Formulation Development of Ursolic Acid Nanosuspensions with Different Particle Sizes and In-vitro Anti-cancer Activity Evaluations

by

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UNIVERSIDADE DE MACAU
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碩士學位論文

不同粒徑熊果酸納米混懸液的製備、表徵和體外抗腫瘤活性評價

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澳門大學

中文摘要

不同粒徑熊果酸納米混懸液的製備、表徵和體外抗腫瘤活性評價

宋 菊

指導老師：鄭 穎 副教授

熊果酸（Ursolic acid, UA）是在自然界廣泛分于布陸英、女貞子等植物中的一種五環三萜類化合物，具有抗腫瘤、抗氧化、抗炎、保肝等多種藥理作用。但由於水溶性差、溶出速率慢，生物利用度低，因而臨床運用受到限制。文獻報導曾製備過熊果酸滴丸、片劑、固體分散體等，不過迄今為止，市場上尚未有熊果酸單體藥品上市。

納米技術將藥物粒徑減小到納米級別、增大藥物的比表面積，從而提高了相對飽和溶解度，加快溶出速率，最終達到提高生物利用度的目的，因此將納米化技術應用難溶藥的開發有重要的意義。納米混懸劑是納米製劑中常見的一種形式，目前用於製備納米混懸液的技術有反溶劑沉澱、球磨法和高壓均質，雖然可結合其中兩種方法以製備粒徑小於 100 nm 的納米混懸液，但是操作複雜，耗時較長，所以急需研發一種製備小粒徑納米混懸液的新技術。

本實驗以 UA 作為模型藥物，採用反溶劑沉澱法製備 UA 納米混懸液 A，并與同濃度下 UA 的 DMSO 溶液進行體外抗腫瘤活性比較，考察藥物納米化后體外抗腫瘤活性的變化。通過單因素考察法考察了不同穩定劑、有機相中藥物濃度以及有機相和水相的體積比對粒徑大小的影響，結果得到 UA 納米混懸液 A 的製備工藝如下：稱取一定量的 UA 溶解在無水乙醇中，得到 3 mg/mL 的熊果酸乙醇溶液作為有機相，之後保持攪拌速度為 1000 rpm，用注射器將 2 mL 有機相

注入到 20 mL Milli-Q 水中，攪拌 5 min。制得的 UA 納米混懸液 A 的平均粒徑為（188.0 ± 4.4）nm，多分散係數為 0.154 ± 0.022，zeta 電位為（-25.0 ± 5.9）mV；在 4 ℃ 條件下放置 7 周後，平均粒徑變化為（199.5 ± 9.2）nm，PdI 值為 0.169 ± 0.059，說明該納米製劑物理穩定性良好。透射電鏡下觀察到納米混懸液中的熊果酸為球狀；差示掃描量熱法結果顯示，與原料藥相比，UA 在納米化後熔點未發生變化，但熔融熱焓值降低到原料藥的 84%，提示結晶度發生下降。溶出實驗表明 UA 納米混懸液中的藥物可於 120 min 內在溶出介質中釋放完全，而相同條件下原料藥只能溶出 50%，說明藥物經納米化后，溶出速率加快。制得的納米混懸劑與同濃度下 UA 的 DMSO 溶液進行體外 MTT 試驗，結果顯示 UA 對 MCF-7 細胞的增長抑制作用依賴於藥物濃度和孵育時間，而且 UA 納米混懸液 A 的細胞毒性強於 UA 溶液，UA 溶液的 IC_{50} 值是納米混懸液 A 的 2 倍；細胞攝取結果顯示在相同濃度、相同孵育時間下 MCF-7 細胞對 UA 溶液的攝取量是對 UA 納米混懸液 A 攝取量的 2 倍；細胞週期分析結果與 MTT 結果相印證，在相同濃度下（5 μM），與 UA 溶液相比較（14.05% - 14.59%）UA 納米混懸液 A 能誘導更多細胞阻滯在 G_2/M 期（25.44% - 29.36%）。以上實驗結果說明 UA 經納米化后，溶出速率加快，抗腫瘤活性增強。

多通道快速混合儀是 2007 年報導的用於製備載藥聚合物納米粒的新方法，可製備粒徑遠小於 100 nm 的顆粒，但目前很少有人將其應用於製藥業。本實驗使用多通道快速混合儀製備 UA 納米混懸液，考察不同粒徑納米混懸液體外抗腫瘤活性的差異。單因素考察發現注射速度、藥物濃度和有機相與水相體積比均會影響 UA 納米混懸液的粒徑分佈，從而可以通過對上述參數的調節制得不同粒徑的納米混懸液；選取其中兩種粒徑的工藝進行重複實驗，固定 UA 在有機相中的濃度為 3 mg/mL，0.05% PVP K90 和 0.05% SDS 組成水相；A 泵連接的管道中一條為有機相，一條為水相，注射體積為 1.6 mL；B 泵連接的兩條管道均為水相，注射體積為 8 mL；當 A 泵注射速率為 8 mL/min（B 泵注射速率相應為 40 mL/min）時，制得熊果酸納米混懸液 B，測得平均粒徑（101.2±3.53） nm，多分散係數為 0.205±0.012，zeta 電位（-9.79±0.79）mV；當 A 泵注射速率為 2 mL/min（B 泵注射速率相應為 10 mL/min）時，制得熊果酸納米混懸液 C，測得平均粒徑為（299.8±6.63）nm，多分散係數為 0.150±0.021，zeta 電位（-8.19±0.78）mV；透

射電鏡觀測到熊果酸在兩種粒徑納米混懸液中均呈球狀，分散均勻且無聚集。之後對兩種粒徑的製劑進行體外抗腫瘤活性評價：MTT 結果顯示隨著孵育時間的延長，UA 納米混懸液對 MCF-7 細胞增殖的抑制作用强於 UA 溶液，但兩個製劑組間無顯著差異；IN Cell 2000 觀察到經 UA 處理後 MCF-7 細胞的細胞核發生收縮和變形，線粒體數量減少，推測 UA 能誘導腫瘤細胞發生凋亡；流式細胞儀分析結果證明 UA 能使細胞阻滯在 G_2/M 期，並且能誘導細胞凋亡，其作用效果為：UA 納米混懸液 C > UA 納米混懸液 B > UA 溶液。實驗結果顯示多通道快速混合儀可以製備不同粒徑的納米混懸液，而且不同批次間差異小、重現性高，從而說明多通道快速混合是一種有效控制粒徑、重現性良好的製備納米混懸液的新技術。藥物製備成納米混懸液后，與其溶液相比較，能誘導更多腫瘤細胞發生凋亡，并且大粒徑納米混懸液的作用强於小粒徑製劑。

本研究表明，將 UA 納米化后有助於藥物的溶出，並且能增強體外抗腫瘤活性。而且與小粒徑的 UA 納米混懸液相比較，大粒徑的製劑能誘導更多腫瘤細胞發生凋亡。本實驗為 UA 靜脈注射液的研發奠定了良好的前期基礎。

關鍵字：熊果酸；納米混懸液；反溶劑沉澱；多通道快速混合；溶出度；細胞毒性；細胞攝取；細胞週期；細胞凋亡

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Abstract

Development of Ursolic Acid Nanosuspensions with Different Particle Sizes and In-vitro Anti-cancer Activity Evaluations

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Thesis Supervisor: Dr. Ying Zheng

Ursolic acid (UA), a natural pentacyclic triterpenoid, is widely existed in plants such as *Sambucus chinensis* (Lindl.) and *Ligustrum lucidum*. It has been reported that UA possesses a variety of pharmacological activities, including antitumor, anti-inflammatory, antioxidant, and hepatoprotective effect. Its poor aqueous solubility and dispersibility and dissolution rate result in poor bioavailability which limits its clinical application. Although several kinds of formulations have been developed, such as dropping pills, tablets and solid dispersion, there is no current commercial product of UA.

Nanosuspension is a new technology to increase the bioavailability of poorly-soluble drugs. Decreasing the particle size of drug from micrometer to nanometer and enlarging the surface area would increase the saturated solubilivity, dissolution rate, even bioavailability of drugs. So the nano-technology is widely applied to develop formulation of poorly soluble drugs. There are three methods to prepare nanosuspension: anti-precipitation, ball-milling and high-pressure homogenization. Nanosuspension with particle size smaller than 100 nm could be prepared by combining anti-precipitation with either ball-milling or high-pressure homogenization. However, the process is complex, costly and time-consuming. It is nessasary to develop a new method to prepare nanosuspension with particle size smaller than 100 nm.

In this study, UA was selected as a model drug to prepare UA nanosuspension by anti-precipitation method and evaluate the in-vitro anti-cancer activity of UA nanosuspension compared with UA solution. It was found that stabilizer type, drug concentration in organic phase and ratio of organic phase to aqueous solution influenced the mean particle size. Based on the investigation of single factor, UA nanosuspension A without stabiliser was prepared as follows: UA was dissolved in ethanol to obtain organic phase with the UA concentration at 3 mg/mL, 2 mL of the organic phase was injected into 20 mL of Milli-Q water at room temperature under stirring at 1000 rpm for 5 min. The particle size, polydispersion index (PdI) and zeta potential values of UA nanosuspension A was 188.0 ± 4.35 nm, 0.154 ± 0.022, and -25.0 ± 5.85 mV, respectively. After storage for 7 weeks at 4 ℃, the particle size was 199.5 ± 9.2 nm, and the PdI was 0.169 ± 0.059, which suggested the samples were physically stable. The morphology of the UA nanosuspension was studied by TEM and the UA nanosuspension were generally spherical in shape. Compared to raw material, the endothermic peak of the UA nanosuspension was similar with raw material, while the enthalpy value decreased to 84%, which indicated that UA was kept in a crystalline form in the nanosuspension with crystallinity decreased. The UA nanosuspension could be completely dissolved in 0.5% SDS solution within 120 min, while raw material just released 50% under same condition, which indicated that the dissolution rate was increased by nano-technology. Then the in-vitro anti-cancer activity of UA nanosuspension A was studied compared with UA solution. The MTT results showed the growth inhibition effect of UA on MCF-7 cells was in a concentration and time dependent manner, and the cytotoxicity of UA nanosuspension A was stronger than that of UA solution and the IC_{50} values of UA solution were twice higher than that of UA nanosuspension A. However, the cell uptake of UA solution was twice higher than UA nanosuspension A at the same concentration and same incubation time. The cell cycle analysis results were consistent with MTT results and UA nanosuspension A significantly induced more cells aressting at G_2/M phase (25.44% - 29.36%) compared with free drug (14.05% - 14.59%) at the concentration

of 5 μM. The above results suggested the dissolution rate and anti-cancer activity of UA were enhanced by nanocrystallization.

Multi-inlet vortex mixer (MIVM) is a new technology to develop drug encapsuled polymer nanoparticles which was proposed in 2007 and the nanoparticles with particle size less than 100 nm were easy to prepare by MIVM. However, there is little application of MIVM in pharmaceutical industry. In this study, UA nanosuspensions with different particle sizes were prepared by MIVM in order to study whether the particle size influenced the in-vitro anti-cancer activity. The single factor investigation results indicated that the injection rate, drug concentration in organic phase and ratio of organic phase to aqueous solution could influence the particle size and the target particle size would be achieved by modifying the parameters. The concentration of UA in absolute ethanol was 3 mg/mL, aqueous solution consisted of 0.05% PVP K90 and 0.0% SDS solution. One of the inlets controlled by A pump was filled with organic phase and the other one was filled with aqueous solution, and the injection volume was maintained at 1.6 mL; two inlets controlled by B pump were both filled with aqueous solution and the injection volume was maintained at 8 mL. When the injection rate of A pump was 8 mL/min (B pump was correspondingly 40 mL/min), UA nanosuspension B was prepared and the mean particle size, PdI and zeta potential was 101.2 ± 3.53 nm, 0.205 ± 0.012 and -9.79 ± 0.79 mV, respectively; When the injection rate of A pump was 2 mL/min (B pump was correspondingly 10 mL/min), UA nanosuspension C was prepared and the mean particle size, PdI and zeta potential was 299.8±6.63nm,0.150±0.021 and -8.19±0.78mV, respectively. Transmission electron microscope photos showed the UA nanosuspension were in spherical. Then in-vitro anti-caner evaluations were performed for UA nanosuspensions with different particle sizes. The MTT results indicated that there was no significant difference between UA nanosuspension B and UA nanosuspension C in inhibition the growth of MCF-7 cells. Under IN Cell 2000, it was found that after treatment with UA the cell nucleus of MCF-7 cells shrank, changed shape and produced apoptosis body, and the amount of mitochondria sharply decreased, which indicated that UA induced MCF-7 cells apoptosis. Flow cytometer

analysis results revealed that UA did induce MCF-7 cells arresting at G_2/M phase and induce apoptosis, and the order of different groups was as follow: UA nanosuspension C > UA nanosuspension B > UA solution. The above results suggested that UA nanosuspensions with different particle sizes were prepared by MIVM and there was little difference among different batches of nanosuspensions, which indicated that nanosuspensions with different particle sizes would be prepared by MIVM and there was little difference among different batches of formulations. MIVM was a new technique with high controllability and reproducibility to prepare nanosuspesnion. And UA nansuspensions with lager particle size induced more MCF-7 cells apoptosis compared with UA nanosuspension with small particle size.

In conclusion, the present study suggested reduction of drug particle size to nanometer significantly increased the dissolution rate as well as the in-vitro anti-tumor activity of UA. And UA nanosuspension with large particle size induced more MCF-7 cells apoptosis compared with UA nanosuspension with small particle size. UA nanosuspension is a potential formulation strategy to develop parenteral delivery system of UA.

Key words: ursolic acid; nanosuspension; anti-precipitation; multi-inlets vortex mixer; dissolution rate; cytotoxicity; cell uptake; cell cycle analysis; cell apoptosis.

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縮略詞列表

縮寫	英文全稱	中文全稱
UA	Ursolic Acid	熊果酸
GA	Glycyrrhetic acid	甘草次酸
HPLC	High performance liquid chromatograph	高效液相色譜
PVP	Polyvinyl pyrrolidone	聚乙烯比咯烷酮
F68	Poloxamer 188	泊洛沙姆 188
HPMC	Hydroxypropyl methylcellulose	羥丙基甲基纖維素
SDS	Sodium dodecyl sulfate	十二烷基硫酸鈉
Tween 80	Polysorbate 80	吐溫 80
DMEM	Dulbecco Modified Eagle Medium	培養基
FBS	Fetal bovine serum	胎牛血清
FTIR	Fourier transform infrared spectroscopy	傅氏轉換紅外線光譜
DSC	Different scanning calorimetry	差示掃描量熱法
TGA	Thermo gravimetric analyzer	熱重分析儀
TEM	Transmission electron microscope	透視電鏡
MIVM	Multi-inlet vortex mixer	多通道快速混合儀