Physical Stability of Ethyl Diatrizoate Nanocrystalline Suspension in Steam Sterilization

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Purpose. To study the effects of formulation variables on the physical stability of a submicron crystal (nanocrystal) suspension under steam sterilization conditions.

Methods. Suspensions of ethyl diatrizoate nanocrystals were prepared by wet milling in the presence of the surfactant poloxamine 908. Particle size distribution and zeta potential were measured by photon correlation spectroscopy.

Results. On heating, the mean particle size of the nanocrystal suspension remained essentially unchanged up to 110°C, the cloud point of the stabilizing surfactant, but increased significantly above that temperature. The increase in particle size was a result of particle aggregation rather than crystal growth. Adding a cloud point booster to the suspension significantly minimized the particle aggregation at high temperatures. The purity of poloxamine 908 and the tonicity agent and buffer salt used also affected the heat stability of the suspension, the latter agents apparently through altering the surfactant cloud point.

Conclusions. The aggregation of the ethyl diatrizoate nanocrystalline suspension under steam sterilization conditions was a result of phase separation of the stabilizing surfactant at its cloud point. When formulated with a cloud point booster to prevent the phase-separation, the suspension maintained its physical stability under steam sterilization without any significant change in particle size distribution.

KEY WORDS: nanocrystals; submicron crystals; suspensions; steam sterilization; physical stability; surfactants; cloud point; ethyl diatrizoate.

INTRODUCTION

Suspensions are found frequently in pharmaceutical products with different routes of administration. While most of the existing suspension products have particle sizes in the micron range, technology development in recent years has extended the range down to the submicron region (1–5). Suspensions in the latter category have several potential advantages over the conventional ones including more favorable biodistribution (5), better bioavailability for drugs with low solubilities (6,7), and the possibility of intravenous administration (1,5,8). These factors have given significant impetus to research in this area. Currently, two types of submicron suspensions are of great interest for pharmaceutical use. The first consists of "nanoparticles", "nanocapsules", or "nanospheres" which are submicron size polymeric particles as drug carriers. The drug is usually adsorbed onto or embedded or encapsulated within the particles (1). The second type of submicron suspension is composed of submicron size drug particles, either amorphous or crystalline, directly without a polymer carrier (2–4,6–8). In the case of crystalline drug particles, the suspension has been referred to as "nanocrystal suspension" (6–8). Nanocrystal suspensions can be prepared by either an attrition process, such as wet milling (3,6–8), microfluidization (2,9), and high pressure homogenization (10), of crystalline drugs, or by a controlled precipitation process where submicron size drug particles are formed *in situ* (2,4). All types of submicron suspensions are usually stabilized by a surfactant adsorbing on the particle surface. Typically, polymeric nonionic surfactants are used by virtue of their strong steric stabilization effect (1,3,5,11).

In the development of submicron suspensions for parenteral use, product sterilization represents a major challenge. Sterile filtration of submicron suspensions can be difficult due to large particle size, high viscosity, and high solid content, resulting in poor filtration rates and filter clogging. Terminal steam sterilization is most desirable from the point of view of sterility assurance but can lead to significant increases in particle size as a result of heat-induced crystal growth, particle aggregation, or both (11). The growth in crystal size on heating and cooling, referred to as Ostwald ripening, is known to be dependent on the solubility-temperature profile of the drug and the polydispersity of the particle size of the suspension. On the other hand, the aggregation of submicron particles at high temperatures is an issue of colloidal stability and could be dependent on formulation parameters such as the surfactant used, its concentration, purity, and cloud point, and the presence of salts and other excipients. We report here the results of studies to understand the effects of the surfactant cloud point and cloud point boosters (CPBs) on the physical stability of a nanocrystal suspension under steam sterilization. The model nanocrystalline suspension used was prepared from ethyl diatrizoate, a radiopaque with a very low aqueous solubility, and stabilized by the surfactant poloxamine 908.

MATERIALS AND METHODS

The poly(ethylene glycols) (PEGs) used were from Baker (Phillipsburg, NJ) and Union Carbide (Danbury, CT). 2-Hydroxypropyl-β-cyclodextrin (HPBCD) was purchased from American Maize (Hammond, IN). The phospholipids were from Avanti (Alabaster, AL). Poloxamine 908 was purchased from BASF (Parsippany, NJ). Ethyl diatrizoate was crystallized in dimethylformamide and had a monoclinic crystal lattice and a powder density of 2.24 g/ml as measured by a helium pycnometer. The typical mean particle size of the drug substance was around 20 μm as measured by a Brookhaven field flow centrifuge (Holtsville, NY). The acetate, citrate, and phosphate buffers were prepared from the sodium salts and the tromethamine buffer was prepared from the chloride salt.

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ABBREVIATIONS: CPB, cloud point booster; HLB, hydrophile-lipophile balance; DOSS, dioctyl sulfosuccinate; DMPG, dimyristoylphosphatidyl glycerol; HPBCD, 2-hydroxypropyl-β-cyclodextrin; CTAB, cetyltrimethylammonium bromide; DTAB, dodecyltrimethylammonium bromide; POPS, 1-palmitoyl-2-oleoyl-phosphatidylserine; DPPA, dipalmitoyl-sn-glycerol-3-phosphatidic acid; DSPE, 1,2-distearoyl-sn-phosphoethanolamine; DPPC, 1,2-dipalmitoyl-sn-glycerol-3-phosphocholine; SDBS, sodium dodecylbenzenesulfonate; SDS, sodium dodecyl sulfate.

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Preparation of Nanocrystalline Suspensions

Nanocrystalline suspensions were prepared by wet milling of ethyl diatrizoate in a 100-ml glass bottle on a roller mill manufactured by US Stoneware (East Palestine, OH). The milling mixture contained 20–25 ml slurry of 20–30% (w/v) ethyl diatrizoate and 4–6% (w/v) poloxamine 908. 100 g of zirconium silicate beads with 0.6 to 0.8 mm diameter (SEPR, Maple Heights, OH) was added as grinding media. The mill was adjusted to give a bottle rotating speed of 80–90 rpm and the milling typically lasted 3–4 days. The final suspension was separated from the milling beads by passing through a 5-μm filter. In subsequent studies, solid additives were dissolved in water before adding to the nanocrystalline suspension and all suspensions were adjusted to 15% (w/v) ethyl diatrizoate and 3% (w/v) poloxamine 908.

Steam Sterilization and Sonication

Samples were filled in 2-ml glass vials, sealed with rubber stoppers and aluminum caps, and steam sterilized in a Castle Model M/C 3500 autoclave sterilizer (Lakewood, NJ). Unless otherwise stated, steam sterilization was at 121°C for 20 min.

Sonication was conducted with a Branson Model 5210 bath sonicator (Danbury, CT). Samples were immersed in water at room temperature. A test showed that the sample temperature increased only about 5°C after 40 min sonication.

Determination of Particle Size Distribution and Zeta Potential

The size distribution and zeta potential of nanocrystalline suspensions were determined by photon correlation spectroscopy using a Zetasizer 3 from Malvern Instruments (Malvern, Worcestershire, UK). The accuracy of the instrument was checked with 220 nm standard polystyrene beads obtained from Duke Scientific (Palo Alto, CA) and AZ55 electrophoresis standard purchased from Malvern Instruments. Sample vials were shaken by hand for 1 min and aliquots of 10 μl were diluted to 3 ml with either a 0.003% poloxamine 908 solution in particle size measurement or the Malvern buffer in zeta potential determination. All size distributions are expressed as volume-weighted. Zeta potentials were measured with triplicate samples.

Solubility and Concentration Determination

The change in solubility of poloxamine 908 at temperatures near the surfactant cloud point was studied. 5 ml of a 3% poloxamine solution was filled in a 10-ml glass vial and sealed with a rubber stopper and aluminum cap (12). At each temperature, samples in triplicate were heated in a bath for 20 min which allowed the phase-separated surfactant to precipitate. While in the bath, a 0.2 ml aliquot was carefully withdrawn from the top layer of each sample with a syringe. They were analyzed by SE-HPLC for concentration of poloxamine 908 (12).

The solubility of ethyl diatrizoate was measured by adding an excess amount of ethyl diatrizoate to screw-cap vials containing different solvents. The vials, in triplicate for each sample, were shaken under various temperatures for two days. After centrifugation to remove the remaining solid, the supernatants were analyzed by reversed phase HPLC to determine the solubility.

RESULTS

Solubility of Ethyl Diatrizoate

The solubilities of ethyl diatrizoate in water at 22, 40, 60, 80, and 100°C were determined to be 5, 14, 20, 41, and 81 μg/ml, respectively. The solubility at 22°C was not affected by the presence of 3.5% poloxamine 908, 0.1% DOSS, or the two surfactants combined, and only increased to 11 μg/ml in 10% PEG 400.

Heat Stability of Nanocrystalline Suspension Without Additive

The heat stability of the ethyl diatrizoate nanocrystalline suspension was first examined without any additive. Figure 1a shows typical particle size distributions before and after the steam sterilization. Before sterilization, the mean particle size of the nanocrystal suspension was in the range of 180 to 200 nm with a polydispersity index below 0.2 and 95% of the particles smaller than 500 nm. After steam sterilization, the mean particle size increased to 400–800 nm, the polydispersity index was in the range of 0.3 to 0.6, and the 95 percentile size increased to the micron range. Visually, the suspension appeared as a light, free flowing colloid before steam sterilization, but

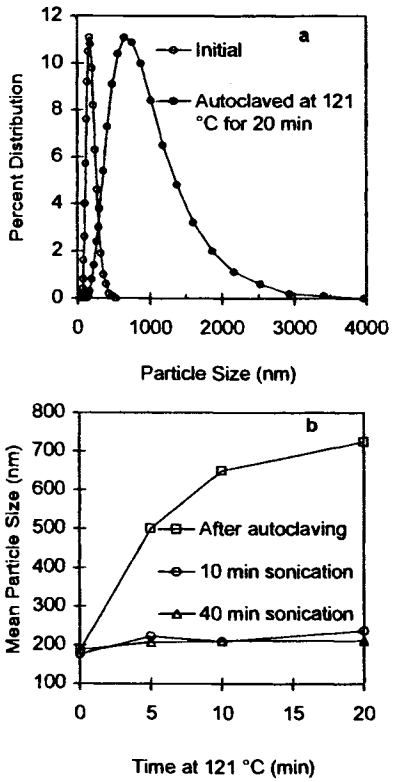

Fig. 1. (a) Particle size distributions of the ethyl diatrizoate nanocrystal suspension before and after steam sterilization. (b) Mean particle size of ethyl diatrizoate nanocrystal suspension as a function of heating time at 121°C in an autoclave. The autoclaved samples were then sonicated for 10 and 40 min.

became more viscous with some clumping and sedimentation after autoclaving.

Effect of Heating Time

A study was conducted to investigate the dependence of the particle size of the nanocrystal suspension on heating time at 121°C. The results plotted in Fig. 1b show that most of the increase in particle size occurred within the initial 5 min of steam sterilization.

Effect of Sonication

The nature of the particle size increase brought by steam sterilization was investigated by sonication. The samples from the heating-time-dependent study described in the preceding section were sonicated in a bath sonicator for 10 and 40 min. The results plotted in Fig. 1b show that most of the size increase was reverted by 10 min sonication. Therefore, the particle size increase in steam sterilization was likely a result of weak particle aggregation and not crystal growth.

Effect of Temperature

The heat-induced increase in particle size was further studied as a function of temperature. To achieve more precise and accurate temperature control, samples were heated in a water bath rather than an autoclave. The results plotted in Fig. 2a show that the mean particle size of the nanocrystal suspension remained essentially unchanged from room temperature to about 110°C, but increased sharply at higher temperatures. The onset temperature of the particle size increase agreed quite well with the cloud point of poloxamine 908 (110 ± 1°C).

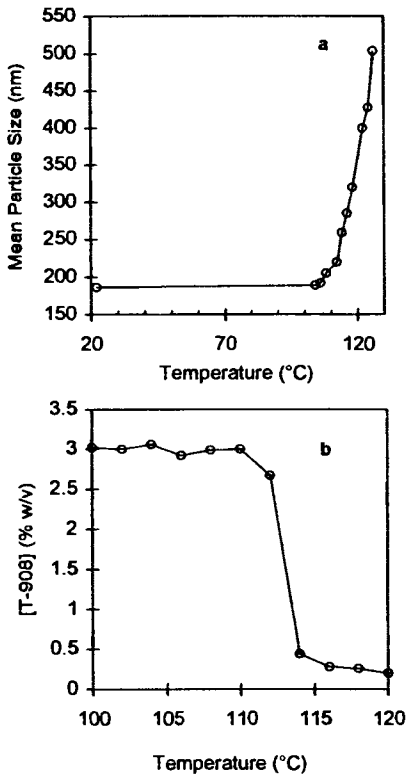

Fig. 2. (a) Temperature-dependence of the mean particle size of the nanosuspension. The samples were heated in a water bath with a dwell time of 5 min at each temperature. (b) Solubility of poloxamer 908 at temperatures near the cloud point.

Solubility of Poloxamine 908 as a Function of Temperature

The change in solubility of poloxamine 908 at temperatures near the cloud point of the surfactant was studied. Due to difficulties conducting centrifugation and filtration at such high temperatures, a simple gravity sedimentation method was used. Since the method did not completely separate the phase-separated surfactant, the results of this study should be taken as upper limits of the solubility. As seen in Fig. 2b, the concentration of poloxamine 908 at the top layer of the samples decreased sharply from 3% to less than 0.5% as the temperature exceeded the cloud point. These results indicate that the majority of the surfactant in the nanocrystalline suspension would phase-separate and precipitate at temperatures above the cloud point.

Effect of Surfactant Purity

A size exclusion HPLC analysis of poloxamine 908 showed an early-eluting parent peak and a late-eluting impurity peak (12). The effect of the impurity in poloxamine 908 on the heat stability of the nanocrystal suspension was examined. Three lots of poloxamine 908 with 88%, 92%, and 99% purity were prepared by diafiltration against water (12) and used to prepare ethyl diatrizoate nanocrystal suspensions. 10% PEG 400 was added to the suspensions as a heat stabilizer. The mean particle sizes before steam sterilization were nearly the same (230 ± 10 nm) for the three suspensions. After steam sterilization the mean particle sizes became 423, 314, and 260 nm (n = 3) for the suspensions prepared from 88, 92, and 99% purity poloxamine 908, respectively.

Effects of Cloud Point Boosters

The effects of the CPBs listed in the preceding paper (12) on the physical stability of the nanocrystal suspension under steam sterilization were studied.

Nonionic CPBs

Among the nonionic CPBs we identified, poly(ethylene glycols) (PEGs) were studied most extensively. As seen in Fig. 3, the suspension containing 10% PEG 400 showed only a small increase in particle size in steam sterilization, in great contrast to the control without PEG 400. The inset of Fig. 3 depicts the mean particle size before and after steam sterilization as a function of PEG 400 concentration. PEG 400 at about 3 to 4% concentration raised the cloud point of poloxamine 908 to above 121°C (12). It is in the same concentration range that the post-autoclaving mean particle size showed the most pronounced decrease.

Three other PEGs with higher molecular weights and nonionic CPBs in other structural classes were also tested for heat stabilization effect. Figure 4 depicts the mean particle size of the nanocrystalline suspension after steam sterilization versus the cloud point of poloxamine 908 in the presence of the CPBs.

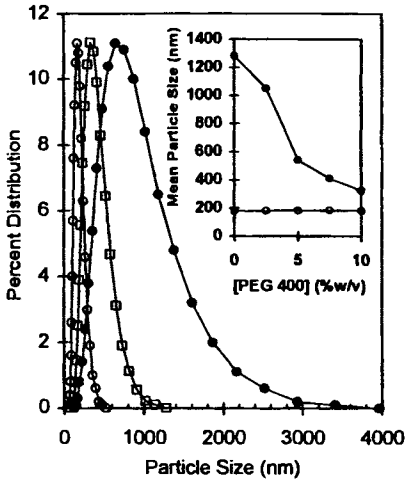

Fig. 3. Effect of PEG 400 on the particle size distribution of ethyl diatrizoate nanocrystal suspension in steam sterilization. The symbols used are: control sample before steam sterilization (○), control sample after steam sterilization (●), and a sample with 10% PEG 400 after steam sterilization (□). The inset shows the mean particle size as a function of PEG 400 concentration before (○) and after (●) steam sterilization.

It shows a decreasing mean particle size with increasing surfactant cloud point. Effective cloud point boosters, such as propylene glycol, ethanol, and 2-hydroxypropyl-β-cyclodextrin, were very effective heat stabilizers, and vice versa for ineffective cloud point boosters such as sugars and polyalcohols.

Ionic CPBs

Ionic CPBs from the three structural classes, ionic surfactants, charged phospholipids, and fatty acids, were tested for effect on the heat stability of the nanocrystal suspension. The ionic surfactant dioctyl sulfosuccinate (DOSS) and charged phospholipid dimyristoyl phosphatidyl glycerol (DMPG) were studied in greater detail and the particle size distributions before and after steam sterilization are plotted in Fig. 5. Both DOSS and DMPG were able to significantly minimize the increase in particle size incurred in steam sterilization. Also, the heat stabilization effect was observed in the millimolar concentration range (Fig. 5 inset) where DOSS and DMPG were noted as effective cloud point boosters (12).

Other ionic CPBs described in the preceding paper (12), including two anionic surfactants (SDS and SDBS), two cationic surfactants (CTAB and DTAB), four phospholipids (POPS, DPPA, DSPE, and DPPC), two bile salts (taurodeoxycholate and taurocholate), and seven fatty acids with chain lengths from C-6 to C-18, were tested at 0.2% (w/v) concentration for heat stabilization effect and the results are plotted in Fig. 4 together with the nonionic CPBs. As seen, the ionic CPBs followed the same trend of decreasing post-autoclaving mean particle size with increasing surfactant cloud point.

Effect of Other Pharmaceutical Excipients

Buffer Salts

The effect of pH buffers on the heat stability of the nanocrystal suspension was investigated. Various concentrations of acetate (pH 4), citrate (pH 5), phosphate (pH 7), or tromethamine (pH 8) buffer were added to the nanocrystalline suspension containing 10% PEG 400 as a heat stabilizer. Figure 6 shows the mean particle size of the samples after steam sterilization. The results indicate that the tromethamine and acetate buffers can be added up to 100 m*M* to the nanocrystal suspension without causing a significant increase of the mean particle size in steam sterilization. In contrast, the phosphate and citrate

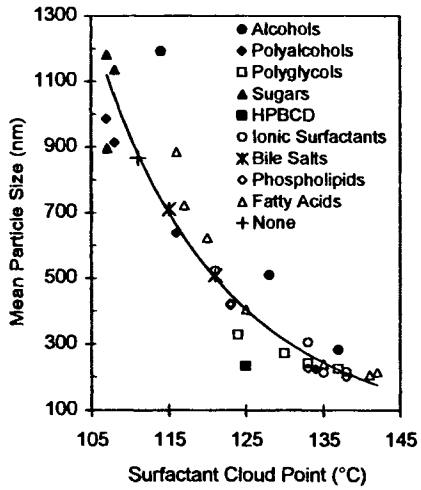

Fig. 4. Mean particle size of ethyl diatrizoate nanocrystal suspension after steam sterilization at 121°C versus cloud point of poloxamine 908. The nonionic CPBs were at 10% (w/v) concentration and the ionic ones at 0.2% (w/v), except the zwitterionic phospholipids DSPE and DPPC which were tested at 0.05% due to low solubilities. The data were grouped by the structural classes of the CPBs and depicted by different symbols. The cloud points of poloxamine 908 (1% w/v) in the presence of the CPBs were taken from the preceding paper (12).

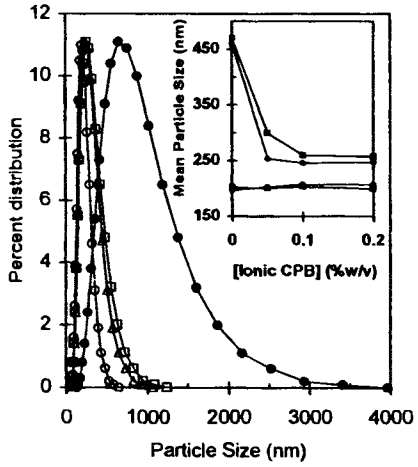

Fig. 5. Effects of ionic cloud point boosters on the particle size distribution of ethyl diatrizoate nanocrystal suspension in steam sterilization. The symbols used are: control (without additive) before steam sterilization (○) and after steam sterilization (●), and samples with 0.2% DMPG (△) and 0.2% DOSS (□) after steam sterilization. The inset shows the dependence of the mean particle size on the concentration of the ionic CPB. The symbols used in the inset are DMPG (○) and DOSS (□) before steam sterilization and DMPG (●) and DOSS (■) after steam sterilization.

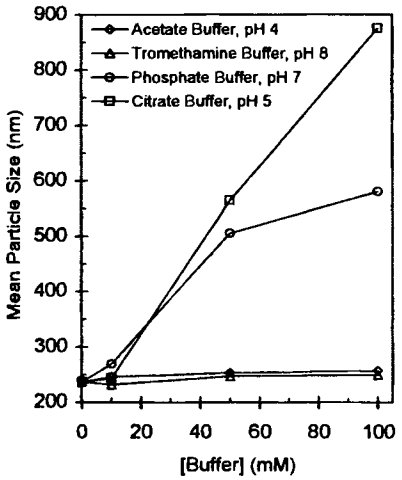

Fig. 6. Effects of four pH buffers on the heat stability of the ethyl diatrizoate nanocrystal suspension in steam sterilization. All samples contained 10% PEG 400 as a heat stabilizer. The salts used in these buffers are described in Materials and Methods.

buffers at 50 and 100 m*M* caused substantial increases in the post-autoclaving mean particle size.

Tonicity Adjustment Agents

Four frequently used tonicity adjustment agents were tested for effect on the heat stability of the ethyl diatrizoate nanocrystal suspension. In this study, 0.2% (w/v) DOSS was added to all suspensions as a heat stabilizer. After steam sterilization, the nanocrystalline suspensions containing 5% dextrose, mannitol, or sorbitol as tonicity agent showed essentially the same particle size distribution as the control without the tonicity adjustment agent. In contrast, the nanocrystalline suspension with 0.9% NaCl added as tonicity adjustment agent showed a mean particle size in the micron range.

Zeta Potentials of Nanocrystal Suspensions

The zeta potentials of ethyl diatrizoate nanocrystalline suspensions containing various CPBs were determined and are listed in Table I. The ethyl diatrizoate nanocrystals without any additive showed a slightly negative zeta potential of -3.4 ± 0.2 mV. The zeta potential was unaffected by 10% PEG 400 but became more negative with the addition of anionic CPBs and turned slightly positive with the addition of the cationic cloud point booster CTAB.

Table I. Effects of Ionic and Nonionic CPBs on the Zeta Potential of the Ethyl Diatrizoate Nanocrystals

Additive and concentration	Zeta potential (mV)[a]
None	-3.4 ± 0.2
PEG 400, 10%	-3.2 ± 0.3
DOSS, 0.2%	-7.2 ± 0.6
SDS, 0.2%	-5.9 ± 0.8
POPS, 0.2%	-6.9 ± 0.4
DPPA, 0.2%	-10.6 ± 0.4
CTAB, 0.2%	$+2.2 \pm 0.3$

[a] All samples were autoclaved at 121°C for 20 min except the control (without any additive) which was not autoclaved due to poor heat stability. Samples were prepared in triplicate and the averages and standard deviations are listed.

DISCUSSION

The objectives of the studies described here were to understand the effect of different formulation variables on the physical stability of the model nanocrystalline suspension under steam sterilization conditions and to achieve a formulation of the suspension that can withstand terminal steam sterilization without a significant increase in particle size. The drug ethyl diatrizoate showed a very low solubility in water under high temperatures and in the presence of the surfactant and CPBs tested. Low drug solubilities, including under high temperatures, are necessary to avoid massive drug solubilization and recrystallization in the steam sterilization process. However, low equilibrium solubility in itself does not assure against recrystallization as pointed out later in the discussion. Crystal polymorphism apparently plays a key role. The lack of crystal growth of the ethyl diatrizoate nanosuspension allowed us to focus on the aggregation of particles under high temperatures.

Our results demonstrated that the phase-separation of the stabilizing surfactant at its cloud point is the main cause of particle aggregation of ethyl diatrizoate nanocrystalline suspension in steam sterilization. Both the ionic and nonionic CPBs described in the preceding paper (12) were effective in maintaining the physical stability of the ethyl diatrizoate nanocrystal suspension in steam sterilization. The effective concentrations of the CPBs also agreed well with the concentrations needed to raise the cloud point above the steam sterilization temperature, further substantiating the causal relationship between the surfactant phase separation and the particle aggregation.

To understand the mechanism of aggregation of nanocrystals under steam sterilization conditions, we measured the solubility of poloxamine 908 as a function of temperature. The results in Fig. 2b show that majority of the surfactant in the nanocrystalline suspension would coalesce and precipitate out of solution at temperatures above the cloud point. In preparing the nanocrystalline suspension by milling, we noticed that a minimum concentration of poloxamine 908 of about 2–2.5% was needed to reach the small particle size. Thus, at temperatures above the cloud point, the amount of surfactant remaining in solution or bound to the particle surface is likely to be too low to provide adequate steric stabilization, and this could be the main cause of the heat-induced aggregation.

Although the ionic and nonionic CPBs are both effective heat stabilizers, they impart different electrostatic charges to the nanocrystals which merits consideration in formulation development. As shown in Table I, ethyl diatrizoate nanocrystals have a zeta potential of -3.4 ± 0.2 mV. This rather weak zeta potential reflects the nonionic nature of the drug and the charge-screening effect of the polymeric surfactant poloxamine 908 (5). The zeta potential changed with the charge of the ionic CPBs added, suggesting that the latter did adsorb onto the nanocrystals. The differences were relatively small and not likely to affect the physical stability of the suspension. However, the zeta potential is also known to affect the biodistribution and clearance of nanoparticles administered intravenously (5). Thus, nanocrystalline suspensions with different CPBs could

have quite different biopharmaceutical properties on intravenous administration.

To achieve a nanocrystalline suspension formulation stable to steam sterilization, the excipients need to be selected with consideration for effect on the surfactant cloud point. Thus, for tonicity adjustment, the sugars and polyalcohols, known to exert little effects on the surfactant cloud point (12), are preferred over sodium chloride. Likewise, for pH buffering, the trometha-mine and acetate buffers, known to suppress the cloud point much less (12), are favored over the phosphate and citrate buffers.

The heat stability of the ethyl diatrizoate nanocrystal suspension improved with increasing purity of poloxamine 908 which cannot be explained in terms of an increased surfactant cloud point (12). Size exclusion HPLC analysis showed that the impurities in poloxamine 908 are fragments of the parent compound with less than half the molecular weight. The impurities could compete with poloxamine 908 in coating the nanocrystal surface but, due to their lower molecular weights, provide weaker steric stabilization and poor heat stability.

It should be emphasized that the cloud point and surfactant purity are only part of the parameters affecting the physical stability of nanocrystal suspensions under autoclaving conditions. We have studied the heat stability of nanocrystal suspensions of several analogs of ethyl diatrizoate with comparable low solubilities. Despite the low solubilities, the suspensions of certain analogs showed polymorph changes and the formation of very large crystals in steam sterilization. The suspensions of some other analogs showed particle aggregation in steam sterilization despite the absence of polymorph change and the presence of adequate amounts of CPB. Apparently, the drug polymorphism and the binding energetics of the surfactant to the crystal surface are also important factors in determining the heat stability of the suspension. In our experience, elevation of the cloud point of the surfactant above the steam sterilization temperature is a necessary but not always sufficient condition to maintain physical stability in steam sterilization.

In summary, the physical stability of the ethyl diatrizoate nanocrystalline suspension under steam sterilization conditions was dependent on the cloud point of the stabilizing surfactant. By using cloud point boosters and being cognizant of the effect of excipients on the cloud point, the suspension was formulated to withstand terminal steam sterilization with little change in its particle size distribution.

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