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Note

Comparison between high-performance thin-layer chromatography–densitometry and high-performance liquid chromatography for the determination of ajmaline, reserpine and rescinnamine in *Rauwolfia vomitoria* root bark

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A number of alkaloids have been isolated from Rauwolfia root barks[1]. Reserpine and rescinnamine are ester alkaloids related to the principal medicinal use of the drug, *i.e.*, the treatment of hypertension and mental disorders[2]; ajmaline is a non-esterified alkaloid and is widely used as an anti-arrhythmic compound, but also as a precursor of semi-synthetic drugs[3].

A number of methods have been proposed for the standardization of Rauwolfia. [They include: total alkaloids gravimetry[4,5], spectrometry of the reserpine-like ester alkaloids[5,6], separation by paper chromatography and spectrometric measurement of reserpine and rescinnamine[7,8]. For these last compounds, thin-layer chromatography (TLC)[9,10], electrophoresis[11], TLC–densitometry[12–14], high-performance liquid chromatography (HPLC)[15,16] have also been used. Assays of ajmaline have been performed by TLC–densitometry[14] and by gas–liquid chromatography (GLC)[3].] Most of these methods were unsatisfactory so that rapid and reliable methods had to be developed in order to study the effects of ecophysiological conditions on the alkaloid production of *Rauwolfia vomitoria* Afz.

An extensive degradation of reserpine and rescinnamine occurs on silica gel so that the addition of α-tocopherol acid succinate to standard solutions was found essential in order to perform the accurate determination of these two alkaloids. Most of the previously described TLC assays for reserpine and rescinnamine were probably biased because of the non-consideration of this degradation.

A new HPLC method, performed on an octadecylsilane column, has allowed the analysis of the three alkaloids in crude plant extracts and the ascertainment of the results obtained by the described HPTLC densitometric methods. All methods

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required a two-step analysis because of the wide difference in the chromatographic behaviours of ajmaline and reserpine–rescinnamine.

EXPERIMENTAL

As far as possible, all the described operations were effected in darkness.

The Perkin-Elmer chromatograph was equipped with a pump (Model 601), a sample loop (Rheodyne 7105), an UV detector (Model LC-55 equipped with the scanner LC-55S, operating at 250 nm for the analysis of reserpine–rescinnamine and at 292 nm for ajmaline) and a 250 × 4 mm I.D. Lichrocart cartridge prepacked with LiChrosorb RP-18, 7 μm (Merck, Darmstadt, F.R.G.). The flow-rate was 1 ml/min. For the analysis of reserpine–rescinnamine, the mobile phase was phosphate buffer (0.04 *M* NaH_2PO_4 and 0.01 *M* Na_2HPO_4)–acetonitrile–1-propanol–tetrahydrofuran (70:13:13:4) and the column was maintained at 40°C while for the ajmaline assays the mobile phase was phosphate buffer (0.04 *M* NaH_2PO_4 and 0.01 *M* Na_2HPO_4)–1-propanol–tetrahydrofuran (95:1:4) and the column temperature was 45°C.

HPTLC pre-coated plates of silica gel 60 F 254 (20 × 10 cm) were obtained from Merck. The solutions (1 μl, standards or crude extracts) were applied 15 mm from the lower edge of the plates and then developed with petroleum benzine (b.p. 40–60°C)–acetone–diethylamine (70:20:10), for reserpine–rescinnamine analysis, or with chloroform–cyclohexane–diethylamine (70:20:10) for ajmaline assays; these mobile phases were allowed to migrate a distance of 100 mm when the plates were placed in unsaturated tanks at a temperature of 20°C. After drying at 105°C for 15 h (reserpine/rescinnamine) or for 2 h (ajmaline), the spots were measured with a Shimadzu high speed TLC scanner CS-930 equipped with a fluorescence attachment. The following settings were used: zigzag stroke width, 9 mm; swing width, 0.2 mm; beam size, 0.4 × 0.4 mm; fluorescence-reflection mode with λ (excitation) = 365 nm, filter 3 (UV cut-off = 480 nm) for reserpine–rescinnamine analysis; absorption-reflection mode with λ (absorption) = 292 nm for ajmaline assays. In both cases, the mean values were calculated by integration of nine spots corresponding to three different standard concentrations, each analysed twice, and three spots of the solution of unknown concentration.

A 100-mg amount of a *Rauwolfia vomitoria* root bark powder (315 μm) was weighed in a 10-ml glass-stoppered centrifuge-tube and 10 mg of calcium hydroxide, 0.2 ml of water and 5 ml of methanol were added. This suspension was shaken for 15 min and centrifuged at 2000 *g*, these two steps being repeated thrice with 5 ml methanol. The supernatants were combined and evaporated to dryness under reduced pressure; the residue was dissolved in 1 ml of methanol and filtered through a Millipore HV-4 filter.

Standards for HPLC were 1/10 to 8/10 dilutions in methanol of a stock solution prepared by dissolving either 10 mg of reserpine base (Boehringer) and 5 mg of rescinnamine base (Inverni & Della Beffa) or 25 mg of ajmaline (Sigma, St. Louis, MO, U.S.A.) in 50 ml of methanol–chloroform (96:4).

For HPTLC, the same standard solutions could be used in the case of ajmaline, whereas for reserpine and rescinnamine other solutions were prepared according to the same modus operandi but using 2% α-tocopherol acid succinate in "hydro-methanolic solution" instead of methanol.

RESULTS AND DISCUSSION

HPLC

There have been few reports on the HPLC separation of Rauwolfia alkaloids in plant material, probably because of low efficiency and peak tailing, both on normal and reversed phases. The chromatographic conditions initially proposed by Robinson[15] were modified so as to obtain the reserpine and rescinnamine separation in

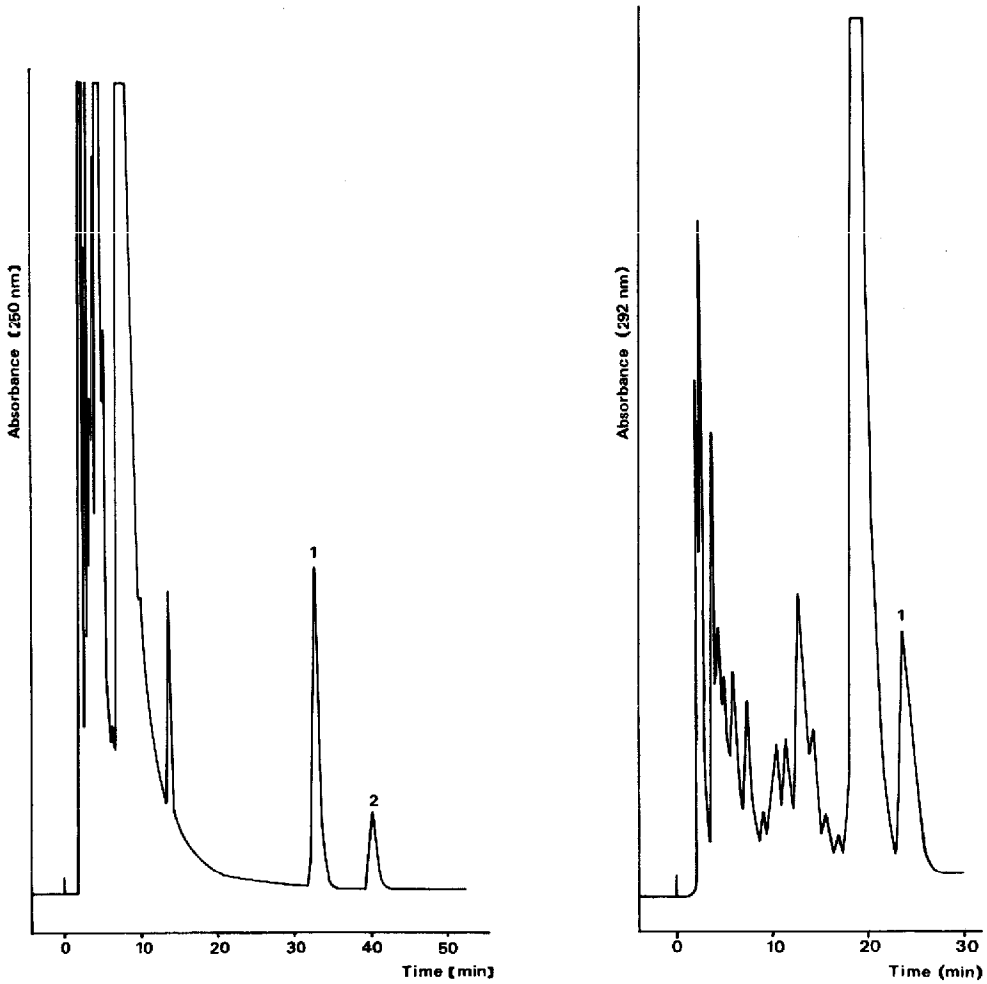


Fig. 1. HPLC chromatogram of a *Rauwolfia vomitoria* stem bark extract. Column packing: Lichrosorb RP-18, 7 μm. Mobile phase: phosphate buffer (0.04 *M* NaH_2PO_4 and 0.01 *M* Na_2HPO_4)–acetonitrile–1-propanol–tetrahydrofuran (70:13:13:4) at a flow-rate of 1 ml/min. Column temperature: 40°C. Detection: UV at 250 nm. Peaks: 1 = reserpine; 2 = rescinnamine.

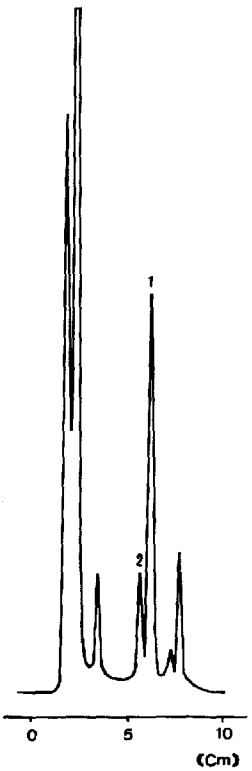
Fig. 2. HPLC chromatogram of a *Rauwolfia vomitoria* stem bark extract. Column packing: Lichrosorb RP-18, 7 μm. Mobile phase: phosphate buffer (0.04 *M* NaH_2PO_4 plus 0.01 *M* Na_2HPO_4)–1-propanol–tetrahydrofuran (95:1:4) at a flow-rate of 1 ml/min. Column temperature: 45°C. Detection: UV at 292 nm. Peak: 1 = ajmaline.

crude plant extracts (Fig. 1) and further adapted to allow an efficient ajmaline retention on the RP-18 column (Fig. 2).

For the analysis of reserpine–rescinnamine, the insufficient selectivity of binary systems such as acetonitrile–buffer was improved by addition of 1-propanol to the mobile phase; the introduction of tetrahydrofuran and higher temperatures allowed an important reduction of the peak tailing without any loss of resolution.

Concerning the analysis of ajmaline, the described conditions are to be considered as "limit conditions", leading to a rapid saturation of the columns with random peaks from previous injections which interfere in the chromatograms, so that frequent washings of the column with methanol–water (50:50) were required, at least twice a day. As described, these assays were only employed to confirm the HPTLC results.

The purity of ajmaline, reserpine and rescinnamine peaks obtained from plant extracts was tested by UV spectroscopy.



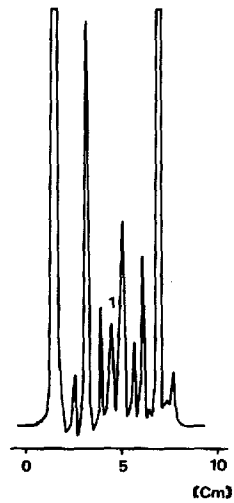


Fig. 3. Scanning profile of an HPTLC chromatogram of a *Rauwolfia vomitoria* stem bark extract. Adsorbent: silica gel 60 F 254. Mobile phase; petroleum benzine (b.p. 40–60°C)–acetone–diethylamine (70:20:10). λ(excitation) = 365 nm; filter 3 (UV cut-off 480 nm). Peaks as in Fig. 1.

Fig. 4. Scanning profile of an HPTLC chromatogram of a *Rauwolfia vomitoria* stem bark extract. Adsorbent: silica gel 60 F 254. Mobile phase: chloroform–cyclohexane–diethylamine (70:20:10). λ(absorption) = 292 nm. Peaks as in Fig. 2.

HPTLC

From the previous studies on the TLC of Rauwolfia alkaloids[17], it became evident that it would not be possible to determine all three alkaloids with a one-step chromatographic development because of the large basicity and polarity differences between ajmaline and reserpine–rescinnamine.

Reserpine and rescinnamine were resolved with one of the solvent systems described by Court and Hubib[18] but using unsaturated tanks at 20°C (Fig. 3). The separation was extremely dependent on the saturation conditions and development temperature.

The purity of the ajmaline spot was uncertain because of the number of substances having similar R_F values and UV spectra; this explains the need for an HPLC method allowing comparison of assay results. An efficient separation was realized

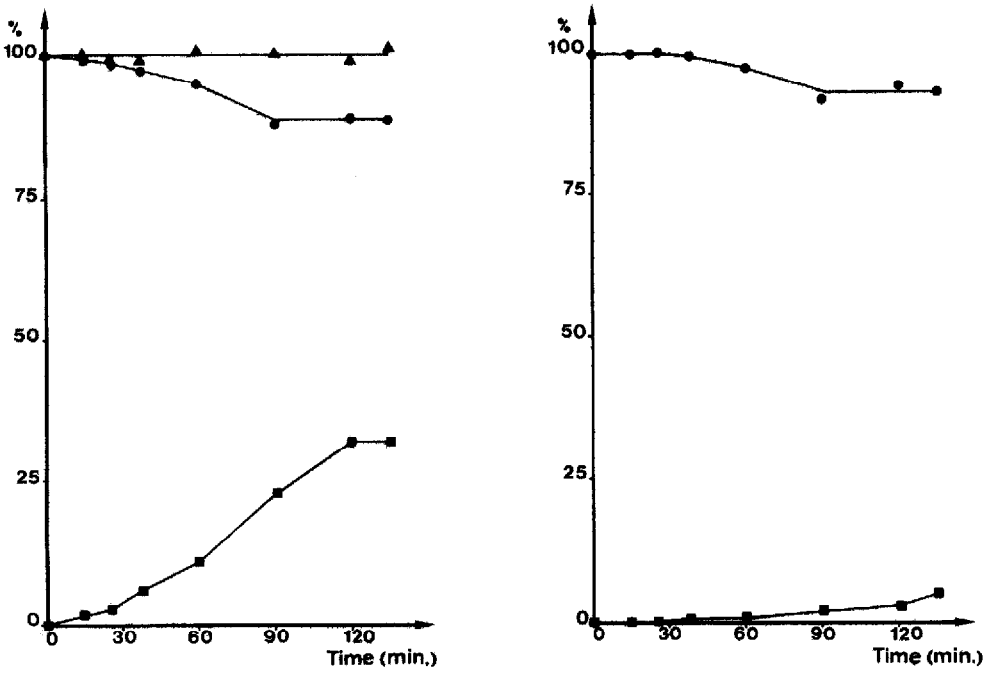


Fig. 5. Degradation of reserpine between the time of application on the silica gel plate and development (time 0): Chromatographic conditions as in Fig. 3; between each application of spots, the plates were left at ambient temperature. ▲—▲; The ratio of the reserpine signal at time t to the reserpine signal at time 0 (percentage) for a *Rauwolfia vomitoria* stem bark extract as a function of the time between application and development. ●—●; Plot as above for a standard. ■—■, The ratio of the signal at time t of the reserpine degradation product (3-dehydro according to Frijns[12]) and to the reserpine signal at time t (percentage) for a standard.

Fig. 6. Degradation of reserpine, in the presence of α-tocopherol acid succinate, between the time of application on the silica gel plate and development (time 0). Chromatographic conditions as in Fig. 3; between each application of spots, the plates were left at ambient temperature. ●—●, The ratio of the reserpine signal at time t to the reserpine signal at time 0 (percentage) for a standard containing α-tocopherol acid succinate as a function of the time between application and development. ■—■, The ratio of the signal at time t of the reserpine degradation product (3-dehydro according to Frijns[12]) to the reserpine signal at time t (percentage) for a standard containing α-tocopherol acid succinate.

TABLE I

DETERMINATION OF RESERPINE, RESCINNAMINE AND AJMALINE IN *RAUWOLFIA VOMITORIA* Afz. EXTRACTS: COMPARISON OF DENSITOMETRIC AND HPLC RESULTS

Alkaloid	*Sample*	*HPTLC with densitometry*		*HPLC*	
		Mean alkaloid (dried powder) (%)	*R.S.D. (%)*	*Mean alkaloid (dried powder) (%)*	*R.S.D. (%)*
Reserpine	1	0.16	6	0.19	2
	2	0.20	2	0.20	4
	3	0.60	3	0.69	4
	4	1.07	2	1.15	2
	5	0.86	1	1.00	4
Rescinnamine	1	0.04	5	0.04	3
	2	0.13	2	0.13	3
	3	0.10	5	0.11	5
	4	0.50	5	0.53	2
	5	0.33	1	0.37	5
Ajmaline	1	0.37	2	0.38	4
	2	0.68	4	0.69	2
	3	1.34	5	1.31	4
	4	0.29	3	0.26	7
	5	0.91	3	0.93	4

using a solvent system described by Kaess and Mathis[19], once again working with unsaturated tanks at 20°C (Fig. 4).

The fluorescence of the reserpine and rescinnamine spots was best developed by keeping the plates at 105°C in the dark for 15 h which yielded stable fluorescent derivatives, probably 3-dehydro[12]; this was preferred to Frijns' method[12] (exposure to light for 2 h), because of its easier reproducibility.

To our knowledge, Frijns[12] is the only author to have noticed the degradation of reserpine and rescinnamine that occurs between application of the spots and the development of the silica gel plates. This degradation probably does not occur during the chromatographic process because no tailing of the spots could be observed; it was noticed only for standard solutions and not for the plant extracts (Fig. 5). From our results, the addition of α-tocopherol acid succinate to the standard solutions was found essential to delay this degradation (Fig. 6). Thus assay errors are reduced if the standard spots are applied moments before development, the time between the first standard spot and development being less than 20 min. Curves similar to those shown in Figs. 5 and 6 were obtained for rescinnamine.

Concentrations of 20–200 ng reserpine, 20–100 ng rescinnamine and 50–500 ng ajmaline per μl spotted afforded linear calibration graphs with r values (correlation coefficients) typically greater than 0.999; the detection limits were 5, 5 and 20 ng respectively.

Extraction procedure and comparison between HPTLC–densitometric and HPLC results

A four-step extraction procedure assured that at least 99% of the total alkaloids were extracted without any degradation. The proposed densitometric HPTLC methods were applied to reserpine, rescinnamine and ajmaline determination in the root bark of *Rauwolfia vomitoria* from Africa. The data were compared (Table I) with those obtained by HPLC, from which it was concluded that these HPTLC–densitometric methods are of interest for the rapid determination of these alkaloids in plants.

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