

Preparation of All-Trans Retinoic Acid Nanosuspensions Using a Modified Precipitation Method

X. Zhang, Q. Xia and N. Gu

State Key Laboratory of Bioelectronics, Jiangsu Key Laboratory of Biomaterials and Devices, Southeast University, Nanjing, China

ABSTRACT All-Trans Retinoic Acid (ATRA) nanosuspensions were prepared with a modified precipitation method. The ATRA solution in acetone was injected into pure water by an air compressor under the action of ultrasonication. Photon correlation spectroscopy results showed that the mean particle size of ATRA nanoparticles in nanosuspensions reduced from 337 nm to 155 nm as the injection velocity increased and the polydispersity index was 0.45–0.50. The morphology of ATRA nanoparticles varied with the different concentration of ATRA solution in acetone. ATRA nanoparticles showed an amorphous state and stable in 6 months. It could be concluded that this modified precipitation method could produce stable and controllable ATRA nanosuspension to a certain extent, thus benefit for higher saturation solubility.

KEYWORDS All-trans retinoic acid (ATRA), Precipitation, Crystallization, Nanosuspension, Saturation solubility

INTRODUCTION

An increasing number of newly developed drugs are poorly soluble in water. Generally, poor solubility results in low bioavailability and/or erratic absorption (Müller, 2001). All-trans retinoic acid (ATRA) is a promising anticancer agent in the chemoprevention and treatment of cancer (Orlandi, 2003). ATRA is known to have poor solubility in water and be unstable under certain circumstances (Barua, 2004). While exposed to light, heat and oxidants, ATRA will occur trans-cis isomerization, which leads to the great decrease of effect of medicine. In addition, a low dissolution velocity limits sufficiently high blood levels as a result of its low saturation solubility (Brisaert, 2000). In order to increase the saturation solubility, an alternative and promising approach is the form of nanosuspension.

Generally, there are three ways for nanosuspension preparation: precipitation produced by Sucker et al in 1980s (List & Sucker, 1995), pearl milling developed by Liversidge et al. in 1990 (NanoCrystals®), high-pressure homogenization

Address correspondence to N. Gu, Southeast University, Nanjing, China; E-mail: guning@seu.edu.cn

developed by Müller et al. in 1994 (DissoCubes®) (2001). According to Müller's review (2001), the essential characterization parameters for nanosuspensions are size and size distribution, particle charge (zeta potential), crystalline status, as well as dissolution velocity and saturation solubility.

Pearl milling leads to broad size distribution and might cause heat-induced isomerization of ATRA. Due to the high pressure, high temperature, and high energy, isomerization might also occur in high-pressure homogenization. Precipitation was performed by dissolving the drug in an organic solvent and adding this solution to water while stirring vigorously (List & Sucker, 1995). A main advantage of precipitation is the avoidance of high temperature, which is quite suitable for the preparation of some temperature-sensitive drugs. In this study, ATRA suspension was prepared by a modified precipitation method, which introduced ultrasonication into precipitation process. The influence of several preparation parameters was studied, including injection velocity and concentration of ATRA solution in acetone. In addition, some characteristics were also examined, including particle size distribution, zeta potential, morphology, and crystalline status.

MATERIALS AND METHODS

Materials

ATRA was purchased from Shanghai Chemical Reagent Corporation, China. Acetone was bought from Nanjing No.1 Chemical Reagent Factory, China. MiliQ water was used in all experiments.

Equipment for Preparation

Figure 1 showed the schematic diagram of the experimental equipment for the improvement of precipitation method. The handspike of a syringe was removed and the rear of the syringe was connected with a silent air compressor (A-8067, Jiaying Minjian Machine Shop) through an air valve. The diameter of its pinhead was 100 μm , which was very important due to its proper resistance to acetone. A wider pinhead would allow acetone to rush into water too quickly and a narrower pinhead dramatically restrained the injection velocity. Therefore, the pinhead with the diameter of 100 μm was the most suitable for this equipment.

X. Zhang et al.

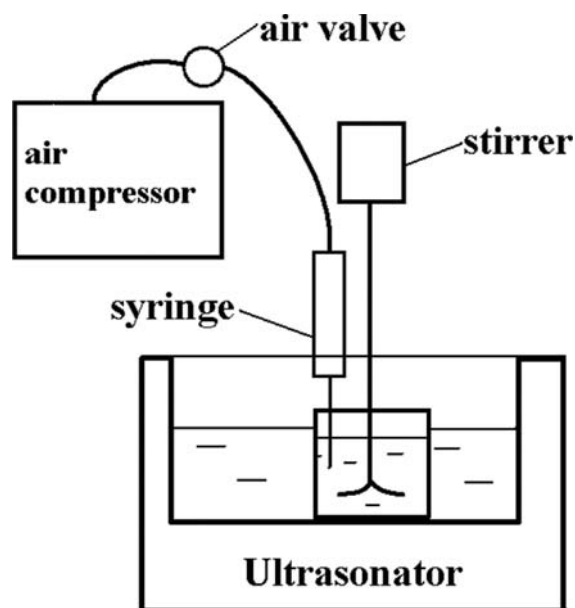


FIGURE 1 Schematic Diagram of the Equipment for the Preparation of Nanosuspension.

In order to estimate the injection velocity, 10 mL (10000 μL) acetone was injected into water with different air pressures. The injection time was taken down. The injection velocity $v = V/t$ (V was the injected volume, here $V = 10000 \mu\text{L}$. And t was the injection time) could be calculated. The relation between air pressure and injection velocity was roughly linear within the air pressure range of 0–0.6 MPa. The linear equation was $v = -0.96586 + 134.47 \cdot P$ (v was the injection velocity and P was the air pressure). The linear relation was benefit for the adjusting of injection velocity.

Preparation of ATRA Nanosuspensions

Ten milligrams of ATRA were dissolved in a certain amount of acetone to obtain a primrose ATRA solution. The silent air compressor pulsed ATRA solution into 100 mL pure water while stirring or ultrasonication using an EQ-250E Medical Ultrasonicator (Kunshan Ultrasonic Instrument Corporation, China). The injection velocity was adjusted by changing air valve pressure from 0 Mpa to 0.6 MPa. The velocity was calculated according to the total acetone volume and the injection time. After injection, ultrasonication or stirring was continued for 30 min for the sake of uniformity. At last, the suspension was vacuumized by water-circulation multifunction vacuum pump (SHB-□,

Zhengzhou Great Wall Scientific Industry and Trade Corporation, China) for 2 h to remove the acetone. All the experiments were strictly operated in the dark due to the photo-instability of ATRA.

Particle Size and Zeta Potential Measurements

The mean size of ATRA nanoparticles (ATRA-NPs) in nanosuspension was determined by photon correlation spectroscopy (PCS) on a Submicron Particle Size Analyzer (N4 plus, Beckman-Coulter, USA) at a scattering angle of 90°. It gives information about the mean size of the bulk population and the polydispersity index (P.I.). Each experimental value was gained by PCS results from three independent experiments, each performed in triplicate. The measurement temperature was chosen as 20°C.

Zeta potential measurements were performed on a Delsa 440SX (Beckmann-Coulter, USA). All measurements were carried out at 25°C.

Optical Microscopy (OM)

The commercial ATRA powder was spread on a clean slide. Then the powder was observed through a Nikon ECLIPSE TS100 optical microscopy.

Tunneling Electron Microscopy (TEM)

The samples were diluted for 10 times and negative stained before TEM measurements due to the poor conductivity of organic samples. A copper grid was placed on the wax plate. Then a drop of diluted ATRA nanosuspension prepared for TEM measurements was added on the surface of copper grid. And a drop of 2% phosphotungstic acid solution was also added on the wax plate. After drying in the air, the copper grid was placed on the top surface of the spreading phosphotungstic acid dispersion to dye for 5 min before TEM measurements. The sample was transferred into a TEM (JEM-2000EX, Japan Electron Optics Laboratory Corporation, Japan) operated at 120 kV.

X-Ray Diffraction (XRD) Measurements

The ATRA-NPs in 100 mL of nanosuspension were gathered by a Sorvall Biofuge Stratos freezing centrifuge (Kendro) at the temperature of 4°C and the rotate

speed of 15000 r/min for 60 min. The upper clear water was removed. The remained nanosuspension, in a step by step manner, was dropped onto a silicon flake and dried in a ZK-82BB electrical heated vacuum desiccator at the temperature of 40 °C. The process was repeated several times to yield a thick film of ATRA-NPs before XRD measurements.

The X-ray diffraction (XRD) analysis of the resulting precipitates was carried out on a D/MAX-RA X-ray diffractometer using Cu (40 kV, 30 mA) radiation.

Differential Scanning Calorimeter (DSC) Examination

The ATRA-NPs in nanosuspension were gathered by a Sorvall Biofuge Stratos freezing centrifuge (Kendro) at the temperature of 4°C and the rotate speed of 15000 r/min for 60 min. The upper clear water was removed and the remained nanosuspension was dried in a ZK-82BB electrical heated vacuum desiccator at the temperature of 40°C to yield ATRA-NPs before DSC determination.

The crystallization structure was assessed by a Q10 DSC (TA Instrument, USA). The scanning velocity was 10°C/min and the scanning temperature range was 5–300°C.

RESULTS AND DISCUSSION

Influence of Ultrasonication

Acetone could miscible with water. It quickly goes up to the water surface when it is injected into water because it's lighter than water. To obtain a homogeneous mixture, some outside forces were introduced. Sucker and coworkers used vigorous stirring during the precipitation preparation (1995). In our experiment, ultrasonication was adopted to enhance the diffusion rate of acetone. In order to avoid the crystallization on surface, acetone should quickly diffuse. The results of two preparation conditions were compared, which were stirring only and stirring associated with ultrasonication. In the case of stirring only, some ATRA particles appeared on the water surface. While in the latter case, a transparent and clear buff suspension was obtained, proving the acetone could diffuse into water very quickly and completely during the process of going up under ultrasonication.

The PCS results of ATRA-NPs in nanosuspensions under two preparation conditions were different. When the stirring was performed, the mean particle size was 176 nm and the P.I. was 0.488, while in case of stirring associated with ultrasonication, the mean particle size was 155 nm and P.I. was 0.482. The results showed ultrasonication also slightly reduced particle size. The similar P.I. results meant similar size distribution. According to Mersmann's review (1999), nanoparticles can be obtained by applying very high nucleation rates, which require very strong supersaturations. Ultrasonication accelerated the diffusion of acetone into water, leading to stronger supersaturations. Therefore, ultrasonication tends to reduce the mean particle size.

So the following experiments were all performed under ultrasonication associated with stirring.

Influence of Injection Velocity

The acetone solution of ATRA was driven into pure water by air pressure. The air pressure given by the silent air compressor varied from 0 MPa to 0.6 MPa, corresponding to various injection velocities. Figure 2 showed that the mean particle size decreased from 337 nm to 155 nm, when the velocity increased from 8 $\mu\text{l/s}$ to 78 $\mu\text{l/s}$. ATRA solution formed drops when it came out of the pinhead at lower injection velocity (8 $\mu\text{l/s}$) and flows at higher velocity (25 $\mu\text{l/s}$). The drops went up to the surface slowly, while the flows quickly rushed into water. So the flows tended to diffuse quicker, leading to the formation of much smaller

nanoparticles. On the other hand, the quicker the flow was, the better the diffusion was. Therefore, quicker flows yielded smaller nanoparticles.

Smaller nanoparticles are significant in the respect of saturation solubility. The saturation solubility is a function of the particle size (Müller et al., 2001). This size-dependency comes only into effect for particles having a size below approximately 1 μm (Müller, Benita, et al., 2001). For a fast dissolution of the drug in the blood, small nanoparticles with the size of approximately 100–200 nm are preferred (Müller et al., 2001). When the high-pressure homogenization was performed, the mean particle size of paclitaxel was 330 nm (Müller et al., 2001), for buparvaquone was 600 nm, and for the drug RMKP22 was 502 nm. In term of our modified precipitation preparation, when the injection velocity was high enough to form flows, the mean particle size was within the range of 100–200 nm. Therefore, in order to achieve different dissolution rate, the velocity of injection could be adjusted to gain nanosuspensions with various mean particle sizes within a certain range.

Influence of Concentration of ATRA Solution in Acetone

ATRA is soluble in acetone and the solubility was about 5 mg/mL. Ten milligrams ATRA were dissolved into 10 mL acetone (solution a), 5 mL acetone (solution b), and 2.5 mL acetone (solution c), respectively. Therefore, the concentration was 1 mg/mL (solution a), 2 mg/mL (solution b) and 4 mg/mL (solution c). The three solutions were injected into 100 mL pure water at the speed of 78 $\mu\text{l/s}$, respectively. The PCS results were showed in Fig. 3. The mean particle size reduced dramatically with the increase of the concentration of ATRA solution while P.I. varied slightly. Perhaps the reason for this is that a higher concentration meant a higher supersaturation, while the forming of nanoparticles requires very strong supersaturations. Therefore, a higher initial concentration led to smaller nanoparticles.

Morphology of ATRA-NPs

Figure 4 showed the TEM images of ATRA-NPs in ATRA nanosuspensions. It could be seen that the morphology of ATRA-NPs were different with the different concentration of ATRA solution. When the

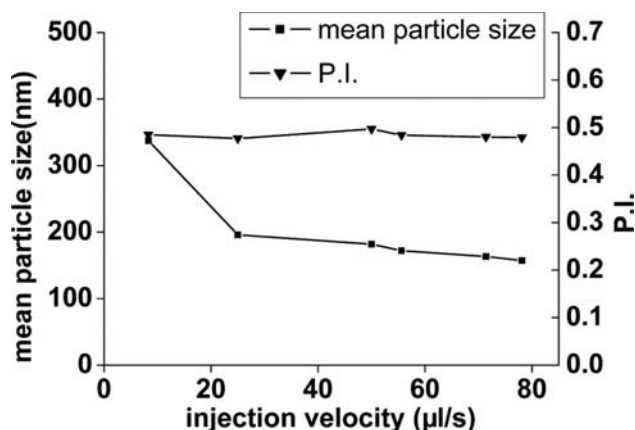


FIGURE 2 Relation Between Injection Velocity and Particle Distribution (2.5 mL 4 mg/mL Acetone Solution of ATRA Was Injected into 100 mL Pure Water).

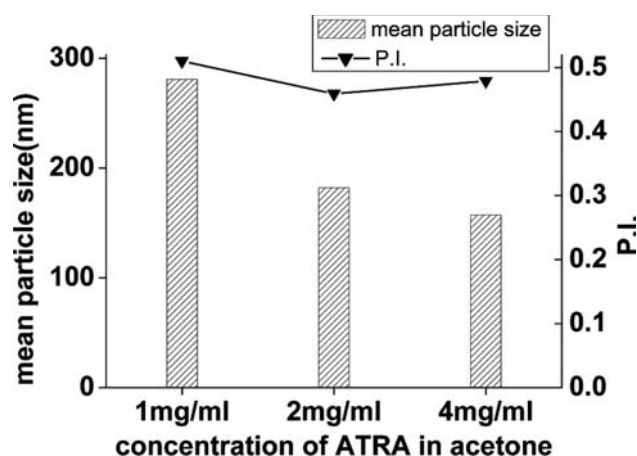


FIGURE 3 Relation Between the Concentration of ATRA Solution and Particle Distribution.

concentration was 1 mg/mL, most of the particles were rhombus, while only very few particles were claviform or spherical. In case of 2 mg/mL, the proportion of claviform particles increased. Furthermore, when the concentration reached 4 mg/mL, the particles were primarily spherical with a diameter around 150 nm, which was consistent with PCS results.

The difference of initial ATRA solution resulted in a difference of supersaturation. In comparison with the TEM image, the image of initial ATRA powder taken by OM (Fig. 5) showed a perfect claviform crystal with the length of about 30 μm . It could be concluded that the morphology of particle could be influenced by the supersaturation. According to Mersmann's review (1999), the high initial concentration results high supersaturation, leading to high rates of

primary nucleation. Perhaps the high supersaturation led fast nucleation, thus influencing crystal morphology.

Crystalline Status of ATRA-NPs

Figure 6 reported the X-ray diffraction (XRD) spectra of the samples. The XRD spectra of commercial ATRA powder showed many strong diffraction peaks, which conformed to PCDFD file. In contrast, that of ATRA-NPs showed almost a smooth line. So it could be suggested that precipitation process promoted the amorphous state.

The crystalline structure of the ATRA-NPs can also be assessed by differential scanning calorimetry (DSC) (Müller et al., 2001). This is especially important when a drug exists in different polymorphic forms. An outstanding feature of nanosuspensions is the increase in saturation solubility and consequently an increase in the dissolution velocity. This increase is promoted not only by the enlargement of the surface area but also by the crystalline structure, which means solubility is best for the polymorphic modification that is characterized by a highest energy and lowest melting point (Müller et al., 2001).

The dried ATRA-NPs were determined compared with commercial ATRA powder. Figure 7 showed that there were two sharp peaks at the DSC curve of ATRA powder. One major sharp peak was 183.61°C, which corresponded to the melting point. The other endothermic peak of 145.79°C before melting would be due to the monoclinic–triclinic transition. In contrast, there was only one tempered peak at 172.61°C at DSC

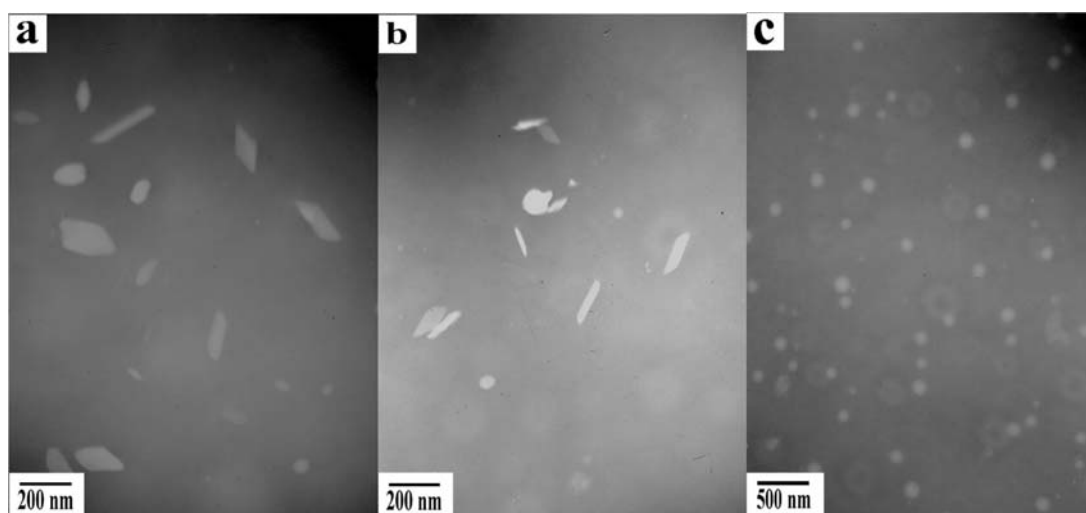


FIGURE 4 TEM Image of ATRA-NPs (the Concentration of ATRA Solution is 1 mg/mL (a), 2 mg/mL (b), 4 mg/mL (c), Respectively).

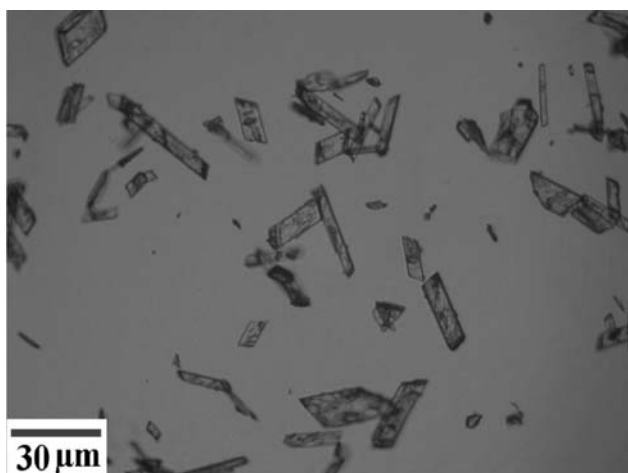


FIGURE 5 Optical Image of Initial ATRA Powder Observed by OM ($\times 100$).

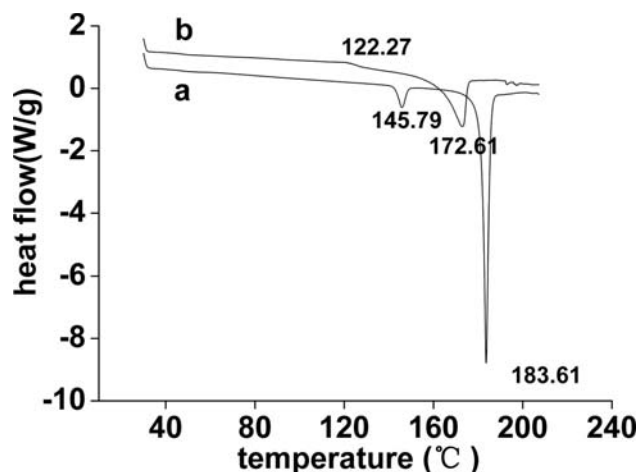


FIGURE 7 DSC Thermal Trace of Commercial ATRA Powder (a), ATRA-NPs (b).

Furthermore, the cool process of precipitation was more suitable than high-pressure homogenization or milling.

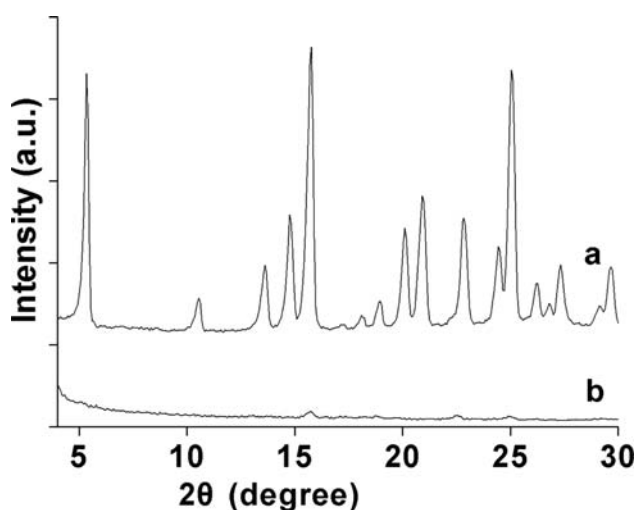


FIGURE 6 XRD Pattern of Commercial ATRA Powder (a) ATRA-NPs (b).

curve of ATRA-NPs, which was lower than 183.61°C by 11°C and the melting range was wider. Besides, the curve declined slowly from the point of 122.27°C, which was dramatically different from the peak of 145.79°C of ATRA powder. This supported the view that the crystalline structure of ATRA changed a lot after the process of precipitation. Müller's review mentioned the application of high pressures during the production of nanosuspensions was found to promote the amorphous state (Müller et al., 2001). And for NanoCrystals® prepared by a milling process, a completely crystalline structure was reported (Liversidge et al., 1992; Liversidge & Cundy 1995). The DSC results of our experiments were a proof of amorphous state.

Stability

Zeta potential is relative to the double electric layer on the surface of colloidal particles. Generally, zeta potential could be used to predict the stability of colloidal particles in aqueous phase. The zeta potential range of -20 to -11 mV corresponds to the threshold of agglomeration in dispersions, according to the definition of Riddick (Liversidge & Cundy, 1995). The higher value of zeta potential means the higher stability with the same other conditions. As described in a review by R. H. Müller (2001), zeta potential value of ± 30 mV is the minimum for the physically stable nanosuspension solely stabilized by electrostatic repulsion and the corresponding value is about ± 20 mV in the case of a combined electrostatic and steric stabilization. In our experiment, the zeta potential of ATRA-NPs in nanosuspensions was -37.9 ± 2.0 mV. This was high enough for a sufficient electrostatic stabilization, which was favorable for long-term stability.

After 6 months of storage at 4°C, the ATRA nanosuspensions were still homogeneous and clear. The particle distribution and crystalline status of ATRA nanosuspensions were determined to access its stability. The mean particle size was 171 nm and P.I. was 0.628, slightly higher than the data of initial nanosuspension (155 nm and 0.482), demonstrating the ATRA-NPs

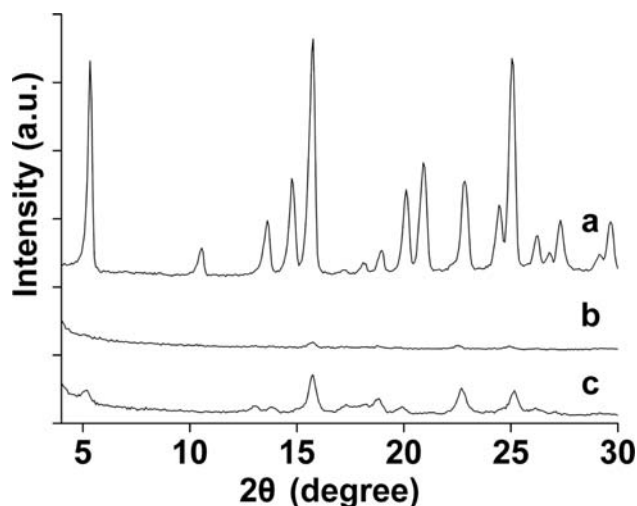


FIGURE 8 XRD Pattern of Commercial ATRA Powder (a), Initial ATRA-NPs (b), ATRA-NPs Stored for 6 Months (c).

were stable in 6 months. Figure 8 showed the XRD spectra of the stored nanosuspension. After 6 months of storage, the weak peaks of initial ATRA-NPs grew stronger. However, they were still much weaker than commercial ATRA powder. Therefore, most of ATRA-NPs remained amorphous status after 6 months.

CONCLUSIONS

ATRA nanosuspensions were prepared by means of a modified precipitation method. An air-pressure-driven equipment was assembled to inject acetone solution of ATRA into pure water. This cool process could protect ATRA against isomerization and the injection velocity could be controlled. Meanwhile, outside forces such as ultrasonication and stirring were performed to speed up the diffusion of acetone into water. Ultrasonication associated with stirring was relatively better condition for preparation. When the injection velocity increased from 8 $\mu\text{l/s}$ to 78 $\mu\text{l/s}$, the mean particle size of ATRA-NPs reduced from 337 nm to 155 nm and the P.I. remained almost the same, which was 0.45–0.50. As the initial concentration of acetone solution of ATRA increased, the mean particle size decreased and the morphology of ATRA-NPs varied from rhombus, claviform to spherical according to TEM image. Compared with the perfect crystallization of initial ATRA powder, the ATRA-NPs showed an amorphous status according to XRD spectra and DSC results. Both the small particle size and the amorphous status were benefit for higher saturation solubility. In terms of long-term stability, the zeta potential

was -37.9 ± 2.0 mV, which was high enough for a sufficient electrostatic stabilization. After 6 months of storage, the nanosuspensions remained homogeneous while the mean particle size and P.I. slightly grew.

In conclusion, this modified precipitation method could produce stable and controllable ATRA nanosuspensions to a certain extent. This is valuable for the future application of ATRA.

ACKNOWLEDGEMENTS

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REFERENCES

- Barua, A. B., McGowan, S. E., Ivanoff, K. D., Goswami, B. C., & Olson, J. A. (2004). Elevation of retinyl ester level in the lungs of rats following repeated intraperitoneal injections of retinoic acid or retinoyl lucuronide. *Pulm. Pharmacol. Ther.* 17, 113–119.
- Berbenni, V., Marini, A., Bruni, G., & Cardini, A. (2001). Thermoanalytical and spectroscopic characterisation of solid-state retinoic acid. *Int. J. Pharm.* 221, 123–141.
- Brisaert, M., Gabriels, M., & Plaizier-Vercammen, J. (2000). Investigation of the chemical stability of an erythromycin-tretinoin lotion by the use of an optimization system. *Int. J. Pharm.* 197, 153–160.
- Grau, M. J., Kayser, O., & Müller, R. H. (2002). Nanosuspensions of poorly soluble drugs — reproducibility of small scale production. *Int. J. Pharm.* 196, 155–157.
- List, M., & Sucker, H. (1995). Hydrosols of pharmacologically active agents and their pharmaceutical compositions comprising them. U.S. Patent 5,389,382, Feb.14.
- Liversidge, G. C., Cundy, K. C. (1995). Particle size reduction for improvement of oral bioavailability of hydrophobic drugs. I. Absolute oral bioavailability of nanocrystalline danazole in beagle dogs. *Int. J. Pharm.* 127, 91–97.
- Liversidge, G. G., Cundy, K. C., Bishop, J., & Czekai, D. (1992). Surface modified drug nanoparticles. U.S. Patent 5,145,684, Sep.8.
- Mersmann, A. (1999). Crystallization and precipitation. *Chem. Eng. Process.* 38, 345–353.
- Müller, R. H., Benita, S., & Böhm, B. (2000). Emulsions and Nanosuspensions for the Formulation of Poorly Soluble Drugs. *Int. J. Pharm.* 212, 143–144.
- Müller, R. H., & Jacobs, C. (2002). Buparvaquone mucoadhesive nanosuspension: preparation, optimisation and long-term stability. *Int. J. Pharm.* 237, 151–161.
- Müller, R. H., Jacobs, C. O., & Kayser (2001). Nanosuspensions as particulate drug formulations in therapy rationale for development and what we can expect for the Future. *Adv. Drug. Deliver. Rev.* 47, 3–19.
- Orlandi, M., Mantovani, B., Ammar, K., Avitabile, E., Dal Monte, P., & Bartolini, G. (2003). Retinoids and cancer: antitumoral effects of ATRA, 9-cis RA and the new retinoid IIF on the HL-60 leukemic cell line. *Med. Princ. Pract.* 12, 164–169.
- Riddick, T. M. (1968). *Zeta-Meter Manual*. New York: Livingston Publishing Company.

