RESEARCH ARTICLE

Pharmaceutical Development of a Parenteral Lyophilized Formulation of the Novel Antitumor Agent Aplidine

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ABSTRACT: Aplidine is a naturally occurring cyclic depsipeptide isolated from the Mediterranean tunicate Aplidium albicans. Aplidine displays promising in vitro and in vivo antitumor activities against various solid human tumor xenografts and is therefore developed now for clinical testing. The aim of this study was to develop a stable parenteral pharmaceutical dosage form for clinical Phase I testing. Aplidine raw material was characterized by using several chromatographic and spectrometric techniques. These experiments showed that aplidine exists as two isomers. A stability-indicating HPLC assay was developed. Solubility testing showed that aplidine exhibits very poor aqueous solubility. Because solubilized aplidine showed substantial degradation under heat and light stress testing conditions, it was decided to develop a lyophilized dosage form. Freeze-drying was carried out with a 500 µg/mL solution of aplidine in 40% (v/v) tert-butanol in Water for Injection (WfI) containing 25 mg/mL D-mannitol as a bulking agent. Differential scanning calorimetry was applied to determine the optimal freeze-drying cycle parameters. The prototype, containing 500 µg aplidine and 25 mg D-mannitol per vial, was found to be the optimal formulation in terms of solubility, length of lyophilization cycle, and dosage requirements in the forthcoming Phase I clinical studies. Quality control of the freeze-dried formulation demonstrates that the manufacturing process does not affect the integrity of aplidine. The optimal reconstitution solution was found to be 15/15/70% (v/v/v) Cremophor EL/ethanol/WfI (CEW). Both reconstituted product and dilutions of the reconstituted product with normal saline (up to 1:100 v/v) appeared to be stable for at least 24 hours after preparation. Shelf-life data, available thus far, show that the lyophilized formulation is stable for at least 1 year when stored at +2–8°C in the dark.

Introduction

Aplidine (dehydrodidemnin B (DDB), MW 1109, Fig. 1) is a novel representative of an evolving group of anticancer agents derived from marine sources (1, 2). This naturally occurring cyclic depsipeptide is isolated from the Mediterranean tunicate Aplidium albicans and belongs to the didemnin family, a class of marine-derived compounds which exhibit antiviral, antitumor, and immunosuppressive activity. All didemnins share a common macrocyclic peptide structure and differ only in the side-chain attached to the backbone by the amino group of threonine (3). Aplidine exists in two conformers or rotamers referring to the cis and trans isomers of the pyruvoyl-proline amide bond (Fig. 2) (4).

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Didemnin B (DB), the most potent representative of the didemnin class up to now, was the first marine-derived anticancer compound to enter clinical trials in the early 1980's. Although interesting results were seen in this phase I/II programme sponsored by the National Cancer Institute (NCI), the occurrence of dose-limiting neuromuscular and cardiac toxicity hindered further dose-escalation and repeated cycles of therapy (2). The dehydro-derivative of DB, dehydrodidemnin B (DDB) or aplidine, displays even more potent *in vitro* and *in vivo* antitumor activity against various solid human tumor xenografts. As its parent compound, the antitumor effect of aplidine is believed to be primarily mediated through inhibition of the cell cycle progression in the G1 phase by binding to elongation factor 1α in the presence of GTP, thus interfering with protein synthesis (5, 6). *In vitro* experiments revealed that aplidine exerts less neurotoxicity and cardiotoxicity at antitumor concentrations than didemnin B. On the basis of these results, aplidine has been identified as a new generation of the didemnin class, with a possibly significantly higher therapeutic index (2, 7, 8, 9).

In animal toxicology studies a maximum tolerated dose (MTD) of 1250 µg/kg body weight in mice and a MTD of 570 µg/kg body weight in rats were determined, respectively. Taking the 1/10 MTD mouse-equivalent, the initial dosage level in Phase I clinical trials was therefore defined at 350 µg/m^2 (10, 11). A suitable parenteral formulation containing 500 µg aplidine per dosage unit was required to start early clinical studies. Because of its very poor aqueous solubility (< 0.1 mg/mL), an adequate vehicle had to be found to administer aplidine to the patient. The work described here was directed towards developing a suitable parenteral formulation for toxicological and clinical evaluation according to the EORTC/CRC/NCI Joint Formulation Working Party (JFWP) guidelines (12, 13, 14). Aplidine bulk drug substance was fully structurally and analytically characterized. The formulation approach involved the use of a cosolvent-surfactant system to enhance the solubility, and lyophilization to improve the stability of the compound.

Materials and Methods

Chemicals and Materials

Aplidine was obtained from natural didemnin A by a three-step synthesis under the responsibility of Pharma Mar s.a. (Tres Cantos, Madrid, Spain) and provided through the New Drug Development Office-Oncology (NDDO-Oncology, Amsterdam, The Netherlands). All chemicals used were of analytical grade and were used without further purification. Distilled water was used throughout. Excipients, including *tert*-butanol, and primary packaging materials used in the manufacturing of aplidine lyophilized product and reconstitution solution were of European Pharmacopeia III (Ph. Eur. III) or United States Pharmacopeia 24 (USP24) grade and provided by the supplier with a Certificate of Analysis. Substances were approved on the basis of in-house quality controls carried out according to monographs in the mentioned pharmacopeias. Normal saline (0.9% w/v sodium chloride in WfI) was manufactured in-house at the Department of Pharmacy of the Slotervaart Hospital (Amsterdam, The Netherlands).

Characterization of Aplidine Bulk Drug

An interim reference standard of the aplidine bulk drug material, i.e., batch of highest purity available, was defined (Lot APL-297) and structurally characterized by fast-atom bombardment mass spectrometry (FAB-MS), nuclear magnetic resonance (NMR), and infra-red (IR) spectroscopy, and analytically characterized by high performance liquid chromatography (HPLC) and ultraviolet/visible (UV/VIS) spectrophotometry. On the basis of these results, specifications were drawn up for the aplidine raw material.

FAB-MS: The FAB mass spectrum was obtained with a Model JMS-SX/SX 102A tandem mass spectrometer (BEBE; JEOL, Tokyo, Japan). The acceleration voltage was 10 kV. A xenon source with an energy of approximately 6 keV was used, and the matrix was glycerol. Positive ion spectra were recorded over a mass range of 10-1500 D.

NMR-spectroscopy: ^{1}H NMR spectra were recorded with a Gemini 300 BB instrument (Varian Assoc., Palo Alto, CA USA) at 300.1 MHz. The sample (1 mg) was dissolved in deuterochloroform ($CDCl_3$) or hexadeuterodimethylsulfoxide (DMSO-d_6). In $CDCl_3$, TMS was used as an internal reference; in DMSO-d_6 the central DMSO line was set at 2.50 ppm.

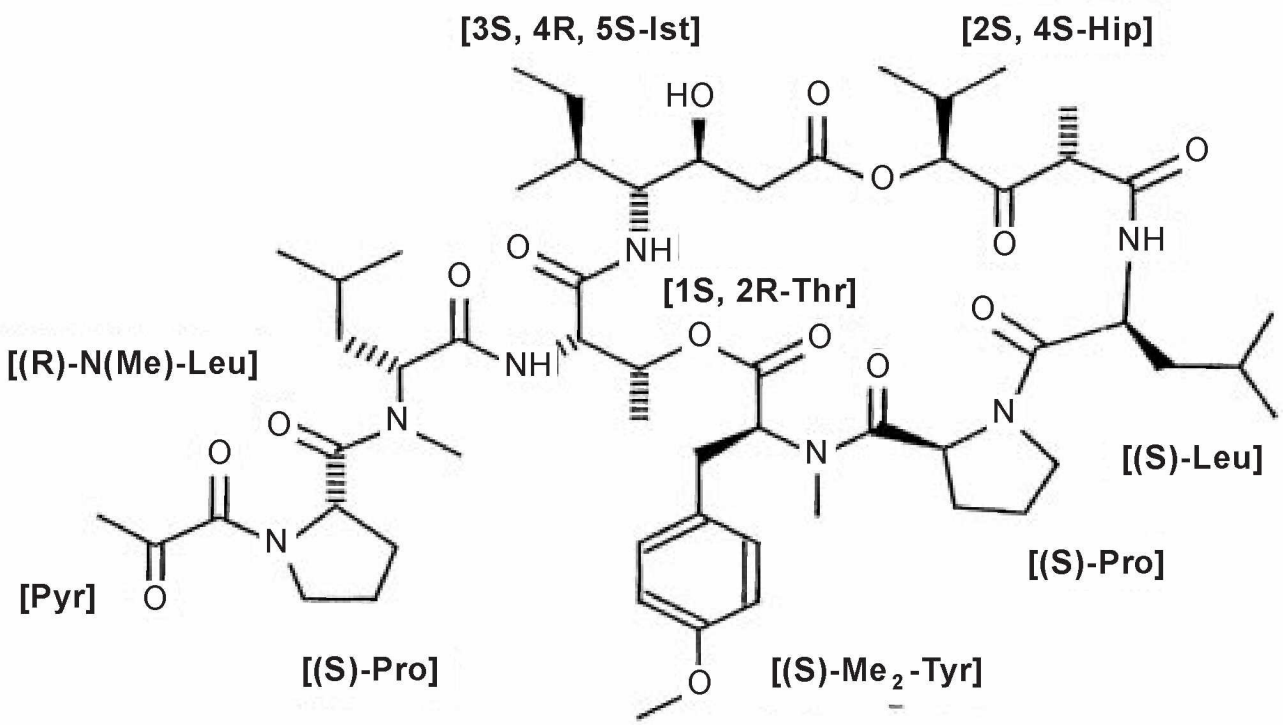
Infra-red spectroscopy: Infra-red (IR) spectra (4000–600 cm^{-1}) were recorded on a Model PU 9706 IR spectrophotometer (Philips Nederland B.V., Eindhoven, The Netherlands) with the potassium bromide (KBr) pellet technique. The pellet consisted of 1 mg of aplidine bulk drug and 200 mg of KBr. The ratio recording mode was set on autosmooth and the scan time was 8 minutes.

UV/VIS spectroscopy: UV/VIS spectra (800–200 nm) of aplidine bulk drug (50 µg/mL in 79% (v/v) methanol/H_2O) were recorded with a Model UV/VIS 918 spectrophotometer (GBC Scientific Equipment, Victoria, Australia) equipped with an LEO personal computer and an Epson LX-400 plotter.

Formulation of Aplidine

Solubility studies: The solubility of aplidine in various solvents at ambient temperature (20–25°C) was examined by accurately weighing approximately 1 mg of aplidine in a glass test tube and adding subsequent solvent volumina of 100 µL, 1 mL, and 10 mL to the bulk drug. After each addition the mixture was vigorously shaken for 30 seconds, placed in an ultrasonic bath for 15 minutes and examined visually under polarized light for complete dissolution of the aplidine drug substance. In this way, the solubilities of aplidine in the various solvents were selectively distributed over four solubility ranges ($s < 0.1$ mg/mL, 0.1 mg/mL $\leq s <$

Figure 1: Chemical structure of aplidine (Hip: hydroxyisovalerylproprionyl; Ist: isostatine; Leu: leucine; Pro: proline; Pyr: pyruvoyl; Thr: threonine; Tyr: tyrosine).



1 mg/mL, 1 mg/mL ≤ s < 10 mg/mL and s > 10 mg/mL, respectively). In addition, solvents in which aplidine dissolved were examined by diluting the solutions 1:1, 1:5, 1:10, 1:50, and 1:100 (v/v) with normal saline for infusion in glass test tubes. After gentle agitation, each of the dilutions was examined visually under polarized light over a one day period for any sign of precipitation.

Reconstitution of aplidine lyophilized product with solutions composed of 40/10/50% (v/v/v) propylene glycol 400/ethanol/polysorbate 80 (PET), 40/10/50% (v/v/v) propylene glycol 400/ethanol/WfI (PEW), and 5/5/90% (v/v/v), 15/15/70% (v/v/v), and 30/30/40% (v/v/v) Cremophor EL/ethanol/WfI was studied by adding increasing volumes of the reconstitution solutions to 500 µg aplidine lyophilized cake. After each addition, the resulting mixture was agitated and subsequently examined visually under polarized light. Quantitative analysis was carried out by diluting 50 µL samples of reconstituted product with 950 µL of acetonitrile and subsequent injection onto the HPLC system. Furthermore, in case of complete dissolution, the reconstituted solution was diluted 1:10 or 1:100 (v/v) with normal saline for infusion in glass test tubes. Stability of the reconstituted solution and the infusion solutions were examined visually under polarized light and by HPLC without further dilution, over a 24-hour period at room temperature (20–25°C) and a normal day-night light cycle.

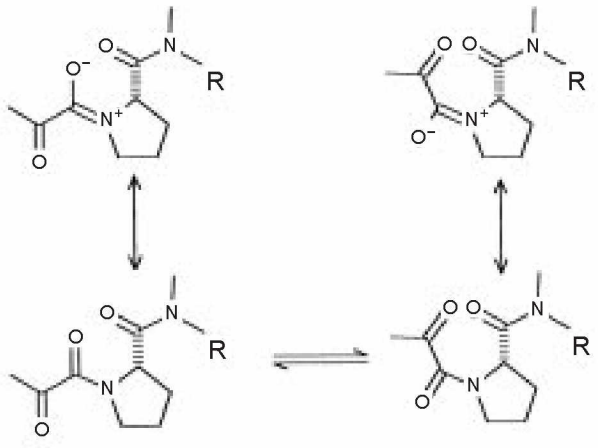
Differential scanning calorimetry: Transition temperatures and freezing characteristics of tert-butanol, D-mannitol, and aplidine solutions were examined by differential scanning calorimetry (DSC). The DSC experiments were carried out with a TA Instruments DSC 2920 (TA Instruments, New Castle (DE), USA) equipped with an LNCA for low temperatures. Samples were sealed in a closed aluminium pan with an empty pan as reference. Temperature scale and heat flux were calibrated with indium. Samples were cooled to -50°C at a rate of 5°C/min. The DSC heating rate was 2.5°C/min. Analyses were performed under a helium purge.

Formulation process: Aplidine lyophilized product was aseptically prepared from a 500 µg/mL aplidine solution in 40% (v/v) tert-butanol in WfI containing 25 mg/mL D-mannitol as a bulking agent. The formulation solution was prepared by weighing aplidine and D-mannitol and dissolving the substances by magnetic stirring in 40% (v/v) tert-butanol in WfI. To make a final concentration of 500 µg/mL aplidine, 40% (v/v) tert-butanol in WfI was added. The formulation solution was sterile filtered through a 0.2 µm Midisart 2000 filter (Sartorius, Nieuwegein, The Netherlands). Subsequently, 1 mL aliquots of the formulation solution were filled into 10 mL type 1 glass vials with a Model 501Dz peristaltic pump (Watson Marlow, UK). After filling, vials were partially closed with grey butyl rubber stoppers, placed in a Model Lyovac GT4 freeze-dryer (AMSCO/FinnAqua, Germany), and lyophilized. The freeze-dryer was equipped with a cold trap filled with liquid nitrogen attached to the condensor to ensure complete condensation of tert-butanol. After completion of the freeze-drying cycle, sterile filtered medical grade nitrogen gas was leaked into the freeze-drying chamber to reach a final vacuum of 100 mbar. Subsequently, the vials were pneumatically closed, capped, and labeled. In-process-controls consisted of integrity testing of the filter unit, weight variation of the filling volume, and determination of the aplidine concentration of the formulation solution before and after filtration. Only clean, sterile inert materials and glassware were used throughout the manufacturing process. All critical manipulations took place under a class 100 (A) down-flow condition with a class 100 (B) background (Interflow, Wieringerwerf, The Netherlands). Air particle counts in the critical areas as well as microbiological contamination of the area and personnel were monitored at operating state.

Reconstitution solution 15/15/70% (v/v/v) Cremophor EL/ethanol/WfI (CEW) was prepared by mixing the appropriate volumes of excipients by magnetic stirring. The solution obtained was filtered through a 0.2 µm Midisart 2000 filter unit (Sartorius, Goettingen, Germany) and subsequently 2 mL

aliquots were filled into type 1 glass ampoules with a Model R910 ampoule fill-and-seal machine (Rota den Boer B.V., The Netherlands). After filling, the solution was sterilized by autoclaving (Model 6.6.15 autoclave, Koninklijke Ad Linden B.V., The Netherlands) for 20 minutes at 120°C. All manipulations involving an "open" solution or excipient were conducted under class 100 (A) conditions. The manufacturing of both aplidine lyophilized product and its reconstituted solution were performed according to the Good Manufacturing Practice (GMP) guidelines (15).

Figure 2: cis/trans isomerism of the pyruvoyl-proline amide bond.



Quality Control of Aplidine Lyophilized Product

Quality control of aplidine lyophilized product consisted of identification by visual inspection of appearance and colour of the pharmaceutical product; determination of reconstitution characteristics and pH of the reconstituted product; content, content uniformity, and purity determination by HPLC analysis; residual moisture determination with the Karl-Fischer titration method; and residual tert-butanol content by gas chromatographic (GC) analysis. Furthermore, sterility of the pharmaceutical product was checked by the filtration method and the presence of bacterial endotoxins with the limulus amoebocyte lysate (LAL) test, both carried out according to the European Pharmacopeia III.

HPLC analysis: Aplidine was assayed by a validated, stability-indicating reversed phase-HPLC method. The HPLC system consisted of a Model SP8800 pump (Thermo Separation Products, USA), a Model Spectra 200 UV-VIS detector (Spectra-Physics, San Jose, USA), and a Model SP8880 autosampler (Thermo Separation Products, USA). Analyses were carried out with a Zorbax SB-C18 analytical column (4.6 mm ID x 150 mm, particle size 3.5 mm, Waters, USA) held at a constant temperature of 80°C with a Model 7971 column heater (Jones Chromatography, USA). The mobile phase at a flow of 0.6 mL/min consisted of a linear gradient of acetonitrile (ACN) containing 0.04% trifluoroacetic acid (TFA) 35% to 70% in 15 minutes and water containing 0.04% TFA. An injection volume of 20 µL and a total run time of 30 minutes were used. UV detection was performed at 225 nm. Under these conditions the chromatogram of aplidine consisted of a single peak eluting at 21 minutes. A series of standard solutions of aplidine in ACN in the concentration range of 5 µg/mL to 300 µg/mL were prepared in duplicate from a stock solution of 1 mg/mL aplidine in ACN and injected into the HPLC system. Quality control samples at concentrations of 7.5, 100, 250, and 275 µg/mL of aplidine in ACN were prepared in quadruplicate from another stock solution with separate weighing of aplidine and injected into the HPLC system. Aplidine lyophilized product was diluted to a test concentration of 250 µg/mL by dilution with a solution of 1:1 (v/v) ACN/water. Least squares regression analysis was used to calculate the slope and intercept for the standard calibration curve from measured peak areas versus concentration. Sample concentrations were calculated from the corresponding peak areas using the regression equation. All aplidine chromatograms were electronically stored in the computer system LABNET (Spectra-Physics, San Jose, USA). Reprocessing of aplidine chromatograms was performed using PC1000 software (Thermo Separation Products, USA).

Residual moisture content: Residual moisture levels in aplidine lyophilized product were determined with the Karl-Fischer titration method. The content of a vial was quantitatively transferred to the titration unit of a Model 658KF titrino apparatus (Metrohm Herisau, Switzerland) with previously dried methanol, and subsequently titrated using Hydranal® Titrant 2.0 mg H_2O/mL (Riedel-de Haen, The Netherlands). The end-point of the titration was determined biamperometrically.

Residual tert-butanol content: Residual tert-butanol levels in aplidine lyophilized product were determined by gas chromatographic (GC) analysis. The GC system consisted of a Model 5890 gas chromatograph (Hewlett Packard, USA), equipped with a flame ionization detector (FID), a HP-5 cross-linked phenyl-methyl silicone capillary column (25 m x 0.32 mm x 0.52 mm film thickness, Hewlett Packard, USA) and a Model 6890 series autosampler (Hewlett Packard, USA). The column temperature was held at 60°C for 2 minutes and was subsequently linearly ramped to 180°C at a heating rate of 30°C/min and kept at this temperature for 2 minutes. The injector temperature was kept at 275°C and the detector temperature at 250°C. An injection volume of 1 µL and a run time of 12 minutes were employed. tert-Butanol standard solutions were prepared from a 2 mg/mL stock solution in 40% (v/v) propylene glycol in water in a concentration range of 20–100 µg/mL. Least square regression analysis was used to calculate the slope and intercept of the standard calibration curve from the measured peak areas versus tert-butanol concentration. Sample solutions were prepared by reconstituting a vial of aplidine lyophilized product with 3 mL of 40% (v/v) propylene glycol in water and injecting 1 µL onto the GC system. The tert-butanol concentrations in the sample solutions were calculated from the corresponding peak areas using the regression equation. All tert-butanol chromatograms were electronically stored in the computer system LABNET (Spectra-Physics, San Jose, USA). Reprocessing of tert-butanol chromatograms was performed using PC1000 software (Thermo Separation Products, USA).

Quality Control of Aplidine Reconstitution Solution

Quality control of 15/15/70% (v/v/v) Cremophor EL/ethanol/WfI reconstitution solution consisted of visual inspection of the appearance of the pharmaceutical solution and presence of particulate matter, determination of the pH, refractive index, relative density, and ethanol content. Also, as aplidine lyophilized product, sterility and the presence of bacterial endotoxins were examined according to the European Pharmacopeia III using the filtration method and LAL test, respectively.

The specificity of the tests for the refractive index, relative density, and ethanol content were examined by comparing the results of the tests performed on solutions composed of varying ratios of Cremophor EL, ethanol and WfI. The refractive indices and relative densities of solutions composed of 5/5/90%, 10/10/80%, 15/15/70%, 20/20/60%, 25/25/50% (v/v/v) Cremophor EL/ethanol/WfI were measured and the ethanol content of 10/10/80%, 15/15/70%, 20/20/60% (v/v/v) Cremophor EL/ethanol/WfI solutions was determined. Also, to simulate erroneous compounding of the excipients, the refractive index and relative density of solutions composed of 15/70/15% and 70/15/15% (v/v/v) Cremophor EL/ethanol/WfI were examined.

Refractive index: The refractive indices of the various Cremophor EL/ethanol/WfI solutions were determined on a Model Abbé 302 refractometer (Atago, Japan) at 20°C.

Relative density: The relative densities of the various Cremophor EL/ethanol/WfI solutions were determined by accurately weighing 1.00 mL of the respective solutions on a Model 440 balance (Mettler Toledo) at 20°C.

Ethanol content: Ethanol content of Cremophor EL/ethanol/WfI solutions was determined by GC analysis. The GC system consisted of a Model HP5710A gas chromatograph (Hewlett Packard, USA) equipped with a flame ionization detector (FID) and a Tenax GC 60–80 Mesh 1 m x 2 mm

column. The column temperature was held at 105°C. The injector and detector temperatures were kept at 200°C. An injection volume of 1 µL and a run-time of 5 minutes were employed. Standard solutions containing approximately 0.75‰, 2.5‰ and 4‰ ethanol were prepared by weighing accurately approximately 75 mg, 250 mg and 400 mg of absolute ethanol, respectively, into a 100 mL volumetric flask and adding distilled water to reach a final volume of 100.0 mL. As an internal standard, a solution containing approximately 3.5‰ 2-propanol was prepared by weighing approximately 350 mg of 2-propanol into a 100 mL volumetric flask and adding distilled water to reach a final volume of 100.0 mL. Before injection into the GC system, each ethanol standard was diluted 1:1 (v/v) with the internal standard solution. Subsequently, 1 µL of the resulting solution was injected into the GC system. From the obtained ethanol/2-propanol peak ratios, a calibration curve was calculated using least squares regression analysis. 10/10/80%, 15/15/70%, 20/20/60% (v/v/v) Cremophor EL/ethanol/WfI solutions were diluted to a theoretical ethanol concentration of 1.6‰ with distilled water. Before injection onto the GC system, each sample was diluted 1:1 (v/v) with the internal standard solution. Subsequently, 1 µL of the resulting solution was injected into the GC system. From the obtained ethanol/2-propanol peak ratios, the ethanol concentration (‰) was calculated with the standard calibration curve. The ethanol content of the Cremophor EL/ethanol/WfI solution was calculated by dividing the obtained ethanol concentration (‰) by the theoretical ethanol concentration (‰), times 100%.

Shelf-life Studies

Long-term stability of aplidine lyophilized product was studied at +2–8°C in the dark. Content and purity of the lyophilized product were determined by HPLC analysis.

Figure 3: Chromatogram obtained with an isocratic HPLC system showing the double-peak for aplidine due to cis/trans isomerism of the pyruvoyl-proline amide bond (concentration of 60µg/mL aplidine in ACN).

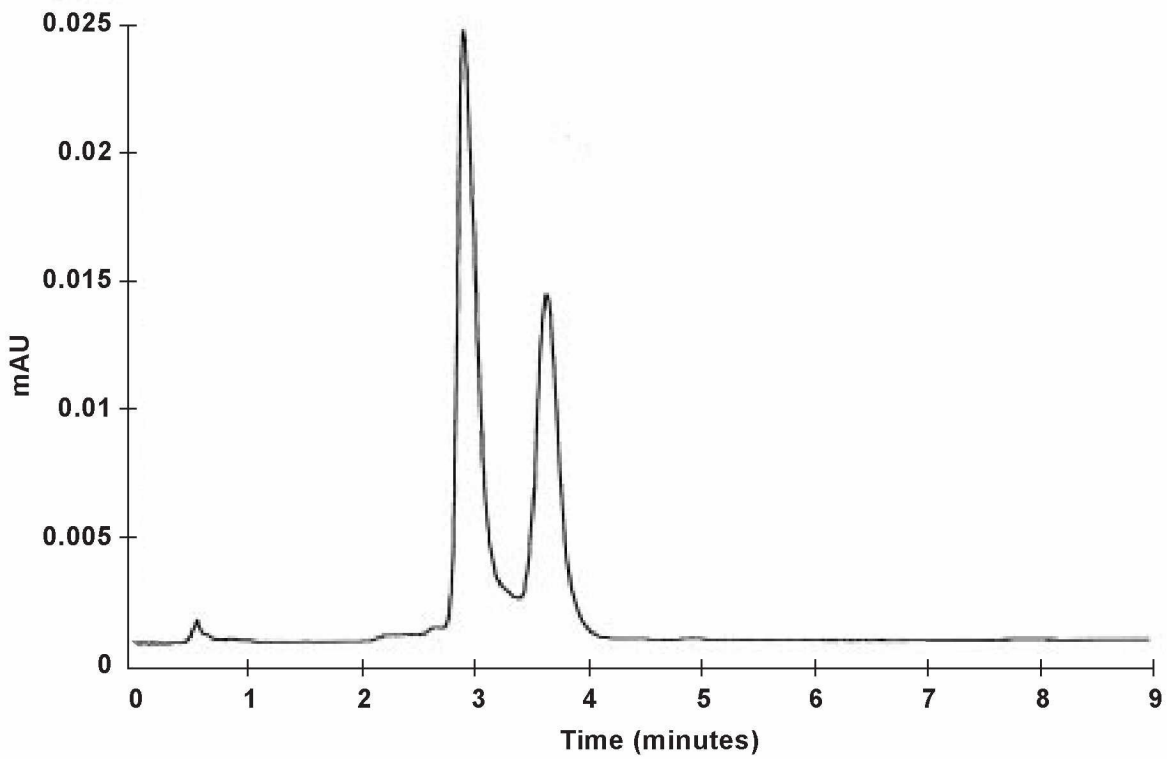


Figure 4: Representative chromatogram of aplidine (R_t of 21 minutes) obtained with stability-indicating gradient HPLC system (concentration of 250 μg/mL aplidine in ACN).

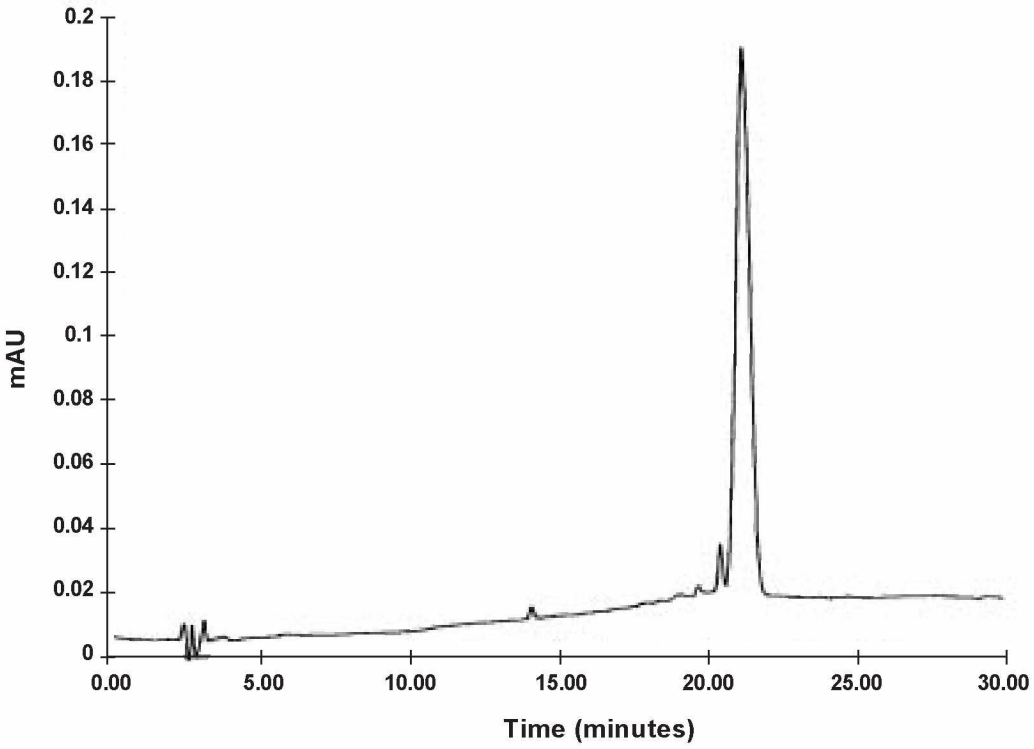
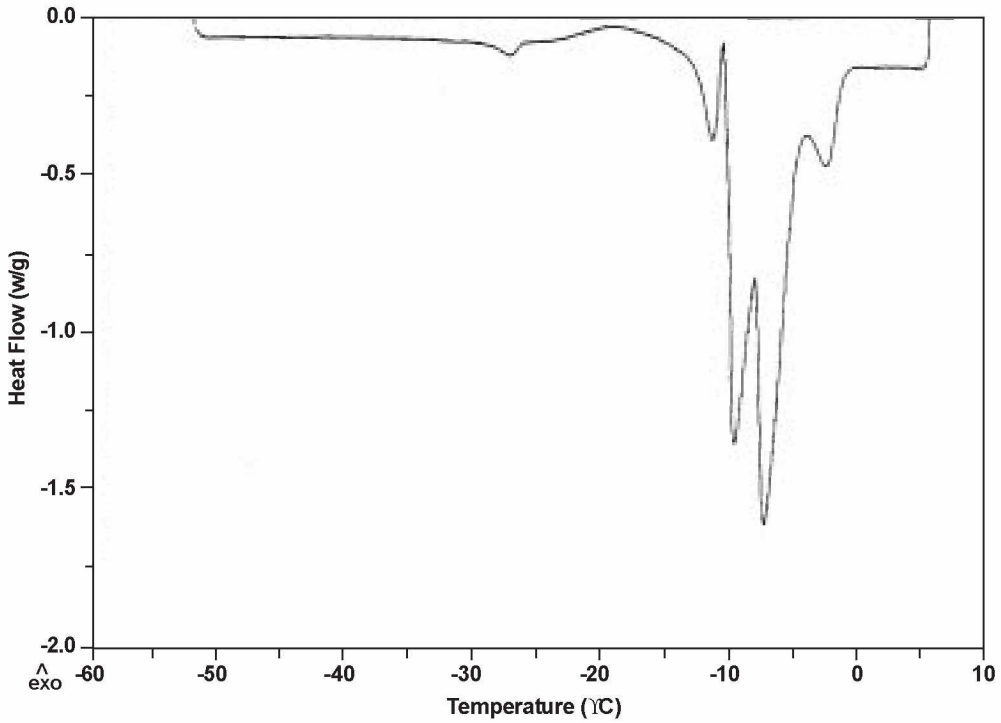


Figure 5: DSC thermogram of aplidine formulation solution (500μg/mL aplidine and 25 mg/mL D-mannitol in 40% v/v tert-butanol/water).



Results and Discussion

Characterization of Aplidine

FAB-MS: The FAB-MS shows the protonated molecular ion $[M+H]^+$ at m/z 1110.7. The major fragmentations are represented in Table 1 and are indicative of the structure of aplidine.

^{1}H-NMR: Table 1 gives the partial assignments for the ^{1}H-NMR spectrum in $CDCL_3$, based on 1D proton and 2D HH-COSY spectra. Two conformers appear to be present in an approximately 1:1 ratio (NH protons and methyl protons of the pyruvoyl group). The existence of two conformers relating to cis/trans isomerism of the pyruvoyl-proline amide bond was also shown with ^{13}C NMR (4). The spectrum in DMSO-d_6 shows the presence of even four conformations in approximately a 35:35:15:15 ratio (methyl protons of the pyruvoyl group). The additional signals probably originate from cis/trans isomerism of the second proline amide bond. This hypothesis was examined by carrying out temperature experiments in DMSO-d_6 at 105°C which showed that only the signals for the pyruvoyl methyl group are still present at this elevated temperature. In the $CDCL_3$ spectrum, the threonine NH signal of one conformer is more shielded than that of the other conformer. This indicates that the two conformers arise, indeed, as a result of cis/trans isomerism at the pyruvoylproline residue (Fig. 2). From the literature the occurrence of more than one conformation in proline-containing peptides is well-known (16,17). The proline residue and cis/trans isomerization is extremely important in the folding, denaturation, and renaturation of polypeptides and proteins, and might also be a factor in the biological activity of aplidine.

UV/VIS spectrophotometry: The UV spectrum of aplidine shows absorption maxima at 212 and 273 nm, respectively (Table 1).

Infra-red spectroscopy: Major assignments are presented in Table 1.

HPLC analysis: The existence of multiple conformations of aplidine as described in the NMR section complicated the development of a stability-indicating HPLC method. The initial assays, both normal phase and isocratic and gradient reverse phase high-performance liquid chromatographic (HPLC) methods, showed the presence of two aplidine peaks in the chromatograms referring to the equilibrium between the cis and trans isomers of the pyruvoyl-proline amide bond (18). At ambient temperature (20–25°C) this equilibrium appears too slow to elute both isomers as one peak but also too fast to elute them as two baseline-separated peaks (Fig. 3). By lowering the column temperature this equilibrium is frozen, thus resulting in improved resolution. Collection of one of the individual conformations at the detector outlet and reinjection of the component results again in a double-peak, indicating a continuous interconversion of both conformers. Although quantification of aplidine concentrations on the total area of both isomer peaks was satisfactory, the stability-indicating capability of the assay was not. It was proven that upon stress-testing of aplidine solutions degradation products co-eluted with the aplidine isomer peaks. Therefore, at present a gradient HPLC method with a column temperature of 80°C is employed. Due to this high column temperature aplidine elutes as a single peak. Furthermore, this method shows good selectivity for impurities and degradation products. A representative chromatogram of aplidine is given in Fig. 4. Aplidine elutes at a retention time (R_t) of approximately 21 minutes. The peaks eluting at approximately 14 and 20 minutes, respectively, are impurities of the aplidine bulk drug. Identification of these impurities is ongoing.

Table 1: Identification and characterization of aplidine bulk drug (Lot APL-297)

Analytical Method	Results
Appearance	Pale beige, amorphous powder
FAB-MS	Molecular formula: $C_{57}H_{87}N_7O_{15}$; Protonated molecular ion $[M+H]^+$ 1110.7; 1040.7 $[M+H]^+$-70, CH_2COCO; 985.6 $[M+H]^+$-125, Pro-CO; 943.6 $[M+H]^+$-167, pyruvoyl-Pro; 859.5 $[M+H]^+$-251, Leu-diMe-keto-OH-hexanoic acid-H_2O; 816.0 $[M+H]^+$-294, pyruvoyl-Pro-*N*-Me-Leu; 701.4 $[M+H]^+$-409, Leu-diMe-keto-OH-hexanoic acid-NH_2-OH-Me-heptanoic acid-NH; 295.2, pyruvoyl-Pro-*N*-Me-Leu; 177.1, Tyr-CH_2 (*N*-CH_3); 169.1, pyruvoyl-Pro
^{1}H NMR ($CDCl_3$)	Proposed assignments: Amino acid residue: *Proline (1)*: δ 4.70 H-α; 2.10 H-β_1; 1.95 H-β_2; 3.66 H-δ_1; 3.84 H-δ_2; 2.54/2.52 CH_3CO; *N-Me-Leucine*: δ 3.15/3.11 *N*-CH_3; 4.62 H-α; 2.15 H-β_1; 1.78 H-β_2; 1.28 H-γ; 0.90 CH_3; *Threonine*: 7.61/ 7.05 NH_4; 4.57/4.65 H-α; 5.29/5.17 H-β; 1.41/1.40 γ-CH_3; *4-Amino-3-hydroxy-5-methylheptanoic acid*: 7.20/7.19 NH_4; 3.25 H-α_1; 2.62 H-α_2; 4.08 H-β; 4.08 H-γ; 1.80 H-δ; 1.64 δ-CH_3; 0.90 H-ε; 0.90 e-CH_3; *2,5-diMe-hydroxy-3-ketohexanoic acid*: 4.23/4.19 H-α; 1.33/1.32 α-CH_3; 5.40 H-γ; 1.70 H-δ; 0.90 δ-CH_3; *Leucine*: 7.86/7.81 NH_4; 4.80 H-α; 1.60 H-β_1; 1.20 H-β_2; 0.90 CH_3; *Proline (2)*: 5.10 H-α; 2.90 H-β_1; 1.90 H-β_2; *N,O-diMe-tyrosine*: 3.80 *O*-CH_3; 2.54 *N*-CH_3; 3.57 H-α; 3.36 H-β_1; 3.16 H-β_2; 7.08 H-1,4; 6.85 H-2,3
UV/VIS spectrophotometry	Absorption maxima at 212 and 273 nm
IR spectroscopy	Characteristic absorption bands (approximately): 3340 cm^{-1}: N-H stretching (amide); 2960/2880 cm^{-1}: C-H stretching (aliphatic); 1740 cm^{-1}: C=O stretching; 1640 cm^{-1}: N-C=O stretching (amide); 1520 cm^{-1}: C=C stretching (aromatic); 1450 cm^{-1}: C-H deformation; 1250 cm^{-1}: phenolic C-O; 1170/1070 cm^{-1}: C-O stretching; 820 cm^{-1}: C-H bending of paradisubstituted benzene

Solubility Studies

Table 2 gives the approximate solubilities of aplidine in various solvent systems. Solvents were chosen on the basis of current use and experience in clinical practice (14, 19). Aplidine is very poorly soluble in water, but dissolves well in a number of organic solvents. Furthermore, aplidine has high solubilities in 60/40/10% (v/v/v) polyethylene glycol 400/ethanol/polysorbate 80 (PET) and 50/50% (v/v) Cremophor EL/ethanol cosolvent/surfactant systems. However, upon dilution with normal saline up to 1:100 (v/v), only aplidine solubilized in 50/50% (v/v) Cremophor EL/ethanol did not precipitate over a 24-hour period. Apparently, a cosolvent in combination with a micelle-forming agent is necessary to solubilize aplidine and make it suitable for further dilution in infusion solution.

Initial stability studies showed that aplidine in solution degrades under the influence of light and heat. Therefore, to increase the stability, it was decided to develop a lyophilized dosage form. As the aqueous solubility of aplidine is very poor, another suitable freeze-drying medium had to be found. On the basis of previously reported studies,

50% (v/v) polyethylene glycol 4000 in water and various concentrations of tert-butanol in water were selected as alternative lyophilization media (20, 21). Aplidine shows very good solubility in tert-butanol/water mixtures (up to 40% (v/v) tert-butanol). tert-Butanol has properties which make it particularly interesting as a co-solvent in the freeze-drying of poorly water soluble, unstable compounds as often is the case with anticancer agents. tert-Butanol is completely miscible with water, has a high melting point of 25.3°C, and even at low temperatures it has a vapour pressure higher than water (22). With respect to stability, the addition of an organic co-solvent may also reduce the degradation rate of a drug in water (23, 24). tert-Butanol has been reported to enhance freeze-drying efficiency by modifying the crystal habit of ice, forming large needle-shaped ice crystals resulting in a more porous and thus less resistant cake (24, 25). Because of the significantly higher surface area of the dried product, the reconstitution of the lyophilized cake is believed to be positively influenced. Furthermore, the broad spectrum antimicrobiological activity of tert-butanol offers an additional sterility assurance in an aseptic manufacturing process (25). A formulation solution consisting of 500 µg/mL aplidine and 25 mg/mL D-mannitol as bulking agent in 40% (v/v) tert-butanol/water was selected for the lyophilization of aplidine.

Differential scanning calorimetry (DSC): In the DSC curve of the formulation solution several thermal events can be distinguished (Fig. 5). The DSC curve proved to be mainly governed by the 40% (v/v) tert-butanol concentration in the tert-butanol/water mixture. DSC thermograms of solutions consisting of 40% (v/v) tert-butanol/water with or without aplidine (500 µg/mL) appeared to be similar. With the addition of 25 mg/mL D-mannitol to the 40% (v/v) tert-butanol in water solution containing aplidine, a small endotherm appears in the thermogram at approximately -30°C. When applying an annealing step (freezing to -50°C followed by heating to -20°C and refreezing to -50°C), this endotherm disappears as a consequence of complete crystallization of D-mannitol. The remaining thermal events are likely due to the melting of eutectic, tert-butanol-hydrate, and ice, respectively. The first endotherm in this complex is attributed to the melting of metastable eutectic, since

Table 2: Solubility of aplidine in various solvents.

Solvent	Solubility (mg/mL)
Water for injection (Wfl)	< 0.1
ethanol absolute	> 10
dimethylacetamide (DMA)	> 10
dimethylsulfoxide (DMSO)	> 10
polyethylene glycol 400/ethanol/polysorbate 80 60/30/10% v/v/v (PET)	> 10
propylene glycol/ethanol/Wfl 40/10/50% (v/v/v) (PEW)	0.33 ≤ s < 0.4
0.5% (w/v) polysorbate 80 in normal saline	< 0.1
DMA/arachis oleum	1 ≤ s < 10
Cremophor El/ethanol 50/50% (v/v)	> 10
polyethylene glycol 400	1 ≤ s < 10
50% (w/v) polyethylene glycol 4000 in Wfl	< 0.25
tert-butanol	> 10
50% (v/v) *tert*-butanol in Wfl	> 5
40% (v/v) *tert*-butanol in Wfl	> 4

it disappears completely upon thawing of the sample followed by immediate refreezing. Apparently, this thermal treatment results in crystallization of metastable to stable eutectic. Similar profiles for tert-butanol/water mixtures have been reported by Kasraian et al. and Wittaya-Areekul et al. (24, 25). The lyophilization cycle selected for the manufacturing of aplidine 500 µg/vial lyophilized product consisted of freezing the formulation solution to -43°C in 1 hour, a freeze-hold for 2 hours followed by a primary drying phase at a chamber pressure of 0.1 mbar for 32.5 hours, and a subsequent secundary drying phase by a linear temperature rise to 25°C in 5 hours and maintaining this temperature for 8 hours. A temperature rise of the shelves during the freeze-drying cycle was allowed only after the primary drying phase to moderate the sublimation rate, thus preventing explosion of the product from the vials. Lyophilization of the formulation solution resulted in a solid white cake with excellent appearance.

Reconstitution Studies

On the basis of the initial solubility studies, the cosolvent/surfactant system 50/50% (v/v) Cremophor EL/ethanol was used as a starting point for the development of a suitable reconstitution solution for aplidine lyophilized product. The presence of D-mannitol in the pharmaceutical product, however, necessitated the addition of another solvent to the vehicle in order to dissolve the excipient. Water for Injection (WfI) was supplemented to the 50/50% (v/v) Cremophor EL/ethanol vehicle in mounting concentrations to obtain solutions composed of 30/30/40% (v/v/v), 15/15/70% (v/v/v), and 5/5/90% (v/v/v) Cremophor EL/ethanol/WfI. Suitability of the resulting mixtures for reconstitution of aplidine 500 µg/vial lyophilized product was examined in terms of dissolution rate, completeness of dissolution, and stability of the reconstituted solution, as well as after dilution with normal saline (Table 3). All three vehicles tested were able to reconstitute aplidine lyophilized product, although the dissolution rate of the lyophilized cake with 30/30/40% (v/v/v) Cremophor EL/ethanol/WfI was considerably slower compared to the other two vehicles. Furthermore, visual precipitation occurred in the solution obtained with 30/30/40% (v/v/v) Cremophor EL/ethanol/WfI within 24 hours after reconstitution. Apparently, the obtained reconstitution solution is supersaturated, resulting in slow precipitation of solubilized compo-

Table 3: Dissolution rate and stability of aplidine in reconstitution solutions and upon dilution with normal saline at room temperature (20 - 25°C) and ambient light.

Reconstitution vehicle	Dissolution rate	Aplidine concentration (µg/mL) in reconstituted solution ± SD (n = 2)		% of theoretical aplidine concentration in infusion solution ± SD (n = 2)			
				1:10 (v/v)		1:100 (v/v)	
		0h	24h	0h	24h	0h	24h
30/30/40% (v/v/v) Cremophor EL/ethanol/WfI	+/-	564.7 ± 10.0	precipitation	101.5 ± 2.5	96.1 ± 2.0	94.0 ± 9.8	93.7 ± 11.2
15/15/70% (v/v/v) Cremophor EL/ethanol/WfI	+	520.2 ± 3.2	534.7 ± 3.2	99.9 ± 3.3	95.8 ± 2.5	101.1 ± 1.3	100.5 ± 4.6
5/5/90% (v/v/v) Cremophor EL/ethanol/WfI	+	558.8 ± 4.7	508.0 ± 0.5	97.7 ± 4.2	94.8 ± 2.7	96.5 ± 4.6	78.2 ± 7.7

+ = immediate and complete reconstitution after manual shaking for 30 seconds
+/- = reconstitution after vigorous vortex-mixing for 1 minute

nents over time. In the solution obtained after reconstitution of aplidine 500 µg/vial lyophilized product with 5/5/90% (v/v/v) Cremophor EL/ethanol/WfI approximately 90% of the initial aplidine content remains after 24 hours of storage at ambient temperature (20–25°C) and a normal day-night light cycle. In conclusion, of the reconstitution vehicles examined, only reconstitution of aplidine 500 µg/vial lyophilized product with 15/15/70% (v/v/v) Cremophor EL/ethanol/WfI results in a solution stable for at least 24 hours at ambient temperature and light.

Using a surfactant, it is important that its concentration remains above the critical micelle concentration (CMC) in order to keep the solubilized components in solution. Therefore, in case of further dilution of the reconstituted lyophilized product in normal saline, the Cremophor EL concentration should be maintained above its CMC of 0.0086% (v/v) (14,19). The concentrations of Cremophor EL in all three vehicles at the maximum dilution of 1:100 (v/v) examined are still well above this level (0.05%, 0.15%, and 0.30% (v/v) for the 5/5/90%, 15/15/70%, 30/30/40% (v/v/v) Cremophor EL/ethanol/WfI vehicles, respectively). From Table 3, it is clear that all 1:10 (v/v) dilutions of aplidine 500 µg/vial lyophilized product reconstituted with 5/5/90% (v/v/v), 15/15/70% (v/v/v), and 30/30/40% (v/v/v) Cremophor EL/ethanol/WfI are stable under the same conditions. For the 1:100 (v/v) dilutions in normal saline, however, only aplidine lyophilized product reconstituted with 30/30/40% and 15/15/70% (v/v/v) Cremophor EL/ethanol/WfI are stable for 24 hours of storage at room temperature (20–25°C) and ambient light. Relative aplidine concentrations were calculated with respect to the theoretical aplidine concentration after dilution of reconstituted product. On the basis of these results, it was decided to select 15/15/70% (v/v/v) Cremophor EL/ethanol/WfI as reconstitution solution for aplidine 500 µg/vial lyophilized product. Cremophor EL is related to the occurrence of anaphylactic reactions upon parenteral administration (26). On the basis of the expected maximum tolerated dosage level of 6 mg/m^2 body surface area (MTD mouse-equivalent) in clinical Phase I studies, corresponding to a total dose of approximately 10 mg of aplidine, a maximum dose of 3 mL of Cremophor EL will be administered intravenously to the patient at the highest dosage level. In comparison, up to 40 mL of Cremophor EL have been administered parenterally to patients treated with Taxol® (paclitaxel) (27). No problems are expected with the intravenous administration of the 15/15/70% (v/v/v) Cremophor EL/ethanol/WfI vehicle. This is supported by initial results of toxicology studies in mice and rats, in which no formulation-related toxicity occurred (personal communication).

Quality Control of Aplidine Lyophilized Product and Reconstitution Solution

The results and specifications of the quality control of two batches of aplidine 500 µg/vial lyophilized product are presented in Table 4. Aplidine is a product of natural origin, which implies a higher risk for the presence of impurities due to the extraction process of the substance from the parent tunicate than in a synthetically prepared compound. Therefore, it was decided to initially set broad range specifications for content and purity of the lyophilized product. It is intended to narrow these specifications for future batches on the basis of the growing experience in the production of aplidine raw substance and lyophilized product. As can be seen in Table 4, the results of the quality control tests of the initial batches of aplidine lyophilized product are well within the specifications.

From the experiments conducted to examine the specificity of the refractive index and relative density tests for the quality control of 15/15/70% (v/v/v) Cremophor EL/ethanol/WfI, linear relationships between the Cremophor EL/ethanol content of the solution and the refractive index as well as the relative density were found with least squares regression analysis (correlation coefficients of 0.99 and 0.98, respectively). Furthermore, the refractive index and relative density of solutions composed of 15/70/15% and 70/15/15% (v/v/v) Cremophor EL/ethanol/WfI differed significantly from the values obtained with 15/15/70% (v/v/v) Cremophor EL/ethanol/WfI. Therefore, both the

refractive index and relative density tests proved to be selective as an identity test for 15/15/70% (v/v/v) Cremophor EL/ethanol/WfI reconstitution solution. On the basis of the calibration curves obtained and a maximal deviation of 10% in the Cremophor EL/ethanol content of the reconstitution solution, specifications for the refractive index and relative density test were set at $1.359 < n_D^{20} < 1.365$ and $0.932 < d_D^{20} < 0.949$, respectively. A specification for the ethanol content of 15/15/70% (v/v/v) Cremophor EL/ethanol/WfI reconstitution solution of 90–110% of the labeled content of 15% ethanol was set. The initial two batches of 15/15/70% (v/v/v) Cremophor EL/ethanol/WfI reconstitution solution prepared thus far fully comply to these specifications.

Shelf-life Studies

Stability data obtained thus far, determined on two separate batches, show that aplidine 500 µg/vial lyophilized product is stable for at least 1 year when stored at +2–8°C in the dark, with 99.1% (sd=0.5) of the initial content remaining after this storage period and no appearance of degradation products.

Conclusions

The investigational, marine-derived cytotoxic agent aplidine shows poor aqueous solubility and stability characteristics. An innovative pharmaceutical formulation was developed combining a lyophilized product containing 500 µg aplidine and a reconstitution solution composed of 15/15/70% (v/v/v) Cremophor EL/ethanol/water. As a lyophilization medium 40% (v/v) tert-butanol/water was used. Shelf-life data available thus far show that aplidine lyophilized product is stable for at least 1 year when stored at +2–8°C in the dark. Aplidine is currently under Phase I clinical trials in Europe and Canada. The preliminary data indicate that the proposed formulation is fully applicable in the clinical setting.

Table 4: Quality control results of two batches aplidine 500 µg/vial lyophilized product.

Test method / item	Specification	Lot 020299BN10	Lot 050299BN11
Visual inspection/appearance	White, lyophilized cake	Conforms	Conforms
HPLC analysis:			
1. Identification	Rt reference standard = Rt lyophilized product	Conforms	Conforms
2. Content	95.0 -105.0% of labeled content	99.27%	99.30%
3. Content Uniformity	Contents of 10 dosage units are within 85.0-115.0% of the labeled content, relative standard deviation ≤ 6.0%	Conforms rsd = 0.95%	Conforms rsd = 0.77%
4. Purity	≥ 95%	98.5%	98.06%
pH after reconstitution	6-7	6.7	6.7
Residual moisture	< 3.0% (w/w)	1.06%	1.10%
Residual tert-butanol	< 3.0% (w/w)	0.78%	0.78%
LAL-test	< 5 EU/vial	Conforms	Conforms
Sterility	Sterile	Conforms	Conforms

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