



METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS QUANTIFICATION OF OLEANOLIC ACID AND B-SITOSTEROL FROM ACHYRANTHES ASPERA AND FROM MARKETED FORMULATION BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

*Mansee M. Pathak¹ | Vikas V. Vaidya¹ | Deepal D. Ghadge¹

¹ Department of Chemistry, Ramnarain Ruia College, Matunga, Mumbai-400019, Maharashtra, India. (*Corresponding Author)

ABSTRACT

Objective: To develop simple, accurate, precise and reproducible High Performance Liquid Chromatographic (HPLC) method for simultaneous quantification of Oleanolic acid and β -sitosterol in the whole plant extract of *Achyranthes aspera*.

Methodology: The methanolic extract of the plant powder of *A. aspera* was separated on HPLC column (Phenomenox, Luna® 5 μ m C18, LC Column 150 x 2 mm) with gradient mixture of HPLC grade Acetonitrile and Water as mobile phase. The separated components were detected using Photo Diode Array (PDA) detector at 210 nm.

Results: Linear response was found in the concentration range of 10-300 μ g/mL for β -sitosterol and 20-400 μ g/mL for Oleanolic acid respectively. The relative standard deviation for inter-day and intra-day precision was found to be <2%. The validated method was successfully applied for quantification of the two components in a commercially available polyherbal formulation "Cystone" containing *A. aspera* extract.

Conclusion: A precise, accurate and reproducible HPLC method is developed and validated for simultaneous quantification of two bio-active components from *A. aspera*. The method can be used as quality control tool in standardization of raw materials and marketed formulations.

KEY WORDS: HPLC, Oleanolic acid, β -sitosterol.

INTRODUCTION

The overuse of synthetic drugs with impurities resulting in higher incidence of adverse drug reactions and development of immunity to these drugs in more advanced communities have motivated mankind to go back to nature for safer remedies. It is being globally recognized that medicinal plants play a significant role in providing health benefits to human beings. The increase in the demand for plant based medicines and their eventual commercialization has brought about a major shift in their status from providing strictly individually tailored hand-picked plant products based health care to availability of over the counter health products.

Validation of analytical methods is mandatory in implementing a quality control system in any analytical laboratory. It provides an assurance of reliability during normal use and can be referred as a process of providing documented evidence of quality for several herbal and traditional drugs. Separation techniques such as chromatography and electrophoresis have been extensively used for quality control of herbal medicine because of their high efficiency and speed.^[3]

Achyranthes aspera commonly known as prickly chauff flower or apamarga is a species of plant found in amaranthaceae family. It is distributed throughout the tropical world. The plant is reported to have several medicinal properties and used as purgative, diuretic, antimalarial, antihyperlipidemic, anti-inflammatory, antispasmodic, antibacterial, and antiviral agents in traditional systems of medicine.^[20,16] In addition, Traditional healers claim that addition of *A. aspera* would enhance the efficacy of any drug of plant origin and is therefore used in numerous commercially available herbal formulations and dietary supplements.^[14]

Scientists have identified different components of *A. aspera* to be responsible for its activity. Chemical investigations of the seeds of *Achyranthes aspera* reported the isolation & identification of Saponins and oleanolic acids.^[21,5,6] Ecdysterone was isolated from the methanolic extract of roots of *Achyranthes aspera* by chromatography on silica gel column.^[7,8] β -sitosterol was isolated from the shoots of the plant.^[9] β -sitosterol, the principal phytosterol appears to have important immunomodulatory activity in human and animal physiology. Oleanolic acid has been reported to have anti-inflammatory activity.^[10] The phytosterol- β -sitosterol has been reported to have anti-inflammatory and anti-pyretic activity.^[11]

In view of these wide therapeutic effects a need was felt for simultaneous quantitation of oleanolic acid and β -sitosterol in the plant of *A. aspera*. Literature survey revealed that HPLC method for quantification of Betaine from *A. aspera* is available.^[12] Also, HPLC and HPTLC methods have been reported for estimation of Oleanolic acid from the extracts of *A. aspera*.^[13,14] However, no HPLC method has been reported for simultaneous estimation of Oleanolic acid and β -sitosterol from the extracts of *A. aspera*.

In this research work, a simple, precise and accurate HPLC method has been established for simultaneous quantitation of oleanolic acid and β -sitosterol in the

dried plant powder of *A. aspera*. Further, the proposed method has been validated as per ICH guidelines and applied as a quality control tool for standardization of a commercially available polyherbal formulation containing plant extract of *A. aspera*. Such a study would not only facilitate standardization of the raw material and commercial products but also facilitate future pharmacological studies and quality control.

MATERIALS AND METHODS

Chemicals

HPLC grade methanol, acetonitrile and water were procured from E.Merck, Mumbai, India. Reference standards of Oleanolic acid (purity >97%) and β -sitosterol (purity >97%) were purchased from Sigma-Aldrich Chemie (Aldrich Division; Steinheim, Germany).

Plant material

Plant specimen of *A. aspera* was collected from Mumbai. Herbarium samples of *A. aspera* was prepared and authenticated by Botanical Survey of India (BSI), Pune, India. A voucher specimen numbered MP-01 has been retained in the herbarium section of BSI, Pune for future reference. The whole plant was washed with water to remove any dust particles, dried in shade, powdered and then sieved through BSS mesh size 85 and stored at 25°C in an airtight container.

Polyherbal formulation "Cystone tablets, Himalaya, India" were procured from Mumbai.

Preparation of stock solutions

Preparation of stock (A) solution of Oleanolic acid (500 μ g/mL)

A stock solution of Oleanolic acid (500 μ g/mL) was prepared in methanol. 50.0 mg of standard Oleanolic acid was accurately weighed and transferred to 100.0 mL standard volumetric flask. The contents of the flask were initially dissolved in about 35.0 mL of methanol, followed by sonication and then diluted up to the mark with methanol.

Preparation of stock (B) solution of β -sitosterol (1000 μ g/mL)

A stock solution of β -sitosterol (1000 μ g/mL) was prepared in methanol. 50.0 mg of standard β -sitosterol was accurately weighed and transferred to 50.0 mL standard volumetric flask. The contents of the flask were initially dissolved in about 35.0 mL of methanol, followed by sonication and then diluted up to the mark with methanol.

Sample Preparation

About 2 gm of dried whole plant powder of *A. aspera* was weighed into a round bottom flask. 30 mL of methanol was added to the flask and the mixture was refluxed on a boiling water bath for about 30 min. The extract was then filtered through Whatman filter paper no. 41 (E. Merck, Mumbai, India). The same procedure was performed twice and filtrate obtained was combined together and made up to 100 mL with methanol. This solution was used for assay.

Dietary supplement: For analysis of 2 gm polyherbal formulation Cystone tablets containing *A.aspera* was accurately weighed into a round bottom flask. 30 mL of methanol was added to the flask and the mixture was refluxed on a boiling water bath for about 30 min. The extract was then filtered through Whatman filter paper no. 41 (E. Merck, Mumbai, India). The same procedure was performed twice and filtrate obtained was combined together and made up to 100 mL with methanol. This solution was further used for assay.

Chromatography

HPLC was performed on Shimadzu UFLC Prominence system. The system was equipped with binary pumps (LC – 20 AD), autosampler (SIL – 20 AC HT) and Degasser (DGU – 20 A₃). The column oven (CTO – 20 AC) temperature was maintained at 40°C throughout the analysis. The detection was carried out using SPD – M20 A photo diode array detector.

The method involves use of a Phenomenex, Luna® 5 µm C18, LC Column 150 x 2 mm as stationary phase. Different compositions of solvents were tried as mobile phase in both isocratic and gradient mode. Finally a gradient of acetonitrile: water was selected which gave a good resolution between the sample components. The concentration of acetonitrile was increased from 20% to 75% in the first five minutes and was maintained at the same level till 7 minutes. The concentration of the organic solvent was then increased to 100% in 10 minutes and was maintained at the same level till 18 minutes to allow complete elution of the components of plant sample from the column. Passing of 100% organic solvent ensured that no traces of the plant or formulation sample remained in the column which might have otherwise interfered with the next sample analysis and also may decrease the life of the column. The flow rate was maintained at 1 mL/min and the separated components were detected at 210 nm.

Validation of the Method

ICH harmonized tripartite guidelines were followed for the validation of the developed analytical method [18].

Selectivity and Specificity: During the experiments an UV scan ranging from 200 to 400 nm in the time window of the analytes using PDA detector was performed with the aim of revealing eventual interfering compounds and evaluating the selectivity of the method. Specificity of the intended method was established by comparing the HPLC retention time and absorption spectra of target peaks from the analysed samples with those of the reference compounds.

System suitability: System suitability experiment was performed by injecting six consecutive injections (100 µg/mL) of each bioactive marker, namely Oleanolic acid and β-sitosterol during the start of the method validation. Values with % CV of ≤2% were accepted.

Calibration curves: Linearity of the components was determined in triplicate at nine different concentrations for Oleanolic acid and β-sitosterol. Calibration curve was plotted as mean peak area versus concentration. The linear regression equation was obtained using a least-square method and used to estimate the concentration of the three components in the analysed samples. RSD of standard peak areas for solutions of the same concentration were less than 2%, indicating there was no statistically significant variation.

Limit of Detection (LOD) and Limit of Quantification (LOQ): Sensitivity of the method was evaluated by determining the values of LOD and LOQ. Stock solution of each standard was serially diluted with methanol to prepare the series of samples with least concentration and injected into the HPLC system. The limit of detection (LOD) and quantification (LOQ) for Oleanolic acid and β-sitosterol were determined by measuring the signal to noise ratio (S/N). LOD and LOQ was considered at S/N of 10:1 and 3:1 respectively.

Precision: Variability of the method was studied by analyzing quality control samples of Oleanolic acid (25, 50, 150 µg/mL) and β-sitosterol (50, 100, 200 µg/mL) on the same day (intra-day precision) and on different days (inter-day precision) and the results were expressed as % RSD. Accuracy values within the range of 85 – 115% and % CV of ≤2% were accepted.

Recovery: Recovery tests were carried out to further investigate the accuracy of the method by adding three different concentration levels of the mixed standard solutions to known amounts of *A.aspera* samples and dietary supplement prior to extraction. The resultant samples were then extracted and analyzed with the described method. The mean percentage recoveries were calculated using the formula: Recovery (%) = [(amount found – original amount) / amount added] x 100. Values within the range of 85 – 115% were accepted.

Ruggedness: Ruggedness of the method was studied by determining the effects of small variations of mobile phase composition (±2%), and flow rate (1.00 ± 0.05 mL/min). Effect of these deliberate changes on the response (area) and retention time of QC samples of Oleanolic acid and β-sitosterol was observed during the analysis. The results were expressed in terms of % mean difference. Values within a difference range of ±5% were accepted.

Stability: The stability of the stock solutions of all the two standards was evaluated by storing the solutions in refrigerator at 2-8°C for 72 hours and then com-

paring the results against freshly prepared stocks for each standard. Samples in triplicate were also subjected to bench top stability at 0.0 h and 12.0 h respectively. Values within a difference range of ±5% were accepted.

Estimation of Oleanolic acid and β-sitosterol in *A.aspera* plant powder and dietary supplement containing *A.aspera* plant powder.

The extract of *A.aspera* and Cystone tablets were injected separately and analysed using the optimized chromatographic conditions. Peak areas were recorded for each analyte of interest and the amount of all the three analytes (corosolic acid, asiatic acid and β-sitosterol) was calculated by use of the calibration plot.

RESULTS AND DISCUSSION

Optimization of the Chromatography

Initial trial experiments were conducted to select a suitable mobile phase for accurate analysis of the standards. Of the various mobile phases tried in both isocratic and gradient mode, acetonitrile:water in gradient mode gave the best resolution between the two analytes Oleanolic acid and β-sitosterol. These standards were also resolved from other components present in the sample extract enabling simultaneous quantification. HPLC chromatograms corresponding to the two standards, *A.aspera* plant extract and marketed formulation are represented in Figure 1 to 3. All the analytes exhibited the UV maximum absorption at 210 nm and hence this wavelength was chosen for the detection of components eluting out from the column.

Method Validation

Selectivity and Specificity: During the UV scan no appreciable difference was found in the spectra of reference standards and the analysed samples. Hence, the method demonstrated a high degree of selectivity.

System suitability: System suitability tests are used to verify whether the resolution and reproducibility of the chromatographic system are adequate for the analysis. For Oleanolic acid and β-sitosterol the % CV values for area and retention time was found to be < 2% indicating that the system was suitable to carry out further analysis.

Linearity: The method was found to be linear from 20-400 µg/mL for Oleanolic acid and 10-300 µg/mL for β-sitosterol respectively. The correlation coefficient was found to be ≥0.995 for the two components. Results of regression analysis are summarized in Table 1.

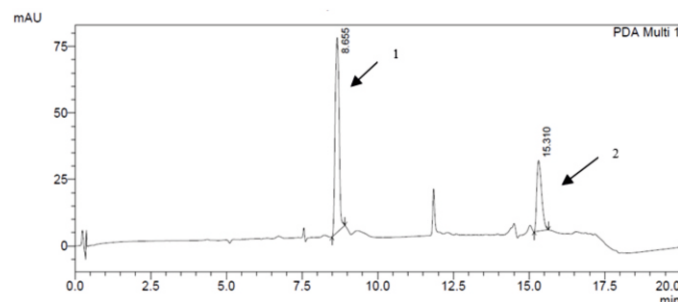


Fig. 1: Typical HPLC chromatogram of mixed standards

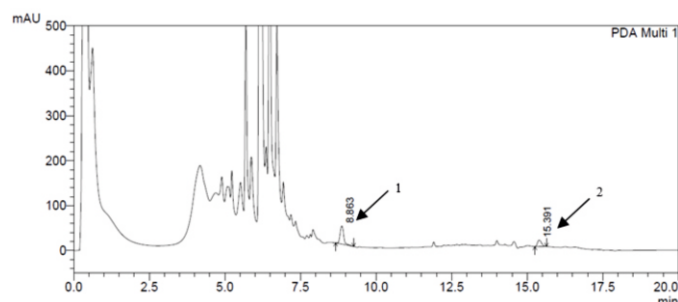


Fig. 2: HPLC chromatogram of plant extract of *A.aspera*

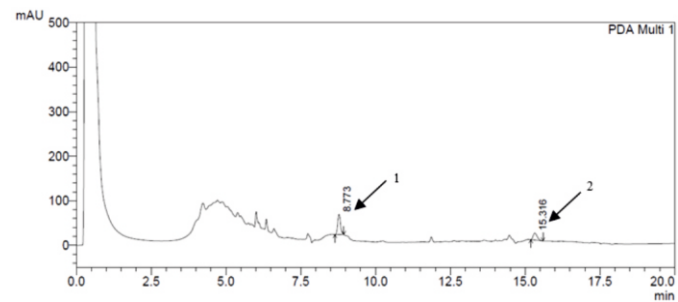


Fig. 3: HPLC chromatogram of marketed formulation "Cystone Tablets"

Key: 1 – β sitosterol; 2 – Oleanolic acid

Sensitivity: Sensitivity of the method was affirmed in terms of LOD and LOQ for Oleanolic acid and β -sitosterol respectively. The results are represented in Table 1. The values for both LOD and LOQ were low when compared to other published works,^[15,16,19] which indicated that the method is capable of detecting and quantifying trace amount of the two components in plant samples.

Precision: In the repeatability study intra-day and inter-day precision of the HPLC method were investigated using replicate injection ($n=3$) of quality control samples of the two standards. The developed method was found precise with % CV < 2%.

Stability: Stock solution stability study of the two standards stored for the period of 72 hours at 2-8°C showed % CV < 2% with the % mean difference within $\pm 5\%$. During bench top stability study, similar results were obtained. Stability studies showed that Oleanolic acid and β -sitosterol were found stable for at least 12.0 h at room temperature and 72 hours at 2-8°C of storage condition.

Recovery: The recovery values for the two components were within acceptable limits (85.0 to 115.0%). This indicated that the method was reliable and accurate.

Ruggedness: Proposed method was not influenced by the factors considered for ruggedness study. Change in flow rate and mobile phase composition affected the retention time of the two analytes but the results were satisfactory since % CV was < 2% with % mean difference < 5%.

Assay: The assay value for samples of *A. aspera* plant powder was found to be 0.14% and 0.08% for Oleanolic acid and β -sitosterol respectively, while for the dietary supplement it was found to be 0.16 % and 0.11% for Oleanolic acid and β -sitosterol respectively. The method is specific for the two components because it resolved all standards well in the presence of other phytochemicals in *A. aspera*. The proposed HPLC method was found to be suitable for qualitative and simultaneous quantitative analysis of oleanolic acid and β -sitosterol in the methanolic extract of *A. aspera*.

Table 1: The regression data, linearity, LOD and LOQ and retention times for two bioactive compounds by HPLC

Com-pounds	Reten-tion time	Regression equation	r ²	Linear range ($\mu\text{g/mL}$)	LOD ($\mu\text{g/mL}$)	LOQ ($\mu\text{g/mL}$)
Oleanolic acid	8.655	$y = 193.54x + 84.514$	0.9998	20-400	10.0	20.0
β -sitosterol	15.310	$y = 285.03x + 347.9$	0.9993	10-300	5.0	10.0

CONCLUSION

A precise, accurate and reproducible HPLC method is validated for simultaneous quantification of two bioactive markers Oleanolic acid and β -sitosterol. Proposed HPLC method can be used as an analytical tool for quality evaluation of plants and formulations containing Oleanolic acid and β -sitosterol as chemical markers. It is an efficient method to screen *A. aspera* plant samples in order to assess its quality and authenticity. Hence, it can be demonstrated that HPLC is a powerful practical tool for comprehensive quality control of plant raw materials and its formulations.

REFERENCES

1. ICH Harmonised Tripartite Guideline, Validation of Analytical Procedures: Text and Methodology Nov. 2005; Q2 (R1).
2. Agharkar SP. Medicinal plants of Bombay presidency, PBI, Scientific Publishers, Jodhpur (India), 1991, 230.
3. Pagliarussi R., Freitas L., Bastos J., A quantitative method for the analysis of xanthine alkaloids in Paullinia cupana (guarana) by capillary column gas chromatography. J. Sep Sci 2002;25:371-378.
4. Girach RD, Aminuddin A, Khan SA. Ethnomedicinal uses of *A. aspera* in Orissa (India). Int J Pharmacog 1992; 30: 113-115.
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. A. Banerji, M.S. Chadha. Phytochemistry, 1970, 9(7), 1671.
8. Wadhwa V, Singh MM, Gupta DN, Singh C, Kamboj VP. Contraceptive and hormonal properties of *Achyranthes aspera* in rats and hamsters. Planta Medica, 5, 1986, 231-233.
9. Pagliarussi R., Freitas L., Bastos J., A quantitative method for the analysis of xanthine alkaloids in Paullinia cupana (guarana) by capillary column gas chromatography. J. Sep Sci 2002; 25:371.
10. V. Hariharan, S. Rangaswami. Phytochemistry, 1970, 9, 409-414.
11. T.N. Misra, R.S. Singh, H.S. Pandey, C. Prasad, S. Singh. Indian Journal of Chemistry - Section B Organic and Medicinal Chemistry, 1996, 35B(6), 637-639.

12. R. Ikan, U. Ravid, D. Trosset, E., Shulman. Experientia, 1971, 27(5) 504-505.
 13. Banerji, M.S. Chadha. Phytochemistry, 1970, 9(7), 1671.
 14. C. P. Khare; 2008 Indian Medicinal Plants – An Illustrated Dictionary; Springer.
 15. Am. J. PharmTech Res. 2013; 3(4) ISSN: 2249-3387 Method Development and Validation for Quantification of Betaine in *Achyranthes aspera* by HPLC VB Krishna1, Naira Nayeem1*
 16. Inter. J. of Phytotherapy / Vol 4 / Issue 1 / 2014 / 16-21 Validated chromatographic method for the determination of Oleanolic acid in
- Achyranthes aspera* Linn. B.N. Suhagia, I.S. Rathod, S.A. Shah, Sindhu Sunil*
17. International Journal of Pharmacy and Pharmaceutical Sciences Vol 2, Suppl 1, 2010 A validated HPLC method for the quantification of Oleanolic acid in the roots of *Achyranthes aspera* Linn. And marketed formulation Vidhu Aeri, M.I. Khan, S. AlaM.
 18. Ayurvedic Pharmacopoeia of India, Ministry of Health and Family Welfare, Govt of India, New Delhi 1999.
 19. A.K. Gupta, Neeraj Tondon, Madhu Sharma, Quality standards of Indian medicinal plants, vol 2; 261, Indian council of medicinal research, New Delhi 2005.
 20. *Achyranthes aspera*-An important medicinal plant: A review Saurabh Srivastav1*, Pradeep Singh1, Garima Mishra1, K. K. Jha1, R. L. Khosla2 J. Nat. Prod. Plant Resour., 2011, 1(1):1-14.