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Development and validation of an HPLC method for determination of oleanolic acid content and partition of oleanolic acid in submicron emulsions

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Received April 26, 2008, accepted October 1, 2008

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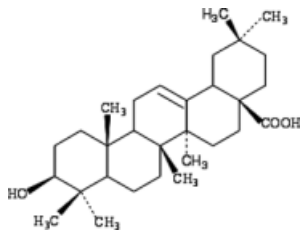
Pharmazie 64: 491–494 (2009) *doi: 10.1691/ph.2009.8123*

The aim of this study was to develop and validate a simple HPLC method for the quantitative determination of the oleanolic acid (OA) content and partition coefficient of OA in a submicron emulsion-based formulation (SE-OA). A Diamonsil™ C_{18} (150 mm × 4.6 mm, 5 μm) column was eluted with a mobile phase consisting of methanol/water (95 : 5, v/v). The analyses were performed at 35 ± 1 °C with a flow rate of 1.0 mL/min and variable wavelength detector (VWD) at 210 nm. The calibration curve was linear over a concentration range of 2–100 μg/mL with a correlation coefficient of 0.999. The LOD and LOQ were 0.1 and 1 μg/mL, respectively. The individual spike recovery of OA ranged from 99.88 to 100.28%. The percent relative standard deviations (% R.S.D.) of intra-day and inter-day analyses were less than 3.1%. The validation results confirmed that the method is specific, linear, accurate, precise, robust and sensitive for its intended use. The present method was successfully applied to the determination of the OA content and partition coefficient of OA in SE-OA during the early stage of formulation development.

1. Introduction

Oleanolic acid (OA; 3β-3-hydroxyolean-12-en-28-oic acid) is a pentacyclic triterpenoid acid that is widely distributed in plants, and triterpenoid derivatives are aglycones of many naturally occurring saponins (Liu 1995). OA is known to have anti-viral (Chiang et al. 2003), hepatoprotective (Kinjo et al. 1999; Liu et al. 1995), anti-proliferative (Liu 1995; Suh et al. 1999; Tokuda et al. 1986; Zhang et al. 2007), and anti-inflammatory properties (Cheng and Tsai 1986; Liu 1995; Suh et al. 1999). However, in spite of the various biological activites of OA, its extremely low solubility in both aqueous and organic media has restricted its clinical application (Jager et al. 2007).



Oleanolic acid (OA)

Emulsion systems offer potential advantages for the delivery of poorly-soluble drugs because of the high volume fraction of the oil phase that can be used (Lundberg et al. 2003; Zhao et al. 2007) and submicron emulsions (SEs) for parenteral use are relatively easy to produce on a large scale. Moreover, submicron emulsions may be promising candidates for sustained-release and targeted delivery systems (Zhang and Kirsch 2003). The release of incorporated drugs from SEs is an important factor in achieving efficient drug delivery (Kawakami et al. 2000). However, the partition of drugs in SEs affects their release from emulsion systems. Submicron emulsions are complex systems where besides oil, water and the interface, other structures like liposomes and various bilayer structures (vesicles) (Ferezou et al. 2001; Groves and Herman 1993; Westesen and Wehler 1992), as well as specific bicompartmental structures (Teixeira et al. 2000), depending on the composition, have been identified. The release of drugs from SEs depends on where they are located in the above mentioned structures. In view of the complexity of the system, it is necessary to determine the drug content and partitioning of a drug in SEs in order to support early stage formulation development.

In our work, a novel and well characterized submicron emulsion-based OA formulation (SE-OA) was developed to overcome the aforementioned problems. To support

Table 1: Results of the particle size, zeta potential measurements, polydispersity index, OA concentration and visual appearance for SE-OA (n = 3)

	Before sterilization	After sterilization
Particle size (nm)	197.97 ± 6.01	201.24 ± 5.92
Polydispersity index	0.18 ± 0.02	0.180 ± 0.01
Zeta potential (mV)	−27.83 ± 3.31	−29.53 ± 4.23
pH	7.38 ± 0.15	6.95 ± 0.23
OA content (mg/mL)	3.43 ± 0.02	3.38 ± 0.03
Visual appearance	Homogenous	Homogenous

Table 2: Precision and accuracy of the method (n = 6)

Theoretical concentration (μg/mL)	Experimental concentration (μg/mL)	R.S.D (%)	Accuracy (%)
Intra-day			
2	1.99 ± 0.01	0.51	99.64
25	25.46 ± 0.24	0.93	101.84
100	101.29 ± 0.80	0.79	101.29
Inter-day			
2	1.99 ± 0.04	2.10	99.70
25	25.07 ± 0.77	3.07	100.29
100	100.79 ± 2.21	2.20	100.79

ongoing formulation development and other preclinical studies, a specific and sensitive analytical method for the partitioning of the drug in SEs was needed. The objective of this study was to develop a simple HPLC method for the determination of OA content and partitioning of OA in SEs to support early stage formulation development.

2. Investigations, results and discusion

2.1. Characteristics of SE-OA

First, the general physico-chemical properties of SE-OA were evaluated (Table 1). As expected, the microfluidizer produced a monodisperse SE-OA (Polydispersity index <0.2) with a typical droplet size in the range 190−230 nm. Regarding zeta potential, SE-OA presented negative values due to the presence of negatively-charged phospholipids in egg-lecithin compounds such as phosphatidylserine and phosphatidic acid at neutral formulation, pH as previously reported in the literature (Cappra Silva et al. 2007; Shi and Benita 2000). The mean drug loading of SE-OA was 3.43 mg/mL and no crystallization was subsequently detected by optical microscopy at this loading level. Apart from pH, no physico-chemical properties of SE-OA were significantly affected by sterilization. The sterilization process decreased the pH by 0.45 units. This pH reduction is explained by hydrolysis of the phospholipids during the heat-sterilization process and formation of free fatty acid (Herman and Groves 1993; Klang et al. 1994).

2.2. Chromatographic specificity

To assure the specificity of the current method, a blank SEs formulation containing the same concentration of lipids and other excipients as the SE-OA formulation was evaluated. The chromatograms are shown in the Figure with the specific chromatographic conditions. Separation of OA from SEs was achieved using a C_{18} column. There were no interfering peaks co-eluting with OA (Figure C). The results showed that the lipid components and other excipients in the formulation did not interfere with the OA peak. The retention time of OA was 10.28 min.

2.3. Calibration curves

The relationship between OA concentration and detector response was evaluated to confirm linearity. OA standards at six different concentrations were included in the study. Peak areas (y) of OA were measured and plotted against the concentration (x) of OA. It was found that in the concentration range of 2–100 μg/mL, the detector response of OA was linear with a correlation coefficient greater than 0.999. The regression equations of the calibration curves were $y = 11.124x + 2.009$.

2.4. Validation of the method

The LOD and LOQ of the current method were determined to be 0.1 μg/mL (% R.S.D. = 5.4), and 1 μg/mL (% R.S.D. = 1.5), respectively, indicating the method was sufficiently sensitive to be used for the drug. The precision and accuracy were calculated using the calibration curve and are shown in Table 2. The results showed that the intra- and inter-day coefficients of variation were less than 3.1% (R.S.D.). The recovery of the assay was between 99.64 and 101.84%. OA spike samples were prepared by the addition of OA standard solution to blank SEs with addition of methanol to give final concentrations of 2, 25 and 100 μg/mL. The percent recoveries of OA in the spiked samples at the three different levels were 99.88 ± 2.11%, 100.11 ± 2.06% and 100.28 ± 1.75%, respectively (n = 5). The results indicate that the method had a high level of accuracy.

2.5. Robustness

Robustness of the present method for analysis of OA was evaluated under intentionally altered conditions including the proportion of mobile phase (± 5%), column temperature (± 5%), flow rate (± 20%) and wavelength (± 1 nm). R.S.D.s were found to be in the range of 0.18–0.52%.

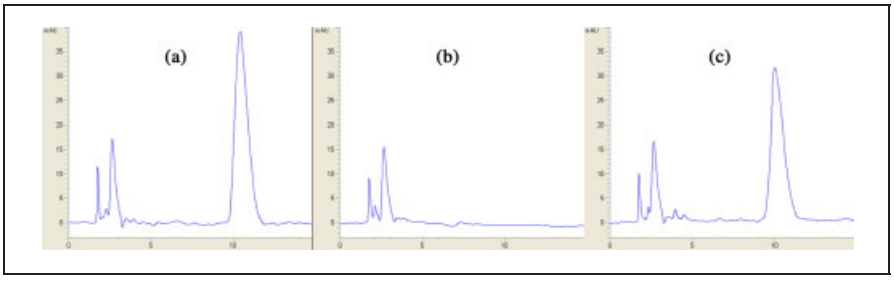


Fig.:
HPLC chromatograms of standard solution (a), blank SEs sample solution (b), SE-OA sample solution (c)

Table 3: Distribution of oleanolic acid in SEs

Compartment	Oleanolic acid amount (μg/g SEs)	Percent oleanolic acid (%)
Oil	1238.26 ± 41.59	35.58
Water	113.48 ± 3.79	3.26
Liposome (or micelle)	461.12 ± 11.96	13.25
Droplet interface	1665.13 ± 45.68	47.85

Therefore, the current method has high toleration of separation conditions, and is reliable for the analysis of OA.

2.6. Determination of OA content and partition in SEs

The current method was used to determine OA content and partitioning in SEs. OA content in SE-OA samples was 3.48 ± 0.54 mg/mL (n = 3). The soyaoil/water partition coefficient was 107.14. The soyaoil/water partition coefficient indicates that, in simple oil/water mixtures, approximately 99% of OA is in the oily phase.

The SEs is much more complex, as it consists of four separate environments which can contain OA. These are the external water phase, the oil droplets, the phospholipid-coated interfaces, and liposomes/micelles formed in water by excess surfactant (lecithin and Poloxamer 188). The masses of OA in the external water phase (m_w), oil droplets + phospholipid-coated interfaces (m_{oi}), and liposomes/micelles (m_l) were directly determined after the phases were separated using ultracentrifugation-ultrafiltration. The masses of OA in the oil droplets (m_o) and the phospholipid-coated interfaces (m_i) were obtained by the following equations.

$$m_o = C_w V_o P \qquad m_i = m_{oi} - m_o$$

where C_w is the concentration of OA in the aqueous phase obtained by ultrafiltration and ultracentrifugation; V_o is the volume fraction of oily phase; and P is the partition coefficient of OA between oil and water.

The fractions of OA in each phase of the SE-OA are given in Table 3. The results indicate that OA is mainly distributed in the phospholipid-coated interfaces and the oily phase. The amount of OA in the phospholipid-coated interfaces is significantly ($p < 0.05$) larger than that in the oily phase, although the lipophilicity of OA is high. The accumulation of drug in the interface is related to the oil/lecithin ratio (Watrobska-Swietlikowska and Sznitowska 2006). Thus, it is crucial for early stage formulation development to determine drug partitioning in SEs. In order to further understand the relationship of OA partitioning in each phase of SE-OA and in drug release, the in vitro release of different submicron emulsion-based OA formulations must be investigated at the same time.

In conclusion, this paper shows a useful HPLC method to determine OA content and OA partitioning in submicron emulsion-based formulations to support formulation development and other pre-clinical studies.

3. Experimental

3.1. Chemicals and reagents

OA (purity >98.6%) was provided by Guizhou Pharmaceutical Co., Ltd (Guiyang, China). Methanol of HPLC grade was obtained from Siyou Chemical Reagent Co., Ltd (Tianjin, China). Lipoid E80 was purchased from Lipoid GmbH (Ludwigshafen, Germany). Soybean oil was purchased from Tieling Beiya Pharmaceutical Oil Co., Ltd (Liaoning, China). Poloxamer 188 was purchased from BASF (Germany). All other chemicals were of analytical grade from a variety of suppliers.

3.2. Preparation and characterization of SE-OA

SE-OA containing OA (0.35%, w/v), soybean oil (10%, w/v) as oily phase and Lipoid E80 as emulsifier (1.2%, w/v) were prepared by a previously described technique (Klang et al. 1994). Briefly, Poloxamer 188 (2%, w/v) and glycerol (22.5%, w/v) were dissolved in the aqueous phase. OA, soybean oil, Lipoid E80 and α-tocopherol (0.08%, w/v) were dissolved in a mixed organic solvent of absolute ethanol and dichloromethane (1 : 1, v/v) which was then removed in a rotary evaporator under vacuum giving the oily phase. Both phases were heated separately to 70 °C, and then the two phases were mixed and emulsified in a homogenizer (FJ-200, Shanghai Specimen and Model Factory, Shanghai, China) at 10,000 rpm for 5 min. The pH of the crude emulsion was adjusted to 7.4 with sodium hydroxide (0.1 M). The crude emulsion was then passed through a Microfluidizer (M-110L, Microfluidics, USA) at 10,000 PSI for 6 discrete volume cycles to produce a fine emulsion. The blank SEs formulation was prepared in a similar manner without drug. The final submicron emulsion was sterilized by autoclaving at 121 °C for 15 min.

The droplet size, polydispersity index and zeta potential were measured using a Malvern Zetasizer Nano-ZS90 (Malvern Instruments, UK). A pH meter (PHS-3C Shanghai REX Instrument Factory, China) was used to determine the pH value of SE-OA at room temperature (21 °C ± 2). The OA content of SE-OA was measured using an HPLC method.

3.3. HPLC determination

3.3.1. Chromatographic equipment and conditions

The HPLC system used for quantification of OA consisted of Agilent 1100 modules (Wilmington, DE, USA), a quaternary pump, mobile phase degasser, auto-sampler with thermostat and a column heater compartment. Agilent software, ChemStation, was used for data acquisition and analysis. For OA content assay, the chromatographic conditions were as follows: Agilent Zorbax SB-C_{18} guard column (4.6 × 12.5 mm, 5 μm); analytical column (4.6 mm × 150 mm, Diamonsil™ C_{18}, 5 μm, from Dikma Technologies Inc); mobile phase, 95 : 5 (v/v) methanol and water, flow-rate, 1.0 mL/min; degas with helium on line, 30 mL/min; variable wavelength detector, 210 nm; injection volume, 20 μL; temperature 35 ± 1 °C. The OA content in the formulation was quantitatively determined using external standards.

3.3.2. Preparation of OA stock and standard solutions

Stock standard solution of OA was prepared at a concentration of 1 mg/mL in methanol and stored in a brown flask at −20 °C until analysis. OA standard solution: The stock solution was diluted with methanol. The solution was equilibrated at ambient temperature for 30 min and was used on the day of preparation.

3.3.3. Calibration curves

To prepare calibration curves, standard samples of OA were added to methanol to give final concentrations of 2, 5, 10, 25, 50 and 100 μg/mL. Calibration curves were constructed by performing a linear regression analysis of peak area versus OA concentrations.

3.3.4. Validation of the method

The limit of detection (LOD) was established by analyzing OA standards in six replicate injections at different concentration levels in decreasing order until signal to noise (S/N) ratio reached about 3. The limit of quantitation (LOQ) was established in the same way as for the LOD, except that the S/N ratio was about 10. The intra-day precision of the assays performed in replicate (n = 6) was tested using three OA concentrations, namely, 2, 25 and 100 μg/mL. The inter-day precision of the assays was estimated from the results of replicate assays on 6 different days within 1 week. The precision was evaluated by the relative standard deviation (R.S.D.).

3.4. Analysis of the total content of OA in SEs

For assay, 0.1 mL of SE-OA was diluted to 1 mL with chloroform–methanol (1 : 1) solution. Final dilution was made with mobile phase and OA content was estimated by the HPLC method.

3.5. Analysis of OA partition in SEs

Oleanolic acid partition between soyabean oil and water was measured by dissolving OA in soyabean oil, adding an equal volume of aqueous solution (pH 7.4), and rotating the samples in a sealed 10 mL bottle to bring OA concentrations to equilibrium. Samples were withdrawn after 24 h and the concentration of OA in the oily phase was analyzed. The partition coefficient was calculated from the concentrations of OA in the saturated soyabean oil and the concentration determined after the procedure.

Analysis of OA partition in SEs was performed according to the method reported in the literature with some modifications using ultracentrifugation-

ultrafiltration (Han and Washington 2005; Watrobska-Swietlikowska and Sznitowska 2006). The aqueous phase of SEs was separated by ultracentrifugation (Hermle Z323K Germany) at 16,000 rpm for 1 h (25 °C). The superstratum (i.e. oil + interface fraction) was then collected and the drug content was measured by HPLC. The collected aqueous phase was opalescent due to liposomal/micellar dispersion, and was subsequently subjected to centrifugal ultrafiltration using Microcon YM-100 (Millipore, Bedford, USA), resulting in a liposome/micelle-free aqueous phase. Liposomes/micelles were then collected and dissolved in chloroform–methanol (1 : 1) solution. The drug contents of aqueous phase and liposomes/micelles were measured by HPLC.The ultrafiltration procedure was validated for the recovery of OA.

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