Anti-Cancer Drugs 1999, **10**, pp. 879–887

Clinical report

Compatibility and stability of aplidine, a novel marine-derived depsipeptide antitumor agent, in infusion devices, and its hemolytic and precipitation potential upon i.v. administration

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Aplidine is a novel marine-derived antitumor agent isolated from the Mediterranean tunicate *Aplidium albicans*. The compound is pharmaceutically formulated as a lyophilized product containing 500 μg active substance per dosage unit. Prior to i.v. administration it is reconstituted with a solution composed of Cremophor EL, ethanol absolute and Water for Injection (15/15/70% v/v/v) with further dilution in 0.9% w/v sodium chloride for infusion (normal saline). The aim of this study was to investigate the compatibility of aplidine infusion solutions with polyvinyl chloride (PVC)-containing and PVC-free administration sets, and to determine the stability of aplidine after reconstitution and further dilution in infusion solutions. Furthermore, *in vitro* biocompatibility studies to estimate the hemolytic and precipitation potential of aplidine infusion solutions upon i.v. administration were conducted. In this study we show that sorption of aplidine to PVC and to a lesser extent to PVC-free administration set materials occurs. Also, most probably due to the presence of Cremophor EL in the infusion solution, significant leaching of diethylhexyl phtalate (DEHP) from the PVC administration set occurs. After reconstitution and dilution the drug is stable for at least 24 and 48 h, respectively, in glass containers when stored at room temperature (20–25 C) and ambient light conditions. We found that aplidine should be administered in infusion concentrations equal or above 28.8 μg/ml using a PVC-free administration set consisting of a glass container and PVC-free infusion tubing. After reconstitution it must be diluted further with normal saline within 24 h after preparation and subsequently administered to the patient within 48 h. Additionally, results from the biocompatibility studies show that neither hemolysis nor precipitation of aplidine is expected upon i.v. administration.

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Key words: Aplidine, compatibility, diethylhexyl phtalate, infusion devices, hemolysis, precipitation, polyvinyl chloride, stability.

Introduction

Aplidine (dehydrodidemnin B, DDB, MW 1109, Figure 1) is a promising representative of an evolving group of anticancer agents derived from marine sources.[1] This natural occurring cyclic depsipeptide is isolated from the Mediterranean tunicate *Aplidium albicans* and belongs to the didemnin family, a class of compounds which exhibits antiviral, antitumor and immunosuppressive activities. Aplidine displays potent *in vitro* and *in vivo* antitumor activity against various solid human tumor xenografts, and is therefore developed as a potential antitumor agent for solid tumor therapy.[2]

We have developed a pharmaceutical formulation for aplidine which is a lyophilized product containing 500 μg of aplidine and 25 mg of D-mannitol as bulking agent.[3] Due to the limited aqueous solubility of aplidine the freeze-dried product needs to be reconstituted with a solution composed of polyoxyethylated castor oil (Cremophor EL), ethanol and Water for Injection (Wfl) (15/15/70% v/v/v) resulting in a solution containing 500 μg/ml of aplidine. Before administration, the reconstituted product is diluted further with 0.9% (w/v) sodium chloride for infusion (normal saline).

In this article we present a preclinical *in vitro* pharmaceutical screening program for the novel formulation of aplidine. In order to determine the preparation and administration parameters for use in

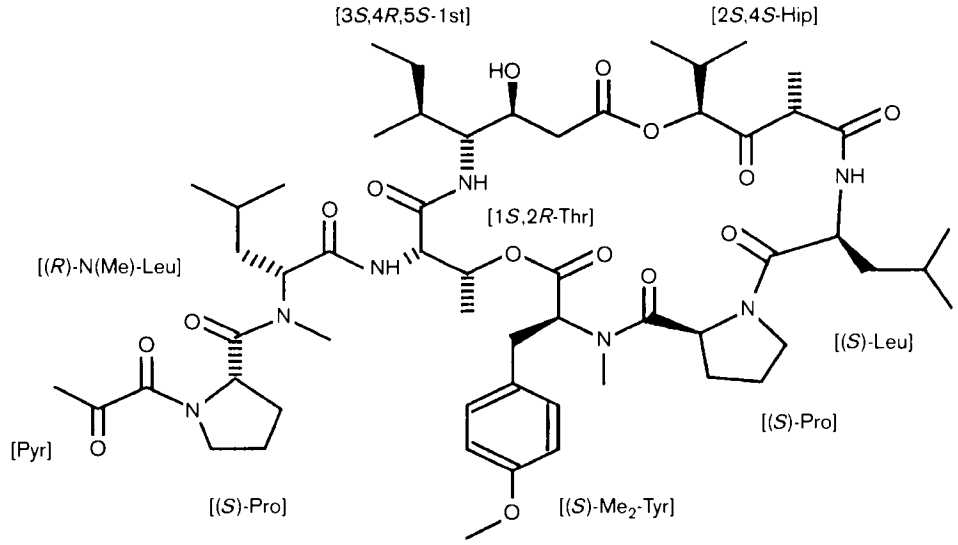

Figure 1. Chemical structure of aplidine (Hip, hydroxyisovalerylproprionyl; Ist, isostatine; Leu, leucine; Pro, proline; Pyr, pyrovoyl; Thr, threonine; Tyr, tyrosine).

the clinical setting, the compatibility of aplidine infusion solutions with different administration sets was investigated. Also, the stability of aplidine after reconstitution and dilution was determined over a 24 and 48 h period, respectively. The administration of aplidine infusion solutions was mimicked *in vitro* by passage of solutions of different, clinically relevant concentrations through two infusion systems composed of polyvinyl chloride (PVC)-containing and PVC-free materials. The aplidine concentrations in the infused solutions in time were assayed using a stability-indicating high-performance liquid chromatography (HPLC) method. Furthermore, any leaching of the plasticizer diethylhexyl phtalate (DEHP) from the administration set materials, a known phenomenon in the presence of a surfactant,[1] was monitored using HPLC analysis. Additionally, in order to determine any adverse effects upon i.v. administration of aplidine infusion solutions as a result of the low aqueous solubility of the drug or the presence of ethanol and Cremophor EL, *in vitro* biocompatibility studies to estimate the hemolytic and precipitation potential of aplidine infusion solutions were conducted.

Experimental

Chemicals and materials

Aplidine was obtained by a three-step synthesis from natural didemnin A under the responsibility of Pharma Mar (Tres Cantos, Madrid, Spain) and provided through the NDDO-Oncology (Amsterdam, The Netherlands). All chemicals used were of analytical grade and were used without further purification. Isotonic Sorensen's buffer was manufactured in-house (Department of Pharmacy of the Slotervaart Hospital). Distilled water was used throughout the experiments. Fresh heparinized blood with a labeled hematocrit of 50% for the whole blood hemolysis study was purchased from the local Blood bank (CLB, Amsterdam, The Netherlands).

Chromatography

Aplidine was assayed by a validated, stability-indicating reversed-phase HPLC method. The HPLC system consisted of a Model SP8800 pump (Thermo Separation Products, San Jose, CA), a Model Spectra 200 UV-VIS detector (Spectra-Physics, San Jose, CA) and a Model SP8880 autosampler (Thermo Separation Products). Analyses were performed on a Zorbax SB-C18 analytical column (4.6 × 150 mm, particle size 3.5 μm; Waters, Etten-leur, The Netherlands) held at a constant temperature of 80 C using a Model 7971 column heater (Jones Chromatography, Hengoed, UK). 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) and water containing 0.04% TFA. The gradient was 35% ACN/0.04% TFA to 70% in 15 min. An injection volume of 20 μl and a total run-

time of 30 min were employed. UV detection was performed at 225 nm. Under these conditions the chromatogram of aplidine consists of a single peak eluting at approximately 21 min. A series of standard solutions of aplidine in ACN in the concentration range of 1.25–125 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, 25 and 100 μg/ml of aplidine in ACN were prepared from a separate stock solution (1 mg/ml aplidine in ACN) and injected into the HPLC system during the analysis.

DEHP was assayed using the same HPLC system as for aplidine with the exception of employing a linear gradient of 65% ACN/0.04% TFA to 100% in 30 min and a flow rate of 1.0 ml/min. Under these conditions the chromatogram of DEHP consists of one main peak eluting at approximately 21.5 min. Aplidine elutes in this system at a retention time of approximately 6.3 min. A series of standard solutions of DEHP in ACN in the concentration range of 1–400 μg/ml were prepared in duplicate from a stock solution of 1 mg/ml DEHP in ACN and injected into the HPLC system. Quality control samples at concentrations of 5.25, 52.5 and 325 μg/ml of DEHP in ACN were prepared in triplicate from a separate stock solution.

For both the aplidine and DEHP analyses, least-squares regression analysis was used to calculate the slope and intercept of the standard calibration curve from measured peak areas versus standard concentration. Sample concentrations were calculated from the corresponding peak areas using the obtained regression equation. All aplidine and DEHP chromatograms were electronically stored in the computer system LABNET (Spectra-Physics). Reprocessing of aplidine and DEHP chromatograms was performed using PC1000 software (Thermo Separation Products).

Simulation of i.v. infusions

Aplidine infusion solutions were prepared at three concentration levels, based on the expected dosage levels in phase I clinical studies. Dosage levels of 350 μg/m^2 (starting dose), 1800 μg/m^2 (fourth dose escalation level) and 6 mg/m^2 [expected maximum tolerated dose (MTD)] were selected. Assuming a body surface area of 1.7 m^2 and a concentration of 500 μg/ml of the reconstituted product, aplidine infusions were prepared by adding the required volume of reconstituted product to 100 ml of normal saline to yield nominal concentrations of 5.9 (1.2 ml of reconstituted product to 100 ml of normal saline), 28.8 (6.1 ml of reconstituted product to 100 ml of normal saline) and 84.7 μg/ml aplidine (20.4 ml of reconstituted product to 100 ml of normal saline), respectively.

Two administration sets composed of PVC and PVC-free materials routinely used in our hospital were selected for the *in vitro* simulation testing of aplidine infusions. The PVC infusion system consisted of a PVC flexible bag containing 100 ml of normal saline (Add-a-Flex; NPBI, Emmer-Compascuum, The Netherlands) and PVC tubing (Model G52703; IVAC, San Diego, CA). The PVC-free system consisted of a clear glass infusion flask containing 100 ml of normal saline (Aluglas, Uithoorn, The Netherlands) and PVC-free, silicone tubing (Model 763324; Codan, Lensahn, Germany). Total contact surface areas (container and tubing) of the PVC and PVC-free administration sets were 440 and 395 cm^2, respectively.

From the 1 and 24 h infusion durations proposed in phase I clinical trials, the 24 h infusion was selected to simulate a 'worst case scenario' assuming that problems like sorption of aplidine to the administration set materials and leaching of plasticizer into the infusion fluid are more likely to occur during a longer contact and transit time in the containers and tubing material.

After addition of the required volume of reconstituted drug solution to normal saline in the infusion container, the resulting solution was gently mixed by manual massaging and shaking during approximately 1 min. Subsequently, the infusion tubing was connected to the container, the infusion flow was adjusted and the simulation test was started. All aplidine infusion solutions were prepared in duplicate at each concentration level immediately before the start of the experiment. All simulation experiments were performed at room temperature (20–25°C) and normal light-dark cycle. Samples of the infusion solutions were taken immediately after preparation, after passage through the infusion system at 2, 5, 15 and 30 min, and 1, 4, 8 and 24 h after starting the infusion simulation. Samples were immediately injected without further dilution into the HPLC system. After quantification of aplidine, all samples were stored at 4°C in the dark until determination of the DEHP concentration. The total amounts of aplidine administered and DEHP leached from the infusion set material were estimated for each aplidine infusion concentration level and administration set from the area under the concentration–time curves (AUC). The AUCs were calculated using the linear trapezoidal rule and for every infusion solution the total amount of aplidine administrated or DEHP leached was estimated using equation (1), with infusion rates of 4.2, 4.4 and 5.0 ml/h for aplidine infusion concentrations of 5.9, 28.8 and 84.7 μg/ml, respectively:

Total amount of aplidine or DEHP released (μg) = AUC (μg/ml·h) × infusion rate (ml/h) (1)

The relative amount of aplidine administered with respect to the theoretical total aplidine dose present in the infusion solutions was calculated using equation (2), with 600, 3050 and 10 200 μg as theoretical aplidine doses for aplidine infusion concentrations of 5.9, 28.8 and 84.7 μg/ml, respectively:

Relative amount of aplidine released (%) = [total amount of aplidine released/ theoretical aplidine dose in infusion solution] × 100% (2)

Stability of reconstituted aplidine lyophilized product and aplidine infusion solutions

To determine the stability of aplidine in reconstitution solution, the lyophilized product was reconstituted in duplicate with 1 ml of 15/15/70% (v/v/v) Cremophor EL/ethanol/Wfl resulting in a solution containing 500 μg/ml aplidine. Immediately after reconstitution and after 24 h of storage at room temperature (20–25°C) and a normal light–dark cycle samples of 50 μl were diluted with 950 μl of ACN (theoretical concentration of 25 μg/ml aplidine) and subsequently injected into the HPLC system.

Infusion solutions were prepared in duplicate and sampled directly after preparation and after 48 h of storage in the administration set container at room temperature (20–25°C) and a normal light–dark cycle. Samples were immediately injected into the HPLC system without further dilution.

Hemolysis studies

The potential of hemolysis upon aplidine infusion was evaluated using both the static and dynamic *in vitro* test models as described by Ward *et al.*[5] and Krzyzaniak *et al.*[6–8] For both models, aplidine infusion solution in a concentration of 84.7 μg/ml in normal saline containing both ethanol and Cremophor EL in a concentration of 2.5% (v/v) was used. Also, blank infusion solutions containing only the aplidine vehicle were tested. All experiments were run in duplicate. Fresh, heparinized human blood with a labeled hematocrit content of 50% was used throughout the experiments. For the static model, to 500 μl of blood 25, 50 and 125 μl of aplidine infusion solution were added, resulting in formulation:blood ratios of 0.05, 0.1 and 0.25, respectively. A blood–formulation contact time of 5 s by manual shaking was employed. For the dynamic model, aplidine solution was infused at a rate of 0.3, 0.6 and 1.5 ml/min using a Model 711 syringe pump (IVAC) in a running blood flow set at 6 ml/min using a Model 501Dz peristaltic pump (Watson Marlow, Rotterdam, The Netherlands) resulting in formulation:blood ratios of 0.05, 0.1 and 0.25, respectively. The contact time of blood and formulation was set at 5 s applying a silicone tube with a length of 25 cm and an internal diameter of 1.6 mm (Watson Marlow). For both the static and dynamic model, the hemolytic reaction was quenched by the addition of 50 ml of normal saline. Subsequently, an aliquot of 3 ml of each solution was centrifuged at 3000 r.p.m. for 10 min and the absorption (A) of the supernatant at 540 nm was determined using a Model UV/VIS 918 spectrophotometer (GBC Scientific Equipment, Victoria, Australia) equipped with a LEO personal computer and an Epson LX-400 plotter. As a positive control, hemolysis induced by 40/10/50% (v/v/v) propylene glycol/ethanol/Wfl (PEW) was determined using the same procedure as for the infusion solution. The baseline hemolysis level was determined by treating normal saline as the infusion solution at all formulation:blood ratios. The 100% hemolysis level was determined by diluting the blood volume applied in the static and dynamic model with distilled water instead of normal saline. The percentage hemolysis induced by the infusion solution and PEW vehicle was calculated using equation (3):

$$\%\ \text{Hemolysis} = (A_{\text{infusion}} - A_{\text{saline}})/(A_{100\%} - A_{\text{saline}}) \times 100\% \quad (3)$$

Precipitation studies

The potential of precipitation of aplidine from infusion solutions was examined based on the dynamic flow model described by Yalkowski *et al.*[9] The testing apparatus consisted of a Model 711 syringe pump (IVAC), a Model 501Dz peristaltic pump (Watson Marlow) and a Model UV/VIS 918 spectrophotometer (GBC Scientific Equipment) equipped with a flow cell cuvet with a fill capacity of approximately 3 ml, and a LEO personal computer and Epson LX-400 plotter. Silicone tubing with a length of 75 cm and an internal diameter of 1.6 mm was applied (Watson Marlow). Isotonic Sorensen's buffer adjusted to a pH of 7.4 was used as a blood simulator and was pumped through the system at a flow rate of 6 ml/min. Light scattering (opacity) due to precipitation was measured at a

wavelength of 400 nm. Aplidine infusion solutions at concentrations of 5.9 and 84.7 μg/ml were infused at rates of 0.15, 0.3, 0.6 and 1.5 ml/min (corresponding to formulation:buffer ratios of 0.025, 0.05, 0.1 and 0.25, respectively) in the running buffer flow at a distance of 25 cm from the flow cell cuvet. Also, blank infusion solutions containing only the aplidine vehicle in the same concentrations as in the aplidine infusion solutions were tested. As a positive control, precipitation induced by diazepam (Diazepam CF; Centrafarm, Etten-Leur, The Netherlands) at a concentration of 5 mg/ml formulated in a mixture of 40/10/50% (v/v/v) PEW was determined using the same procedure as for the aplidine infusion solutions, including an additional formulation:buffer ratio of 0.014. The baseline opacity was determined by infusing normal saline at all formulation:buffer ratios. All solutions were infused in the running buffer flow at a distance of 25 cm from the flow cell cuvet. Precipitation was measured in quadruplicate as the plateau opacity induced by infusion of a formulation solution.

Results and discussion

Chromatography

Both the aplidine and DEHP assay were linear in the range of interest of 1.25–125 and 1–400 μg/ml, respectively, with correlation coefficients above 0.999. A representative chromatogram of DEHP is given in Figure 2.

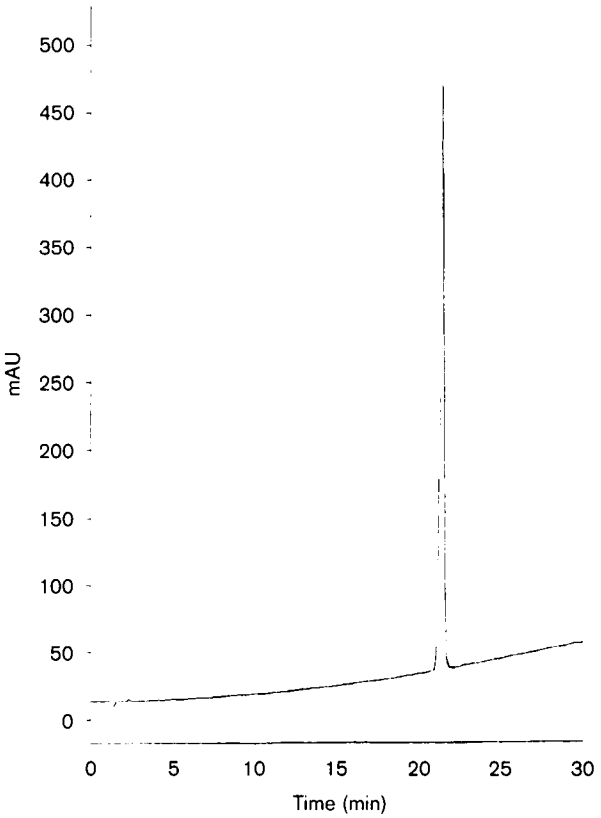

Figure 2. Representative chromatogram of DEHP (4.5 μg on column).

Compatibility with administration sets

In Table 1 the concentrations of aplidine during the infusion simulation experiment and the absolute and relative amounts of aplidine released using a PVC and PVC-free administration set are given. It can be seen that the total amount of aplidine released during the infusion simulation depends on both the infusion set material used and the aplidine concentration of the infusion solution. With the non-PVC administration set and with higher aplidine concentrations, relatively more aplidine becomes available. None of the chromatograms obtained revealed any appearance of degradation peaks, indicating that the decrease in concentration is most likely due to sorption processes. These sorption processes might consist of a combination of adsorption of aplidine onto the surface of the administration set material and absorption into the administration set matrix resulting in loss of drug substance. From Table 1 it becomes clear that the sorption effects are more prominent with lower aplidine concentrations and with the PVC administration set. When calculating the amount of aplidine sorbed per contact surface area of the infusion set materials, it appears that for the PVC administration set the amount sorbed increases linearly ($r > 0.9999$) within the aplidine concentration range tested (5.9 μg/ml: 0.71 μg/cm^2; 28.8 μg/ml: 1.58 μg/cm^2; 84.7 μg/ml: 3.80 μg/cm^2), whereas the amount sorbed for the PVC-free administration set is almost equal for both the 28.8 and 84.7 μg/ml aplidine infusion concentration (28.8 μg/ml: 0.54 μg/cm^2; 84.7 μg/ml: 0.56 μg/cm^2). Apparently, the sorption process becomes saturated with infusion concentrations equal or above at least 28.8 μg/ml of aplidine using the PVC-free administration set.

Table 2 shows the leaching of DEHP from the PVC administration set material. As the PVC-free administration set does not contain any DEHP, no leaching of DEHP from the PVC-free administration set was detected as expected. However, from the PVC-administration set significant DEHP leaching was observed. Several studies reported DEHP leaching

Table 1. Aplidine concentrations (μg/ml) during infusion using a PVC and PVC-free administration set

Time (h)	Aplidine infusion solution concentration					
	PVC administration set			Non-PVC administration set		
	5.9 μg/ml	28.8 μg/ml	84.7 μg/ml	5.9 μg/ml	28.8 μg/ml	84.7 μg/ml
0	4.9±0.4	26.3±0.2	77.4±0.1	6.3±0.3	28.9±0.4	84.7±0.9
0.03	4.6±0.4	25.9±0.2	77.0±0.6	5.2±0.4	28.7±0.03	83.8±2.7
0.08	4.4±0.7	25.7±0.1	76.9±0.1	5.5±0.2	29.2±1.0	84.0±1.6
0.25	4.8±0.8	25.7	77.7±0.9	5.5±0.5	28.6	83.4±2.1
0.5	4.2±1.1	25.6±0.6	76.7±0.5	4.9±0.3	28.0±0.3	83.6±4.0
1	3.5±0.4	24.5±1.2	74.9±1.1	4.7±0.3	27.5±0.6	81.9±2.5
4	2.0± 0.8	21.1±0.4	70.5±0.2	4.2±0.1	27.7±2.5	81.7
8	2.9	20.8±1.0	67.9±2.3	4.1±1.3	26.3±0.8	82.8±1.5
24	2.9±0.5	23.6±0.6	73.2±2.7	5.7±0.6	26.7±0.6	83.2±2.1
Total amount of aplidine released (mg)	0.29 (48.3%)[a]	2.36 (77.4%)	8.53 (83.6%)	0.47 (78.3%)	2.84 (93.1%)	9.98 (97.8%)

[a]With respect to the theoretical total aplidine dose.

from PVC administration set materials under influence of surfactants.[4,10–15] Also, any ethanol present may play a role, although minor, in the DEHP leaching process.[16] In our study, we found total amounts of DEHP extracted from the PVC administration set of 3.5 (7.9), 13.1 (29.7) and 34.9 mg (79.4 μg/cm^2) after a 24 h infusion simulation of solutions containing 0.2, 0.9 and 2.5% v/v Cremophor EL and ethanol, respectively (Table 2). These data show a linear relationship between the concentrations of Cremophor EL and ethanol in the aplidine infusion solutions tested and the amount of DEHP extracted ($r > 0.999$). Furthermore, for the PVC administration set, the amount of aplidine sorbed per contact surface area seems to be correlated with the amount of DEHP leached from the infusion set per contact surface area ($r > 0.99$, $p < 0.05$). An explanation for this might be the more porous contact surface matrix resulting from the migration of DEHP from the administration set material, thus giving opportunities for sorption processes to occur. The concentration-time patterns of DEHP leaching during the infusion simulation of the different aplidine infusion solution concentrations as depicted are similar to those reported earlier by Trissel *et al.* for paclitaxel infusion solutions.[13] At the highest aplidine and Cremophor EL/ethanol/Wfl vehicle concentration the DEHP release from the PVC administration set material reaches a maximum at approximately 8 h after the start of the infusion simulation after which period the DEHP release decreases. The same pattern, although not as clear as for the 84.7 μg/ml aplidine infusion solution, can be observed for the 28.8 μg/ml solution. DEHP release from the PVC administration set under influence of the 5.9 μg/ml aplidine infusion solution, however, reaches a plateau value of approximately 40 μg/ml after 8 h from the start of the infusion which is maintained during the rest of the infusion simulation. The initial increase and subsequent leveling off of the DEHP release is probably due to depletion of accessible DEHP. As more DEHP is leached from the administration set material in time, a point will be reached at which further release is slowed down because less DEHP is available at locations in the administration set matrix which are more difficult to reach for the contact solution to solubilize the plasticizer. The amount of DEHP leached from PVC administration sets is dependent on various parameters like the initial DEHP content of the PVC

Table 2. DEHP leaching (μg/ml) during infusion using a PVC administration set

Time (h)	Aplidine infusion solution concentration		
	5.9 μg/ml	28.8 μg/ml	84.7 μg/ml
0	0	0	0
0.03	1.5±2.1	0	0
0.08	1.8±2.5	1.9±2.7	1.7±2.5
0.25	4.8±1.0	6.2±5.3	8.5±2.5
0.5	3.5±0.9	13.2	26.1±6.1
1	5.7±1.3	21.0±1.0	55.0±1.7
4	31.7±2.4	116.9±48.3	287.5±14.9
8	37.4±1.4	146.2±26.3	419.1±69.3
24	40.4±4.6	130.6±18.5	207.6
Total amount of DEHP leached (mg)	3.5	13.1	34.9

material which may constitute up to 40% of the dry weight of the plastic, contact surface area, contact duration and temperature, volume, and composition of the contact solution. As reported by Trissel *et al.*, an amount of 10 mg of DEHP was released from an FDA-approved paclitaxel administration set containing an IVEX-2 filter set composed partially of PVC material during a 24 h infusion of an 1.2 mg/ml paclitaxel solution containing 10% v/v of Cremophor EL.[15] Apparently, this amount of DEHP administered i.v. is assumed safe and non-toxic. On the basis of the DEHP amounts released in our study, only the 5.9 μg/ml aplidine infusion solution leached less than 10 mg of DEHP during the 24 h infusion simulation. In an earlier report, a 'no observed adverse effect' level of DEHP of 2.5 mg/kg/day was described, corresponding to a total dose of approximately 175 mg of DEHP during a 24 h infusion.[15] On the basis of this non-toxic level, all aplidine infusion solution concentrations tested could be safely administered using a PVC administration set. However, although up to now no specific toxicities have been found as a consequence of DEHP administration to humans, several animal toxicology studies showed changes in hepatocellular structure and liver function, and even development of hepatocellular carcinoma leaving suspicion with respect to the safety of the parenteral administration of this plasticizer. Also, the formation of particulate matter as a consequence of DEHP precipitation has been reported.[11] Additionally, given the number of parameters of influence on DEHP leaching and the abundance of PVC administration sets commercially available at present it is difficult to predict the exact amounts of DEHP leaching for combinations of infusion solutions and PVC administration sets in the actual clinical situation. This is illustrated by the wide variety of DEHP amounts leached from infusion set containers and administration accessories reported in the literature.[16] Therefore, from a pharmaceutical point of view, whenever possible combinations of infusion solutions and administration sets which cause DEHP leaching should be avoided in clinical practice. For aplidine this means that a PVC-free adminstration set should be applied.

Stability of infusion solutions

Aplidine appeared to be stable for at least 24 h after reconstitution of aplidine 500 μg/vial lyophilized product with 1 ml of 15/15/70% (v/v/v) Cremophor EL/ethanol/Wfi when stored at room temperature (20–25°C) and a normal day-night cycle (102.7%, RSD=6.1% of the initial aplidine content remaining after 24 h). The stabilities of aplidine infusion solutions in concentrations of 28.8 and 84.7 μg/ml in the non-PVC infusion container were examined. No degradation was observed in both aplidine infusion solutions and 95.0% (RSD=0.44%) and 99.2% (RSD=1.35%) of the initial aplidine concentration remained after 48 h of storage at room temperature (20–25°C) and a normal day–night cycle for the 28.8 and 84.7 μg/ml aplidine infusion solution, respectively.

Hemolysis studies

Hemolysis or the destruction of red blood cells in the blood stream caused by the i.v. administration of pharmaceutical vehicles results in the release of hemoglobin into the plasma which is associated with vascular irritation, phlebitis, and even anemia, jaundice, kernicterus and acute renal failure.[6] Studies conducted so far to determine the hemolytic potential of pharmaceutical formulations are almost exclusively focused on the i.v. injection and not on the continuous infusion of pharmaceutical solutions.[5–8,17] Although infusion solutions generally contain highly diluted drug vehicles and are therefore less expected to induce blood cell destruction, the hemolytic potential of such a solution infused over a longer period of time should be evaluated as a part of biocompatibility studies of a new formulation. We studied the hematolytic potential of an infusion solution concentration of 84.7 μg/ml of aplidine, being the highest concentration expected in clinical phase I studies, and containing both an ethanol and Cremophor EL infusion concentration of 2.5% (v/v). Instead of a blood-formulation contact time of 1 s, proposed by Krzyzaniak *et al.* as physiologically realistic after an i.v. injection, a 5-fold longer contact time of 5 s was applied to correct for the continuous exposure during an i.v. infusion. Based on a total infusion volume of 120 ml, an infusion duration of 1 h and a venous blood flow of 40 ml/min, a formulation:blood ratio of 0.05 was employed.[6] Also, ratios of 0.1 and 0.25 were tested, to simulate higher formulation:blood proportions occurring during, for example, shorter infusion durations or adjustments in the infusion rate during administration. From Table 3 it can be seen that for both the static and the dynamic model the degrees of hemolysis seen with aplidine infusions did not differ significantly with those obtained with blank infusion solutions (all below 1% hemolysis). Hemolysis levels seen with 40/10/50% (v/v/v) PEW are comparable with percentages described earlier.[6] Also, the higher percentages of hemolysis obtained with the static *in vitro* model compared to the dynamic model are in

Table 3. Results of *in vitro* hemolysis testing

Solution	Model	Mean % hemolysis at formulation:blood ratio		
		0.05	0.1	0.25
Aplidine infusion solution (84.7 μg/ml)	static	0.43±0.2	0.31±0.4	0.50±0.1
	dynamic	0.08±0.1	0.34±0.2	0.05±0.07
Blank aplidine infusion solution	static	0.05±0.05	0.38±0.4	0.18±0.3
	dynamic	0.25±0.2	0.74±0.04	0.46±0
40/10/50% (v/v/v) PEW	static	5.66±1.3	28.42±0.09	53.67±1.5
	dynamic	13.26±0.3	22.82±0.5	39.78±1.7

correspondence with data in the literature.[6] Ward *et al.* reported that the static model may not be appropriate as a predictive model for the hemolytic potential because of the occurrence of false-positive results.[5] For the *in vitro* dynamic hemolysis model it was reported that this method is a good predictor of phlebitis as a result of hemolysis upon injection of pharmaceutical vehicles.[6] In this study hemolysis was detected in neither the static nor the dynamic model with aplidine and blank infusion solutions. Therefore, it is not expected that aplidine infusion solutions will cause red blood cell destruction upon administration, even at the highest expected concentration and a 1 h infusion duration.

Precipitation studies

Infusion of poorly water-soluble drugs formulated in co-solvent/surfactant systems in the bloodstream results in a sudden and fast dilution of the drug solution. This could result in precipitation of the solubilized components, e.g. due to dilution to concentrations below the critical micelles concentration (CMC) of the surfactant. Precipitation is related to pain and phlebitis at the injection site as well as altered bioavailability. Therefore, aplidine infusion solutions at the lowest and the highest concentrations (5.9 and 84.7 μg/ml aplidine, respectively) expected to be administered to the patient in phase I clinical trials were subjected to *in vitro* precipitation testing. Based on a total infusion duration of 1 h, total infusion solution volumes ranging from approximately 100 to 120 ml and a venous blood flow of 40 ml/min, a formulation:buffer ratio of 0.05 was employed. As reference, a diazepam formulation with known precipitation when injected into the bloodstream at rates exceeding 1 ml/min, corresponding to a formulation:buffer ratio of 0.025, was selected. Any opacity detected with diazepam at this formulation:buffer ratio in the *in vitro* simulation experiment was taken as a threshold value above which adverse effects (e.g. pain, phlebitis) are likely to occur. Diazepam precipitation occurred at all formulation:buffer ratios examined with a threshold value of approximately 0.2 at a formulation:buffer ratio of 0.025 (Figure 3). A similar precipitation profile of diazepam was reported by Li *et al.*[18] At all formulation:buffer ratios the opacity induced by the aplidine infusion solutions did not differ significantly from those obtained with blank aplidine infusion solutions or normal saline and were well below the threshold value as determined with the diazepam formulation (Figure 3). On the basis of these results, no adverse effects as a consequence of precipitation from aplidine solutions upon infusion are expected at the tested infusion rates.

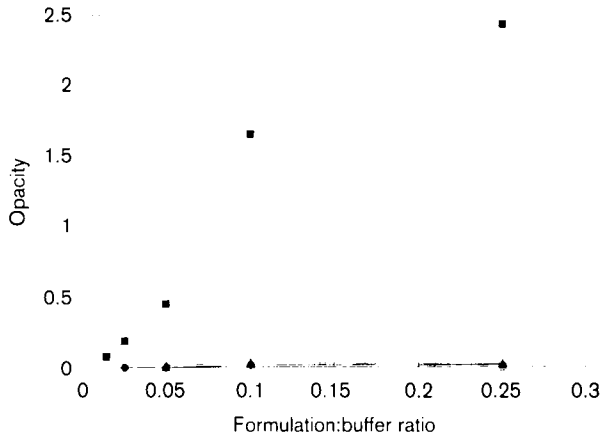

Figure 3. Precipitation as a function of the formulation:buffer ratio (■, 5 mg/ml diazepam; □, 5.9 μg/ml aplidine infusion solution; ◆, 84.7 μg/ml aplidine infusion solution; ▲, blank aplidine vehicle 84.7 μg/ml; ◇, blank aplidine vehicle 5.9 μg/ml).

Conclusions

In conclusion, we have presented data that aplidine should be administered in infusion concentrations equal or above 28.8 μg/ml using a PVC-free adminis-

tration set consisting of a glass container and silicone infusion tubing. After reconstitution, aplidine lyophilized product should be further diluted with normal saline within 24 h which can be subsequently administered to the patient within 48 h. *In vitro* hemolysis and precipitation studies indicate that no biocompatibility problems in these respects are expected upon parenteral administration of aplidine infusion solutions. Aplidine is currently under phase I clinical trials in Europe and Canada.

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(Received 5 August 1999; accepted 19 August 1999)