Research Paper E-ISSN No : 2455-295X | Volume : 2 | Issue : 6 | June 2016

SEPERATION AND QUANTIFICATION OF PHARMACOLOGICALLY ACTIVE MARKERS OLEANOLIC ACID, 20-HYDROXYECDYSONE AND B-SITOSTEROL FROM ACHYRANTHES ASPERA AND FROM MARKETED FORMULATION BY HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY

*Mansee M. Pathak [1] | Vikas V. Vaidya [1] | Saurabh H. Patil [1]

[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 Thin Layer Chromatographic (HPTLC) method for simultaneous quantification of Oleanolic acid, 20-hydroxyecdysone and β-sitosterol in the whole plant extract of Achyranthes aspera.

Methodology: In the present work a precise, accurate and reproducible HPTLC method is developed and validated for simultaneous quantification of three pharmacologically active markers Oleanolic acid, 20-hydroxyecdysone and β-sitosterol from the whole plant of Achyranthes aspera and from marketed Cystone® tablets of Himalaya herbal healthcare. These markers were extracted from the whole plant and from Cystone® tablets using Soxhlet extraction followed by chromatographic separation on HPTLC silica gel 60 F254 pre-coated plates using double development method. The analysis was carried out using a double development method. The first development up to 50 mm was carried out using a mixture of dichloromethane- ethanol (8:2) (v/v) as mobile phase and the second development up to 80 mm using a mixture of petroleum ether- ethyl acetate- methanol (8.5:1:1) (v/v/v), as mobile phase with chamber saturation of 20 minutes. Quantification was carried out at 540 nm for all the three markers.

Results: Linear responses for Oleanolic acid, 20-hydroxyecdysone and β-sitosterol were obtained over the concentration ranges of 20-400 µg/mL , 40-400 µg/mL and 40-400 µg/mL respectively.

Conclusion: This HPTLC method can be used as a quality control tool for quantification of these markers simultaneously from raw material as well as marketed formulation.

KEY WORDS: HPTLC, Oleanolic acid, 20-hydroxyecdysone , β-sitosterol.

INTRODUCTION

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. 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. [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 reprted 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 HPTLC method has been reported for simultaneous estimation of Oleanolic acid, 20-hydroxyecdysone 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*.

This HPTLC method can thus help to check for the adulteration in the raw material, as well as serve as a quality control tool for quantification of these markers simultaneously from raw material as well as marketed formulation.

MATERIALS AND METHODS

Chemicals

HPLC grade ethanol, ethyl acetate and methanol were procured from E.Merck, Mumbai, India. Analytical grade petroleum ether and dichloromethane were procured from RFCL Limited (RANKEM) Mumbai, India.

Reference standards of Oleanolic acid (purity >97%), β-sitosterol (purity >97%) and 20-hydroxyecdysone 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 solution (A) of β-sitosterol (1000 µg/mL)

A stock solution of β-sitosterol (1000 µg/ 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 15.0 mL of methanol, followed by sonication and then diluted up to the mark with methanol.

Preparation of stock solution (B) of 20-hydroxyecdysone (1000 µg/mL)

A stock solution of 20-hydroxyecdysone (1000 µg/ mL) was prepared in methanol. 50.0 mg of standard 20-hydroxyecdysone was accurately weighed and transferred to 50.0 mL standard volumetric flask. The contents of the flask were initially dissolved in about 15.0 mL of methanol, followed by sonication and then diluted up to the mark with methanol.

Preparation of stock solution (C) of Oleanolic acid (500 µg/mL)

A stock solution of Oleanolic acid (500 µg/ 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 70.0 mL of methanol, followed by sonication and then diluted up to the mark with methanol.

Sample Preparation

About 2 gm of dried 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 Whatmann 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 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 Whatmann 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.

Chromatographic procedure

The stationary phase was HPTLC pre-coated silica gel aluminum plate 60F254 with 250µ thickness, prewashed with methanol. The sample application was performed as per the chromatographic conditions mentioned in table 1. This plate was now derivatised using Anisaldehyde sulphuric acid. This was followed by detection at 540 nm for all the three markers (refer Figure 1).

Validation of the Method

ICH harmonized tripartite guidelines were followed for the validation of the developed analytical method [18]. The summary of the validation parameters have been tabulated in Table 2.

Specificity: Specificity was ascertained by analyzing standard compounds and samples. The bands from sample solutions were confirmed by comparing the Rf and spectra of the bands to those of the standards. The peak purity of all the compounds was analyzed by comparing the spectra at three different levels, i.e. start, middle, and end positions of the bands.

Inter: Day and Intra-Day Precision: Variability of the method was studied by analysing quality control samples of β-sitosterol (40, 100, 300 µg/mL), 20-hydoxyecdysone (40, 100, 300 µg/mL) and Oleanolic acid (20, 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.

Calibration curves: Linearity of the components was determined in triplicate at six different concentrations for Oleanolic acid, 20-hydroxyecdysone 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. LOD and LOQ were calculated using the formula

$$LOD = 3.3\ \sigma/s \qquad LOQ = 10\ \sigma\ /S$$

Where σ = Standard deviation of the responses of calibration curve S = Slope of the calibration curve

Recovery: The accuracy of the method was assessed by performing recovery study at three of different levels (80, 100 and 120%, spiking Oleanolic acid, 20-hydroxyecdysone and β-sitosterol in plant matrix and market formulation). The percent recovery and the average percent recovery for each were calculated.

Robustness: The robustness of the method was studied, during method development by determining the effects of small variation of mobile phase composition (±2%), duration of plate pre-washing, chamber saturation period, development distance and scanning time (10% variation of each). No significant change in Rf or in response of β-sitosterol, 20-hydroxyecdysone and Oleanolic acid were observed, indicating the robustness of the method.

Stability: The stability of the master stocks of all the three standards was evaluated by storing the stocks in refrigerator at 2-8°C for 72 hours. This was followed by comparing these concentrations of these stocks against freshly prepared stocks for each standard.

Table 1: Instrumentation and Chromatographic Conditions

Parameters	Description
Stationary phase	Silica gel 60 F254 pre-coated on aluminum sheet
Mobile phase	1st development upto 50mm Dichloromethane: Ethanol (8:2)(v/v) 2nd development upto 80mm Petroleum ether: Ethyl acetate: Methanol (8.5:1:1)(v/v/v)
Prewashing of plate	Methanol and activated at 100°C for 15 min
Development Chamber	CAMAG Twin Trough Chamber
Chamber Saturation	20 min.
Sample Applicator	CAMAG LINOMAT V
Application Volume	10 µl
Band	8 mm
Space	7 mm
Speed	0.5 µL/sec
Development Distance	8.0 cm
Drying of plate	At 110oC for 5 min
Densitometric scanner	CAMAG TLC SCANNER III
Derivatization	Anisaldehyde sulphuric acid
Lamp	Tungsten
Wavelength	540 nm
Chromatograhic evaluation	CAMAG TLC software winCATS3

RESULTS AND DISCUSSION

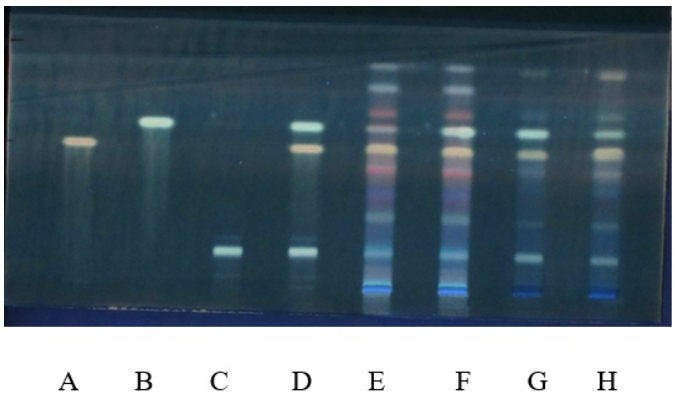

Figure1: Photograph of developed TLC plate

A: Standard Oleanolic acid
B: Standard β-sitosterol
C: Standard 20-hydroxyecdysone
D: Mixture of standards
E, F: Methanolic extract of whole plant powder of A.aspera
G,H: Methanolic extract of formulation containing extracts of A.aspera (Cystone tablets)

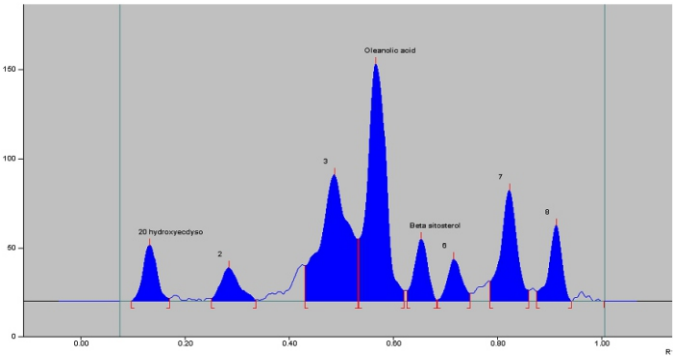

Figure 2: A typical densitogram of plant of Achyranthes aspera

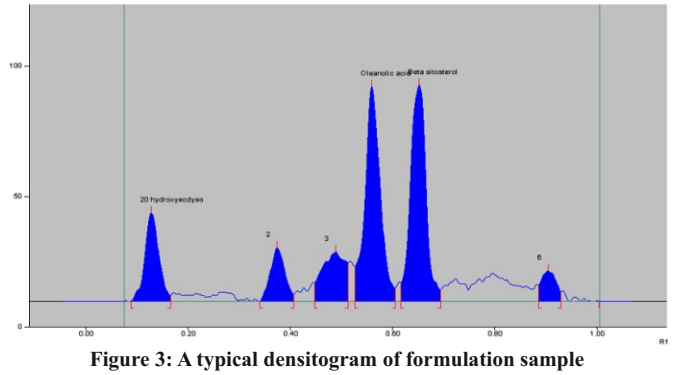

Figure 3: A typical densitogram of formulation sample

Different mobile phases containing various ratios of dichloromethane, petroleum ether ,ethanol, methanol, ethyl acetate, were tried. Among the different solvents systems investigated, a double development method with mobile phase Dichloromethane: Ethanol (8:2) (v/v) upto 50 mm and second development with mobile phase Petroleum ether: ethyl acetate: methanol (8.5:1:1) (v/v/v) upto 80 mm demonstrated compact spots with typical Gaussian shaped peaks for β-sitosterol, 20-hydroxyecdysone and Oleanolic acid with good resolution between other peaks of the extract. The above mobile phase composition enabled the peak shape and elution of the component. Also in this separation, the UV detector gave good spectra of the separated components. The detection wavelength was confirmed at 540 nm.

The method was validated for linearity, precision, specificity, recovery, robustness and stability. The method was found to be linear from 40-400 µg/mL for β-sitosterol and 20-hydroxyecdysone and from 20-300 µg/mL for Oleanolic acid. The correlation coefficient was found to be ≥0.99 for all the three components. The precision (%RSD) of the method was found to be ≤ 2%, indicating that the proposed method is precise. The recovery values for all the three components were within acceptable limits (90.0 to 110.0%). Solution stability were evaluated by monitoring the peak area response. Standard solutions were analysed right after its preparation and after 72 hrs. There was no significant change (% RSD ≤ 2%) in the Rf and area values of standard peak.

Table 2: Summary of method validation parameters

Parameter		β-sitosterol	20-hydroxyecdysone	Oleanolic acid
Specificity		Specific	Specific	Specific
Linearity(µg/ml)		40-500	40-500	20-300
Correlation coeff		0.9983	0.9998	0.9983
LOD (µg/mL)		12.88	13.21	6.32
LOQ (µg/mL)		40.34	40.16	20.36
Precision (RSD)		≤ 2 %	≤ 2 %	≤ 2 %
Assay	Plant	0.09%	0.90%	0.18%
	Formulation	0.12%	1.02%	0.22%
Stock soln. stability (2-8°C)		Stable till72hrs	Stable till 72hrs	Stable till 72hrs
Robustness		Robust	Robust	Robust

CONCLUSION

A precise, accurate and reproducible HPTLC method is validated for simultaneous quantification of three pharmacologically active markers 20-hydroxy ecdysone, Oleanolic acid and β-sitosterol. This HPTLC method can aid in confirming adulteration in the raw material as well as serve as a quality control tool for quantification of these markers simultaneously from raw material as well as marketed formulation.

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 Pharmacog1992; 30: 113-115.
5. Jeong HG. Inhibition of cytochrome P450 2E1 expression by oleanolic acid: hepatoprotective effects against carbon tetrachlorideinduced hepatic injury. Toxicol Lett 1999; 105: 215-222.