A rapid and simple high performance thin layer chromatographic method for simultaneous analysis of β-sitosterol-D-glucoside, gallic acid, withaferin A and withanolide A in *Ashvagandharishta*

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ABSTRACT

A new, simple, precise, accurate and robust high-performance thin-layer chromatographic (HPTLC) method has been developed for simultaneous estimation of β-sitosterol-D-glucoside, gallic acid, withaferin A and withanolide A in an Ayurvedic formulation, Ashvagandharishta. The plates were developed in mobile phase consisting of toulene: ethyl acetate: formic acid: methanol (6:3:0.1:0.6, v/v/v/v). The retention factors of β-sitosterol-D-glucoside, gallic acid, withaferin A and withanolide A were 0.18 ± 0.01, 0.26 ± 0.02, 0.37 ± 0.02 and 0.57 ± 0.02, respectively. Linearity was in the range of 50-400 ng/band for β-sitosterol-D-glucoside, 150-500 ng/band for gallic acid, 50-450 ng/band for withaferin A and 50-500 ng/band for withanolide A. Accuracy study showed recoveries of 99.04 - 102.67, 98.77 - 101.96, 99.64 - 102.67 and 100.07 - 101.96 % for β-sitosterol-D-glucoside, gallic acid, withaferin A and withanolide A, respectively. The results obtained in validation assays indicated accuracy and reliability of HPTLC method for simultaneous quantification of β-sitosterol-D-glucoside, gallic acid, withaferin A and withanolide A in Ashvagandharishta. The percent content of β-sitosterol-D-glucoside, gallic acid, withaferin A and withanolide A in Ashvagandharishta were found to be 0.012, 0.064, 0.016 and 0.312 %, respectively.

INTRODUCTION

Last few decades have seen a tremendous increase in the use of traditional medicines globally (Patwardhan *et al.,* 2005; Raj *et al.,* 2011; Patwardhan and Vaidya *et al.,* 2009). According to an estimate about 80% of population the world over depends on traditional medicines (Bodekar *et al.,* 2005; Kunle *et al.,* 2012; Farnsworth *et al.,* 1985). Ayurveda, the system of Indian medicines is one of the best known ancient disciplines throughout the world and well-known for poly-herbal formulations (Anonymous, 2008). Poly-herbalism in Ayurveda is of a peculiar view, although it is challenging to explain it in terms of modern parameters (Yadav *et al.,* 2008; Klein *et al.,* 2013). Ayurvedic literature vouches for the phenomenon of synergism behind polyherbal formulations and admired for its clinical prophesies (Wagner *et al.,* 2009). However, the concerns linked with quality of the formulations have become an important issue, considering the increasing demand and large scale production of formulations (Mosihuzzaman *et al.,* 2008). It is also a pressing need to abide by modern standards of evaluation using rapid, economic and reliable methods of evaluation (Patwardhan *et al.,* 2003; Fabricant and Farnsworth *et al.,* 2001).

The key challenge in integrating Ayurvedic medicines with the current clinical practice is lack of scientific data and better understanding of efficacy and safety of the herbal formulations. The need of the hour is to evolve a systematic approach and to develop well-designed methodologies for standardization of raw material as well as herbal formulations (Vaidya *et al.,* 2003; Marcus and Grollman *et al.,* 2002).

Ashvagandha (*Withania somnifera*) is one of the most important herbs that have long been considered as an excellent rejuvenator and immunomodulatory agent(Sharma *et al.,* 1999; Chatterjee and Pakrashi, 1995; Bone *et al.,* 1996). Several formulations are prepared using Ashvagandha (Anonymous *et al.,* 2008). It is also scientifically proven that Ashvagandha extract has

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strong antioxidant and anti-inflammatory effect attributed mainly to the biologically active compounds such as alkaloids (ashwagandhin, cuscohygrine anahygrine and tropine) and steroids (β-sitosterol-D-glucoside, withaferin A, withasomniferin-A, withasomidienone, withasomniferols A-C and withanone) (Rastogi and Mehrotra *et al.,* 1998; Abraham *et al.,* 1968; Ali *et al.,* 1997). We earlier have prepared *Ashvagandharishta*, a fermented formulation, and have assessed it with different physicochemical parameters such as physical appearance, pH, total reducing sugar, self-generated alcohol, specific gravity and presence of methanol. Its biological action on diseased rat model also has been checked (Bhondave *et al.,* 2014). In the present communication, simple, precise and rapid HPTLC method for simultaneous estimation of β-sitosterol-D-glucoside, gallic acid, withaferin A and withanolide A from *Ashvagandharishta* has been described. Previously, different methods have been reported for HPTLC analysis for individual estimation of β-sitosterol-D-glucoside, gallic acid, withaferin A and withanolide A (Jirge *et al.,* 2010; Jirge *et al.,* 2011; Pathak *et al.,* 2004; Sharma *et al.,* 2007; Shinde *et al.,* 2011). To the best of our knowledge this is the first report on a method for concurrent analysis and estimation of these marker compounds.

MATERIALS AND METHODS

Solvents and chemicals

Standard β-sitosterol-D-glucoside, gallic acid, withaferin A, and withanolide A were procured from Natural Remedies, Bangalore, India. All chemicals and reagents used in the study were of analytical grade and purchased from Merck specialties Pvt. Ltd. Mumbai, India. Double distilled water was used in the present research study. Raw material for *Ashvagandharishta* was purchased from Green Pharmacy, Pune, Maharashtra, India. All raw materials procured were of quality mentioned in the Ayurvedic Pharmacopoeia of India. All the herbal material was authenticated in the Department of Dravya Guna Vigyan, College of Ayurved, Bharati Vidyapeeth Deemed University, Pune, India.

Ashvagandharishta formulation using consortium of the yeasts isolated from *Woodfordia fruticosa*

Ashvagandharishta is a fermented liquid preparation wherein traditionally, fermentation process relies on use of dried *W. fruticosa* flowers presumed to be a source of wild yeasts. We previously have isolated, identified and characterized six yeast cultures from flowers of *W. fruticosa*, and have demonstrated their importancein fermentation process of Asava/Arishta (Bhondave *et al.,* 2014). *Ashvagandharishta* prepared using consortium of yeast in the present communication wherein simultaneous estimation of β-sitosterol-D-glucoside, gallic acid, withaferin A and withanolide A was attempted. *Ashvagandharishta* (50 mL) was vacuum dried to remove the self-generated alcohol. Distilled water (50 mL) was added to dissolve the extract. Extract was then partitioned successively with n-hexane (3 x 50 mL) followed by chloroform (3 x50 mL). The chloroform extracts were combined, dried over anhydrous sodium sulphate and evaporated under vacuum. The extract was weighed and dissolved in chloroform ($CHCl_3$) for further analysis.

HPTLC instrumentation and chromatographic conditions

HPTLC analyses were carried out on precoated pre-washed and activated silica gel aluminium HPTLC plates $60F_{254}$ (20 cm × 10 cm with 250 μm thickness, in the form of bands of 6 mm width with a Camag syringe (100 μL) and Camag Linomat V (Switzerland) sample applicator. The slit dimension were kept at 5mm × 0.45 mm with 10 mm/s scanning speed. HPTLC plate was then developed at constant temperature with 20 mL mobile phase consisting of toulene: ethyl acetate: formic acid: ethanol (6:3:0.1:0.6, v/v/v/v). Linear ascending development was carried out in 20 cm x 10 cm twin trough glass chamber (Camag, Muttenz, Switzerland) saturated with the mobile phase. The chamber saturation time for mobile phase was 15 min at room temperature (25±2 °C) at relative humidity of 60±5%. Based on the current literature survey, several derivatizing agents have been examined such as 10% sulphuric acid, vanillin-sulphuric acid reagent, anisaldehyde sulphuric acid etc. Out of those 5% sulphuric acid in ethanol reagent showed promising and reproducible results with chromophore stability for 25 min and hence was selected for derivatization. It was prepared by mixing 5 mL conc. sulphuric acid in 70 mL methanol in 100 mL volumetric flask and final volume was adjusted with ethanol. The length of chromatogram run was 8 cm. After chromatography, developed plates were dried in a current of air and plates were dipped into 5 % sulphuric acid reagent, heated at 110 °C for 05 min in a pre-heated oven. Densitometric scanning was performed with in 10 min after derivatization using Camag TLC scanner III with winCATS software version 1.4.4 in the reflectance absorbance mode at 474 nm.

Selection of analytical wavelength

Analytical wavelength selection was performed at a concentration level of 200 ng/band for all phytoconstituents. After HPTLC development and derivatization, bands were scanned over the range of 400-700 nm and the spectra were overlaid. All marker compounds showed considerable absorbance at 474 nm (Figure 1) and hence was selected for densitometric analysis.

Preparation of standard solutions

Standard stock solutions of β-sitosterol-D-glucoside, gallic acid, withaferin A and withanolide A were prepared separately by dissolving 10 mg each in 10 mL methanol to get a stock solution of 1000 μg/mL. From the resulting solutions 1 mL solution, was further diluted with methanol to obtain a working solution of 100 μg/mL.

Development of the optimized mobile phase

HPTLC procedure was optimized with the aim to develop as assay method. Initially, mobile phase was selected on the basis of previous reports. All the above markers solutions were

spotted on HPTLC plates and different individual solvents as well as combination of solvents were tried to get a good separation and resolution. The mobile phase containing toluene: ethyl acetate: formic acid: methanol (6:3:0.1:0.6, v/v/v/v) gave good resolution of compounds. The identities of the bands from the *Ashvagandharishta* extract were confirmed by overlapping the densitograms of standard with that of samples.

METHOD VALIDATION

The HPTLC method was validated as per International Conference on Harmonization (ICH) guidelines, 2005.

Linearity and range

Linearity was evaluated by applying working solutions on HPTLC plate in the range of 50 - 400 ng/band for β-sitosterol-D-glucoside, 150-500 ng/band for gallic acid, 50-450 ng/bandfor withaferin A and 50-500 ng/band for withanolide A. Peak area versus concentration was subjected to calculate least square linear regression analysis and the slope, intercept and correlation coefficient for the calibration were estimated. Correlation coefficient alone is not suitable to prove linearity; therefore residual analysis was also performed. Sensitivity of the method was determined by estimating the limit of detection (LOD) and limit of quantitation (LOQ). They were calculated as 3.3 σ/S and 10 σ/S, respectively, where σ is the standard deviation of the response (y-intercept) and S is the slope of the calibration plot.

Precision studies

Precision of the method was verified by intra and intermediate precision studies. Repeatability studies were performed by analysis of three different concentrations 100, 200, 300 ng/bandof the four compounds, six times on the same day. Intermediate precision of the method was checked by repeating the study with above mention concentrations on three successive days.

Accuracy studies

Ashvagandharishta was spiked with known amount of standard markers and the percent ratios between the recovered and expected concentrations were determined. The analyzed samples were spiked with 80, 100 and 120 % of 100 ng/band of β-sitosterol-D-glucoside, withaferin A and withanolide A and 200 ng/band of gallic acid (standard addition method). Accuracy was calculated from the following equation:
[(spiked concentration-mean concentration) / spiked concentration] × 100.

Robustness of the Method

Robustness of an analytical method is a measure of its capacity to remain unaffected by small but deliberate variation in the experiment. Variation in retention factor and peak areas of the four compounds was examined. Factors changed were mobile phase composition (±0.1 mL), amount of mobile phase (±5%), time from banding to chromatography (+10 min) and time from

chromatography to scanning (+ 15 min), one factor was varied at a time, to study the effect. The robustness of the HPTLC method was studied at concentration of 200 ng/band for β-sitosterol-D-glucoside, gallic acid, withaferin A and withanolide A in triplicate. The standard deviation of peak areas and %relative standard deviation (% RSD) were calculated for each variable.

Specificity

Specificity is a measure of the degree of freedom from other active ingredients, excipients, impurities and possible degradation products. In specificity studies, β-sitosterol-D-glucoside, gallic acid, withaferin A and withanolide A solutions and the *Ashvagandharishta* sample solutions were applied on a HPTLC plate and the plate was developed, derivatized and scanned as described above. The peak purity of β-sitosterol-D-glucoside, gallic acid, withaferin A and withanolide A was assessed by comparing the visible spectra of markers at peak start, peak apex and peak end positions of the band i.e., r (start, middle) and r (middle, end).

Solution Stability

The stability of standard solutions was verified after 0, 6, 12, 24 and 48 h of storage. The stability of the standard solutions was determined by comparing peak area percentage and peak purity at 200 ng/band.

RESULTS AND DISCUSSION

HPTLC method optimization

A simple HPTLC method was optimized with the view to develop an assay method for simultaneous estimation of β-sitosterol-D-glucoside, gallic acid, withaferin A and withanolide A. Initially, different mobile phases were tried containing various ratios of toluene, ethyl acetate and methanol. The mobile phase consisting of toluene: ethyl acetate: formic acid: methanol in the ratio of (6:3:0.1:0.6, v/v/v/v),gave good resolution and reproducible results and was selected for further study. The optimum wavelength selected for detection and quantitation was 474 nm. The retention factors for β-sitosterol-D-glucoside, gallic acid, withaferin A and withanolide A were found to be 0.18±0.01, 0.26±0.02, 0.37±0.02 and 0.57±0.02, respectively (Fig. 2).

HPTLC method validation

Linearity, limit of detection and limit of quantitation

The results were found to be linear in the range of 50-400 ng/band for β-sitosterol D-glucoside, 150-500 ng/band for gallic acid, 50-450 ng/band for withaferin A and 50-500 ng/band for gallic acid. The square of correlation coefficients (r^2) for the plots of β-sitosterol-D-glucoside, gallic acid, withaferin A and withanolide A were found to be 0.9998, 0.9983, 0.9994 and 0.9996 respectively. Linearity of the method was ascertained by residual analysis (Data not shown here).Slope was significantly different from zero. The LOD and LOQ values indicate good sensitivity of the HPTLC method (Table 1).

**Table. 1:** Summary of validation parameters of proposed method.

Parameters	A	B	C	D
Linearity range[a] (n=6)	50-400	150-500	50-450	50-500
Correlation coefficient (r)	0.9998	0.9991	0.9996	0.9997
LOD[a]	7.4299	17.7444	12.0487	11.0456
r^2	0.9998	0.9983	0.9994	0.9996
Slope	5.657	6.235	7.251	6.994
Intercept	295.3	429.94	182.01	188.19
LOQ[a]	22.5150	53.7711	36.5114	33.4717
Precision (% RSD)				
Intra-day (n=3)	0.61 - 0.73	0.64 - 0.78	0.80 - 0.89	0.86 - 0.91
Inter-day (n=3)	0.66 - 0.74	0.66 - 0.79	0.81 - 0.93	0.87 - 0.94
Robustness	Robust	Robust	Robust	Robust
Specificity	Specific	Specific	Specific	Specific

a) a Concentration in ng/band

b) **A**: β-sitosterol D-glucoside, **B**: Gallic acid, **C**: Withaferin A, **D**: Withanolide A.

Table . 2: Results of recovery studies (n=6).

Amount added[a]				Amount found[a]/ ±S.D.				% Recovery / ±% R.S.D.			
A	B	C	D	A	B	C	D	A	B	C	D
80	160	80	80	80.70 / ±1.20	160.86 / ±1.42	81.14 / ±1.37	80.52 / ±1.56	100.87 / ±1.48	100.53 / ±0.88	101.42 / ±1.68	100.65 / ±1.93
100	200	100	100	101.23 / ±1.41	201.81 / ±1.33	100.68 / ±1.51	99.63 / ±1.47	101.23 / ±1.39	100.90 / ±0.65	100.68 / ±1.49	99.63 / ±1.47
120	240	120	120	119.84 / ±1.31	239.61 / ±1.18	120.19 / ±1.93	120.95 / ±1.93	99.86 / ±1.09	99.83 / ±0.49	100.15 / ±1.60	100.79 /±1.59

a) a Concentration in ng/band

b) **A**: β-sitosterol D-glucoside, **B**: Gallic acid, **C**: Withaferin A, **D**: Withanolide A.
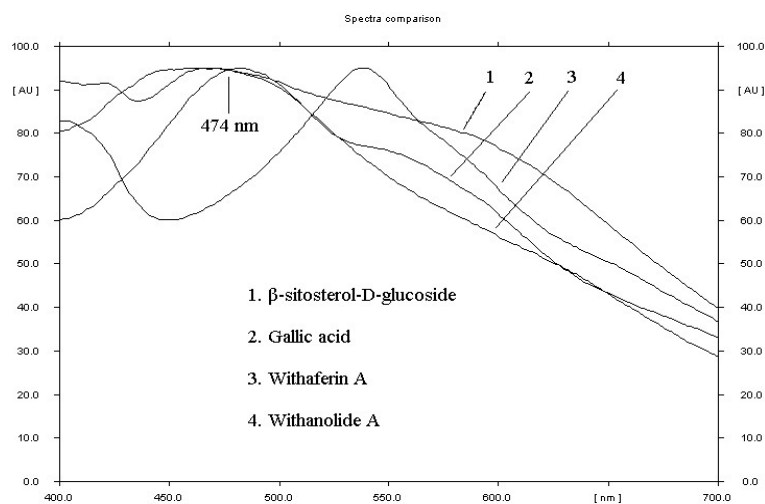
Fig. 1: Overlain visible spectra of β-sitosterol-D-glucoside, gallic acid, withaferin A and withanolide A**.**
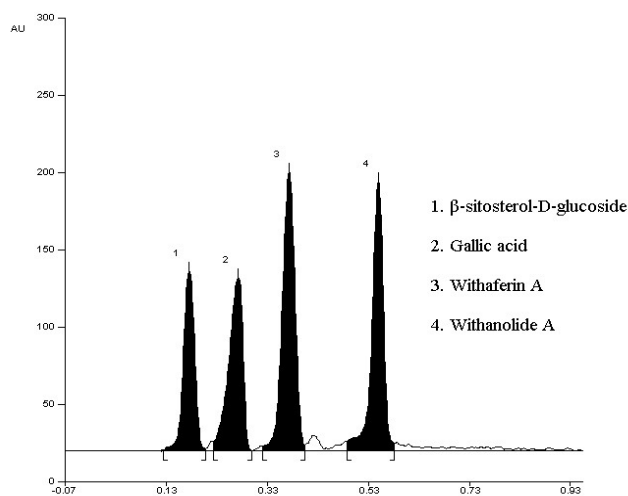
Fig. 2: Densitogram obtained from mixed standard solution of β-sitosterol-D-glucoside, gallic acid, withaferin A and withanolide A.

Precision

Precision of the developed method was investigated with respect to both repeatability and reproducibility. It was performed by using sample concentration of 100, 200 and 300 ng/band. The results are shown in Table 2. The developed method was found to be precise as the % RSD values for repeatability and intermediate precision were less than 2%, as recommended by ICH guidelines.

Specificity

Assessment of peak purity of β-sitosterol-D-glucoside, gallic acid, withaferin A and withanolide A was done by comparing the visible spectra of marker compounds at peak start, peak apex and peak end positions of the band, which were found to be. r (start, middle) = 0.998, 0.998 and r (middle, end) = 0.998, 0.998 respectively. Good correlation was also obtained between markers and sample spectra of β-sitosterol-D-glucoside, gallic acid, withaferin A and withanolide A.

Accuracy

Accuracy study showed recoveries of 99.04 - 102.67, 98.77 - 101.96, 99.64 - 102.67 and 100.07 - 101.96 % for β-sitosterol-D-glucoside, gallic acid, withaferin A and withanolide A, respectively indicating the reliability of the proposed densitometric method (Table 1).

Analysis of *Ashvagandharishta* formulation

Suggested validation methodology was applied for standardization of *Ashvagandharishta*. The shape of the peaks was not altered by other substances present in *Ashvagandharishta*. The percent content of β-sitosterol-D-glucoside, gallic acid, withaferin A and withanolide A in *Ashvagandharishta* were found to be 0.012, 0.064, 0.016 and 0.312 % respectively.

Robustness

Robustness of the given method was checked after measured alterations of the analytical parameters indicated that areas of peaks of interest and retention factor remained unaffected by small changes in the operational parameters (% RSD < 2). The summary of validation parameters of proposed method are given in Table 1.

CONCLUSION

A validated HPTLC method was developed which is simple, fast, accurate, precise and robust that can be used for simultaneous estimation of β-sitosterol-D-glucoside, gallic acid, withaferin A and withanolide A from *Ashvagandharishta*. This method can easily be used for routine analysis and standardization of this and other formulations containing Ashvagandha.

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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