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Pharmaceutical development of a parenteral lyophilized formulation of the antimetastatic 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 (TG) analysis and stability studies at various conditions for 1 year. Minimal degradation during the production process is achieved with a formulation solution of pH 3–4. Of the acids tested, only hydrochloric acid (HCl 0.1 mM) both stabilized the formulation solution and was compatible with the lyophilized product. This product was stable for at least 1 year when stored at −20 °C, 25 °C/60% relative humidity (RH) and 40 °C/75% RH, and was also photostable.

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Keywords: NAMI-A; Pharmaceutical formulation; Stability

1. Introduction

NAMI-A (imidazolium *trans*-tetrachloro(dimethylsulfoxide)imidazoleruthenium(III), H_2im-[*trans*-$RuCl_4$(DMSO)Him], Fig. 1 (Sava et al.,

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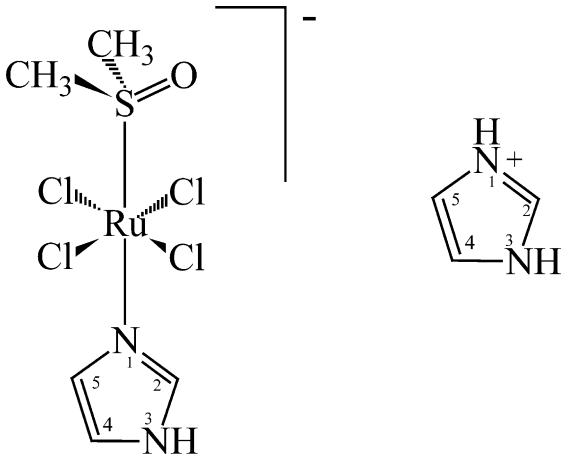

Fig. 1. Chemical structure of NAMI-A, molecular weight 458.18 g/mol.

1999a) is a novel ruthenium anticancer agent. Preclinical pharmacological and toxicological studies showed selective activity against lung metastases of murine tumors (Mestroni et al., 1994; Sava et al., 1998, 1999a,b) and low toxicity in mice and dogs (Bergamo et al., 1999; Cocchietto and Sava, 2000; Sava and Cocchietto, 2000). Its action seems to be independent of the origin (type of primary tumor) and stage of growth of the metastases (Bergamo et al., 1999; Sava et al., 1999b). NAMI-A possesses no direct tumor cell cytotoxicity (Sava et al., 1998; Bergamo et al., 1999; Sava et al., 1999b, 2000), 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 (Sava et al., 1998, 2000; Zorzet et al., 2000). Based on the promising activity and toxicity profile, NAMI-A was developed as an antimetastatic agent.

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 five times 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 (Davignon et al., 1988).

2. Material and methods

2.1. Chemicals and materials

NAMI-A drug substance was supplied by SIGEA Srl (Trieste, Italy). NAMI-A lyophilized products were manufactured in-house (Department of Pharmacy and 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 substitution 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.).

2.2. ^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-d_6 (1–2 mg in 0.65 ml). The central DMSO line at 2.505 ppm was used as the reference line.

2.3. 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 30 V.

2.4. Infrared (IR) spectroscopy

IR spectra of NAMI-A were recorded with a Model PU 9706 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 min.

2.5. 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.

2.6. 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 min was employed for the standard samples (calibration curves and quality controls) and a run time of 30 min 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 min. The method was proven to be stability-indicating, precise, and accurate (Bouma et al., 2002c).

2.7. Melting point

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

2.8. Preformulation studies

2.8.1. 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 s, if not dissolved placed in an ultrasonic bath for 15 min, 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 and analyzed by HPLC. Furthermore, UV/VIS analysis was performed on the same samples: their absorption was measured directly at 390 nm and quantified in relation to a standard calibration curve (20–120 μg/ml NAMI-A in distilled water). The two analytical methods were proven to be equivalent. The observed rate constants at the various temperatures were calculated from the slopes of the ln concentration–time plots (multiplied by −1).

2.8.2. pH-rate profile

A pH-rate profile of NAMI-A in buffered aqueous solution was constructed as described previously (Bouma et al., 2002a).

2.8.3. 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 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.

2.8.4. 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 min was built in. As a reference, all formulation solutions (without NAMI-A) were subjected to the same DSC procedures.

2.9. 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) 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 HCl (pH 3.8),
E) 5 mM citric acid in 2.5% (w/v) mannitol (pH 2.8).

2.9.1. 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 (Münnerstädter 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 h, after which the temperature was increased to −20 °C in 2 h, then decreased to −40 °C in 2 h (annealing step). This temperature was maintained for 1.5 h. After this period, the primary drying phase was started by establishing a vacuum of 0.2 mbar in 1 min and raising the temperature to −20 °C in 1 h. This temperature and pressure were maintained for 55 h. Subsequently, the secondary drying phase was started by linearly raising the temperature to 15 °C in 5 h while maintaining a chamber pressure of 0.2 mbar. After reaching 15 °C, this shelf temperature was maintained for 2 h. Subsequently, the chamber pressure was lowered in 1 min to 0.02 mbar while maintaining the temperature at 15 °C. This temperature and pressure were maintained for another 5 h, 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 (European Commission, 1997).

2.10. NAMI-A lyophilized product

2.10.1. 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.

2.11. Stability of NAMI-A lyophilized product

2.11.1. Thermogravimetric analysis

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

2.11.2. 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 1 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.

2.11.3. 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 (ICH Guideline Q1B, 1996) (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/m^2 at <400 nm and 170 klux at >400 nm). The Suntest unit was connected to a Suncool^{TM} 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 (Yoshioka et al., 1994) according to the ICH guidelines (ICH Guideline Q1B, 1996). 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, HELLMA GmbH and Co., Müllheim, Germany).

3. Results and discussion

3.1. 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.

3.2. Preformulation studies

3.2.1. 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 (t_{90} approximately 5 h 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 ($r^2 > 0.92$, measured over 24 h, seven points in time). Fig. 2, showing the Arrhenius plot for NAMI-A in water, demonstrates that

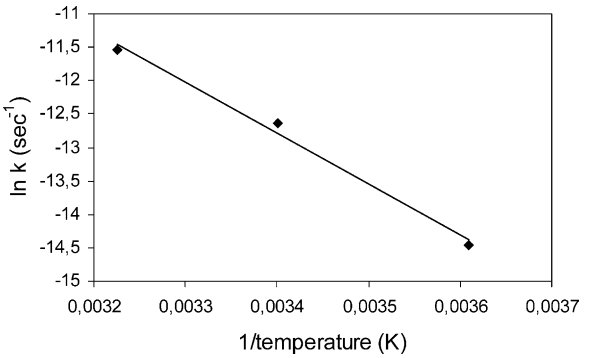

Fig. 2. Arrhenius plot for NAMI-A in water (pH 5).

Table 1
Identification and characterization of NAMI-A drug substance (Lot O9800905)

Analytical method	Results
Appearance	Orange to red crystalline powder
Melting point	172–174 °C
^{1}H NMR spectroscopy (DMSO-d_6)	Proposed assignments: δ 14.16 ppm = ImH$^+$ H_1 and H_3; δ 9.09 ppm = ImH$^+$ H_5; δ 7.73 ppm = ImH$^+$ H_2 and H_4; δ −2.45 ppm = Im–Ru H_5; δ −6 ppm = Im–Ru H_2 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 δ −13, 39 ppm = DMSO CH_3's
MS	Molecular formula: M = $C_8H_{15}Cl_4N_4OruS$, Positive ion spectrum (the values for the most abundant signals in a cluster are given): *m/z* 597.9: $[M+2ImH]^+$; 528.1: $[M+ImH]^+$; 491.9: $[M+ImH]^+ -$ HCl, Negative ion spectrum: *m/z* 439.1: *m/z* 371 + 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: $[M-ImH]^-$ 371.0: see 439.1; 355.0: $[M-ImH]^-$Cl; 321.9: $[M-ImH]^- -$Im; 287.0: $[M-ImH]^- -$Im−Cl; 243.9: $[M-ImH]^- -$Im−DMSO; 209.0: $[M-ImH]^- -$Im−Cl−DMSO
IR spectroscopy	Characteristic absorption bands (approximately): 3340 per cm:amine stretching; 3150–3100 per cm: aromatic stretching (imidazole); 2980–2900 per cm: CH_3 stretching; 1570 per cm: amine deformation; 1540–1480 per cm: aromatic stretching (imidazole); 1440–1370 per cm: CH_3 group deformation; 1330–1280 per cm: C–N stretching; 1159 per cm: S=O stretching; 830 per cm: aromatic C–H deformation (imidazole)
HPLC analysis	t_R = 4.1 min, purity >99.0%
UV/VIS spectrophotometry	Absorption maxima at 288, 390 and 452 nm

for every 10 °C rise in temperature, the reaction rate constant approximately triples. Upon degradation, the pH of an aqueous solution of NAMI-A drops from five immediately after preparation to approximately 3.5 in 54 h (Bouma et al., 2002a).

Fig. 3 shows the pH-rate profile of NAMI-A. As was observed and discussed previously (Bouma et al., 2002a), 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 (Bouma et al., 2002a,c). NAMI-A solutions are most stable between pH 3 and 4, requiring the use of an acidifier for NAMI-A's formulation solution.

3.2.2. *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 (Rajewski and Stella, 1996). However, HP-β-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.

Table 2
Stability of 10 mg/ml NAMI-A in different solvents with and without addition of excipients when stored at room temperature in the dark

Solvent	Excipient	Stability ratio[a]
Water	None (pH 4.4)	1.00
	Hydroxypropyl-β-cyclodextrin 1:1 (pH 4.3)	0.94
	Hydroxypropyl-β-cyclodextrin 2:1 (pH 4.3)	0.91
Acetate buffer	1 mM (pH 2.9)	1.06
	0.1 mM (pH 4.0)	1.12
Citric acid	1.5 mM (pH 3.2)	1.19
	0.15 mM (pH 3.9)	1.25
	0.15 mM+5% DMSO (pH 4.0)	1.29
	0.15 mM+2.5% mannitol (pH 4.2)	1.03
	0.15 mM+5% DMSO+2.5% mannitol (pH 4.0)	1.27
HCl	1 mM (pH 3.2)	1.20
	0.1 mM (pH 3.9)	1.25
	0.1 mM+5% DMSO (pH 4.0)	1.31
	0.1 mM+2.5% mannitol (pH 4.2)	1.13
	0.1 mM+5% DMSO+2.5% mannitol (pH 4.1)	1.27

[a] Stability ratio, percentage NAMI-A remaining in solvent under investigation after 48 h/percentage NAMI-A remaining in water after 48 h.

The addition of three different acidifiers was examined. Citric and acetic acid are commonly used to lower the pH of parenteral products (Strickley, 1999), whereas HCl 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

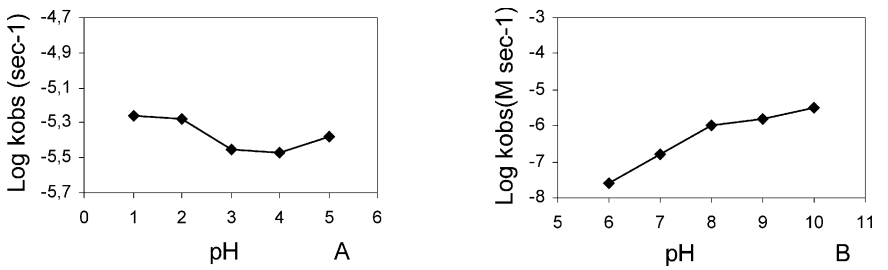

Fig. 3. (A) pH-rate profile of NAMI-A at pH 1-5 ((pseudo-) first-order kinetics) (B) pH-rate profile of NAMI-A at pH 6–10 (zero-order kinetics)

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 (Bouma et al., 2002a). Citric acid and HCl 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 HCl.

Addition of DMSO to solutions containing citric and HCl 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 (European Pharmacopoeia, 1997; Decision Trees for the Selection of Sterilisation Methods, 2000), or have pharmaceutically acceptable shelf-lives at feasible storage conditions. Therefore, it was decided to develop a lyophilized product.

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 (Shalaev et al., 2002). HCl 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.

3.3. Formulation studies

3.3.1. Differential scanning calorimetry and lyophilization

Fig. 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, −1.7, −1.9, −1.0 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 Fig. 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. 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 (Hatley, 1991; Her and Nail, 1994; Lueckel et al., 1998). As Her and Nail (1994) 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

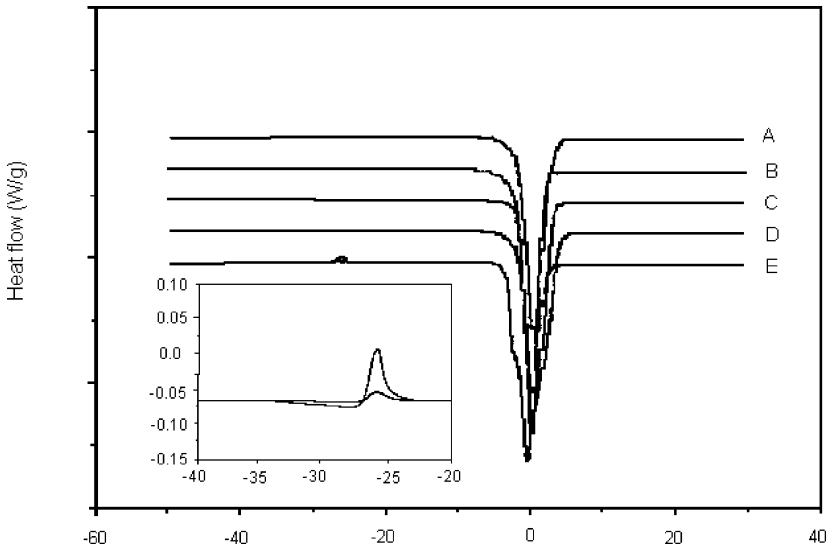

Fig. 4. DSC thermograms of 10 mg/ml NAMI-A in water (A), 0.15 mM citric acid (B), 1.5 mM citric acid (C), 0.1 mM HCl (D), and 5 mM citric acid with 2.5% (w/v) mannitol (E).

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.

3.4. 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 (Fakes et al., 2000).

3.4.1. Stability of NAMI-A lyophilized product

3.4.1.1. 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 (Ford and Timmins, 1989). Fig. 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

Table 3
Quality control of NAMI-A lyophilized products

Test item	Drug substance (Lot 0200)	Formulation A	Formulation B	Formulation C	Formulation D	Formulation E
Appearance	Orange–red powder	Dark yellow sphere	Dark yellow spheres	Dark yellow spheres	Dark yellow spheres	Dark yellow cakes
pH after reconstitution	–	4.4	3.9	3.2	4.4	2.8
Foreign insoluble matter	–	None	None	None	None	None
UV/VIS analysis	λ_{max} 288, 390, 452 nm[b]	λ_{max} 288, 390, 452 nm[b]	λ_{max} 288, 390, 452 nm[b]	λ_{max} 288, 390, 452 nm[b]	λ_{max} 288, 390, 452 nm[b]	λ_{max} 288, 390, 452 nm[b]
HPLC analysis						
A. Identity	A. $t_R = 4.1$ min	A. $t_R = 4.1$ min	A. $t_R = 4.1$ min	A. $t_R = 4.1$ min	A. $t_R = 4.1$ min	A. $t_R = 4.1$ min
B. Content	B. –	B. 93.2%	B. 94.7%	B. 90.0%	B. 95.8%	B. 94.0%
C. Purity[a]	C. 99%	C. 99.2%	C. 99.2%	C. 99.4%	C. 99.1%	C. 99.2%
Residual moisture content	–	$<$LOD[c]	$<$LOD[c]	0.1%	0.2%	1.2%

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 HCl, 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] Wavelength of maximum absorption.

[c] Limit of detection.

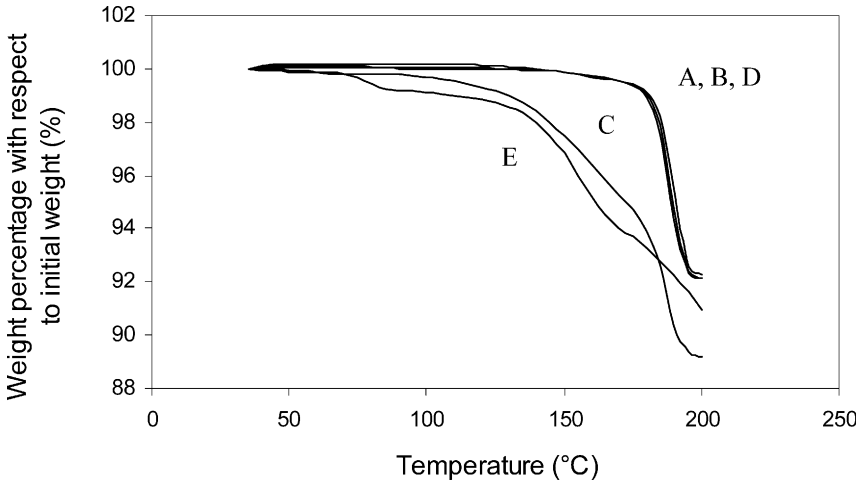

Fig. 5. TG profiles of NAMI-A lyophilized product.

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 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.

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

Formulation	Storage condition	
	25 °C/60% RH, dark	40 °C/75% RH, dark
A	100.2 ± 0.2	99.1 ± 0.9
B	93.3 ± 6.3	98.5 ± 0.3
C	86.8 ± 5.6	92.1 ± 0.7
D	99.3 ± 0.1	96.0 ± 5.2
E	82.2 ± 8.0	70.6 ± 5.7

3.4.1.2. Stability upon storage. All five formulations of NAMI-A were stable when stored at −20 °C. Table 4 shows the results obtained after 1 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. Formulations 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 HCl stabilized the formulation solution during the production process (t_{90} approximately 6.3 h).

3.4.1.3. 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 (Bouma et al., 2002b).

4. 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 which underwent stability testing, the product lyophilized from a formulation solution containing 0.1 mM HCl was selected for further development. It is photostable and stable for at least 1 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.

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