HPTLC FINGERPRINTING AND QUANTIFICATION OF SHATAVARIN IV IN EXTRACT AND POLYHERBAL FORMULATIONS

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ABSTRACT

A new, simple, sensitive, precise and robust high-performance thin layer chromatographic (HPTLC) method was developed for the estimation of Shatavarin IV in herbal extracts and polyherbal formulation. Analysis of Shatavarin IV was performed on TLC aluminium pre-coated plates with silica gel 60F-254 as stationary phase. Linear ascending development was carried out in twin trough glass chamber saturated with mobile phase consisting of Chloroform : Methanol, (7 : 3 v/v) for Shatavarin IV at room temperature ($25\pm2^{\circ}$C). After development the plate was derivatized with Anisaldehyde H_2SO_4. Camag TLC scanner III was used for spectrodensitometric scanning and analysis of plate in absorbance mode at 560 nm for Shatavarin IV. The system was found to give compact spots for Shatavarin IV (R_f 0.46). The data for calibration plots showed good linear relationship with $r2 = 0.9993$ in the concentration range of 100–600 ng for Shatavarin IV. According to international conference on harmonization (ICH) guidelines the present method was validated for precision, repeatability and recovery. The limits of detection and quantification were determined. Statistical analysis of the data showed that the method is reproducible and selective for estimation of Shatavarin IV.

KEYWORDS: High-performance thin layer chromatography; Method validation; Shatavarin IV; Polyherbal formulations.

1. INTRODUCTION

Herbal medicine has been enjoying revitalization among the customers throughout the world. However, one of the impediments in the acceptance of the ayurvedic/herbal medicines is the

lack of standard quality control profiles. The quality of herbal medicine i.e. the profile of the constituents in the final product has implication in efficacy and safety. Due to the complex nature and inherent variability of the chemical constituents of plant-based drugs, it is difficult to establish quality control parameters. To overcome these problems modern analytical techniques are expected to help in circumventing this problem.[1, 2]

The dried roots and stolons of *Asparagus racemosus* wild *from the* Family: Liliaceae is an annual herb cultivated in tropical and subtropical part of India. Grows up to 1000 ft. in the Himalayan track from Kashmir eastwards. Besides India it is also cultivated in Tropical Africa, Java and Australia.[3] The literature survey revealed for the variety of therapeutic actions of *Asparagus racemosus* including bitter, sweet; oleaginous, cooling, indigestible; appetiser; alterative, stomachic, tonic, aphrodisiac, galactogogue, astringent to bowels; useful in dysentery, inflammations, biliousness, disease of the blood and the eye, throat complaints, tuberculosis, leprosy, epilepsy, night blindness. The root of this plant is used medicinally as a galactogogue, demulcent, diuretic, antispasmodic and anti diarrhoeatic. It is used chiefly as a demulcent in veterinary medicine.[4–8] The principle substance is Shatavarin I to Shatavarin IV. (Fig.1) It also contain Saponin A [Sarsasapogenin-glu-rha], β-sitoserol and Stigmasterol glucoside, Diosgenin & Quercitin-3-glucuronide, Undecanylcetanoate, mucilage, starch, flavones, tannins, proteins, Saponin.[9]

Certain herbal extracts and polyherbal formulations containing active constituents were standardized by high-performance thin layer chromatography (HPTLC) method. HPTLC method was reported to be the most suitable for the estimation of active constituents of extracts, plant species (raw material) and polyherbal formulations. Therefore, an attempt has been made to develop accurate, specific, repeatable and reproducible HPTLC method for the determination of Shatavarin IV in herbal extracts and Polyherbal formulation.
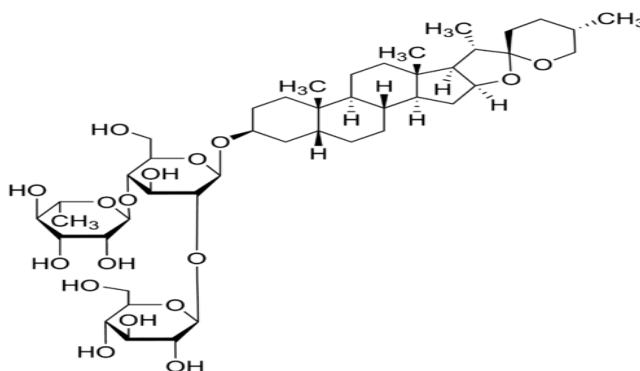

Fig. no. 1: Structure of Shatavarin IV.

2. EXPERIMENTAL

2.1. Materials

Standard Shatavarin IV was purchased from Indus extract, Mumbai. Spry dried aqueous extracts of *Asparagus racemosus* was obtained from Prashant Pharmaceuticals (ISO 9001 herbal unit) Rajpipla, Gujarat, India. All chemicals including solvents were of analytical grade from E. Merck, India. The HPTLC plates Silica 60F254 (20 cm x 20 cm) were purchased from E. Merck (Darmstadt, Germany).

2.2. Instrumentation and chromatographic conditions

The samples were spotted in the form of bands of 6 mm with Camag microlitre syringe on pre-coated silica gel aluminium Plate 60F-254 (20 cm ×10 cm) with 200 μm thickness (E. Merck, Germany) using a Camag Linomat V (Switzerland) sample applicator. A constant application rate of 150 nL s−1 was employed and space between two bands was 14 mm. The slit dimension was kept at 2mm×0.45mm and 20 mm s−1 scanning speed was employed. The composition of mobile phase is Chloroform: Methanol, (7: 3 v/v) for Shatavarin IV. Linear ascending development was carried out in a twin trough glass chamber saturated with mobile phase. The optimized chamber saturation time for the mobile phase was 23 min for Shatavarin IV at room temperature (25 ± 2^0C). The length of chromatogram run was 80 mm. Then the plate was allowed to dry at room temperature. Derivatized the plate with Anisaldehyde H_2SO_4 reagent by dipping for 5 s and dried the plate for 5 min at 45^0C in an oven. The separated bands on the HPTLC plates were scanned over the wavelength of 560 nm.

2.3. Calibration curve of Shatavarin IV

A stock solution of Shatavarin IV 200 μg/ml in methanol was prepared. Different volumes in the range 0.5 – 3 μl of Shatavarin IV were applied on TLC plate to obtain concentration of 100 - 600 ng of Shatavarin IV. (Fig. no. 2).
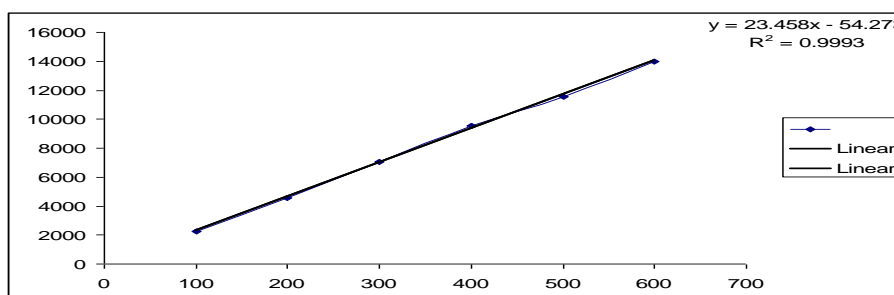

Fig 2: Calibration Curve for Shatavarin IV

Y= 23.458 X – 54.273

Coefficient of correlation = 0.9993;

Slope = 23.458; Intercept = 54.273

2.4. Method validation

2.4.1. Instrumental precision and inter-day and intra-day precision

Precision of the method was determined as intra-day and inter-day variations. Intra-day variations was determined by analyzing 200, 300, 400 ng/spot of standard solution of Shatavarin IV for three times on the same day. Inter-day precision was determined by analyzing 200, 300, 400 ng/spot of standard solution of Shatavarin IV for three consecutive days over a period of a week.

Table 1: Intra- and Inter-day precision study.

Marker compounds	Concentration (ng/spot)	Intra-day precision*	Inter-day precision*
Shatavarin IV	200	1.15	1.65
	300	1.53	1.79
	400	0.92	1.09

* Relative standard déviation (% CV, n=3)

2.4.2. Accuracy (recovery study)

Recovery experiments were performed at three different levels i.e. 80, 100 and 120 %. To the pre-analysed sample solutions; a known amount of standard drug solution of Shatavarin IV was over spotted at three different levels. The chromatogram was developed and scanned as discussed in instrumentation condition.

Table 2: Recovery study.

Marker compounds	Amount present in the sample, (ng)	Amount added (ng)	Amount found (ng)	Recovery (%)	Average recovery (%)
Shatavarin IV	50	40	90.37 ±0.59	99.50	
	50	50	100.42 ±0.80	99.69	99.57 ± 1.27
	50	60	110.29 ±0.96	99.52	

*Mean ± standard deviation (SD, n=3)

2.4.3. Sensitivity, limit of detection and limit of quantification

The sensitivity of measurements of Shatavarin IV by the use of the proposed method was estimated in terms of the Limit of Detection (LOD) and Limit of Quantitation (LOQ). The LOD and LOQ were calculated using equation LOD = 3.3 x N/B and LOQ = 10 x N/B,

where, 'N' is standard deviation of the peak areas of the drugs (n = 3), taken as a measure of noise, and 'B' is the slope of the corresponding calibration curve. The linearity equation was found to be Y= 23.458 X – 54.273. The LOD and LOQ for Shatavarin IV for were found to be 7.94 ng and 24.08 ng, respectively [where, N =56.49, B = 23.458].

2.4.4. Repeatability

Repeatability of sample application was assessed by spotting 1.5 μL contain 300 ng/spot of standard Shatavarin IV for on TLC plate in triplicate; develop and scanned as described in instrumentation and chromatographic condition.

2.4.5. Specificity

The specificity of the method was ascertained by analyzing the standard drug and extract. The spot for Shatavarin IV in the sample was confirmed by comparing the R_f value and spectra of the spot with that of the standard. The peak purity of Shatavarin IV was assessed by comparing the spectra at three different levels, viz. peak start (S), peak apex (M), peak end (E), position of the spot.

Table 3: Method validation parameters of marker compounds by HPTLC.

Parameters	Shatavarin IV
Instrumental precision (% CV)(n=3)	0.45
Repeatability	1.42
Limit of detection (ng/spot)	7.94
Limit of quantification (ng/spot)	24.08
Specificity	Specific
Linearity (Correlation of coefficient)	0.9993
Range (ng/spot)	100-600

2.5. Analysis of Shatavarin IV in herbal extracts

50 mg aqueous extract of *A. Racemosus* was transferred in 10 ml volumetric flask and volume make up with 10 ml methanol. Both resulting solution was filtered using Whatmann filter paper 41. An appropriate volume of 5 μL aqueous extract was spotted on TLC plate. The analysis was repeated in triplicate.

2.6. Analysis of Shatavarin IV in polyherbal formulation

Polyherbal formulation (Tablet) prepared using the combination of aqueous extract of *A. Racemosus*, *T. terrestris, G. glabra, and Anethum sowa,* all these extract added in fixed

quantity and increase the bulk by adding excipients like flavoring agent, color, binding agent etc. and this polyherbal formulation analyzed with the marker compound Shatavarin IV. To determine the content of Shatavarin IV in polyherbal formulation; require amount of formulation was weighed and crushed into fine powder. Then it was transferred to 10 ml volumetric flask containing 10 ml of methanol. Resulting solution was filtered using Whatmann filter paper 41. An appropriate volume 10 µL was spotted for analysis of Shatavarin IV, The concentration was determined by linear regression equation. The analysis was repeated in triplicate.

Table 4: Marker compounds quantified by HPTLC from aqueous extract and poly herbal formulation.

Sr. no.	Marker compounds	Aqueous extract (%)	Poly herbal formulation (%)
1.	Shatavarin IV	0.18	0.074

3. RESULTS AND DISCUSSION

3.1. Selection of mobile phase

The standard solution and the test solution were spotted on HPTLC plates and different solvents as well as combination of solvents have been tried to get a good separation and stable peak. The optimized mobile phase for Shatavarin IV was Chloroform : Methanol, (7 : 3) (*v/v*) was selected for estimation of the drug by HPTLC method, which gave good resolution with R_f = 0.46 (Shatavarin IV) (Fig. 5.) and Well defined spots were obtained when the chamber was saturated with mobile phase for 23 min at room temperature.

3.2. Calibration curves

The present HPTLC method for estimation of Shatavarin IV showed a good correlation coefficient ($r2$ = 0. 0.9993) in the concentration range of 100–600 ng /spot with respect to the peak area. The mean value (±S.D.) of slope and intercept were 23.458 & 54.273. No significant difference was observed in the slope of standard curves (ANOVA, $P > 0.001$).

3.3. Method validation

The real goal of validation process is to determine limits of allowed variability for the conditions needed to run the method. The accuracy of the method was determined by recovery experiments. The recovery studies were carried out three times and the percentage recovery were calculated and presented. From the data obtained, recoveries of added standard drugs were found to be accurate (Table 2). Three repeated standard solutions were made and

response factors of drug peaks and %R.S.D. were calculated and presented. From the data obtained, the developed HPTLC method was found to be precise. The calibration curves were plotted using the response factors versus concentration of standard solution. These data demonstrate that the methods have adequate sensitivity to the concentrations of the analytes. The LOD and LOQ of the developed method were determined by injecting progressively low concentrations of the standard solutions using the developed methods. The LOD is the smallest concentration of the analyte that gives a measurable response. The LOD and LOQ were found to be 7.94 ng and 24.08 ng [where, N = 56.49, B = 23.458] which indicate adequate sensitivity of the method. The LOD and LOQ values determined are affected by the separation conditions, i.e., HPTLC plates, reagent; instrumentation and detection wavelength and data system solvents other than AR grade solvent can result in large changes in single to noise ratio due to base line noise and drift (Table 3). The precision (Table 1) of the methods were studied by carrying out experiments by changing conditions. It was observed that there were no marked changes in the chromatograms. The values obtained demonstrated the suitability of the system for the analysis of the above drug system suitability parameters might fall within ±3% standard deviation range, during routine performance of the method.

3.4. Estimation of Shatavarin IV in herbal extracts and in polyherbal formulations

Spot at R_f 0.46 was observed in the chromatogram for Shatavarin IV in extracts and polyherbal formulation respectively. The content of Shatavarin IV in aqueous extract and poly herbal formulation were found to be 0.18 % and 0.074% (Table 4).
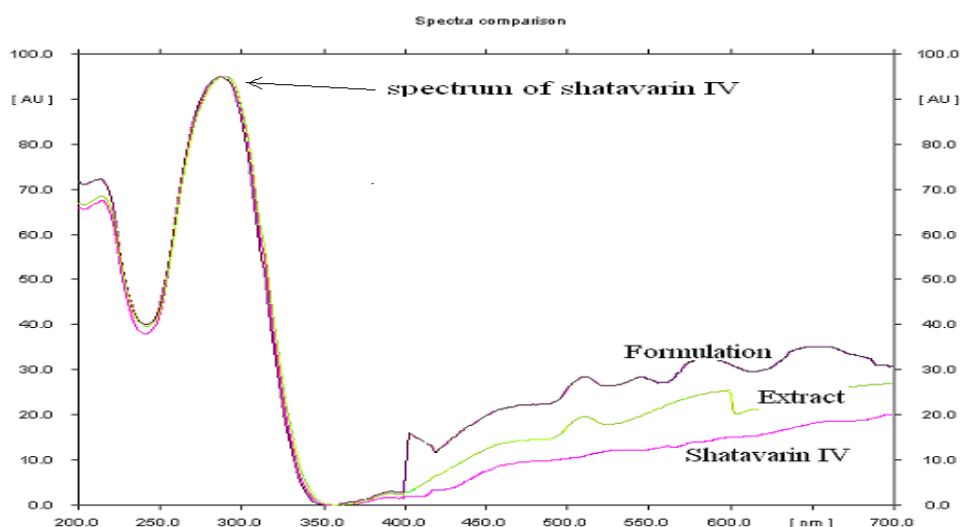

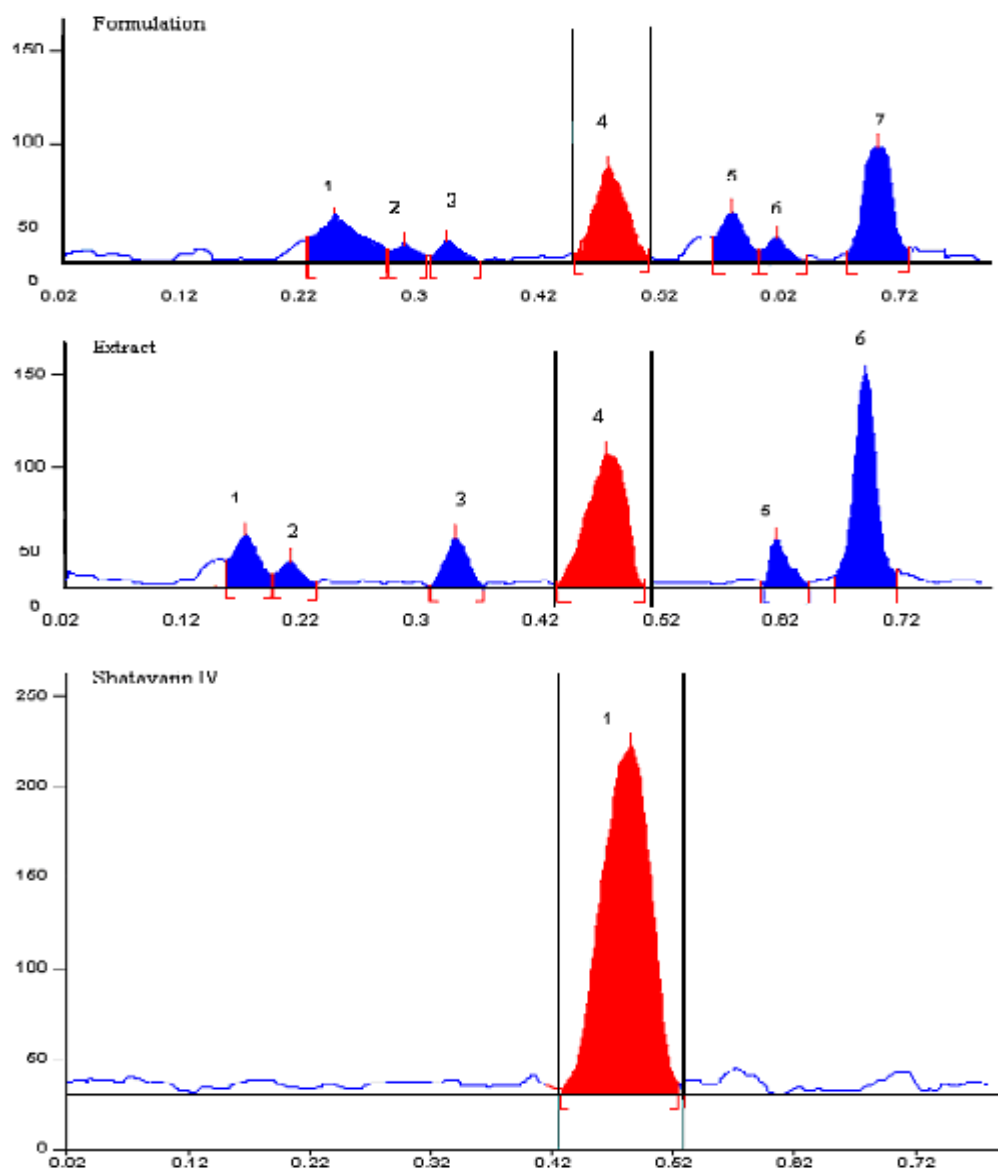**Fig 3: Comparative spectral study of Shatavarin IV, *A. racemosus* extract and Mamalait granules formulation.**

Fig. 4: Comparative HPTLC peak of standard Shatavarin IV, formulation, and *A. racemosus* extract.

4. CONCLUSION

From the above studies, it can be concluded that HPTLC technique can be successfully used for estimation of Shatavarin IV in extracts and polyherbal formulations. The developed HPTLC method for this estimation of Shatavarin IV is simple, accurate, linear and rapid. Statistical analysis proves that the method is reproducible and selective for the analysis of Shatavarin IV.

5. REFERENCES

1. L.V. Asokar, K.K. Kakkar, O.J. Chakra, *Glossary of Indian medicinal plants with active principles*, Publication and Information Directorate, New Delhi, 1992; 122.

2. M.N. Ravishankara, N. Shrivastava, H. Padh, M. Rajani, *Planta Med,* 2001; 67: 294-296.

3. Anonymous, Medicinal plant. Vol.-IV, Orient Longman series, New Delhi, 1994; 139-140, 241-243, 84-86, 311-313.

4. Tripathi, K., Prajapati, V., Kishan, K., Aggarwal, S., Kumar, S. Insecticidal and ovicidal activity of the essential oil of *anethum sowa* kurz against *callosobruchus maculatus* f., Journal of Ethnopharmacology, 2001; 59: 218–222.

5. Agrawal, K., Khanuja, S., Ateeque Ahmad., Santa Kumar., Gupta, S. Antimicrobial activity profiles of the two enantiomers of limonene and carvone isolated from the oils of Mentha spicata and Anethum sowa, Insect Sci. Applic*,* 2001; *21*(1): 61-66.

6. Elmastaş, M., Ibrahim,H., Isildak,N., Omer,A., Hassan, G. Antioxidant Activity of Carvone Isolated from Spearmint (Mentha Spicata L. Fam Lamiaceae), Journal of Liquid Chromatography & Related Technologies, 2006; 29(10): 146-151.

7. Shashikumar J.M., Palniswamy, M., Dossan, A., Geetha, V. Antibacterial and anti-fungal activity of The fruit of *Anethum sowa*, Journal of Pharmaceutical Sciences, 2005; 7: 765-766.

8. Khare, G., Negi, P.S., Jayaprakash, G.K., 1999.Unsaponifiable matter of fixed oil from seeds showed anti-bacterial, cardiac depressant and hypoglycemic activity, Journal of Ethanophamacolog, 48: 294-298.

9. Oludotun, A., Koyippalli, T., Mabayoje, A. Antihypertensive and vasodilator effects of methanolic and aqueous extracts of *Tribulus terrestris* in rats, Journal of Ethnopharmacology, 2006; 104: 351–355.

10. Kirtikar, K.R., Basu, B.D. Indian Medicinal Plants. Vol. I, 2nd Ed., International Book Distributors and Publishers, Dehradoon, India, 1996; 113-135, 419-421.

11. Anonymous, The Ayurvedic Pharmacopoeia of India. 1996. Vol.-II, 1st Ed., Govt. of India, Ministry of Health and Family welfare, New Delhi, 153.

12. Michael, A.,. Flavonoids-rich nutrients with potent antioxidant activity prevent atherosclerosis development: the licorice example, Journal of Ethnophramacology, 2007; 116: 377–380.

13. Kirtikar, K.R., Basu, B.D. Indian Medicinal Plants. Vol. IV, 2nd Ed., International Book Distributors and Publishers, Dehradoon, India, 1996; 2501-2502.

14. Ansari,M., Houlihan, L., Hussain, B., Pieroni, A. Glycyrrhizin, a Triterpenoid glycoside and licorice from G. glabra and ammonium salt of Glycyrrhizinic acid were tested for antiviral activity on the three strains of Japanese encephalitis virus, Indian Journal of pharmaceutical science, 1977; 60(5): 287-289.

15. Gupta, V., Atiya, A., Negi, A., Shankar, K., Rahuja, N., Sisodia, B. Antimicrobial potential of *Glycyrrhiza glabra* roots, Journal of Steroid Biochemistry & Molecular Biology, 2004; 91: 241–246.

16. Hye gwang, J., Sung Jun, P., Ran moon, A., Young, C., Shin, K. Hepatoprotective effects of 18β-glycyrrhetinic acid on carbon tetrachloride-induced liver injury: inhibition of cytochrome p450 2e1 expression, Pharmacological Research, 2002; 46(3): 34-37.

17. Dhingra, D., Parle, M., Kulkarni, S. Memory enhancing activity of *Glycyrrhiza glabra* in mice Ind J Expt Bio, 2004; 9: 68-71.

18. Rajpal, V. Standardization of Botanicals, Testing and Extraction Methods of Medicinal Herbs. Vol. 1, Eastern publishers, 2005; 115-131, 223-227.

19. Dhalwal, K., Biradar, Y., Shinde, V., Mahadik, K., Rajani, M., 2008. Phytochemical evaluation and validation of a polyherbal formulation using HPTLC. Phcog Mag, 4: 89-95.

20. Peishan Xie., Sibao Chen., Yi-zeng Liang., Xianghong Wang., Rutano Tian., Roy Upton. Chromatographic fingerprint analysis – a rational approach for quality assessment of traditional Chinese herbal medicine. Journal of chromatography a, 2006; 1112: 171-179.