Quantitative Determination of Reserpine, Ajmaline, and Ajmalicine in *Rauvolfia serpentina* by Reversed-Phase High-Performance Liquid Chromatography

A. Srivastava, A.K. Tripathi, R. Pandey, R.K. Verma, and **M.M. Gupta***
Analytical Chemistry Division, Central Institute of Medicinal and Aromatic Plants, P.O.-CIMAP, Lucknow 226015, India

Abstract

A sensitive and reproducible reversed-phase high-performance liquid chromatography (HPLC) method using photodiode array detection is established for the simultaneous quantitation of important root alkaloids of *Rauvolfia serpentina,* namely, reserpine, ajmaline, and ajmalicine. A Chromolith Performance RP-18e column (100 × 4.6-mm i.d.) and a binary gradient mobile phase composed of 0.01M (pH 3.5) phosphate buffer (NaH_2PO_4) containing 0.5% glacial acetic acid and acetonitrile are used. Analysis is run at a flow rate of 1.0 mL/min with the detector operated at a wavelength of 254 nm. The calibration curves are linear over a concentration range of 1–20 µg/mL (r = 1.000) for all the alkaloids. The various other aspects of analysis (i.e., peak purity, similarity, recovery, and repeatability) are also validated. For the three components, the recoveries are found to be 98.27%, 97.03%, and 98.38%, respectively. The limits of detection are 6, 4, and 8 µg/mL for ajmaline, ajmalicine, and reserpine, respectively, and the limits of quantitation are 19, 12, and 23 µg/mL for ajmaline, ajmalicine, and reserpine, respectively. The developed method is simple, reproducible, and easy to operate. It is useful for the evaluation of *R. serpentina*.

Introduction

Rauvolfia (*Rauwolfia serpentina* (L.) Benth. ex kurz; family: Apocynaceae) is a small woody perennial from India and the East Indies. Reserpine, the major alkaloid of the root, was the first major tranquilizer to be used, especially for the treatment of paranoia and schizophrenia. It was also used as a substance that lowers blood pressure and controls hypertension. Interestingly, its roots were long used in India for treating mental illness and snakebite, known to medicine men and peasants as the "Insanity herb" or "snakeroot". The alkaloid is effective against snakebites and scorpion stings. This drug rapidly replaced electric shock and labotomy as a treatment for certain types of mental illnesses. Moreover, knowledge about the chemistry of this natural plant stimulated the synthesis of other similar alkaloids that are now used as major tranquilizers. The other major alkaloid, ajmaline, possesses antihypertensive and antiarrhythmic activity, whereas ajmalicine is useful in circulatory disorders (1–3). Few thin-layer chromatography (TLC) (4–7) and high-performance liquid chromatography (HPLC) (7–9) methods are reported; however, both the TLC and isocratic HPLC methods reported suffer from the drawback of poor resolution and elution of some impurities together with these compounds at the same retention time. In this paper, a reversed-phase (RP)-HPLC method that is suitable for the separation and quantitation of the three important indole alkaloids: ajmaline, ajmalicine, and reserpine (Figure 1) from a single gradient run of a chromatogram using a photodiode array (PDA) detector is reported. The present work is part of a series of our efforts towards developing liquid chromatographic (LC) procedures (10–18) for plant drug analysis.

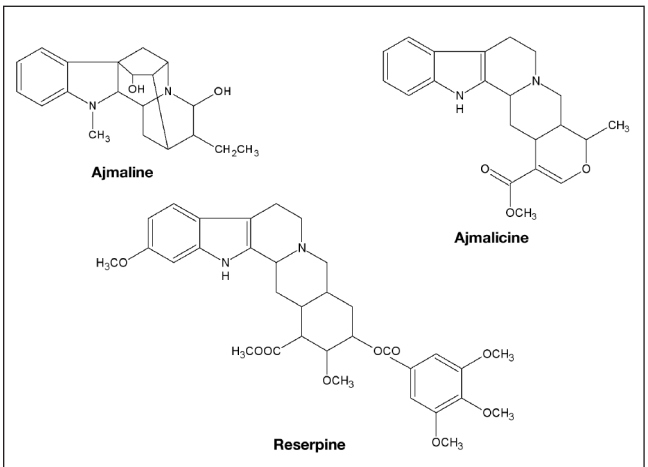

Figure 1. Chemical structures of ajmaline, ajmalicine, and reserpine.

* Author to whom correspondence should be addressed: email guptammg@rediffmail.com.

Experimental

Plant material

R. serpentina roots were collected from the experimental fields of the Central Institute of Medicinal and Aromatic Plants (Lucknow, India), and a voucher specimen of the plant material was deposited in the Gene Bank of this Institute.

Standards and chemicals

The solvents used were of HPLC grade (Merck, Darmstadt, Germany). The standards of ajmaline and ajmalicine were purchased from M/S SRL (Mumbai, India), and reserpine was procured through M/S Sigma (St. Louis, MO). Sodium dihydrogen ortho-phosphate was obtained from Glaxo (Mumbai, India), and HCl (35% GR) was bought from Merck (Mumbai, India). Double distilled water was used after filtering through a 0.45-µm filter before use.

Chromatographic equipment and conditions

A Shimadzu (Kyoto, Japan) LC-8A gradient HPLC instrument equipped with two LC-8A pumps and controlled by a CBM-10A interface module and 7725 I manual injector valve (Rheodyne, Cotati, CA) was used in the HPLC analysis. An SPD-M10Avp (Shimadzu) PDA detector was used to test the peak purity and similarity of the described alkaloids using a class LC-10 work station. The solvents were prefiltered by a Millipore filtration unit (Millipore, Billerica, MA). An RP-18e (100 × 4.6-mm i.d.) RP Chromolith Performance HPLC column (Merck, Darmstadt, Germany) was used for all the analysis. The separation was achieved with a gradient program for pump A (acetonitrile) and pump B (0.01M phosphate buffer containing 0.5% glacial acetic acid; pH 3.5), as given in Table I. The flow rate was 1.0 mL/min throughout the gradient run. Column temperature was maintained at 26°C.

Table I. Gradient Program for the Separation of the Test Indole Alkaloids From the Extract of *R. serpentina* Roots

Time (min)	Acetonitrile conc. (%) (Pump A)	Phosphate buffer containing 0.5% glacial acetic acid conc. (%) (Pump B)	Flow rate (mL/min)
0.01	15	85	1.0
9.00	15	85	1.0
9.01	25	75	1.0
10.00	25	75	1.0
10.01	30	70	1.0
12.00	30	70	1.0
12.01	35	65	1.0
30.00	35	65	1.0
50.00	15	85	1.0

Sample preparation

Air-dried roots of *R. serpentina* (0.1 g) were extracted with methanol for 10 h (3 × 10 mL), filtered, evaporated, defatted with hexane (3 × 5 mL), dried, and redissolved in 1 mL of acidic methanol (methanol–HCl 98;2, v/v) for HPLC analysis.

Calibration graphs

Stock solutions of the compounds (ajmaline, ajmalicine, and reserpine) were prepared in methanol (1 mg/mL) separately, and diluted concentrations were injected for the preparation of calibration graphs and LC analysis. Calibration graphs were plotted by using an area count of each peak (Y) and corresponding concentration (X). The regression equations are presented in Table II.

Results and Discussion

Figure 2 illustrates the separation of ajmaline, ajmalicine, and reserpine in a standard mixture (A) and a plant sample extract (B). For a baseline separation of the test alkaloids, the optimized gradient program for pump A (acetonitrile) and pump B (0.01M phosphate buffer containing 0.5% glacial acetic acid, pH 3.5) was: 0–9 min, 85% of B; 9–10 min, the concentration was maintained at 75% of B; 10–12 min, 70% B; 12–30 min, 65% B; 30–50 min, 85% B; and then the gradient was stopped. The retention times were 6.05, 14.41, and 21.68 min for ajmaline, ajmalicine, and reserpine, respectively. The relative standard deviation (RSD) for the retention times were 1.89%, 0.61%, and 0.65% for ajmaline, ajmalicine, and reserpine, respectively. The selected wavelength, 254 nm, was close to the absorption maxima of all three test compounds. The column performance report for the root extract sample is presented in Table II. As a measure of column performance, the number of theoretical plate counts (N) for ajmaline, ajmalicine, and reserpine were 1720, 33580, and 79482, respectively. Concentration of the analytes was estimated at different intervals, and no change was observed over 24 h.

Table II. The Performance of the RP-18e Chromolith Column for the Separation of the Test Alkaloids From the Extract of *R. serpentina* Roots

Indole alkaloids	t_R* (min)	No. of theoretical plates (N)	Retention factor (k)	Recovery (%)	Separation factor	Linear regression equation [Y = AX ± C]
Ajmaline	6.05	1720	3.01	98.27	1.17	Y = 277.52X + 8.6 (r^2 = 1.0000)
Ajmalicine	14.41	33580	8.54	97.03	1.02	Y = 553.10X + 29.4 (r^2 = 1.0000)
Reserpine	21.68	79482	13.36	98.38	1.02	Y = 166.66X+ 11.2 (r^2 = 1.0000)

* t_R = Retention time.
† r = correlation coefficient

Evaluation of peak purity

A PDA detector with an LC-10 workstation was

used for the peak purity and similarity tests of the three alkaloids. All peaks were found to be pure: both the upwards and downwards slopes of the peaks (Table III). A similarity test of ajmaline, ajmalicine, and reserpine in a sample extract was performed by comparing the similarity of peaks in a sample run to that of library records maintained for the test alkaloids. The similarity of all the compounds was > 0.999 (Table III).

Linearity

To determine the linearity, five different concentrations of each alkaloid were used in a working range of 1–20 µg. Linear regression equations and correlation coefficient (*r*) values for the test compounds are presented in Table II. The plots of peak area versus concentration were linear with *r* values of 1.0000. The calibration plots showed good linearity in the examined concentration range.

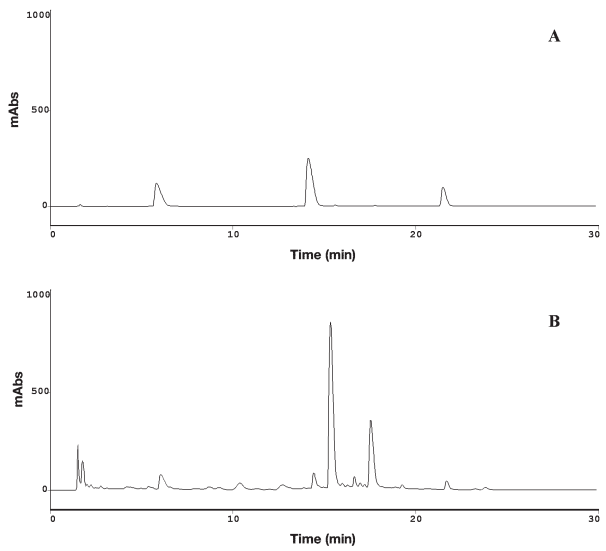

Figure 2. RPLC separation of an artificial mixture of pure ajmaline, ajmalicine, and reserpine, 1 mg/mL each (A) and *R. serpentina* root extract (B).

Table III. Peak Purity Test Results of the Test Alkaloids Using PDA Detector

	Peak Purity		
Alkaloids	Up	Down	Similarity
Ajmaline	0.9998	0.9989	0.9998
Ajmalicine	0.9995	0.9999	0.9998
Reserpine	0.9998	0.9999	0.9997

Limits of detection and quantitation

The limits of detection (LOD) and quantitation (LOQ) values were calculated for the test compounds based on three and 10 times of the noise levels, respectively. The LODs were found to be 6, 4, and 8 µg/mL, whereas the LOQs were found to be 19, 12, and 23 µg/mL for ajmaline, ajmalicine, and reserpine, respectively.

Precision

The precision of the method was measured by repeating each experiment three times. The relative standard deviation values were 2.77%, 2.51%, and 2.38% for ajmaline, ajmalicine, and reserpine, respectively.

Recovery

For the examination of recoveries of ajmaline, ajmalicine, and reserpine, known amounts of stock solutions of authentic test alkaloids were added to the *R. serpentina* root extract, and the quantitation was repeated three times. Recoveries for ajmaline, ajmalicine, and reserpine were 98.27%, 97.03%, and 98.38% with RSDs of 2.14%, 2.05%, and 1.20%, respectively.

Robustness

In order to evaluate the robustness of the method, the influence of small and deliberate variations of analytical parameters on the retention times of the test compounds was studied. The parameters selected were mobile phase composition, flow rate, and temperature. A variation of ± 1% change in the pump B concentration was made to study the effect of slight changes in mobile phase composition. The effect of variations in flow rate and temperature were studied by making changes in the range of ± 0.1% and ± 2°C, respectively. Only one parameter was altered at a time, and the others were kept constant. The RSDs of the retention times and peak area counts were calculated for each parameter, and the RSD was found to be less than 2%. The low RSD values indicated that the method was robust. The results are recorded in Table IV.

Table IV. Robustness Testing for the Alkaloids (*n* = 3)

	RSD % of peak area counts			RSD % of t_R*		
Parameter	Ajmaline	Ajmalicine	Reserpine	Ajmaline	Ajmalicine	Reserpine
Mobile phase concentration	1.25	1.51	1.92	0.83	0.39	0.37
Flow rate	0.36	0.94	0.78	0.28	0.10	0.17
Temperature	0.45	0.82	0.90	0.25	0.17	0.12

* t_R = Retention time (min)

Conclusion

In the reported method, the important alkaloids (i.e., ajmaline, ajmalicine, and reserpine) in the root extracts of *R. serpentina* can be successfully separated, identified, and quantitated by RP-HPLC with the gradient elution mode using a PDA detector. The method, which provides baseline separation of the test alkaloids, is simple, rapid, and precise. The procedure reported here could be used for the rapid screening of the *R. serpentina* plant for genotypic quality assessment, drug analysis, etc.

Acknowledgment

We are thankful to the Director, CIMAP, Lucknow, for providing the facilities and keen interest during our work.

References

1. Anonymous. *The Wealth of India*. CSIR, New Delhi, India, 1969, pp. 377.
2. J.D. Phillipson, and M.H. Zenk. *Indole and Biogenetically Related Alkaloids*. Academic Press, London, UK, 1980, pp. 298.
3. O.P. Virmani, S.P. Popli, L.N. Misra, M.M. Gupta, G.N. Srivastava, Z. Abraham, and A.K. Singh. *Dictionary of Indian Medicinal Plants*. CIMAP, Lucknow, India, 1992, pp. 387.
4. M.S. Habib and W.E. Court. Estimation of Rauwolfia alkaloids by quantitative thin layer chromatography. *J. Pharm. Pharmacol.* **2-3 (Suppl):** 2305 (1971).
5. K.C. Nguyen and I.G. Nikolova. Quantitative determination of alkaloid in root bark of some species of *Rauwolfia* L. by thin layer chromatography. *Rastit. Resur.* **25:** 594–99 (1989).
6. A.G. Vollorovich, I.R. Malva, and E.S. Karbelainan. Improvement of the technique for determination of qualitative composition and quantitative contents of indoline alkaloids in tissue culture of *R. serpentina*. *Rastit. Resur.* **30:** 143–47 (1994).
7. V. Ye. Klyushnichenko, S.A. Yakimov, T.P. Bychkova, Ya. V. Syagailo, I.N. Kuzovkiha, A.N. Bulfson, and A.I. Miroshnikov. HPLC and TLC separation of indole alkaloid from *Rauwolfia serpentina* and *R. vomitoria*. *Khim. Farm. Zh.* **28:** 58–61(1994).
8. U.R. Cieri. Identification and estimation of the alkaloids of Rauwolfia serpentina by high-performance liquid chromatography. *J.—Assoc. Off. Anal. Chem.* **66:** 867–73 (1983).
9. U.R. Cieri. Determination of reserpine and rescinnamine in *Rauwolfia serpentina* preparation by liquid chromatography with fluorescence. *J.—Assoc. Off. Anal. Chem.* **70:** 540–46 (1987).
10. M.M. Gupta, R.K. Verma, G.C. Uniyal, and S.P. Jain. Determination of plumbagin by normal phase high performance liquid chromatography. *J. Chromatogr.* **637:** 209–12 (1993).
11. D.C. Jain, M.M. Gupta, S. Saxena, and S. Kumar. Liquid chromatographic analysis of hepatoprotective diterpenoids from *Andrographis paniculata*. *J. Pharma. Biomed. Anal.* **22:** 705–709 (2000).
12. D.V. Singh, A. Maithy, R.K. Verma, M.M. Gupta, and S. Kumar. Simultaneous determination of *Catharanthus* alkaloids using reversed phase high performance liquid chromatography. *J. Liq. Chromatogr. Relat. Technol.* **23:** 601–607 (2000).
13. D.V. Singh, S. Prajapati, S. Bajpai, R.K. Verma, M.M. Gupta, and S. Kumar. Simultaneous determination of important alkaloids in *Papaver somniferum* using reversed phase high performance liquid chromatography. *J. Liq. Chromatogr. Relat. Technol.* **23:** 1757–64 (2000).
14. D.V. Singh, R.K. Verma, S.C. Singh, and M.M. Gupta. RP-LC analysis of oleane derivatives in *Terminalia arjuna*. *J. Pharma. Biomed. Anal.* **28:** 447–52 (2002).
15. S. Srivastava, R.K. Verma, M.M. Gupta, S.C. Singh, and S. Kumar. HPLC determination of vasicine and vasicinone in *Adhatoda vasica* with photo diode array detection. *J. Liq. Chromatogr. Relat. Technol.* **24:** 153–59 (2001).
16. S. Srivastava, R.K. Verma, M.M. Gupta, and S. Kumar. Reversed phase high performance liquid chromatographic determination of 1,3 benzodioxanes in *Piper mullesua*. *J. Chromatogr.* **841:** 123–26 (1999).
17. R.K. Verma, K.G. Bhartariya, M.M. Gupta, and S. Kumar. Reverse phase high performance liquid chromatography of asiaticoside in *Centella asiatica*. *Phytochem. Anal.* **10:** 191–93 (1999).
18. M.M. Gupta, D.V. Singh, A.K. Tripathi, R. Pandey, R.K. Verma, S. Singh, A.K. Shasany, and S.P.S. Khanuja. Simultaneous determination of vincristine, vinblastine, catharanthine and vindoline in leaves of *Catharanthus roseus* by high-performance liquid chromatography. *J. Chromatgr. Sci.* **43:** 450–53 (2005).

Manuscript received October 13, 2005;
Revision received June 28, 2006.