IJBPAS, March, 2022, 11(3): 1503-1512

ISSN: 2277–4998

International Journal of Biology, Pharmacy and Allied Sciences (IJBPAS)

'A Bridge Between Laboratory and Reader'

www.ijbpas.com

DETERMINATION OF URSOLIC ACID FROM SOLID LIPID NANOPARTICLES USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

MOULI GC AND VEERESHAM C*

University College of Pharmaceutical Sciences, Kakatiya University, Warangal, Telangana, India

***Corresponding Author: Prof. Ciddi Veeresham: Email: ciddiveeresham@gmail.com**

Received 26th April 2021; Revised 24th May 2021; Accepted 30th June 2021; Available online 1st March 2022

https://doi.org/10.31032/IJBPAS/2022/11.3.6411

ABSTRACT

Objective: The purpose of this study was to develop an RP-HPLC method for determining drug content and entrapment efficiency in ursolic acid-loaded solid lipid nanoparticles (UA-SLNs).

Methods: The RP-HPLC method was carried out in the reverse phase (RP) C18 column with a mobile phase of methanol: water (80:20 v/v), at the flow rate of 1mL/min. The λmax was set at 249nm.

Results: The retention time of ursolic acid was found at 5.7 ± 0.02 minutes. The approach was demonstrated to be specific, linear in the range of 0.5-8.0 µg/mL (R^2 0.999), precise at the intra-day and inter-day levels as evidenced by the relative standard deviation values (<1.0%), and accurate at the intra-day and inter-day levels (average recovery 99.02 to 99.59%). The detection and quantification limits were determined to be 0.074 and 0.228 µg/mL, respectively. The method was utilized to determine ursolic acid drug content and entrapment efficiency (EE) in solid lipid nanoparticles were found to be 98.82 ± 0.35% and 87.29 ± 1.12%.

Conclusion: The RP-HPLC method may be useful for the determination of ursolic acid content and entrapment efficiency in solid lipid nanoparticles.

Keywords: Ursolic acid, RP-HPLC, solid lipid nanoparticles, method development, and validation

INTRODUCTION

Ursolic acid (UA, 3-hydroxy-urs-12-ene-28-oic acid) is a hydrophobic pentacyclic triterpenoid with antitumor, anti-inflammatory, antibacterial, antioxidant, and antidiabetic effects **[1]**. UA is found in a variety of plants, including *Rosmarinus officinalis*, *Glechoma hederacea*, *Lithocarpus frutescens*, *Phoradendron juniperinum*, *Syzygium claviflorum*, and *Hyptis capitata*, and can be found as a free acid or as an aglycone of triterpenoid saponin **[2 & 3]**. The structure of UA is shown in **Figure 1**.

Solid lipid nanoparticles (SLNs) have been suggested recently as an interesting for the development of formulations with increased effectiveness and stability. The nanoformulations provide greater stability, these new formulations may allow the modified release and specific drug targeting **[4]**.

HPLC is one of the best and most accurate method for determining UA content and entrapment efficiency (EE) in solid lipid nanoparticles. For the determination of ursolic acid-loaded solid lipid nanoparticles (UA-SLNs), several researchers have researched and employed various approaches. To measure evaluation characteristics such as drug content and entrapment efficiency, adequate and proven quantification methods are necessary to characterize UA solid lipid nanoparticles. The use of the HPLC method to determine and quantify the UA in formulations is described in a few references. Mariana M *et al*. used a high-performance liquid chromatography approach for *in vitro* characterization to determine ursolic acid from polymeric nanoparticles **[5]**. For the UA phospholipid nanopowders, Zhou *et al*. used HPLC to examine the entrapment efficiency and UA concentration *in vitro* and *in vivo* **[6]**. Using a charged aerosol detector (CAD ™), Marc plante *et al*. demonstrated a sensitive HPLC approach for resolving and measuring triterpenoids **[7]**. Using HPLC, Zhitao liang *et al*. determined oleanolic acid and ursolic acid in *Oldenlandia diffusa* and its equivalent **[8]**. Ursolic acid solid lipid nanoparticles have yet to be characterized by any method.

The goal of this study is to develop and validate a simple and optimized RP-HPLC method for measuring the content and entrapment efficiency of UA in solid lipid nanoparticles.

MATERIALS AND METHODS

Materials

Ursolic acid was procured from Yucca enterprises, Mumbai, India. Glycerol

monostearate was supplied by Hi Media Labs, Mumbai. Kolliphor®P188 was provided as a gift sample by Aurobindo Labs, India. HPLC grade methanol was purchased from Merck life sciences Pvt Ltd, Mumbai, India. Double distilled water was prepared by using Milli-Q® (Direct-Q-UV-3). All other chemicals are analytical grade.

Equipment

The method development and quantify the ursolic acid (UA) was performed by the Ultra-Fast Liquid Chromatography (UFLC, Shimadzu Corporation, Kyoto, Japan) system with gradient binary pump (LC-20AD) and the analytical reverse-phase column C_{18}, 250 × 4.6 mm; 5 µm particle size (Luna® 5 µ, Phenomenex), equipped with a UV-Visible wavelength detector (SPD-A20). The results were acquired and processed by the Lab solution software. Milli Q water purification system equipped with 0.22 µm Millipak express filter. Membrane filters of 0.45 µm pore size were used for filtration of the mobile phase.

Chromatographic conditions

In isocratic mode, the chromatographic analysis was carried out. At a flow rate of 1.0 ml/minute, the mobile phase was made up of methanol: water (80:20% v/v). The eluted samples were detected at a wavelength of 249nm with a 20 µL injection volume. The total run time was 10 minutes. The total area of the peak was utilized to quantify ursolic acid in all experiments, which were carried out at a temperature of 25 ± 0.1°C.

Preparation of standard solution

A standard solution of 10 mg/mL of UA was prepared by accurately weighing 10 mg of UA into a 10 mL volumetric flask containing methanol. After solubilization, the volume was made up to the mark by using methanol to give the final concentration (1 mg/mL) of the drug solution. The resulting solution was sonicated in an ultrasonic bath (PCi ™ Analytics) for 15 minutes. Dilution of these solutions with appropriate volumes of the solvent mixture was carried out to obtain concentrations of 0.5, 1, 2, 4, 6, and 8 µg/mL. The standard solutions were filtered through a 0.45 µm membrane filter, 20 µL were injected into the chromatography and the concentrations of UA were determined (n=3) **[9]**.

Method validation

The HPLC method was validated according to the ICH guidelines. The following characteristics were considered for validation those are selectivity, linearity, the limit of detection (LOD) and limit of quantification (LOQ), accuracy, precision, and robustness **[10 & 11]**.

Selectivity

The selectivity of the method was evaluated by comparing the chromatograms obtained

from the samples containing UA and internal standard (Glycyrrhetinic acid; GA) with the retention time (Rt) without any interference.

Linearity

A regression line was calculated from the peak area vs. concentration to determine linearity. UA solutions with concentrations ranging from 0.5 to 8.0 µg/mL were prepared. The calibration standards were injected, and the detector response (peak area) and concentrations exhibited a linear relationship.

Limit of detection (LOD) and limit of quantification (LOQ)

The LOD and LOQ were evaluated from the linearity equation i.e. slope of the calibration curve and the standard deviation (SD) of the peak areas using the following formula:

LOD:

3.3×SD/slope; LOQ: 10×SD/slope

Precision

Precision was assessed by six determinations at three levels of known concentrations corresponding to low (2 µg/mL), medium (4 µg/mL), and high (6 µg/mL) levels in the calibration curve range. To assess the inter-day precision, these experiments were performed for three days under the same HPLC conditions. The relative standard deviation (RSD) was used to calculate both inter-day and intra-day precision. According to ICH guidelines, the % of RSD should be less than 2.

Accuracy (% recovery)

The accuracy was assessed at three distinct concentration levels of UA at low (50%), medium (100%), and high (150%) within the linearity range, and each level was repeated three times (n=3).

Robustness

A robustness study was performed by altering the optimized chromatographic conditions such as by modifying the flow rate and wavelength. It was calculated by estimating % RSD.

Method applicability

Preparation of ursolic acid nanoparticles (UA-SLNs)

The UA-SLNs were developed through a process of hot homogenization followed by ultrasonication. To obtain the oil phase, ursolic acid (10 mg), solid lipid (Glycerol monostearate, 100 mg), and lipoid E 80 (50 mg) were liquefied in a 20 mL mixture of chloroform and methanol (1:1 ratio). A rota evaporator was used to separate the organic solvents, and a heating system set at 5^{o}C above the melting point was used to melt the drug-encapsulated lipid covering layer. The aqueous phase was developed using the surfactants poloxamer 188 and polysorbate 80. These surfactants are dissolved in double

distilled water and heated to the same temperature as the oil phase. The hot aqueous phase was mixed with the oil phase and homogenized for 5 minutes (at 12,000 rpm) using a homogenizer. A probe sonicator (Vibracell, Sonics, 12T-Probe, USA) was used to sonicate the resulting coarse oil in water (O/W) emulsion for 20 minutes. When a heated nanoemulsion was allowed to cool to room temperature, it resulted in the development of ursolic acid-loaded solid lipid nanoparticles **[12]**.

Entrapment efficiency from solid lipid nanoparticles

The entrapment efficiency (EE) of developed UA-SLNs formulations was determined by measuring the concentration of free drug (unentrapped) in the aqueous phase. The aqueous phase was separated by ultrafiltration using Centrisart tubes which were composed of a filter membrane (molecular weight cut-off 20 kDa) at the base sample recovery chamber. About 5 mL of the UA-SLNs formulation sample was kept in the outer chamber, and a sample recovery chamber was placed over the sample and centrifuged at 3500 rpm for 30 minutes. The SLN and encapsulated drug remained in the outer chamber, while the aqueous phase passed through the filter membrane and into the sample recovery chamber. The amount of ursolic acid in the aqueous phase was analyzed by the HPLC method. Entrapment efficiency was calculated by the following equation,

$$\% EE = (W_{Initial\ drug} - W_{Free\ drug}) / W_{Initial\ drug} \times 100$$

Determination of drug content

A 0.1 mL of the UA-SLNs formulation was collected and dissolved in 0.9 mL of chloroform: methanol (1:1) mixture, which was then diluted further with a mobile phase. The amount of drug in diluted samples was determined using HPLC **[13]**.

RESULTS AND DISCUSSION

Method development

The retention time (Rt) of UA was determined to be 5.7 minutes and the internal standard (GA) was found to be 8.2 minutes. Calibration graphs for UA were constructed in the range of 0.5 to 8.0 µg/mL. The regression equation of this curve and its coefficients of determination (R^2) were calculated as follows: Y=268.3 X and -25.35X (R^2=0.999). The limit of quantification (LOQ) was 0.225µg/mL, while the limit of detection (LOD) was 0.074µg/mL, with a relative standard deviation (RSD) of <2.0%.

Method validation

The chromatograms of UA and internal standard (IS) with the retention time (Rt) at

5.7 ± 0.02 minutes and 8.2 ± 0.02 minutes respectively without any interference. The results are depicted in **Figure 2**.

Linearity

The calibration curve was constructed by plotting the mean peak area versus the concentration of analyzing the calibration graph of UA was within the concentration range of 0.5, 1, 2, 4, 6, and 8µg/mL. The correlation coefficient (R^2) for UA is 0.999, indicating good linearity in the proposed range. The peak area (y) is proportional to the concentration of UA (x) following the regression equation y= 268.3 X and -25.35X. The data is represented in **Table 1** and **Figure 3**.

Precision

The % RSD of intra-day and inter-day precision was found to be <2%, which confirms the high repeatability of the method **[14]**. The results are shown in **Table 2**.

Accuracy

The average recovery and the %RSD for each level have been calculated. The results have shown that the current method has good recovery (from 99.02 to 99.59%) from UA at three concentration level studies (2, 4, and 6µg/mL) and within the RSD lower than 1% **[15]**. The results are shown in **Table 3**.

LOD & LOQ

The LOD and LOQ of UA were found to be 0.074 ± 0.005 and 0.228 ± 0.003 µg/mL respectively.

Robustness

The method was found to be robust with a change of ±2% in flow rate and wavelength. But no significant changes were observed **[16]**. The results are shown in **Table 4**.

Method applicability

The UA-SLNs were prepared using a technique of hot homogenization followed by ultrasonication. The polydispersity index (PDI) was within normal ranges, indicating that the particles were uniform in size. The proposed method was employed to investigate the drug content of UA in solid lipid nanoparticles with a size range of 163.8 ± 4.89 nm, PDI of 0.240 ± 0.02, and zeta potential (ZP) of -26.48 ± 2.63 mV.

Drug content and EE of UA solid lipid nanoparticles

An HPLC approach was used to determine entrapment efficiency and drug content in UA-SLNs. In the UA-SLNs formulation, the drug content was found to be 98.82 ± 0.35%. The UA-SLNs formulations had an entrapment efficiency (%) of 87.29 ± 1.12%, showing a higher degree of encapsulation of UA into SLNs [17]. The results are summarised in **Table 5**.

Table 1: Linearity results of UA

Parameters	Results of UA
Range of linearity (µg/mL)	0.5 - 8µg/mL
Regression equation	y = 268.3x – 25.35
Correlation coefficient (R^2)	0.999 ± 0.001
Slope ± SD	268.3 ± 0.416
Intercept ± SD	25.35 ± 0.165

Table 2: Intra-day and inter-day precision results of UA

Concentration (µg/mL)	Intra-day Precision		Inter-day Precision	
	Mean ± SD	% RSD	Mean ± SD	% RSD
2	508.33 ± 5.033	0.79	515.33 ± 3.05	0.59
4	1035.33 ± 3.511	0.33	1043.66 ± 4.58	0.43
6	1576.66 ± 4.725	0.29	1581.33 ± 4.50	0.28

Table 3: Accuracy results of UA

Parameter	Actual concentration (µg/mL)	Recovered concentration (µg/mL)	% Recovery	% RSD
50%	2	1.980	99.021	0.411
100%	4	3.944	98.616	0.443
150%	6	5.975	99.599	0.352

Table 4: Robustness results of UA

Parameters	Robustness of UA (Mean ± SD		
	Modifications	Mean Rt of UA ± SD	% RSD
Flow rate (mL/min)	0.8	5.57 ± 0.05	0.99
	1 (Optimized)	5.75 ± 0.02	0.46
	1.2	5.93 ± 0.04	0.67
		Mean area of UA ± SD	% RSD
	247	1031.33 ± 6.506	0.63
Wavelength (nm)	249 (Optimized)	1044.33 ± 5.033	0.48
	251	1049.66 ± 8.326	0.79

Table 5: Results of UA-SLNs formulation

S. No.	Evaluation parameters	Results
1	Size (nm)	163.8 ± 4.89 nm
2	PDI	0.240 ± 0.02
3	Zeta potential	-26.48 ± 2.63 mV
4	Entrapment efficiency	87.29 ± 1.12%
5	Drug content	98.82 ± 0.35%

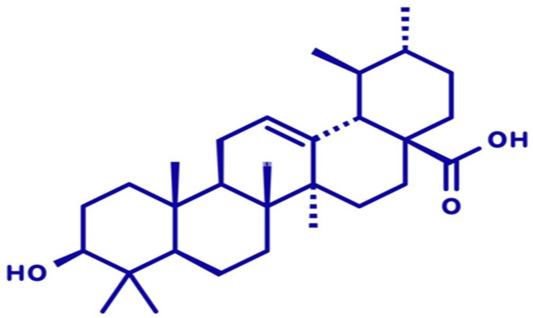

Figure 1: Structure of ursolic acid

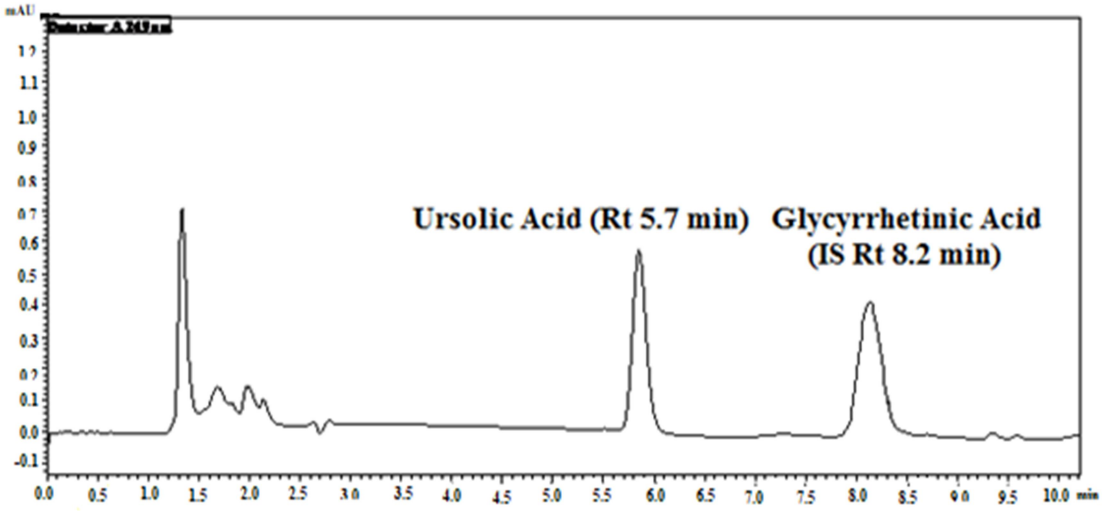

Figure 2: Representative chromatogram of ursolic acid with internal standard (Glycyrrhetinic Acid)

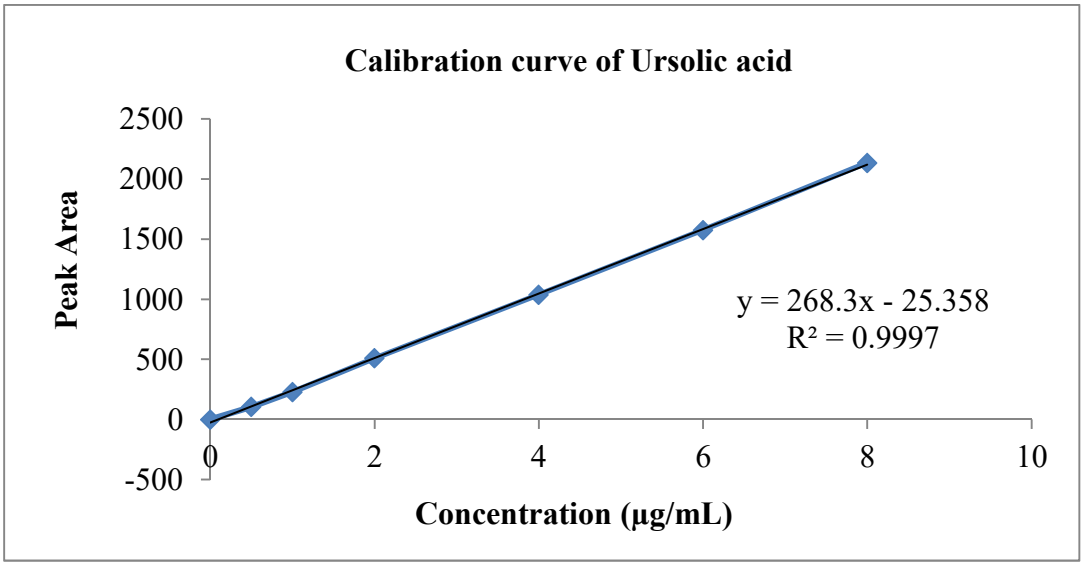

Figure 3: Calibration curve of ursolic acid

CONCLUSION

The RP-HPLC method was successfully applied for the determination of ursolic acid in solid lipid nanoparticle formulation. The proposed RP-HPLC method offers good linearity, precision, accuracy, and robustness, according to the method validation. The detection and quantification limits were also established. The method was found to be suitable for analyzing ursolic acid-loaded solid lipid nanoparticles for drug content and entrapment efficiency assessment.

ACKNOWLEDGMENTS

The author thanks UCPSc, KU, and Warangal for providing the entire requirements to carry out research work.

CONFLICT OF INTEREST

The author declares that there is no conflict of interest.

REFERENCES

[1] Holger T, Thomas K, Melanie NL, Armin S, Pentacyclic triterpene distribution in various plants rich sources for a new group of multi-potent plant extracts, Molecules.,14, 2009, 2016-2031.

[2] Kashyap D, Hardeep ST, Anil KS, Ursolic acid (UA): A metabolite with promising therapeutic potential, Life. Sci.,146(1), 2016, 201-213.

[3] Liu J, Pharmacology of oleanolic acid and ursolic acid, J. Ethnopharmacol.,49, 1997, 57-68.

[4] El-Say KM, Hosny KM, Optimization of carvedilol solid lipid nanoparticles: An approach to control the release and enhance the oral bioavailability on rabbits, PloS. one., 13(8), 2018,2001-2025.

[5] Mariana M, Nadia A, Vanessa F, Andre R, Telma M, Velasco VR, Determination of Ursolic acid polymeric nanoparticles using High Performance Liquid Chromatography Method, Lat. Am. J. Pharma.,31(8), 2012,1217-1221.

[6] Zou S, Chen W, Li K, Preparation and HPLC analysis of ursolic acid from *Eriobotrya japonica* Lindl, Acta. Agri. Univer. Jiangxiensis.,26, 2004,808-810.

[7] Marc P, Bruce B, Christopher C, Ian NA, Sensitive HPLC method for Triterpenoid analysis using charged aerosol detection with improved resolution, Ther. Fish. Sci., 12, 2012, 1-8.

[8] Zhitao L, Zhihong J, David W, Zhongzhen Z, Determination of oleanolic acid and ursolic acid in Oldenlandia diffusa and its substitute using High Performance Liquid Chromatography, J. Food. Drug. Anal.,17(2), 2009,69-77.

[9] Taralkar SV, Chattopadhyay S, A HPLC method for determination of ursolic acid and betulinic acids from their methanolic extracts of *Vitex negundo* Linn, J. Anal. Bioanal. Techniques.,3(3), 2012,1-6.

[10] Priyanka S, Rakhi C, Simultaneous determination and quantification of three pentacyclic triterpenoids-betulinic acid, oleanolic acid and ursolic acid in cell cultures of *Lantana camara* L, In Vitro. Cell. Dev. Biol. Plant.,46, 2010, 549-557.

[11] Xu X, Su Q, Zang Z, Simultaneous determination of oleanolic acid and ursolic acid by RP-HPLC in the leaves of *Eriobotrya japonica* Lindl, J. Pharm. Anal.,2(3), 2012, 238-240.

[12] Chunlei T, Hailin C, Kai Z, Qing L, Lili G, Weidong C, Preparation and characterization of biochanin A loaded solid lipid nanoparticles, Asian. J. Pharm.,6(4), 2012, 275-281.

[13] Xia Y, Wei G, Si D, Liu C, Quantitation of ursolic acid in human plasma by ultra performance liquid chromatography tandem mass spectrometry and its pharmacokinetic study, J. Chromatogr. B. Analyt. Technol. Biomed. Life. Sci.,879(2), 2011, 219-224.

[14] Shuge T, Yang S, Yu Q, Halmurat U, Determination of oleanolic acid and ursolic acid contents in *Ziziphora clinopodioides* Lam. by HPLC method, Pharmacogn. Mag., 6(22), 2010, 116-119.

[15] Mrinmoy N, Amit K, Joydeb C, Pulok K, RP-HPLC analysis of methanol extract of *Viscum articulatum*, J. AIM.,11, 2020, 277-280.

[16] Xie Y, Hang T, Cheng J, Zhang Z, HPLC determination of oleanolic acid and ursolic acid in Chinese medicinal herbs, Chin. J. Chin. Mat. Med.,26, 2001, 615-620.

[17] Yang L, Sun Z, Zu Y, Sun X, Zhonghua Z, Zhang L, Physicochemical properties and oral bioavailability of ursolic acid nanoparticles using supercritical anti-solvent (SAS) process, Food. Chem.,132(1), 2012, 319-325.