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1,3-Dicyclohexyl urea nanosuspension for intravenous steady-state delivery in rats

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Aqueous insolubility is recognized throughout the pharmaceutical industry as a major hurdle for pre-clinical and clinical drug delivery. Pre-clinical, early efficacy, and proof of concept studies oftentimes rely on model compounds that have less than ideal physiochemical properties, and the *in vivo* results from these studies often have critical impact on the future of the project. As such, effective delivery of prototype compounds with sub-optimal properties is important in target validation. 1,3-Dicyclohexyl urea (DCU), a potent inhibitor of soluble epoxide hydrolase (sEH) has been shown to lower systemic blood pressure in spontaneously hypertensive rats. This compound has limited aqueous solubility that makes *in vivo* delivery difficult. In such situations, co-solvents, complexation reagents, and emulsions are commonly used to increase the bioavailability of a prototype compound. However, these approaches are often limited by their capacity to get and keep a compound in solution and can have unwanted placebo effects, which can confound the interpretation of animal efficacy results. Nanosuspension formulations of DCU have been utilized for both intravenous injection and infusion to reach steady-state (C*ss*) plasma concentrations in rat enabling the investigation of the target, chemistry space, and PK/PD in a timely manner without encountering confounding efficacy results.

Keywords: Nanosuspension; 1,3-Dicyclohexyl urea; Poorly soluble; Intravenous injection; Infusion; Steady-state

1. Introduction

In the pharmaceutical industry today, an increasing number of lipophilic drug candidates are providing scientists with the growing challenge of reaching desired exposures *in vivo*. Approaches to deliver poorly soluble molecules have been developed for both clinical and pre-clinical activities [1, 2]. However, in discovery where a large

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number of potential candidates are screened, there is a growing need to provide quick turnaround formulations to evaluate the *in vivo* efficacy of potential drug candidates. In general, formulations made for early discovery need to be prepared on the small scale using common excipients with little lead development time and the assurance of reliable delivery of target concentration levels. Organic solvents, such as polyethylene glycol, ethanol, propylene glycol, and complexation agents such as hyrdoxypropyl-β-cyclodextrin and sulfobutyl ether cyclodextrin, and emulsions are commonly used as solubilization enhancers for preparing IV formulations for both bolus and infusion dosing [1]. However, the degree of solubility enhancement, even within a molecular series, can vary from compound to compound and the ability to increase the solubility to target concentrations may not be attainable with reasonable concentrations of solubility enhancers. Furthermore, the possibility of drug precipitation upon dosing is always a common threat in these formulations [3], and the possibility of unwanted placebo effects on efficacy readouts from the use of high concentrations of surfactants and co-solvents can confound results [4, 5].

In recent years, researchers have established that various epoxyeicosatrienoic acid (EETs) regioisomers cause either vasodilatation or vasoconstriction in a number of vascular beds [6–8] and that they possess anti-inflammatory properties [9]. Based on these findings, soluble epoxide hydrolase (sEH) inhibition is a potentially attractive pharmacological approach to treat human hypertension. It has been reported that 1,3-dicyclohexyl urea (DCU) is a potent sEH inhibitor and inhibits human vascular smooth muscle (VSM) cell proliferation in a dose-dependent manner [10, 11]. Because of the anti-inflammatory and antihypertensive properties of sEH inhibition, DCU can be used as a model sEH inhibitor to further investigate decreased VSM cell proliferation, a key pathologic mechanism in the progression from systemic hypertension to the atherosclerotic state [9, 12, 13]. However, despite having high *in vitro* potency, the utility of DCU to investigate sEH is limited based both on its high *in vivo* clearance in rat (in house results) and its low aqueous solubility, which makes oral delivery of DCU to maintain prolonged and constant exposure difficult. Furthermore, its poor aqueous solubility also limits the option of delivering DCU intravenously without using a formulation that has a high percentage of organic co-solvents. In the sEH animal efficacy model, the use of these co-solvents in IV formulations interferes with antihypertensive readouts confounding data interpretation.

The approach discussed in this work is to use a nanoparticle, intravenous infusion delivery system to achieve the desired steady-state plasma concentration of the model drug over time without using large amounts of co-solvent or surfactants, thereby minimizing placebo effects. The use of nanoparticles and particle size reduction in general to increase exposure for poorly soluble drugs is well precedented [2, 14–20]. Reducing the particle size increases the surface area available to the dissolution media and thus increases the overall apparent dissolution rate. This can be estimated by the equation developed by Noyes and Whitney:

$$\frac{dC}{dt} = \frac{D * S(C_s - C_t(t))}{Vh_d}$$

where:

dC/dt: Dissolution rate (R)

D: Solute diffusion coefficient
V: Volume of dissolution medium
h_d: Diffusion boundary thickness
S: Surface area of solute
C_s: Saturation solubility of solute
$C_t(t)$: Bulk solute concentration

Nano and micro particle drug delivery has been widely used in the pharmaceutical industry and has mainly focused on oral, intraperitonel, intramuscular, or subcutaneous delivery (2). The main advantage of such delivery systems is to take advantage of an increased surface area to enhance the overall dissolution rate and thereby boost the bioavailability of the dose. However, reduction of particle size for IV formulations of poorly soluble drugs is not necessarily sufficient to ensure the desired rate of dissolution in the blood. Thus, to reliably dose a low aqueous solubility drug by IV without the complications from precipitation there should first be some assurance based on theoretical estimations and *in vitro* experiments that the particle size has been reduced to a point where there will be complete dissolution of the drug in the vascular bed shortly after dosing. To this end, reduction to the nanoparticle scale is often required for very poorly soluble drugs.

There are various patents and publications of nanoparticulate drug preparations and applications [14–30]. In practice, nanoparticles can be produced by two general approaches. These are: (1) constructing particles from their molecular state, such as fast precipitation or rapid expansion; or (2) by breaking large particles, such as by milling. The approach of making and maintaining a stable nanoparticulate system is not free of problems. Challenges such as solid form changes and physicochemical stability must be addressed and the formulations need to be well characterized. Furthermore, if a wet milling/suspension system is used, the effect on changing particle size in an aqueous environment needs to be understood.

The potential for particle agglomeration has been addressed by researchers and summarized in great detail [2, 21]. In theory, the new surface area generated by either approach ΔA, requires a ΔG (Gibbs free-energy) and H (Enthalpy) cost and it is defined by;

$\mathrm{d}G = -\mathrm{d}Wnet$: where $Wnet$ is the surface energy
$\Delta G = \gamma_{s/l} * \Delta A$: where $\gamma_{s/l}$ is the interfacial tension.
$H' = G' - T\ (\partial G'/\partial T)_p = \gamma - T(\partial\gamma/\partial T)_p$ where H' is the enthalpy of the surface per unit.

The ΔG increase due to the increase in surface area by either procedure will create a less stable system. Such a system will have a tendency to offset the increase in surface area and thereby reduce ΔG by agglomeration. This phenomena has been effectively controlled by introducing surfactants (reduce $\gamma_{s/l}$) and maintaining good particle size control. The addition of surfactants can provide stabilization at longer times due to an increased energy barrier and by preventing particles from coming close enough to cause agglomeration [2].

The successful use of nanoparticles in IV formulations for water insoluble drugs has been previously studied [5, 22–28]. Despite the use of nano and micro particles for IV injection, utilization of nanoparticles for IV infusion has not been well characterized.

This has limited the use of such technology in pre-clinical research where prolonged and constant exposure is needed to validate targets with 'non ideal' tool compounds. Our effort has focused on developing and characterizing a suitable DCU nanoparticle formulation for both IV bolus injection and infusion targeted for steady state delivery *in vivo* in a spontaneous hypertensive rat model of hypertension.

2. Materials and methods

HPLC grade acetonitrile was obtained from Burdick & Jackson (Muskegon, MI) and reagent grade formic acid was obtained from EM Science (Gibbstown, NJ) and 1,3-dicyclohexyl urea, Cremophor EL, amitriptyline hydrochloride, and polyvinyl pyrrolidone (PVP) were purchased from Sigma-Aldrich (St. Louis, MO).

Lead free glass beads (0.5–0.75 mm) were purchased from Glen Mill (NJ) and pre-conditioned in house. The HPLC-MS system used was a Agilent 1100 HPLC equipped with a diode array detector (DAD) and quaternary solvent delivery system (Palo Alto, CA) coupled with the SCIEX 2000 tandem mass spectrometer from Applied Biosystems (Foster City, CA) and several analytical columns were tested and an Zorbax SB-C8 (5 μm × 150 mm) was selected and used for analysis. The water purification system used was a Millipore Milli-Q system.

A 0.5 μm KrudKatcher disposable pre-column filter was used to prevent on-line precipitation and prolong the column life. The HPLC auto injector was used to accurately inject small volumes (10 μL) of sample solution. A short gradient method was developed and used for the analysis. Solvent line A contained 50 mM formic acid buffer (pH 3.5) and line B contained acetonitrile with 0.5% formic acid. For the method in general, from $T=0$ to $T=4$ minutes, a linear gradient from 60% B to 100% B and hold for 1 minute. At $T=4.01$ minutes, the system was set back to the initial condition and allow to equilibrate for 2 minutes for the next injection. The flow rate was 1 mL/min with a 1:3 post column split for the MS. The Turbo Ion-spray was performed in the positive mode and quantitative analysis was performed in multiple reactions monitoring (MRM). Data were processed with Analyst (version 1.4.1). Amitriptyline hydrochloride (Sigma, St. Louis) was used as internal standard. The spray current was set at 4000 V and temperature was set at 425°C. The collision energy was set at 30 V. For DCU the transition of m/e $225 \longrightarrow 100$ was used and for the internal standard a transition of m/e $278 \longrightarrow 233$ was used.

A standard calibration curve was established by running standards from 0.61 μg/mL to 12.2 μg/mL. For the formulation potency test, a standard curve was constructed based on peak area instead of peak height to achieve a more accurate result. The linear equation of $y=0.0144x$ with a $R^2=0.98$ was obtained. The limit of detection (LOD) for DCU standards was 0.1 μg/mL (S/N = 3) and limit of quantitation (LOQ) was 0.5 μg/mL (S/N = 6). All the formulations were tested in triplicate prior to *in vivo* delivery.

For particle size reduction, a bench scale wet milling (micronization) device was used [29]. To make the stock nanosuspension formulation (10 mg/mL) DCU, an appropriate amount of glass beads, and 1% (w/w) Cremophor EL in phosphate buffered saline (pH 7.4) were added in a scintillation vial. The mixture was then stirred

at 1200 rpm for a period of 48 hrs with occasional shaking. The stock formulation was then harvested and potency was checked by LC/MS/MS/MRM.

Powder X-ray diffraction (PXRD) was done on a Bruker D-8 Advance diffractometer. The system used a copper X-ray source maintained at 40 kV and 40 mA to provide radiation with an intensity weighted average of ($K\alpha_{ave}$) 1.54184 Å. A scintillation counter was used for detection. A Göbel mirror was used to eliminate $K\beta$ radiation. Beam aperture was controlled using a divergence slit of 0.6 mm and a primary 4° Soller. After diffraction, a secondary Soller was used to ensure collimation of the diffracted beams. Data were collected using a step scan of 0.02° per point with a 1 second/point counting time over a range of 3° to 35° two-theta. In house fabricated aluminum inserts or inserts with a Hasteloy sintered filter (0.45 μm) pressed in the center and held in Bruker plastic sample cup holders were utilized for all analyses. Dry DCU was run as received without hand grinding. Suspensions were filtered onto sintered filters under vacuum.

Thermal gravimetric analysis with simultaneous differential thermal analysis (TGA/SDTA) was done on a Mettler TGA/SDTA851e. Samples were sealed in 40 μL pierceable aluminum capsules. The instrument robot was used to pierce the samples before insertion into the furnace. Samples were heated at 5°C/min from 20°C up to a maximum of 400°C. The temperature and simulated heat flow axis were calibrated using indium.

Particle size distribution was determined on a Beckman Coulter LS 230 particle size analyzer using the small volume accessory (Miami, FL). A PIDS obscuration water optical model was employed. Particle size distribution was computed by the software using Mie scattering theory. There was no absorption by DCU at the laser line (750 nm) so the complex index of refraction was determined by finding the average refractive index of DCU (1.57) by microscopy. Index matching fluids from Cargille (Cat 18005) were employed.

The non-stirred dissolution rate was determined by submerging one side of a compressed pellet of DCU still in the compression die into rat plasma such that the exposed surface area could be assumed constant (0.178 cm^2) over the length of the experiment. The system was held at 37°C. Sink conditions over 5 fold were maintained throughout the experiment. Aliquots of the media were taken at 5, 60, 90, 150, 180, 240, and 300 minutes and analyzed by the HPLC method above for concentration of DCU. A linear fit $y = 1.354x$ with a $R^2 = 0.9344$ was obtained.

For the IV bolus dose, the formulation was prepared by diluting the stock with the 1% (w/w) Cremophor EL in phosphate saline to the desired concentration. For the infusion dose, formulation was prepared by first diluting the stock to twice the target concentration with 1% (w/w) Cremophor EL in phosphate saline and then further diluted with 20% PVP (w/w), 1% (w/w) Cremophor EL in phosphate saline to the desired concentration. Several other modifiers were tested (pluronic, dextrose, mannitol, and sucrose) for better consistency of infusion by syringe pump and it was found PVP gave the best consistency. Other delivery devises such as Alzet pump, tubing loops, and IV bag were evaluated for alternative delivery. Three samples from each formulation were taken and assayed by LC/MS/MS/MRM to ensure potency.

For the *in vivo* work, male Sprague-Dawley rats (SD) were purchased from Charles River Laboratories (Wilmington, Mass). This animal study was approved by

the St. Louis Pfizer Institutional Animal Care and Use Committee. The animal care and use program is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International. The detail of *in vivo* work and PK profiling and modeling will be addressed on separate publication. Briefly, for the IV bolus injection, the injection volume is controlled at 1 μL per gram of body weight and infusion is controlled at 1 mL per hr for a period of 3 hrs. Blood samples were obtained periodically and handled with extra caution to prevent particle carrier-over.

3. Results and discussion

In order to show the validity of using a DCU nanoparticle formulation to achieve a desired C*ss* by infusion dosing *in vivo*, several *in vitro* experiments and theoretical estimations were done. The results were then compared with *in vivo* data.

Micronization of DCU was successful in reducing particle size. Figure 1 shows the final distribution for DCU used *in vivo*. The bulk material was reduced from 40 μm to 0.5 μm with a final D_{50} of 0.45 μm. The solid form of the micronized material was checked by PXRD and TGA/SDTA. Figure 2 shows the PXRD pattern of the starting and post micronized material. There was no discernable change in crystal form during the micronization process. Although a small increase in amorphous content would not be readily observed by PXRD, there was no gross increase in amorphous material, as indicated by an increased baseline halo. The TGA/SDTA data for DCU post micronization, figures 3 and 4 were also unchanged. In both cases, the initial melt had an onset of 220°C.

One of the key concerns of utilizing nanoparticles for IV injection and further modeling for infusion to achieve the C*ss* is the dissolution rate of the nanoparticles following injection. Reducing the particle size to improve the overall dissolution rate is critical to making a successful IV suspension formulation that can dissolve quickly and provide the ability to do long term infusion dosing. An estimation of the increase in

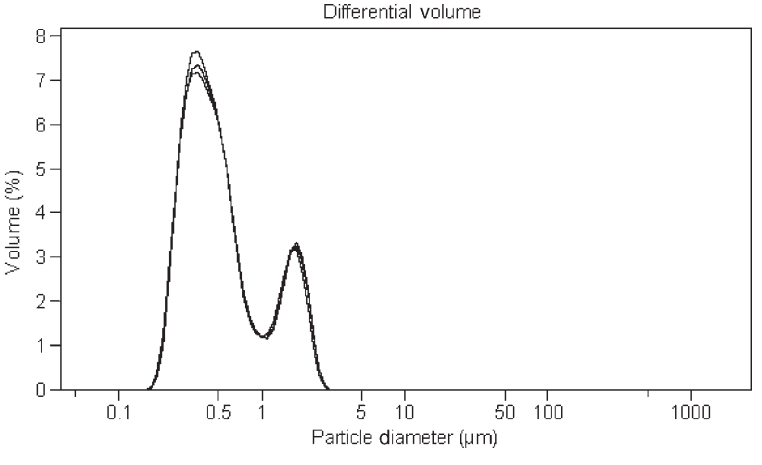

Figure 1. Final particle size distribution for DCU used *in vivo* formulation work.

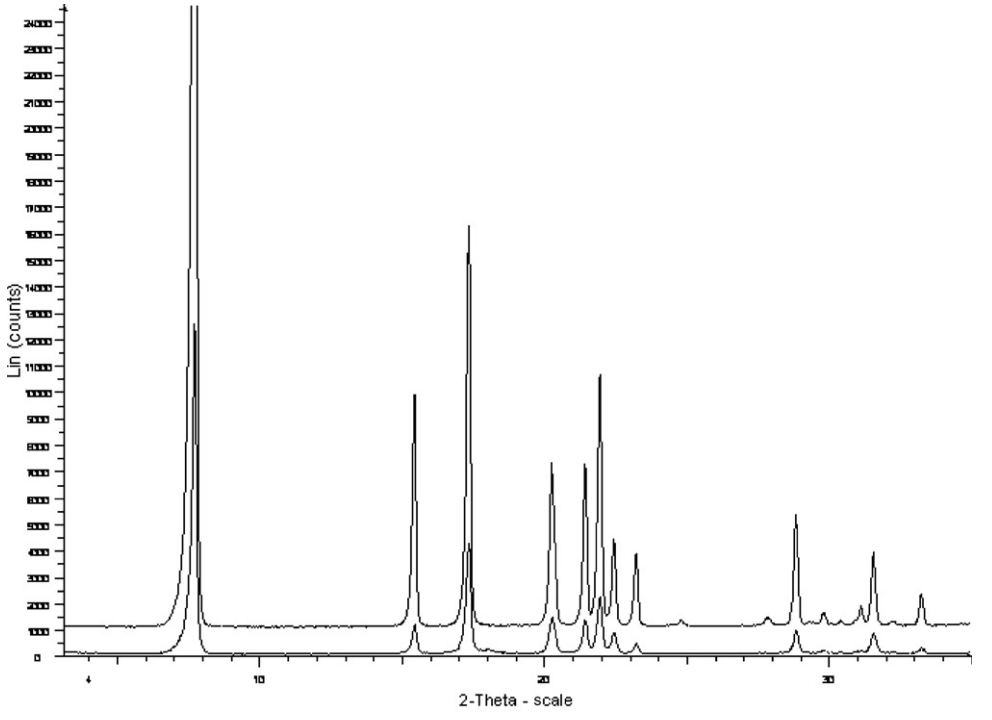

Figure 2. PXRD data for DCU both as received (top) and after micronization for 48 hrs (bottom).

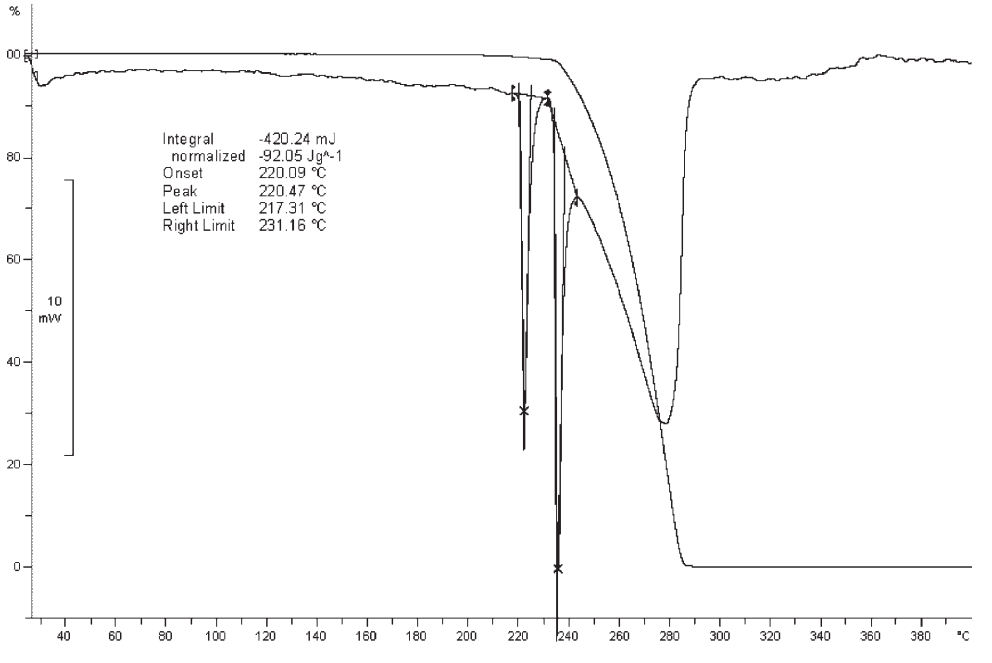

Figure 3. TGA/SDTA data for the as received DCU bulk material from Aldrich.

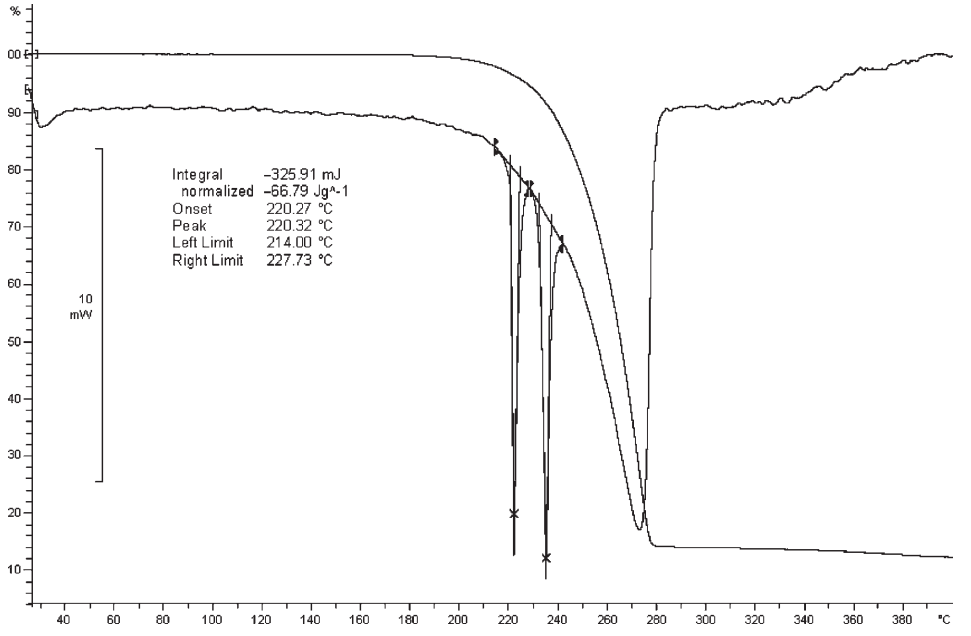

Figure 4. TGA/SDTA data for DCU post milling.

dissolution rate resulting from particle size reduction can be done by using the equation proposed by Noyes and Whitney. A sphere shape model was used and the surface area gain (ΔA) of the micronized vs. non-micronized DCU per unit mass was calculated to be 800 fold. If other factors are held constant, we can assume the degree of rate gain dR is equal to the gain in surface area. Furthermore, the advantage of boundary layer reduction from particle size reduction should further enhance the rate of dissolution.

Researchers have observed changes in pharmacokinetic parameters when using nanoparticles as a tool for IV injection [1, 2, 30]. According to these sources, the use of nanoparticulates may contribute to slow dissolving particle organ accumulation. To estimate if this might be a problem with the DCU nanosuspension, the dissolution rate and solubility of DCU in rat plasma were determined. The solubility of DCU in rat plasma (no-milled DCU stirred at room temp for 24 hours) was approximately 7 μg/mL and the diffusion coefficient of 9×10^{-7} cm^2/sec was obtained by non-stirred dissolution in rat plasma. A non-stirred solution under overall sink conditions was used to model the ''worst case'' scenario for dissolution *in vivo*. Using the assumption that the diffusion layer thickness is close to the mean particle size and assuming all particles are uniformly sized, the following equation derived from the Hixson–Crowell cube root law can be used to calculate the time for complete dissolution [31].

$$\Gamma = \frac{\rho r_o^2}{2DCs}$$

where:

Γ: is the estimate time for complete dissolution

ρ: is the density of the solution
r_o: is the radius of the particle
D: is the diffusion coefficient
Cs: is the solubility

The calculated time for each particle with a mean radius 0.25×10^{-4} cm to dissolve is less than a minute. Furthermore, upon IV injection, turbulent blood flow in the vein should serve to further reduce the diffusion boundary thickness, rapidly disperse the initial injection volume, and minimize local concentration effects. This suggests that upon injection DCU nanoparticles should dissolve quickly, thereby reducing the *in vivo* risk of phlebitis and organ accumulation [2, 3, 19].

If the *in vitro* estimations made above translate to *in vivo* results, the nano-formulation should have a similar performance to the solution dose with regard to such fundamental PK parameters as clearance and mean residence time (MRT). This estimation was demonstrated *in vivo* in the rat. Table 1 shows results processed by Winonlin software (Mountain View, CA) from IV bolus dosing using organic solution and the nanoparticle formulation. Both the MRT and the clearance are similar for each formulation.

Given the success of the IV bolus dose, the formulation was then dosed as an infusion *in vivo*, which was targeted for the target validation study, to test delivery speeds and durations. This determined the ability to utilize nanoparticle IV bolus dose data to estimate infusion and post infusion results. The equation derived from a one-compartment model was used to estimate the plasma concentration as a function of time for infusion and post infusion.

$Css =$ Infusion rate (IR)/Clearance (CL)
$Ct = Css * (1 - e^{-kt1})$ and $Cp = Css * (e^{-kt2})$
where:

Css: Steady state concentration
Ct: Concentration during infusion
Cp: Post dose concentration
k: Elimination rate constant
$t1$: Infusion time
$t2$: Post infusion time

The MIR (maximum infusion rate) was also calculated based on the following equation by Glenn and Daryl [32].

MIR = (Plasma solubility $\times$ Plasma flow rate)

The nanoparticle, infusion, *in vivo* data presented in table 2 are in good agreement with the estimated values based on the nanoparticle bolus dose data. Such a good agreement demonstrates the validity of the modeling work and provides a rationale to further expand the usage of nanoparticles for infusion.

Table 1. DCU IV bolus Rat PK comparing solution vs. nanosuspension.

PK Parameter ($n = 3$)	IV Solution	IV Nanosuspension
MRT (hr)	1.1 ± 0.1	1.5 ± 0.4
CL (mL/min/kg)	34.2 ± 0.9	36.2 ± 1.3

Table 2. DCU IV infusion experiment.

Infusion Time point (minutes)	Calculated μg/mL	*In vivo* ($n=4$) mg/mL
60	1.7	2.1 ± 0.1
120	2.1	2.3 ± 0.1
180	2.2	2.0 ± 0.3
30 (post infusion)	0.8	1.0 ± 0.1

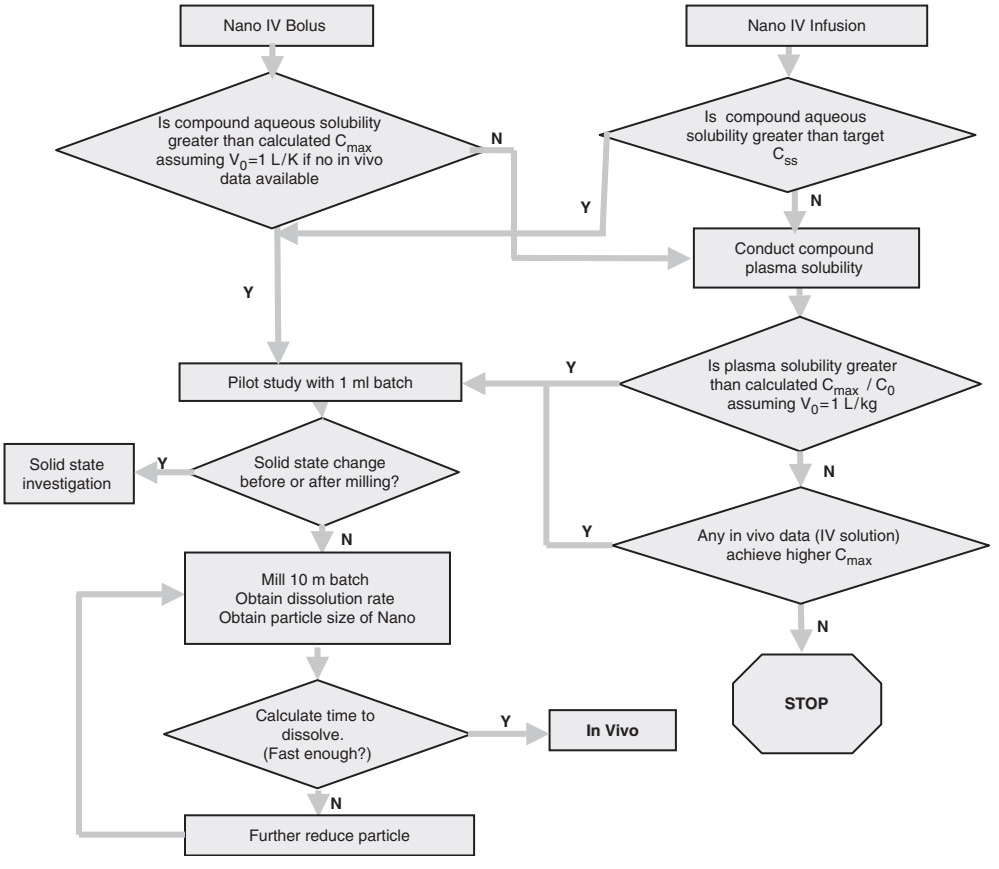

Figure 5. Proposed feasibility test for nano IV when solution formulation not suitable.

This example has demonstrated that with a carefully characterized nanoparticle system that meets the parameters outlined above, it is possible to achieve the desired *in vivo* exposure (target steady-state at 2 μg/mL) with a non-ideal compound to evaluate a target. A systematic approach to evaluating compounds for nanoparticle parental formulation pre-clinically when solution formulation is not suitable with the assumption of volume of distribution at time 0 (V_0) = 1 L/kg has been listed in figure 5. Such an approach has been tested for several in house compounds with good success.

4. Conclusions

One of the biggest challenges facing the pharmaceutical industry today is the need to lower costs and save time to market. Therefore, early target validation without large up front investments is becoming increasingly important. The use of nanoparticle parenteral drug delivery systems for infusion of tool compounds to reach desired steady-state exposures allows researchers to obtain reliable data for decision making early in the discovery process without large investments by utilizing non-ideal, prototype compounds for target validation.

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