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RP-HPLC Analysis of Withanolides in the Flowers, Leaves, and Roots of *Withania somnifera*

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Summary. Ashwaghanda, *Withania somnifera*, is one of the most widely used herbs in Ayurvedic medicine. Leaves and roots are the traditionally used parts of the plant. An RP-HPLC method using a binary acetonitrile–water gradient containing 0.1% acetic acid has been developed for analysis of withaferin A. The method was validated in accordance with ICH guidelines and used for analysis of the withanolide content of the flowers, leaves, and roots of *W. somnifera*. The withanolide content was highest in the flowers.

Key Words: *Withania somnifera*, Solanaceae, RP-HPLC, withanolides, withaferin A, flower

Introduction

Withania somnifera (Solanaceae), known as Ashwagandha, is one of the most valuable plants in traditional Indian medicine. It is known to have adaptogenic, anti-convulsion, and antisedative activity. The plant has been used in the treatment of neurological disorders, geriatric debilities, arthritis, and stress and behaviour-related problems [1–5]. Ashwagandha contains a large and structurally diverse set of withanolides, steroidal molecules based on the ergostane nucleus. Several modern studies have revealed a link between the therapeutic actions of the plant and one or more withanolides [6–9]. Both roots and leaves of the plant are prescribed for medicinal uses in the traditional systems of medicine [10]; the flowers have not attracted much attention.

The objective of this study was, therefore, to develop an optimized, validated, and simple RP-HPLC method for standardization of *Withania somnifera* using withaferin A (*Fig. 1*) as marker. The method was validated for selectivity, linearity, precision, and accuracy, in accordance with ICH requirements, then used for analysis of withaferin A in the flowers, leaves, and roots of the plant. This is the first report of analysis of the withanolide content of the flowers of *Withania somnifera* [11–13].

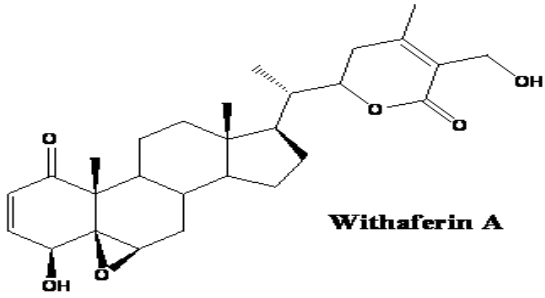

Fig. 1. The chemical structure of withaferin A

Experimental

Materials, Reagents, and Solutions

Authenticated *W. somnifera* plant material was collected in June 2008 in Karachi, Pakistan. A voucher specimen was deposited at the general Herbarium, University of Karachi (no. 72993). Withaferin A standard (99.98%) was kindly provided by Dr Sammer Yousef, ICCBS, Pakistan.

Methanol, acetonitrile, and glacial acetic acid HPLC grade were from Fluka. Double-distilled deionised water was used throughout.

Withaferin A stock solution (10 mg mL^{-1}) was prepared by dissolving 1.00 g pure withaferin A powder, accurately weighed, in 75 mL methanol in a 100-mL volumetric flask. After vigorous shaking the solution was diluted to volume with methanol. Withaferin A working solution (1 mg mL^{-1}) was prepared by diluting 10 mL withaferin A stock solution (10 mg mL^{-1}) to 100 mL with methanol in a volumetric flask.

Sample Preparation

Finely powdered plant material (flowers, leaves, or roots; 1 g) was extracted with methanol (3 × 3.0 mL) by sonication for 10 min. The extracts were combined separately for each part of the plant, centrifuged (10 min at

3000 rpm), transferred to separate 10-mL volumetric flasks, diluted to volume with methanol, then filtered through 0.45-μm membrane filter.

Chromatography

HPLC was performed with a Shimadzu (Japan) LC-20AT system equipped with a photo-diode array (PDA) detector operated at 230 nm. Compounds were separated on a 4.6 mm × 250 mm C_{18} column (Separation Methods Technologies) with a gradient prepared from 99.9:0.1 (*v*/*v*) water–glacial acetic acid (component A) and 99.9:0.1 (*v*/*v*) acetonitrile–glacial acetic acid (component B). The gradient was: 0–5 min, 80% A; 5–30 min, 80–0% A; 30–35 min, 0% A; 35–37 min, 0–80% A; 37–40 min, 80% A. The flow rate was 1 mL min^{-1}. The injection volume was 20 μL.

Validation

For calibration and determination of linearity, different volumes of withaferin A working solution (1 mg mL^{-1}) were transferred to a series of 10-mL volumetric flasks and diluted to volume with methanol, to furnish eight solutions containing from 1.2 to 720 μg mL^{-1}. Each of these solutions was injected in triplicate and peak areas of withaferin A were recorded. A calibration plot was constructed relating peak area to the corresponding concentration, and the regression equation was computed.

Repeatability, as intra-day variation, was determined by triplicate analysis of solutions of concentrations of 10, 100, and 500 μg mL^{-1}. Reproducibility, as inter-day variation, was determined by performing seven replicate analyses of the same solutions on four different days.

The limits of detection and quantification were determined, in accordance with ICH recommendations, on the basis of the standard deviation (SD) of the response and the slope of the calibration plot, by use of the equations LOD = 3.3 × SD/slope and LOQ = 10 × SD/slope.

The accuracy of the method was assessed by use of the standard addition technique. Known amounts of pure withaferin A were added to plant extracts in which the natural concentration was known. The resulting mixtures were assayed and the results compared with the expected results.

Results and Discussion

Method Development

RP-HPLC is the method of choice for analysis of marker compounds in medicinal plants owing to its high selectivity, accuracy, precision, and sensitivity. Obtaining good resolution is regarded as a prerequisite for development of such methods. A validated RP-HPLC method for separation and quantitation of the withanolide content of methanolic extracts of flowers, leaves, and roots of *W. somnifera* was developed and optimized as shown in *Figs 2* and *3*. Withaferin A was chosen as marker compound for standardization purposes. Withaferin A could be separated to baseline from other compounds in less than 20 min.

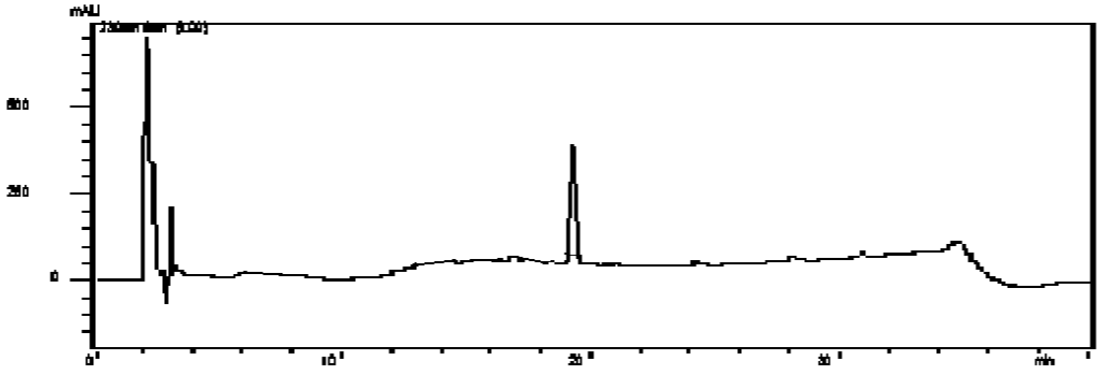

Fig. 2. HPLC chromatogram obtained from withaferin A standard (400 μg mL^{-1})

A linear correlation was obtained between peak area and withaferin A concentration in the range 1.2–720 μg mL^{-1}. The calculated regression equation was $Y = 0.162X - 0.0032$; $r = 0.9998$, where Y and X are, respectively, the peak area and concentration (μg mL^{-1}) of withaferin A and r is the correlation coefficient.

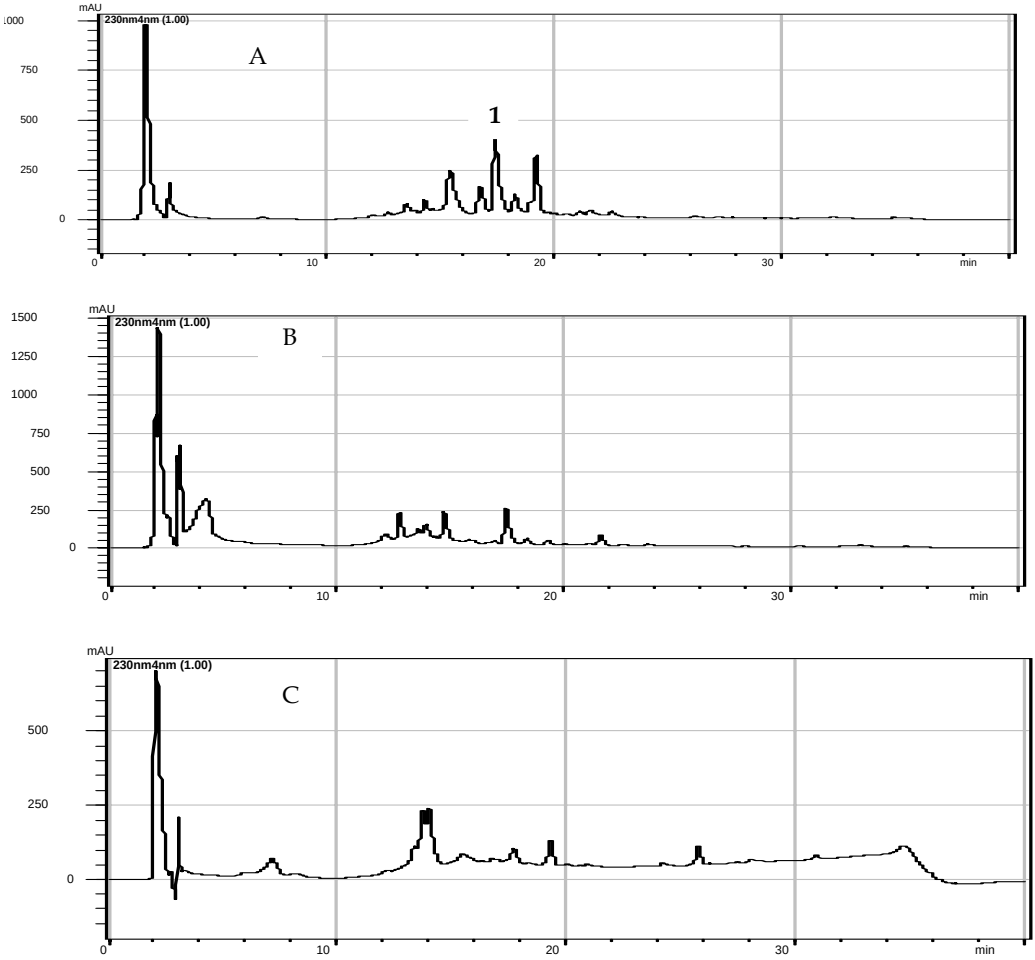

Fig. 3. HPLC chromatograms for analysis of withaferin A (1) in extracts of *Withania somnifera* flowers (A), leaves (B), and roots (C)

Method Optimization

To optimize the method, all of the experimental conditions were investigated. Reversed-phase chromatography was selected in preference to normal-phase because of the drawbacks of the letter, e.g. hydration of silica with water, which can cause peak tailing. The optimum tailing factor was obtained by use of a C_{18} column.

The choice of mobile phase is crucial for successful method development. When methanol was used, withaferin A eluted after 35 min. Because analysis time is an essential aspect of a method, methanol was replaced by

acetonitrile. Acid was added to the mobile phase to reduce peaks tailing and, consequently, increase resolution. The best resolution was achieved by use of the gradient reported above.

The effect of pH was studied by use of buffers of different pH. It was found that changing the pH did not affect peak shape.

Method Validation

The linearity of the calibration graphs are apparent from the high value of the correlation coefficient and the low intercept *(Table I)*. Results from determination of precision, LOD, and LOQ are also given in *Table I* and the

Table I. Method validation data obtained by use of the proposed HPLC method for analysis of pure withaferin A

Linear range (μg mL^{-1})	1.2–720
Limit of detection	1.4
Limit of quantitation	3.7
Slope	0.162
Intercept	0.0032
Mean	99.34
SD	1.76
Correlation coefficient	0.9998
RSD (%)[a]	1.61, 0.77, 0.74
RSD (%)[b]	1.48, 1.48, 1.45

[a]Intra-day RSD for concentrations 10, 100, and 500 μg mL^{-1}
[b]Inter-day RSD for concentrations 10, 100, and 500 μg mL^{-1}

Table II. Results from application of the standard addition technique to analysis of withaferin A in the flowers, leaves, and roots of *Withania somnifera*

Plant material	Amount of pure withaferin A (μg mL^{-1})		Recovery (%)
	Added	Found	
Flowers	20	20.22	101.10
	150	151.98	101.32
	500	498.12	99.62
Mean ± SD			100.68 ± 0.922
Leaves	20	20.32	101.60
	150	148.11	98.74
	500	502.44	100.49
Mean ± SD			100.28 ± 1.441
Roots	20	19.46	97.3
	150	148.88	99.25333
	500	503.44	100.688
Mean ± SD			99.08 ± 1.701

good recovery, and thus accuracy, of the method is apparent from the results listed in *Table II*. No chromatographic changes were observed when withaferin A working solution was stored for 3 days at room temperature, so the solution can be regarded as stable. The suitability of the method is apparent from the good resolution (R = 3.9), relative retention time (α = 2.2), column capacity (K = 2.4), and tailing factor (T = 1.24).

Analysis of Plant Materials

Analysis of *W. somnifera* flowers, leaves, and roots confirmed the presence of withanolides in these three parts of the plant. The withaferin A content was highest in the flowers (*Table III*). It is worthy of note that the withanolide content of the leaves was maximum during the appearance of the flower primordia.

Table III. Application of the proposed HPLC method for determination of withaferin A in flowers, leaves, and roots of *Withania somnifera*

Plant material	Amount of withaferin A (mg g^{-1})
Flowers	4
Leaves	1
Roots	1

Conclusion

The method described herein enables rapid, accurate, and precise analysis of withanolides in *W. somnifera*. The method is characterized by baseline resolution and short analysis time and fulfils all the validation criteria stipulated in the ICH guidelines.

Acknowledgments

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References

[1] R. Schliebs, S.K. Liebmann, A. Kumar, S. Ghosal, and V. Bigl, Neurochem. Int. **30**, 181 (1973)
[2] A.B. Ray and M. Gupta, Prog. Chem. Org. Nat. Prod., **63**, 2 (1994)
[3] S.K. Bhattacharya, K.S. Satyan, and S. Ghosal, Indian J. Exp. Biol., **35**, 236 (1997)
[4] S.K. Kulkarni, B. George, and R. Mathur, Phytother. Res., **12**, 451 (1998)
[5] J.N. Dhuley, Phytother. Res., **15**, 311 (2001)
[6] A.D. Kinghorn, B.N. Su, D.S. Jang, L.C. Chang, D. Lee, J.Q. Gu, E.J. Carcache-Blanco, A.C. Pawlus, S.K. Lee, E.J. Park, M. Cuedei, J.J. Gills, K. Bhat, H.S. Park, E. Mata-Greenwod, L.L. Song, M. Jang, and J.M. Pessuto, Planta Med., **70**, 691 (2004)
[7] C. Tohda, K. Komaatuse, and T. Kuboyama, J. Tradit. Med., **22**, 176 2005
[8] H. Ichikawa, Y. Takada, S. Shishodia, B. Jayaprakasam, M.G. Nair, and B.B. Aggrawal, Mol. Cancer Ther., **5**, 1434 (2006)
[9] M. Kaileh, W.V. Berghe, A. Heyerick, J. Horion, J. Plette, C. Libert, D. De Keukelerie, T. Essawi, and G.Haegeman, J. Biol. Chem., **282**, 4253 (2007)
[10] R.S. Sangwan, N.D. Chaurasiya, L.N. Misra, P. Lal, G.C. Uniyal, N.S. Sangwan, A.K. Srivastava, K.A. Suri, G.N. Qazi, and R. Tuli, US Patent # 7, 108, 870 (2005)
[11] N.D. Chaurasiya, G.C. Uniyal, P. Lal, L. Misra, N.S. Sangwan, R. Tuli, R.S. Sangwa, Phytochem. Anal., **19**, 148 (2008)
[12] S. Dalavayi, S.M. Kulkarni, R.L. Itikala, and S. Itikala, Ind. J. Pharm. Sci., **68**, 253 (2006)
[13] M. Ganzera, M.I. Choudhary, and I.A. Khan, Fitoterapia, **74**, 68 (2003)

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