International Journal of Pharmacy and Pharmaceutical Sciences

Vol 2, Suppl 1, 2010

Research Article

A VALIDATED HPLC METHOD FOR THE QUANTIFICATION OF OLEANOLIC ACID IN THE ROOTS OF *ACHYRANTHES ASPERA* LINN. AND MARKETED FORMULATION

VIDHU AERI*[1], M. I. KHAN, S. ALAM

[1]Department of Pharmacognosy and Phytochemistry, Faculty of Pharmacy, Jamia Hamdard, Hamdard Nagar, New Delhi-110062, India.

*E-mail:vidhuaeri@yahoo.com

ABSTRACT

A new validated, quality evaluation method for *Achyranthes aspera* roots and marketed formulation has been established by a defined high performance liquid chromatography (HPLC). The mobile phase acetonitrile and water (65:35 % v/v), was pumped through a C18 octadecyl silica column at the flow rate, 0.5 ml/min and elute was monitored at 215 nm, which gave a sharp and well defined peak of oleanolic acid at the retention time of 8.303 ± 0.011 min. The linear regression analysis data for the calibration curve showed a good linear relationship with correlation coefficient of 0.9999 in the concentration range of 1-20 µg/ml. The linear regression equation produced is Y= 392850x. The limit of detection (LOD) and quantification (LOQ) were 0.098 and 0.297 µg/ml respectively. The method is precise with relative standard deviation from 0.14 -1.04% (intra-day) and 0.83-1.03% (inter-day). The developed method has been validated for accuracy, precision, reproducibility and robustness, as per ICH guidelines.

Key words: *A. aspera*, Oleanolic acid, HPLC, ICH guidelines

INTRODUCTION

A aspera Linn. (Amaranthaceae) is a well-known Ayurvedic drug. According to the Ayurvedic formulary, *A aspera* is an important ingredient of many formulations. The dried root of the plant is used in tumor, pyrexia, heart diseases, disorders of liver and blood, and diseases of abdomen[1,2] The root was found to contain oleanolic acid as the aglycone isolated from the saponin fraction[3]. Pharmacological studies indicate that oleanolic acid has protective effects for acute chemically injured liver and chronic liver fibrosis and cirrohsis [4-6]. It also inhibits tumor cell differentiation and apaptosis [7-10]. These bioactivities can be related to medicinal functions of *A. aspera*, and oleanolic acid can be suggested as a marker compound for the quality evaluation of *A aspera.* No previous reports are available on the comparative study of oleanolic acid in the samples obtained from different geographical regions. The quantitative analysis of oleanolic acid has been carried out in different plants by HPLC [11,12.] In the present study, a rapid and accurate method has been developed for the quantitative analysis of oleanolic acid in samples of different geographical regions and a market formulation. The method has been validated as per ICH guidelines [13]

MATERIALS AND METHODS

A. aspera roots were collected from three different geographical regions viz: Balarampur (U.P), Alwar (Rajasthan) and herbal garden of Jamia Hamdard, New Delhi in the month of September. The samples were authenticated by the authors and the voucher specimens were deposited in the Department of Pharmacognosy and Phytochemistry, Faculty of Pharmacy, Hamdard University, New Delhi for reference. Oleonolic acid reference standard was purchased from Sigma Aldrich USA. Acetonitrile, methanol and water (HPLC grade) were obtained from E. Merck India Ltd., Potassium dihydrogen phosphate, methanol, toluene, formic acid, hexane, hydrochloric acid, sodium hydroxide flakes and sulphuric acid were purchased from Qualigen India Limited.

The traditional Ayurvedic marketed formulation (Cystone®, Himalaya, India) was used for quantification of oleonolic acid.

Equipment and conditions

A Shimadzu model HPLC equipped with quaternary LC-10A VP pumps variable wavelength programmable UV/VIS detector SPD-10AVP column oven (Shimadzu), SCL10AVP system controller (Shimadzu), Rheodyne injector fitted with a 20 µl loop and Class-VP 5.032 software was used. The chromatographic column used was a reverse phase C18, 250 X 4.6mm, 5mic Zorax RP-HPLC. The column and HPLC system were kept at ambient conditions. The mobile phase was acetonitrile: water (65:35) with the flow rate of 0.5 ml/min. The injection volume was 20 µl and elute was analyzed at a wavelength of 215 nm. The mobile phase after filtration through 0.4 µm membrane filter was

delivered at 0.5ml/min for column standardization and baseline was continuously monitored during the process.

The stability of drug in solution during analysis was determined by repeated analysis of samples during the course of experimentation on the same day (intra-day) and also after 72 h storage of drug solution at laboratory conditions and in the refrigerator (inter-day).

Preparation of sample extracts

The washed and dried samples were ground into powder and passed through a 20-mesh sieve. Each 10g of the powder was refluxed thrice with 250ml of methanol for one hour at room temperature. The extract was filtered and concentrated by vacuum evaporation under reduced pressure. The solid residues were dissolved in methanol and transferred into a 25 ml volumetric flask and made to volume with methanol. The solution was then filtered through 0.45 µm membrane and 20 µm aliquots were analyzed by HPLC.

Sample preparation of market formulation

Six tablets of marketed formulation of *A. aspera* (Cystone®) were crushed in 250 ml of beaker and dissolved in 50 ml of methanol. The methanolic solution was boiled for 10 min and macerated in 50 ml of methanol for 2 h by occasional shaking. This extract was filtered and concentrated by vacuum evaporation under reduced pressure. The filtrate was weighed (260 mg) and was dissolved in 20 ml of methanol (HPLC grade). The solution was prepared, diluted and 20 µl of this sample solution was injected into the HPLC system.

Preparation of standard solutions

Accurately weighed 10 mg of oleanolic acid was dissolved in 100 ml methanol to produce stock solutions. It was successively diluted to prepare standard solutions of concentration: 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 8.0, 10.and 20µg/ml to give calibration solutions. Aliquots of 20 µm of calibration solutions were analyzed by HPLC. Calibration curves were constructed by plotting the peak area of the standards against their respective concentrations.

RESULTS AND DISCUSSION

Selection of mobile phase

The gradient system was studied for various concentrations of acetonitrile: water, from 90-70% and was converted to isocratic mode. Five mobile systems were tried to find the optimum elution conditions: methanol: water (50:50 %); acetonitrile: water (65:35 %); methanol: water (60:40 %); acetonitrile: water (70:30 %) and acetonitrile: water (75:25 %). The suitability of the solvent system: acetonitrile: water (65:35 %) was decided by cost, sensitivity of the assay and time required for the analysis.

Oleanolic acid showed retention time (Rt) 8.298-8.309 min throughout the study (Fig.1). The capacity factor was greater than one and asymmetry of peak was found to be in the range of 0.95-0.99. The number of theoretical plate was more than 2500 for the entire study. The drug was stable for a period of 72 h at laboratory temperature and under refrigeration in acetonitrile: water (65:35 %) mixture.

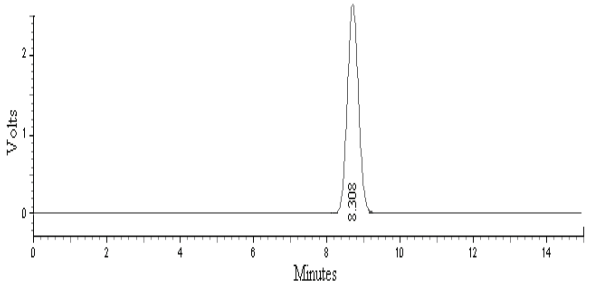

Fig. 1: Chromatogram of oleanolic acid in acetonitrile: water (65:35)

Method validation

Satisfactory linearity for the analysis of oleanolic acid was obtained. It was examined with a series of standard solution in the concentration range of 1-20µg/ml. The response ratio (response factor) was determined by dividing the peak area with the respective concentration. The correlation coefficient 0.9999 was found in the linearity range of 1-20µg/ml. The linearity response ratio for all the concentration was found to be equal. The process parameters are shown in Table 1. The linear regression equations of the calibration curves of oleanolic acid was calculated to be y= 392850x with a correlation coefficient of 0.9999.

Table 1: Area values of oleanolic acid in mobile phase: acetonitrile: water (65:35) at 215 nm using HPLC

Concentration (µg/ml) (X)	Area (Y) (n=3)	Area value by regression	± SD
1.0	403060	392850	10210
2.0	782256	785700	3444
3.0	1175433	1178550	3117
4.0	1563520	1571400	7880
5.0	1954031	1964250	10219
6.0	2398899	2357100	41799
8.0	3110302	3142800	32498
10	3961604	3928500	33104
20	7845325	7857000	11675

Accuracy was determined by standard addition method i.e. A-B/C × 100, where, A is the amount of the detection, B is the amount of sample without added standard, and C is the added amount of the standard.

The preanalyzed samples of oleanolic acid were spiked with the extra percentage (%) of the standard oleanolic acid and the mixtures were reanalyzed by the proposed method. The percentage (%) recovery of samples, percentage relative standard deviation (% RSD), and standard error were calculated at each concentration level. Accuracy studies were done by determining the amount of drug recovered, when known quantity of oleanolic acid was added to the marketed preparation. The % recovery (A-B/C × 100) was in the range of 98.33-99 % which was within the acceptable limit of 98-102% (Table 2).

Table 2: Recovery study of tablet formulation (cystone®)

Initial Amount (mg) [A]	Addition of known quantity (mg) [B]	A+B	Amount recovered (mg)	% Recovery
0.87	1.80	2.67	2.64	98.33
0.87	2.0	2.87	2.85	99.00
0.87	2.5	3.37	3.32	98.00

Precision was determined for repeatability and intermediate precision. Repeatability of sample application was determined at intra-day variation, whereas intermediate precision was determined by carrying out inter-day variation for the determination of oleanolic acid at three different concentration levels of 8, 10 and 20µg/ml. %RSD was found less than ≥2%, which was within the acceptable limit (Tables 3 and 4).

Table 3: Peak area and RSD for determination of system repeatability (intra-day precision)

Concentration (µg/ml)	Area	Mean	SD	RSD%
8.0	3110302	3107014	32498	1.04
	3100365			
	3110376			
10	3961604	3955101	33104	0.83
	3951897			
	3951803			
20	7845325	7838790	11675	0.14
	7835689			
	7835358			

Reproducibility of the method was checked by obtaining precision on a different instrument, which was analyzed by another person in different laboratory. Both intra-day and inter-day precision was calculated at concentration levels of 8 and 10 µg/ml for repeatability and for intermediate precision in triplicates. No significant differences were observed in the % RSD values of intra-day (Table 5) and inter-day precision (Table 6).

Table 4: Determination of intermediate precision-ruggedness

Concentration (µg/ml)	Area	Mean	SD	RSD (%)
8.0	3190343	3179954	32470	1.03
	3185302			
	3164217			
10	3951913			
	3961876	3958824	33104	0.83
	3962684			

Table 5: Reproducibility of the proposed method for repeatability (intra- day) precision

Concentration (µg/ml)	Area	Mean	SD	RSD (%)
8.0	3179636	3176348	30480	0.95
	3169699			
	3179710			
10	3926805	3850702	32450	0.84
	3917098			
	3917004			

Table 6: Reproducibility of the proposed method for intermediate (inter-day) precision

Concentration (µg/ml)	Area	Mean	SD	RSD (%)
8.0	3190312	3160916	32480	1.02
	3172065			
	3120373			
10	3978783	3978975	33104	0.87
	3979243			
	3978899			

The detection limit (DL) was calculated by the following formula: LOD = 3.3σ/S; Where σ = the standard deviation of the response, S= the slop of the calibration curve. The LOD was calculated as 0.098µg/ml.

The quantization limit (LOQ) was expressed as: LOQ = 10σ/S; The LOQ was calculated as 0.297µg/ml,which indicated that the proposed method can be used in wide range for detection and quantification of oleanolic acid effectively. The low values of LOD and LOQ indicate the sensitivity of the proposed method.

Robustness was carried out to evaluate the influence of small but deliberate variation in the chromatographic conditions for the determination of oleanolic acid. Robustness of the method was determined by changing the ratio of acetonitrile and water (65:35 and 63:37). Robustness of method was determined by altering experimental condition like mobile phase composition by 2%. The method was found to be robust and no significant changes were observed. The average %RSD was found to be 0.44% which was within the acceptable limit (Table 7).

Table 7: Determination of robustness by altering the mobile phase composition: acetonitrile:water

Area	Mean	SD	RSD (%)	Area	Mean	SD	RSD (%)
	(65:35)				(63:37)		
782256 781295 785287	782946	3444	0.43	782759 784256 783258	783424	3444	0.44

Quantification of oleanolic acid in root extracts of *A. aspera*

The test samples were injected and chromatogram was obtained in same conditions as that of standard oleanolic acid (Figs. 2-4). The area of the peak corresponding to the Rt of standard oleanolic acid was recorded and content of the same was calculated from the regression equation obtained from calibration curve.

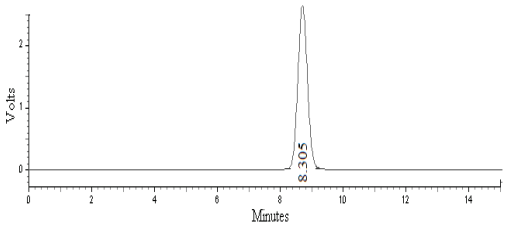

Fig.2: Chromatogram of sample in acetonitrile:water (65:35), (Delhi Region)

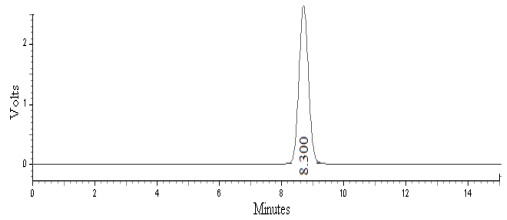

Fig.3: Chromatogram of sample in acetonitrile: water (65:35), (Rajasthan Region)

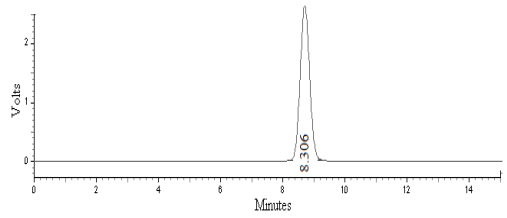

Fig.4: Chromatogram of sample in acetonitrile: water (65:35), (UP Region)

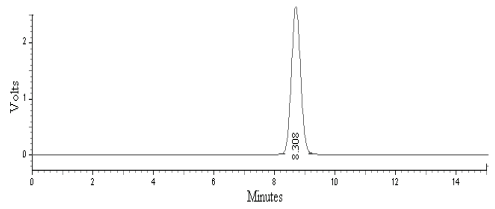

Fig.5: Chromatogram of tablet formulation in acetonitrile:water (65:35) (Cystone®)

The peaks of oleanolic acid from sample solution were identified by comparing their retention times obtained from the peaks with those of standard. HPLC Profile of the 60 % methanolic extract of the *A.aspera* was developed through the same condition as estimation of standard oleanolic acid (Rt 8.300 min). It was observed that extract of *A.aspera* collected from UP zone showed good content of oleanolic acid followed by samples from Rajasthan and New-Delhi regions. The oleanolic acid was quantified in extract of *A.aspera* using regression equation and value was found to be 0.30 %, 0.29 %, and 0.28 % w/w in UP, Rajasthan, and New Delhi samples, respectively (Table 8).

Quantification of oleanolic acid in marketed formulation:

A single HPLC peak was observed at the same retention time in the samples of formulation (fig 5). There was no interaction between the tablets of marketed formulation of *A. aspera* (Cystone®) and other excipients present in the marketed formulations. Oleanolic acid content was found to be 0.33 % w/w. The values of % RSD were also low, which indicated the suitability of this method for the routine analysis of oleanolic acid in marketed formulations (Table 8).

Table 8: Quantification of oleonolic acid by HPLC

Peak area	Root(different regions) and Cystone® extract	Conc. from calibration curve (µg/ml)	Concentration of OA in crude drug (mg/g)	Concentration in percentage (% w/w)
3225327	Delhi	8.2	0.68	0.28
2296768	Rajasthan	5.8	0.58	0.29
3824142	U.P.	9.7	0.77	0.30
4495729	Cystone®	11.4	0.87	0.33

CONCLUSION

An easy method has been developed for the quantitative analysis of the contents of oleanolic acid in *A aspera*. The validation data indicated that the

method is reliable. The developed method was applied for the determination of oleanolic acid in samples obtained from different geographical regions and the comparison has been carried out for the first time. Therefore, this method can be successfully used for the routine analysis of oleanolic acid in both crude drugs and prepared formulations without any interference which can be explored for standardization and quality control of raw materials and marketed herbal products of traditional system of medicine containing roots of *A.aspera* as one of the ingredients.

REFERENCE

1. Ayurvedic Pharmacopoeia of India, Ministry of Health and Family Welfare, Govt of India, New Delhi 1999.
2. Girach RD, Aminuddin A, Khan SA. Ethnomedicinal uses *of A aspera* in Orissa (India). Int J Pharmacog1992; 30: 113-15.
3. Khastgir HN, Sengupta SK, Sengupta P.The sapogenin from seeds of *Achyranthes aspera* Linn. J. Ind Chem Soc 1958; 35: 693-94.
4. Liu J, Liu Y, Mao Q, Klaassen CD. The effects of 10 triterpenoid compounds on experimental liver injury in mice. Fundam Appl Toxicol 1994 22:34-40.
5. Jeong HG. Inhibition of cytochrome P450 2E1 expression by oleanolic acid: hepatoprotective effects against carbon tetrachloride-induced hepatic injury**.** Toxicol Lett 1999; 105: 215-222.
6. Liu J. Pharmacology of oleanolic acid and ursolic acid. J Ethnopharmacol 1997; 49:57-68.
7. Tokuda H, Ohigashi H, Koshimizu K, Ito Y. inhibitory effects of ursolic and oleanolic acid on skin tumor promotion b 12-O-tetradecanoylphorbol-13-acetate. Cancer Lett 1986;33: 279-285.
8. Hsu HY, Yang JJ , Lin CC. Effects of oleanolic acid and ursolic acid on inhibiting tumor growth and enhancing the recovery of hematopoietic system postirradiation in mice. Cancer Lett 1997;111: 7-13.
9. Aparecida Resende F, de Andrade Bareala CA, da Silva Faria MC, Kato FH. Antimutagenicity of ursolic acid and oleanolic acid against doxorubicin-induced clastogenesis in Balb/c mice. Life Sci 2006;79:1268-1273.
10. Ovesna Z, Kozics K, Slamenova D. Protective effects of ursolic acid and oleanolic acid in leukemic cells. Mutat Res 2006; 600: 131-137.
11. Chen JH, Xia ZH, Tan RX. High performance liquid chromatographic analysis of bioactive triterpenes in *Perilla frupescens.* J Phar Biomed Anal 2003; 32: 1175-1179.
12. Zhou C, Chen K , Sun C , Chen Q , Zhang W, Xian Li. Determination of oleanolic acid, ursolic acid and amygdalin in the flower of *Eriobotrya japonica* Lindl by HPLC. Biomed Chromatogr 2007;21: 755-761.
13. International Conference on Harmonization (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use: Harmonized Triplicate Guideline on Validation of Analytical Procedures: Methodology, Recommended for Adoption at Step 4 of the ICH Process on November 1996 by the ICH Steering Committee, IFPMA, Switzerland.