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Standardization of marketed ayurvedic formulations containing Shatavari and Ashwagandha

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ABSTRACT

A sensitive, simple, precise, accurate and reproducible High performance thin layer chromatographic method has been established for quantification of active compound in marketed ayurvedic formulations containing *Asparagus racemosus* (Shatavari) and Withania somnifera (Ashwagandha). The total saponins (shatavarin) and withanolides were isolated from root powder, purified and characterized by various physical methods and spectral analyses like chemical test, UV, and IR. HPTLC finger printing of isolated active compounds was used for identification and quantitation of marketed Ayurvedic formulations. The selected Ayurvedic formulations [G, R, G, and C] were extracted with methanol and known quantities of the extracts were spotted on pre-coated silica gel 60 G_{54} HPTLC plates. The plates developed in solvent system comprising of ethyl-acetate: methanol: water: diethylamine (10:6:5:0.1) and the absorption was measured at 254 nm using the CAMAG's HPTLC densitometer. The calibration curves of active compounds were linear over the range 4-12µg/µl for total saponins and 2-10µg/µl for withanolides. The Ayurvedic formulations G and R contained 57. 85 mg and 86.2 mg of total shatavarin respectively, and withanolides present in formulations G and C were found to be 50.25 mg and 69.4 mg per 5 g of each formulation. The method was precise as indicated by low CV (2%). The recovery studies indicated reproducible results. The proposed HPTLC method can be used for a routine quality control analysis of marketed Ayurvedic formulations containing Shatavari and Ashwagandha.

KEYWORDS

HPTLC;
Shatavarin;
Withanolide;
Asparagus racemosus;
Withania somnifera.

INTRODUTION

Asparagus racemosus Willd (family: Liliaceae) is a plant growing throughout tropical and subtropical regions of India. The root of *Asparagus racemosus* contains starch, mucilage, flavones, tannins, proteins, and saponins[1]. *A.racemosus* contains saponins (shatavarins), which are used as marker substances for assessment of quality of the herb. *A.racemosus* has many pharmacological activities such as nutritive tonic, antidysentric, galactogogue, and adaptogen[2]. *Withania somnifera* (Dunal) of Solanaceae family is widely distributed throughout India[3]. Majority of the constituents are withanolides (steroidal lactones with ergostane skeleton) and alkaloids. The withanolides include withanone, withaferin A, withasomidienone and alkaloids like tropine, psedotropinine, anaferine. The root part of the plant is commonly used as drug in Ayurvedic

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and health care products. *Withania somnifera* has anti-tumor, antibacterial, anti-arthritic, immunosuppressive, nutritive tonic, and adaptogenic property[4].

Since there can be variation in the amount of phytoconstituents, a reliable and practical method of quantification is necessary. An attempt is made to develop HPTLC method for quantification of shatavarin and withanolide in marketed Ayurvedic formulations containing *Asparagus racemosus* and *Withania somnifera*. To avoid conflict of interest, we have coded the formulations containing *Asparagus racemosus* as G and R and formulations of *Withania somnifera* as G and C.

Chemicals

HPLC grade Methanol, ethyl acetate, water, diethylamine, and TLC aluminium Plates Pre-coated with silica gel 60 G54 (0.2 mm thick, Merck, Germany) were used.

Preparation of sample and standard

Two marketed Ayurvedic formulations containing *Asparagus racemosus* (G and R) and two formulations containing *Withania somnifera* (G and C) were selected for the study. The brand names were coded to avoid conflict of interest, the codes represent the manufacturer and the investigators, AAM and NPR were unaware of the codes. Five gram of each formulation was macerated with ethanol (95% v/v, 25 ml for 48 h). Shatavarin and withanolide were isolated from ethanolic (95%) extract of root powder of Shatavari and Ashwagandha as described earlier by Rajpal (2005, 2006). They were purified by column chromatography using neutral alumina and eluted sequentially with hexane, followed by ethyl acetate and methanol. The methanol soluble fraction contained shatavarin and withanolides as characterized by chemical test (Harborne, 1985). UV and IR spectroscopy[6].

HPTLC Method for estimation of active compound

Instruments

A CAMAG Linomat V sample applicator was used for spotting. CAMAG Twin trough glass chamber (10 × 10cm) was used for development of plates and CAMAG TLC scanner equipped with Wincat software was used for interpretation of data.

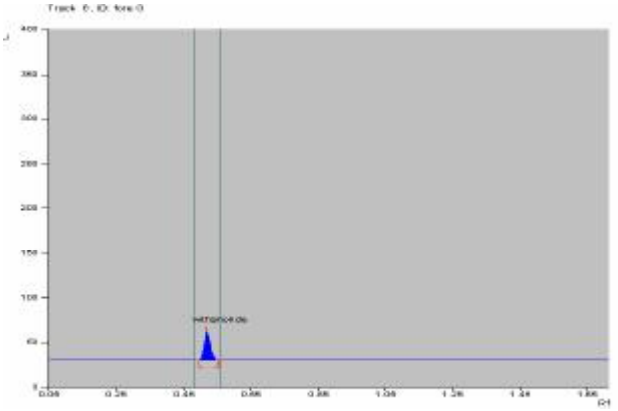

Figure 1: Typical HPTLC chromatogram of Withanolide

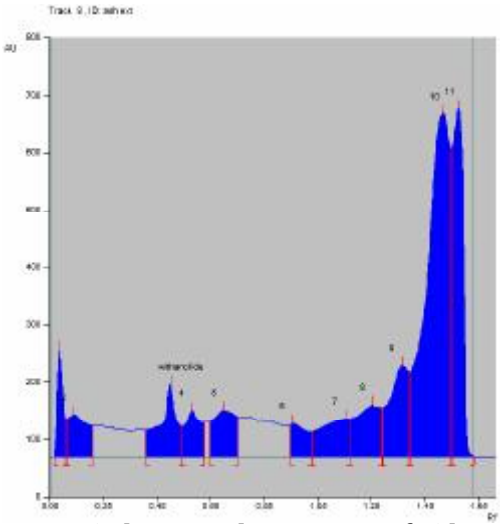

Figure 2: Typical HPTLC chromatogram of withanolide in formulation G

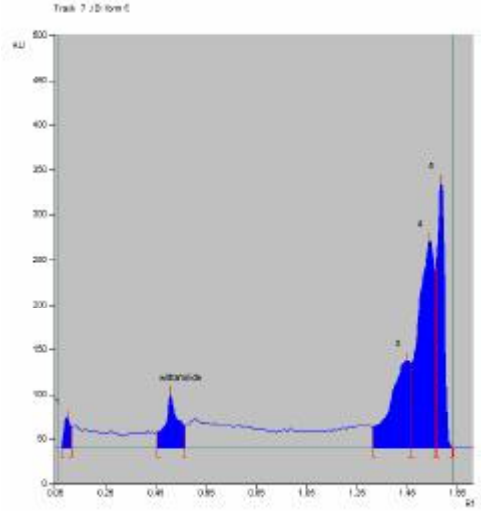

Figure 3: Typical HPTLC chromatogram of withanolide in Formulation C

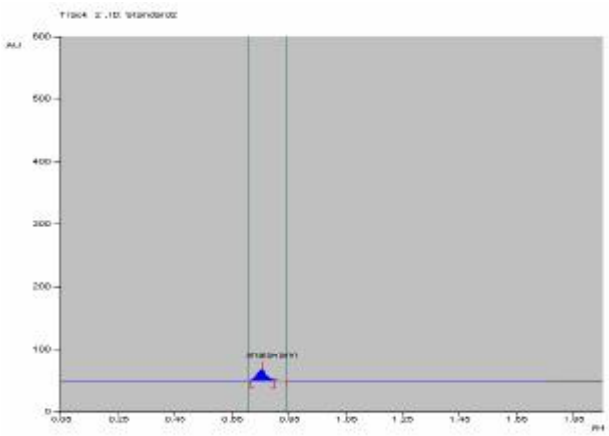

Figure 4: Typical chromatogram of shatavarin in standard

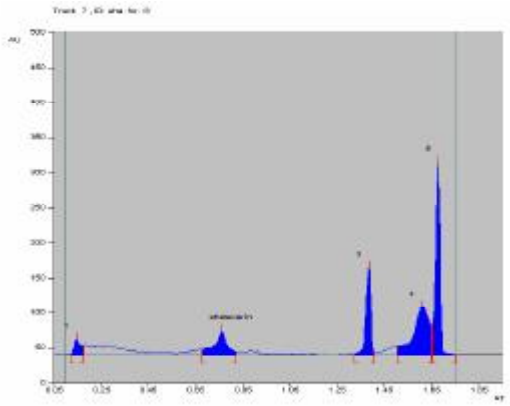

Figure 5:Typical chromatogram of shatavarin in formulation R

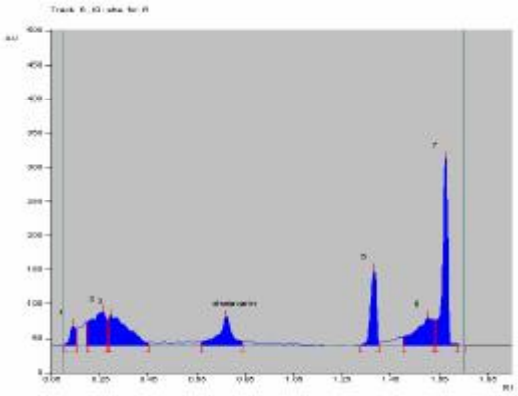

Figure 6:Typical chromatogram of Shatavarin in formulation G

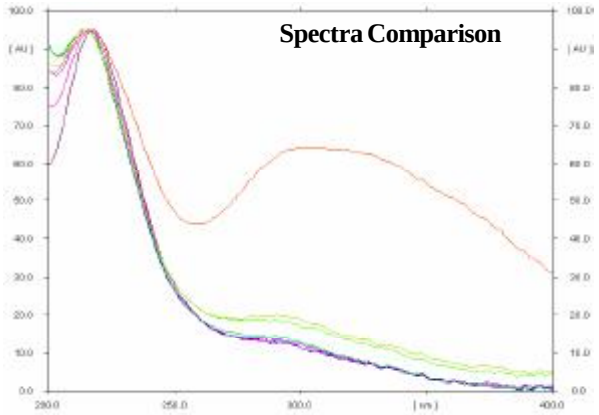

Figure 7: Overlay spectra of standard withanolide and withanolide in formulation

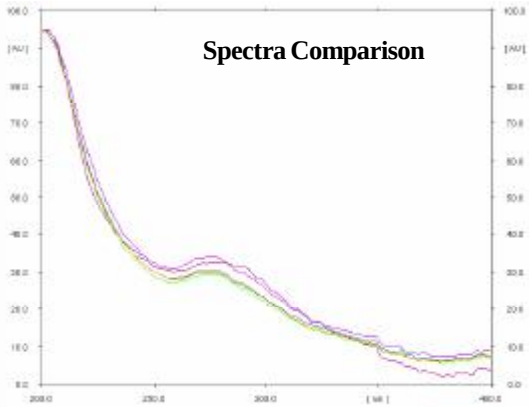

Figure 8: Overlay spectra of spectra of withanolide precision

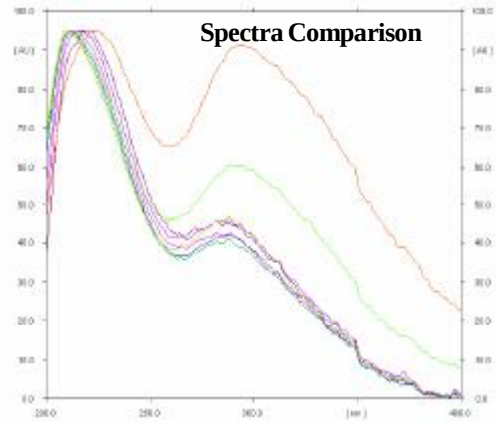

Figure 9: Overlay spectra of shatavarin at concentration 4-12µg

Preparation of standard calibration curve

Preparation of standard stock solution

Twenty five mg of total Shatavarin and withanolides isolated from ethanolic extract of *A.racemosus* and *W.somnifera* were dissolved in 25 ml of methanol to yield stock solution containing 1000µg/ml. The concentration range for the calibration curve was decided on the basis of probable concentration of Shatavarin and Withanolide present in the Ayurvedic formulations. Calibration curve was prepared from 4 to 12µg for shatavari and 2 to 10µg for withanolide per spot and linearity was checked.

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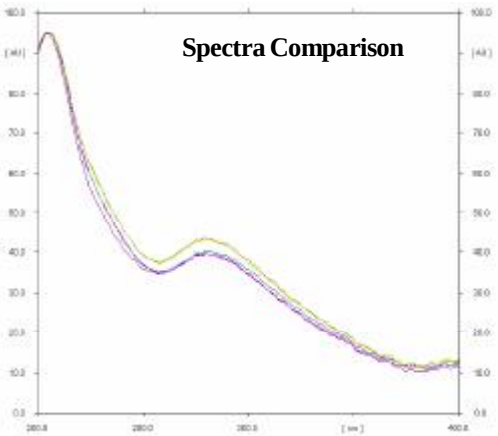

Figure 10: spectra indicating precision of analysis

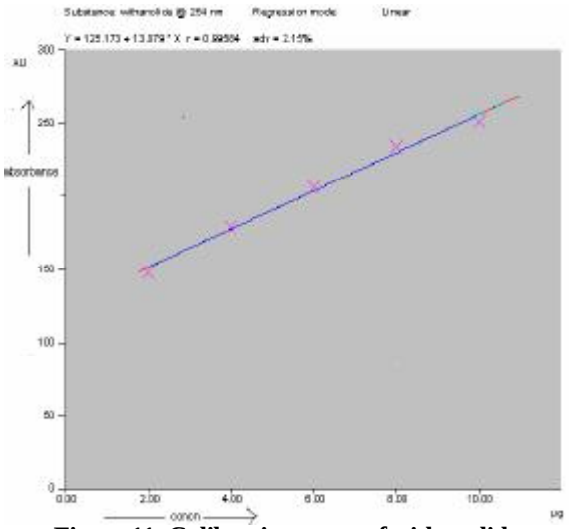

Figure 11: Calibration curve of withanolide

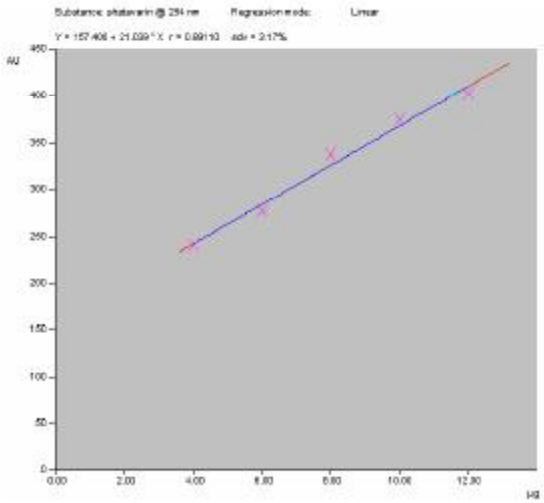

Figure 12: Calibration curve of Shatavarin

Sample preparation

Approximately weighed 5 g of each formulation G, R, G, and C were extracted with ethanol (25 ml ×3) and the extract was concentrated under reduced pressure to 10 ml and this extract was used for further experiment[6].

Method specifications

Chromatography was performed on aluminium sheet pre-coated with silica gel 60 G_{54} HPTLC plate. Before use, plates were pre-washed with the methanol and dried in an oven at 105 ^{0}C for 1 hr. samples were applied on the plates as bands of 6mm width with the help of CAMAG Linomat V sample applicator at the distance of 15 mm from the edge of the plates. The plates were developed to a distance of 80mm in CAMAG twin-trough chamber previously equilibrated with mobile phase for 20 min. The solvent system comprised of ethyl-acetate: methanol: water: diethylamine (10:6:5:0.1).After development plates were dried under current of air at room temperature densitometric evaluation of plates was performed at λ = 254 nm and 366 nm using Deuterium and mercury lamp with Wincat software for quantification. A proper separated peak of Shatavarin and Withanolide were obtained at Rf 0.53 and 0.57 respectively. The content of active compound in the selected marketed Ayurvedic formulations were determined by comparing area of the chromatogram with the help of calibration curve of the working standard of active compounds.

Recovery study

Recovery studies were carried out using 4µg of ethanol extracted formulation (as described above) and the TLC plate was spiked with 4µg of total Shatavarin and Withanolides isolated from the alcoholic extract. The plate was developed in a similar manner and amount of Shatavarin and Withanolide were quantified as described above. The data was analyzed for the amount of Shatavarin and Withanolide recovered. This estimation was repeated three times.

RESULT AND DISCUSSION

Isolated fraction of extract showed four Peaks in Shatavari and three Peaks in Ashwagandha in HPTLC chromatogram. The Peaks having Rf 0.53 and 0.57 were integrated as they were corresponding to the ac-

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TABLE 1: Amount of Shatavarin and Withanolide estimated in formulations

Sample	Amount in µg/µl
Formulation G	11.57 µg
Formulation R	17.24 µg
Formulation G	10.05 µg
Formulation C	13.88 µg

TABLE 2: Experimental data for linearity, accuracy, precision and recovery study

Parameters	Values of Shatavarin	Values of Withanolide
Linear range	4-12µg	2-12µg
Standard derivation	3.17%	2.16%
Correlation coefficient	0.99110	0.9958
Limit of detection	7.210µg	9.42
Limit of quantification	21.84µg	28.54
Slope (m)	21.04	13.78
Intercept (c)	157.4	123.3
Precision:		
1.RSD	0.08165	0.126
2.CV	8.165	12.6
Accuracy:		
Formulation (G,G)		
1.SD	7.43	1.58%
2.RSD	0.01889	0.0390
3.CV	1.889	3.9
Formulation (R,C) 1.SD	10.67	7.5%
2.RSD	0.02033	0.0252
3.CV	2.033	2.5
Recovery study		
For 50% addition	86.30%	91.43%
For 100% addition	100.53%	102.74%
For 150% addition	103.81%	-

TABLE 3: UV absorbance of Shatavarin and Withanolide

Sample name	Wavelength (nm)	Absorbance
Shatavarin	223	0.4126
Withanolide	226.50	0.3480

tive compound. These Peaks were selected because of capacity factor (2 to 11) and its high concentration. The structure of active compound (Shatavarin and Withanolide) was characterized by physical method and spectral analysis like, melting point, chemical test, UV(TABLE 2), and IR (TABLES 3 and 4). The Melting Point of Shatavarin was found to be 142^0C-144^0C and withanolide 251^0C -254^0C .The chemical test like, Foam test, Haemolysis test were positive for Shatavari and Liebermann-Burchard test was positive for Withanolide. The λmax of Shatavarin and Withanolide were found to be 223nm and 226nm respectively. IR spectra of Shatavarin was matching with that reported in the literature with peaks at 699 (C-H bend), 1131

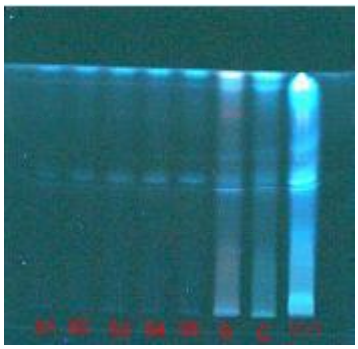

Figure 13: Photodocumentation of HPTLC withanolide at 366 nm

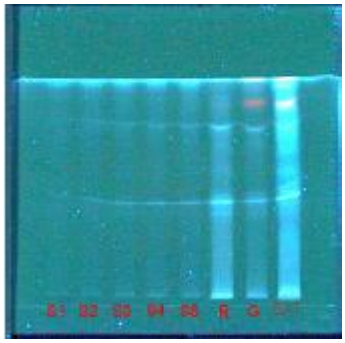

Figure 14: Photodocumentation of HPTLC of Thin of shatavarin at 366 nm

(C-O stretch),1472 (C-C stretch within ring), 3266(-OH stretch) and IR spectra of Withanolide was matching with that reported in the literature with peaks at 1213 (C-O stretch ,ether and ester), 1458(C-H bend, Alkanes), 1693(C=O strech, Carbonyl group), 3020(C-H stretch, Aromatic ring),3327(OH stretch, alcohol)[5].

In HPTLC Chromatogram of Shatavarin and Withanolide the retention factor (Rf) is 0.53 and 0.57 respectively matched with that of standard Shatavarin and Withanolide[7] (Figures 13 and 14). The Calibration curve of Shatavarin and Withanolide were found to be linear in the concentration used in this study (Figures 11 and 12).The spectra of standard active compound and active compound in four different selected ayurvedic formulations (G, R, G, and C) and alcoholic extract were found by measuring by area in HPTLC method (TABLE 1). Data from table revealed that formulation G contains lesser amount of active compound and formulations G and C contains higher amount of active compound. It may be due to various factors like time of collection, age of the plant, processing conditions, incorrect identification of plants, depending upon the soil

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TABLE 4: Important peaks observed in the FTIR of shatavarin

Peak	Intensity	Correlation intensity	Base(H)	Base(L)	Area	Correlation area	Interpretation
699.22	27.2	7.0	710.79	697.29	6.4	0.6	C-H Bend
1131.29	38.9	6.8	1134.18	1114.89	4.6	0.3	C-O Stretch
1472.70	5.9	8.1	1486.20	1467.88	17.0	2.1	C-C Stretch within ring
3266.56	39.0	2.9	3268.49	3261.74	2.6	0.1	OH Stretch

TABLE 5: Important peaks observed in the FTIR of Withanolide

Peak	Intensity	Correlation intensity	Base(H)	Base(L)	Area	Correlation area	Interpretation
1213.27	7.48	75.83	1242.20	1195.91	27.34	23.38	C-O stretch,Ether, ester
1458.23	75.89	13.06	1481.38	1440.87	3.47	1.46	C-H bend(Alkanes)
1693.56	40.90	43.01	1753.35	1658.84	18.12	11.50	C=O Stretch, (Carbonyl group)
3020.63	12.57	71.71	3053.42	3001.34	12.21	8.93	C-H stretch, (Aromatic ring)
3327.32	86.89	0.07	3329.25	3279.10	2.88	0.01	OH stretch, Alcohol

characteristics and geographical conditions.

The HPTLC method was validated using various parameters like linearity, Standard deviation, Co-relation coefficient, LOD, LOQ, Precision, Accuracy, and recovery studies (TABLE 2). Data from table revealed that the percentage of active compound is more in Formulation G and C, and less in G. Thus the study concludes that the ayurvedic formulations differed in amount of active compound indicating need to validate the Formulation procedure.

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