscopy

IR spectra of NAMI-A were recorded with a Model PU 9706 Infrared (IR) spectrophotometer (Philips Nederland BV, Eindhoven, The Netherlands) using the potassium bromide (KBr) pellet technique. The pellet consisted of 2 mg NAMI-A bulk drug or lyophilized product and 300 mg potassium bromide. The ratio recording mode was auto-smooth and the scan time 8 minutes.

Ultraviolet/visible light (UV/VIS) spectrophotometry

UV/VIS spectra were recorded with a Model UV/VIS 918 spectrophotometer (GBC Scientific Equipment, Victoria, Australia). Spectra were recorded from 800 to 225 nm. Samples were prepared by diluting the test solutions of NAMI-A with distilled water to a final concentration of 100 µg/ml.

High Performance Liquid Chromatography (HPLC)

The HPLC system consisted of a model SP8800 ternary pump (Thermo Separation Products (TSP), Fremont, CA, USA), a model 996 photo diode array (PDA) detector (Waters, Milford, MA, USA) and a model SP8880 autosampler (TSP). Chromatograms were processed using Millennium® software (Waters). Separation was achieved with a µBondapak C18 column (Waters), protected with a C8 guard column (Security Guard, Phenomenex, Torrance, CA, USA). The mobile phase consisted of 0.50 mM sodium dodecylsulphate in 3% methanol, acidified to pH 2.5 with trifluoromethanesulfonic acid (triflic acid). The flow rate was 0.5 ml/min and the system was operated at ambient temperature. The detection wavelength was 358 nm and on-line spectral analysis was carried out with the PDA detector. The injection volume was 20 µl. A run time of 10 minutes was employed for the standard samples (calibration curves and quality controls) and a run time of 30 minutes for the samples under investigation. Calibration curves of standard NAMI-A solutions in distilled water were linear (r > 0.98) in the concentration range of interest (10-600 µg/ml). In this HPLC system, NAMI-A produces a peak with a retention time of approximately 4.1 minutes. The method was proven to be stability-indicating, precise, and accurate [11].
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Melting point

The melting point of NAMI-A was determined with a Büchi B-540 melting point apparatus (Mettler-Toledo GmbH, Greifensee, Switzerland).

Preformulation studies

Solubility and stability in water

The solubility of NAMI-A in water at ambient temperature (21±2°C) was examined by accurately weighing approximately 100 mg NAMI-A in a glass test tube and adding water in 100 µl increments. After each addition, the solution was shaken for 30 seconds, if not dissolved placed in an ultrasonic bath for 15 minutes, and examined visually under polarized light for complete dissolution of NAMI-A drug substance.

The stability of 100 µg/ml solutions of the drug substance in distilled water was determined in duplicate at room temperature (21±2°C), elevated temperature (37±2°C) and refrigerated temperature (5±3°C), under all conditions protected from light. Aliquots of the solutions under examination were extracted periodically, their absorption measured directly at 390 nm and quantified in relation to a standard calibration curve (20-120 µg/ml NAMI-A in distilled water). The observed rate constants at the various temperatures were calculated from the slopes of the ln concentration-time plots (multiplied by –1).

pH-rate profile

A pH-rate profile of NAMI-A in buffered aqueous solution was constructed as described previously [12].

Influence of excipients

The stability of a 10 mg/ml solution of NAMI-A in a solution containing hydroxypropyl-β-cyclodextrin in a molar proportion 1:1 and 2:1 was investigated by HPLC. Furthermore, the degradation rate of 10 mg/ml NAMI-A at pH 3 and 4 in acetate buffer (1 mM and 0.1 mM, respectively), hydrochloric acid (HCl) (1 and 0.1 mM for pH 3 and 4, respectively) or citric acid (1.5 and 0.15 mM for pH 3 and 4, respectively), stored at room temperature (21±2°C) in the dark, was investigated by HPLC. The effect of addition of 2.5% (w/v) mannitol and/or 5%
(v/v) DMSO on the stability of NAMI-A in acidic solution (0.15 mM citric acid and 0.1 mM HCl, both pH 4) was investigated at room temperature (21±2°C) in the dark.

**Differential Scanning Calorimetry**

Thermal properties of 5 different formulation solutions of NAMI-A as described under “formulation process” were examined by differential scanning calorimetry (DSC). These experiments were performed using a Q1000 V 6.2 DSC in T4 mode equipped with a refrigerated cooling accessory (RCS) for low temperatures (TA Instruments, New Castle (DE), USA). Samples were placed in an aluminum pan, which was subsequently sealed, and measured against an empty pan as reference. Temperature scale and heat flux were calibrated with indium. Analyses were performed under a nitrogen purge at 50 ml/min. Samples (5-10 mg) were cooled to -50°C at a rate of 5°C/min, after which they were heated at a rate of 2°C/min to -2°C. Subsequently, the samples were cooled again to -50°C and heated to 30°C at the same rates. After each cooling and heating step, an isothermal step lasting 5 minutes was built in. As a reference, all formulation solutions (without NAMI-A) were subjected to the same DSC procedures.

**Formulation process**

Based on the results obtained in the preformulation studies, five different formulations of NAMI-A were prepared by lyophilization and subjected to quality control and stability studies. The formulation solutions consisted of 10 mg/ml NAMI-A in

A. sterile Water for Injections (WfI) (pH 4.4)  
B. 0.15 mM citric acid (pH 3.9)  
C. 1.5 mM citric acid (pH 2.8)  
D. 0.1 mM hydrochloric acid (pH 3.8)  
E. 5 mM citric acid in 2.5% (w/v) mannitol (pH 2.8)

**Lyophilization**

NAMI-A lyophilized product was aseptically prepared. Each formulation solution contained 10 mg/ml NAMI-A and was dissolved with stirring at ambient temperature. After complete dissolution, the solution was adjusted to the final volume with the formulation solution and
sterile filtered through a 0.22 µm Millipak 40 filter (Millipore, Milford, MA, USA). Subsequently, 10 ml aliquots were filled into 30 ml type I glass lyophilization vials (Münnerstädtter Glaswarenfabrik, Münnerstadt, Germany). Siliconized gray bromobutyl rubber stoppers (Type FM 157/1, Helvoet Pharma NV, Alken, Belgium) were positioned on each vial. Two vials were equipped with thermocouples and all vials were loaded into a Model Lyovac GT 4 freeze-drier (STERIS, Hürth, Germany) pre-chilled to 10°C. The products were frozen to -40°C in 1 hour, after which the temperature was increased to -20°C in two hours, then decreased to -40°C in 2 hours (annealing step). This temperature was maintained for 1.5 hours. After this period, the primary drying phase was started by establishing a vacuum of 0.2 mbar in 1 minute and raising the temperature to -20°C in 1 hour. This temperature and pressure were maintained for 55 hours. Subsequently, the secondary drying phase was started by linearly raising the temperature to 15°C in 5 hours while maintaining a chamber pressure of 0.2 mbar. After reaching 15°C, this shelf temperature was maintained for 2 hours. Subsequently, the chamber pressure was lowered in one minute to 0.02 mbar while maintaining the temperature at 15°C. This temperature and pressure were maintained for another 5 hours, after which the lyophilization cycle was stopped. After completion, the vials were pneumatically closed, and sterile filtered medical grade nitrogen was added to lift the vacuum, and the vials were retrieved from the freeze-drier. The product was capped with aluminum caps and labeled.

In-process controls consisted of integrity testing of the filter, weight variation of the filling volume, and determination of the NAMI-A concentration and bioburden before and after filtration. Furthermore, the shelf, product, and condensor temperatures, as well as the chamber pressure were routinely monitored during the lyophilization process. Only clean, sterile, inert materials and glassware were used throughout the manufacturing process. All manipulations took place in a class 100 (A) down-flow cabinet inside a class 100 (B) clean room (Interflow, Wieringerwerf, The Netherlands). Air particle counts in the critical areas and microbiological contamination of area and personnel were monitored during the manufacturing process. Manufacturing was performed in compliance with the Good Manufacturing Practice (GMP) guidelines [13].
NAMI-A lyophilized product

Quality control

NAMI-A lyophilized products were characterized using the methods described for the drug substance as well as by visual inspection of appearance and color and determination of reconstitution characteristics (rate of dissolution and pH after reconstitution, presence of foreign insoluble matter) and residual moisture content.

Stability of NAMI-A lyophilized product

Thermogravimetric analysis

The five lyophilized formulations were subjected to thermogravimetric analysis employing a TGA7 Perkin Elmer thermogravimetric analyzer. Duplicate samples of approximately 2 mg were heated at 10°C/min from 30 to 200 °C in a nitrogen atmosphere.

Stability upon storage

NAMI-A lyophilized products were stored at –20 ± 2°C, 25±2°C/60±5% relative humidity (RH), and at 40±2°C/75±5% RH (both elevated temperatures in HEKK 0057 climate chambers obtained from Weiss Technik Ltd., Buckinghamshire, UK) for a year and duplicate or triplicate samples were taken at different points in time. These samples were visually analyzed for appearance and reconstitution characteristics (rate, presence of foreign substances, and pH after reconstitution) and were subjected to HPLC analysis to determine their content and purity. Furthermore, at the end of the 1-year storage period, the residual moisture content was determined for duplicate samples under each storage condition.

Photostability

NAMI-A lyophilized products in their primary containers underwent photostability testing in a Suntest CPS+ apparatus equipped with a xenon lamp (NXE 1500 B) with a coated quartz dish and window glass filter to allow only light with a wavelength of 320-800 nm and a spectral
distribution similar to the ID 65 standard, as required in the ICH guideline [14] (all Atlas Material Testing Technology LLC, Chicago, IL, USA). Samples and dark controls (samples placed alongside the exposed samples wrapped in aluminum foil) were exposed in duplicate to light at the highest irradiation level (after passage of the filters: 68.9 W/m² at < 400 nm and 170 klux at > 400 nm). The Suntest unit was connected to a Suncool™ chiller (Atlas) and the minimum and maximum temperature during exposure were monitored with a minimum-maximum thermometer (Merck). A 2% (w/v) quinine hydrochloride dihydrate solution served as the actinometric system to monitor the intensity of the ultraviolet (UV) radiation around 330 nm [15] according to the ICH guidelines [14]. The quinine actinometric solution and its dark control were exposed in standard 1 cm quartz cells with two frosted sides and tight-fitting PTFE caps (4 ml total volume, HELLMAM GmbH & Co, Müllheim, Germany).

**Results and discussion**

**Characterization of NAMI-A drug substance**

The results of the characterization of NAMI-A drug substance are shown in Table 1. The results are indicative of the structure of NAMI-A.

**Preformulation studies**

**Solubility and stability in water**

NAMI-A is soluble in water (up to 50 mg/ml). However, when dissolved in water, NAMI-A degrades rapidly (t₉₀ approximately 5 hours at + 20-22°C). Degradation follows (pseudo-)first-order kinetics, as was indicated by the linearity of plots of the natural logarithm of NAMI-A concentration versus time (r² > 0.92). Figure 2, showing the Arrhenius plot for NAMI-A in water, demonstrates that for every 10°C rise in temperature, the reaction rate constant approximately triples. The pH of an aqueous solution of NAMI-A is approximately 5.
Table 1. Identification and characterization of NAMI-A drug substance (Lot O9800905).

<table>
<thead>
<tr>
<th>Analytical method</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Appearance</td>
<td>Orange to red crystalline powder</td>
</tr>
<tr>
<td>Melting point</td>
<td>172-174°C</td>
</tr>
<tr>
<td>$^1$H NMR spectroscopy (DMSO-$_{d6}$)</td>
<td>Proposed assignments: ( \delta 14.16 \text{ ppm} = \text{ImH}^+ \ H_1 \text{ and } H_5; \ \delta 9.09 \text{ ppm} = \text{ImH}^+ \ H_3; \ \delta 7.73 \text{ ppm} = \text{ImH}^+ \ H_2 \text{ and } H_4; \ \delta -2.45 \text{ ppm} = \text{Im-Ru} \ H_3; \ \delta -6 \text{ ppm} = \text{Im-Ru} \ H_2 \text{ or } H_4; ) this signal is very broad and barely visible above the baseline; no other signal was observed for the remaining proton, probably because the corresponding signal was even broader, ( \delta -13, 39 \text{ ppm} = \text{DMSO CH}_3's )</td>
</tr>
<tr>
<td>MS</td>
<td>Molecular formula: ( \text{M} = \text{C}<em>8\text{H}</em>{15}\text{Cl}_4\text{N}_4\text{ORuS} )</td>
</tr>
<tr>
<td>Positive ion spectrum (the values for the most abundant signals in a cluster are given):</td>
<td>( m/z 597.9: [\text{M}+2\text{ImH}]^+ ); 528.1: ( [\text{M}+\text{ImH}]^+ ); 491.9: ( [\text{M}+\text{ImH}]^+-\text{HCl} )</td>
</tr>
<tr>
<td>Negative ion spectrum:</td>
<td>( m/z 439.1: m/z 371 + \text{Im}, 371 ) being NAMI-A in which one Cl has been replaced by OH; this may be present as an impurity, or it may be formed during the ionisation process; 389.9: ( [\text{M-ImH}] ); 371.0: see 439.1; 355.0: ( [\text{M-ImH}]^-\text{Cl} ); 321.9: ( [\text{M-ImH}]^-\text{Im} ); 287.0: ( [\text{M-ImH}]^-\text{Im-Cl} ); 243.9: ( [\text{M-ImH}]^-\text{Im-DMSO} ); 209.0: ( [\text{M-ImH}]^-\text{Im-Cl-DMSO} )</td>
</tr>
<tr>
<td>IR spectroscopy</td>
<td>Characteristic absorption bands (approximately): 3340 cm$^{-1}$: amine stretching; 3150-3100 cm$^{-1}$: aromatic stretching (imidazole); 2980-2900 cm$^{-1}$: CH$_3$ stretching; 1570 cm$^{-1}$: amine deformation; 1540-1480 cm$^{-1}$: aromatic stretching (imidazole); 1440-1370 cm$^{-1}$: CH$_3$ group deformation; 1330-1280 cm$^{-1}$: C-N stretching; 1159 cm$^{-1}$: S=O stretching; 830 cm$^{-1}$: aromatic C-H deformation (imidazole)</td>
</tr>
<tr>
<td>HPLC analysis</td>
<td>( t_R = 4.1 \text{ minutes} )</td>
</tr>
<tr>
<td>Purity &gt; 99.0%</td>
<td></td>
</tr>
<tr>
<td>UV/VIS spectrophotometry</td>
<td>Absorption maxima at 288, 390 and 452 nm.</td>
</tr>
</tbody>
</table>
Figure 3 shows the pH-rate profile of NAMI-A. As was observed and discussed previously [12], at acidic pH, degradation of NAMI-A in aqueous solution follows (pseudo-) first-order kinetics, whereas at neutral and alkaline pH, zero-order kinetics best describe the degradation process. NAMI-A in solution degrades by substitution of its ligands. At neutral and alkaline pH, the chloride groups are replaced by water molecules or hydroxide ions, at acidic pH this type of degradation is accompanied by hydrolysis of the DMSO ligand [11,12]. NAMI-A solutions are most stable between pH 3 and 4, requiring the use of an acidifier for NAMI-A’s formulation solution.

Figure 2. Arrhenius plot for NAMI-A in water.

Figure 3. 
A. pH-rate profile of NAMI-A at pH 1-5 (pseudo-) first-order kinetics.
B. pH-rate profile of NAMI-A at pH 6-10 (zero-order kinetics).
Influence of excipients

The effect of several different excipients on the stability of NAMI-A in solution was investigated (Table 2). Apart from enhancing the solubility, HP-ß-CD has also been reported to increase the aqueous stability of various compounds by preventing interaction between the solvent molecules and the drug that is incorporated within the cyclodextrin cavity [16]. However, HP-ß-CD in either a 1:1 or a 2:1 molar ratio with NAMI-A had a slightly negative effect on the degradation rate of NAMI-A in water (Table 2). Therefore, its use was not further investigated.

The addition of three different acidifiers was examined. Citric and acetic acid are commonly used to lower the pH of parenteral products [17], whereas hydrochloric acid in theory had the additional advantage of containing chloride, which could reduce the rate of hydrolysis of the chloride ligands of NAMI-A. The results in Table 2 show that all acidifiers tested stabilized NAMI-A in solution, and that the solutions at pH 4 were slightly more stable than the solutions at pH 3. This is most likely caused by the concentration of the acidifiers, which is higher for the solutions at pH 3. It was shown previously that higher buffer concentrations decrease the stability of NAMI-A in solution [12]. Citric acid and hydrochloric acid stabilized the solutions to the same extent, but acetate appeared to produce a slightly less stable solution. Therefore, further studies were conducted with citric and hydrochloric acid.

Addition of DMSO to solutions containing citric and hydrochloric acid further stabilized NAMI-A, probably by reducing hydrolysis of the DMSO ligand. The optimal concentration was 5% (v/v) DMSO, whereas addition of higher amounts did not increase the stability any further. Mannitol, a commonly applied bulking agent in lyophilized products, decreased the stability of buffered NAMI-A solutions, but addition of 5% DMSO to the solution containing mannitol countered its negative effect.

From the stability results, it was concluded that none of the examined solutions are suitable for sterilization by (moist) heat in the primary container, the first choice when manufacturing a parenteral product [18,19], or have pharmaceutically acceptable shelf-lives at feasible storage conditions. Therefore, it was decided to develop a lyophilized product.
### Table 2.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Excipient</th>
<th>Stability Ratio*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>none (pH 4.4)</td>
<td>1.00</td>
</tr>
<tr>
<td>Hydroxypropyl-β-cyclodextrin 2:1 (pH 4.3)</td>
<td></td>
<td>1.13</td>
</tr>
<tr>
<td>Acetic acid</td>
<td></td>
<td>1.20</td>
</tr>
<tr>
<td>Water</td>
<td></td>
<td>1.20</td>
</tr>
<tr>
<td>Hydroxypropyl-β-cyclodextrin 1:1 (pH 4.3)</td>
<td></td>
<td>1.25</td>
</tr>
</tbody>
</table>

*Stability ratio = (Percentage NAMI-A remaining in solvent under investigation after 48 hours) / (Percentage NAMI-A remaining in water after 48 hours).
Five formulation solution compositions (A-E) were investigated, all with a NAMI-A concentration of 10 mg/ml. Formulation A contained NAMI-A in only water, as a reference. The other formulations were all acidified in order to stabilize the formulation solution during the initial steps of the production process (i.e. dissolution, sterile filtration, filling, and freezing). Formulation B and C contained different amounts of citric acid to investigate the influence of different buffer concentrations on the stability of the final product. Upon freezing, citric acid in solution does not lead to a pH shift, and in theory this acid was the most suitable acidifier [20]. Hydrochloric acid could sublimate during lyophilization and the corresponding increase in pH might induce degradation of NAMI-A. On the other hand, HCl has the theoretical advantage of containing chloride ions, providing a more stable environment for NAMI-A. Thus, Formulation D was also investigated. Lastly, although mannitol slightly decreased the stability of NAMI-A in solution, its addition was investigated, because in solid state it might prove to be useful as a bulking agent. In order to attain a pH of 3 for this solution, however, a concentration of 5 mM citric acid (instead of 1.5 mM) was added. The addition of DMSO was not further investigated as lyophilization of a test solution of NAMI-A containing 5% DMSO resulted in a product with unacceptable appearance.

**Formulation studies**

**Differential Scanning Calorimetry and lyophilization**

Figure 4 shows the DSC thermograms of 10 mg/ml NAMI-A in the five different formulation solutions. All formulations show an ice melting endotherm, with extrapolated onsets of melting at –1.9°C, -1.7°C, -1.9°C, -1.0°C, and –3.4°C for the NAMI-A solutions in water, 0.1 mM HCl, 0.15 mM citric acid, 1.5 mM citric acid, and 2.5% mannitol in 5 mM citric acid, respectively. Apart from the ice melting endotherm, no other thermal events can be observed in any of the solutions, except the one containing mannitol in citric acid (see inset of Figure 4). This solution shows a glass transition temperature at –31°C, followed by a small endotherm and a subsequent crystallization exotherm at -26°C, which disappears upon repeating the freezing and heating cycle.
Figure 4. DSC thermograms of 10 mg/ml NAMI-A in water (A), 0.15 mM citric acid (B), 1.5 mM citric acid (C), 0.1 mM hydrochloric acid (D), and 5 mM citric acid with 2.5% (w/v) mannitol (E).
This behavior has been described previously for mannitol and was found to occur at slightly higher (2-3°C) temperatures using similar freezing rates but faster heating rates [21-23]. As Her and Nail [22] described, the midpoints of transitions occur at higher temperatures at faster heating rates, and the results obtained here confirm these findings. All blank solvents showed identical DSC thermograms to the formulation solutions (extrapolated onsets of melting within 0.2-1.0°C of the solutions containing NAMI-A), indicating that the freezing and heating properties of the formulation solutions are due to the solvents and that addition of NAMI-A does not influence these properties. As no thermal events were observed in the DSC thermograms of formulations A-D, no special precautions were required in the freeze-drying cycle. However, lyophilization of formulation E required the use of an annealing step to ensure complete crystallization of mannitol. As degradation of NAMI-A is temperature-dependent, the shelves of the freeze-drier were pre-chilled to 10°C to prevent degradation after the production process as much as possible.

**NAMI-A lyophilized product**

Table 3 shows the results of the quality control performed after manufacture of the five batches of lyophilized product. Formulation E had formed a solid cake due to the presence of mannitol, whereas for all other formulations, the product was present as spheres inside the vials. All formulations immediately reconstituted upon addition of sterile WFI to form a clear, dark yellow solution, free from foreign insoluble matter. Formulations A, B, and E had the same pH after reconstitution as the formulation solutions before lyophilization. The pH of formulation C and D was found to be slightly higher after reconstitution. For formulation D, this might be due to some evaporation of HCl upon lyophilization. The residual moisture content of all formulations was low, although formulation E contained significantly higher amounts than the others, probably due to the presence of mannitol [24].
### Table 3: Quality control of NAMI-A lyophilized products.

<table>
<thead>
<tr>
<th>Test Item</th>
<th>Drug substance</th>
<th>Lot 0200</th>
</tr>
</thead>
<tbody>
<tr>
<td>Appearance</td>
<td>Orange-red powder</td>
<td>Dark yellow cakes</td>
</tr>
<tr>
<td>pH after reconstitution</td>
<td>&lt; 4.4</td>
<td>B: 288, 390, 452 nm</td>
</tr>
<tr>
<td>Foreign insoluble matter</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>UV/VIS analysis</td>
<td>A: 1 hr = 4.1 min, B: 94.7%</td>
<td>C. Identity: B. Concentr. A: 1 hr = 4.1 min, B: 94.7%</td>
</tr>
<tr>
<td>HPLC analysis</td>
<td>A. tR = 4.1 min, B: 94.7%</td>
<td>C. Purity: B. 94.7%</td>
</tr>
<tr>
<td>Residual moisture content</td>
<td>&lt; LOD</td>
<td>C. Purity: C. 99.6%</td>
</tr>
</tbody>
</table>

Formulation A = sterile Water for Injections, Formulation B = 0.15 mM citric acid, Formulation C = 1.5 mM citric acid, Formulation D = 0.1 mM hydrochloric acid, Formulation E = 5 mM citric acid + 2.5% (w/v) mannitol. a Purity = percentage of the NAMI-A peak area with respect to the total peak area. b \( \lambda_{\text{max}} \) = wavelength of maximum absorption. LOD = limit of detection.
**Stability of NAMI-A lyophilized product**

*Thermogravimetric (TG) analysis*

TG analysis can be used as a screening tool for incompatibilities between components of a formulation and for prediction of long-term stability, although isothermal formal stability studies are required to confirm indicative results obtained by this method [25]. Figure 5 shows the TG profiles of the five formulations of NAMI-A. Formulations A, B, and D do not show weight loss up to 150°C and degradation for all three samples starts at 180°C. The behavior of these samples is identical to that of NAMI-A drug substance. For formulation C, the onset of the first degradation process is about 130°C, whereas for formulation E, there is a small weight loss at 80°C, followed by the start of degradation at 140°C. TG-IR analysis of the gases evolving from the samples showed that the weight loss at 80°C was due to water evaporation (only observed in formulation E, which contained a higher residual moisture content than the other formulations), and that the onset of degradation corresponded to the emission of hydrochloric acid (HCl) gas. Thus, thermal decomposition of NAMI-A lyophilized product involved the release of a chloride ligand with HCl emission. The presence of citric acid in a concentration higher than 0.15 mM and/or the presence of mannitol apparently decreased the thermal stability of NAMI-A lyophilized substance. It thus appeared that formulation C and E could be expected to show a lower stability upon storage than the other three formulations.

![Thermogravimetric profiles of NAMI-A lyophilized product.](image)
Stability upon storage

All five formulations of NAMI-A were stable when stored at -20°C. Table 4 shows the results obtained after one year of storage at 25°C / 60% RH and 40°C / 75% RH. The long-term storage condition was defined as 25°C / 60% RH, whereas storage at 40°C / 75% RH was considered to be the accelerated condition. Formulation A, B, and D showed no change in content and chromatographic purity for either storage condition, while formulation C and E slowly degraded. Formulation E proved to be the least stable formulation, with 70% of the initial content remaining after 1 year of storage at 40°C/75% RH in the dark. In Formulation E, the presence of mannitol (and/or its related higher moisture content) may have added to the observed instability. The visual appearance of all formulations remained unchanged at all storage conditions, except Formulation E stored at 40°C, which paled in time. These findings confirm the expected lower stabilities of Formulations C and E based on TG analysis.

The results obtained with Formulation C indicate that the presence of citric acid in a concentration higher than 0.15 mM destabilized the lyophilized product. Therefore, it was decided not to use citric acid as acidifier. Although formulations A and D were equally stable, formulation D was chosen as the formulation to be used in the manufacture of NAMI-A lyophilized product, because the presence of hydrochloric acid stabilized the formulation solution during the production process ($t_{90}$ approximately 6.3 hours).

Table 4. Percentage NAMI-A remaining with respect to initial content ± standard deviation after 1 year of storage.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>25°C / 60% RH, dark</th>
<th>40°C / 75% RH, dark</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>100.2 ± 0.2</td>
<td>99.1 ± 0.9</td>
</tr>
<tr>
<td>B</td>
<td>93.3 ± 6.3</td>
<td>98.5 ± 0.3</td>
</tr>
<tr>
<td>C</td>
<td>86.8 ± 5.6</td>
<td>92.1 ± 0.7</td>
</tr>
<tr>
<td>D</td>
<td>99.3 ± 0.1</td>
<td>96.0 ± 5.2</td>
</tr>
<tr>
<td>E</td>
<td>82.2 ± 8.0</td>
<td>70.6 ± 5.7</td>
</tr>
</tbody>
</table>
Photostability

All five formulations of NAMI-A lyophilized product proved to be stable when exposed in their primary container to light of high intensity. No difference was observed between the dark controls and the exposed samples. Thus, NAMI-A lyophilized product does not need to be stored in the dark. However, in solution NAMI-A is sensitive to light and after reconstitution and dilution, it should be protected from light [26].

Conclusion

The development of a stable, lyophilized formulation of NAMI-A is presented. The formulation solution needs to be acidified in order to stabilize it during the production process. Out of the five different formulations of NAMI-A that underwent stability testing, the product lyophilized from a formulation solution containing 0.1 mM hydrochloric acid was selected for further development. It is photostable and stable for at least one year when stored at 25°C/60% RH. TG analysis proved to be predictive of the stability ranking, with the product showing weight loss at the lowest temperature also showing the lowest long-term stability.

References

