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Determination of indole alkaloids from *R. serpentina* and *R. vomitoria* by high-performance liquid chromatography and high-performance thin-layer chromatography☆

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Abstract

The separation of a model mixture of six indole alkaloids by high-performance liquid chromatography (HPLC) and high-performance thin-layer chromatography (HPTLC) on the normal- and reversed-phase Armsorb supports, respectively, was studied. A procedure for the analysis of extracts from plants cell callus culture of *Rauwolfia serpentina* and hairy roots of *R. serpentina* and *R. vomitoria* in the presence of these alkaloids and its derivatives with different chromatographic systems was elaborated. It was found that the alkaloid compositions of plant cell cultures and hairy roots of *R. serpentina* and *R. vomitoria* are identical. With HPLC analysis a higher precision of alkaloid determination in solution and measurements of concentration is achieved. HPTLC analysis gives a qualitative identification of the extracts. The combination of these high-performance methods provides quantitative and qualitative analyses of indole alkaloids such as ajmaline, ajmalicine, reserpine, raucaffricine, serpentine and yohimbine.

1. Introduction

Indole alkaloids such as ajmaline, reserpine, yochimbine and others, which are contained in plants of the *Rauwolfia* family, are biologically active substances, valuable drugs and semi-products which can be extracted [1].

In recent years, there has been widespread developments in biotechnological methods for obtaining indole alkaloids, apart from their isolation from natural plants. Metabolic biosynthesis in the natural plants may be different to that in the intact plants. An interesting technique for the synthesis of indole alkaloids by cell culture hairy root cultures of *Rauwolfia serpentina* was described recently [2,3]. Hence it is essential to develop methods for the identification of indole alkaloids.

Among the methods for the identification of alkaloids in solution, such as spectrometry, elec-

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trophoresis and paper chromatography [4], surface adsorption [5,6], liquid column chromatography and affinity chromatography [7], high-performance liquid chromatography (HPLC) with electrochemical and UV detection [8,9], thin-layer liquid chromatography (TLC) [10] and their combinations have particular value.

For separation by TLC, some techniques exist for the identification of alkaloids such as spectrophotometry [11] and chromogenic reactions with different reagents. Court and Timmins [12] described specific chromogenic reactions for 22 indole alkaloids from *Rauwolfia* on a silica plate. In this case, TLC is suitable owing to the diversity of available elution systems [13,14]. A high selectivity was achieved in two-dimensional separation [15]. It is possible to use HPLC and TLC for the scaled-up isolation of pure alkaloids [16].

The aim of the work was to study the separation of a six-alkaloid mixture by high-performance thin-layer chromatography (HPTLC) and reversed-phase (RP) HPLC on Armsorb supports, and to develop qualitative and quantitative analyses for these alkaloids and their derivatives in biotechnological extracts of cell cultures and intact cell cultures of *R. serpentina* and *R. vomitoria* such as hairy roots.

2. Experimental

2.1. Cell cultures

A callus cell culture of *R. serpentina* was obtained as described [17]. The hairy roots of *R. serpentina* and *R. vomitoria* were grown in non-hormonal B-5 medium without light on a shaker (90 rpm) at 26°C for 21 days as described previously [18], then cells were dried under vacuum. Dried culture was kindly provided by Professor V.G. Winter (Kazan State University). The culture of hairy roots was obtained by genetic transformation of leaves of sterile plants of *R. serpentina* L. and *R. vomitoria* Afz. by Ri-plasmid of *Agrobacterium rhizogenes*, strain A-4. Genetic transformation of the plant occurs, which expresses the character of root formation and growth.

2.2. Alkaloid extraction

Amounts of 5 g of dried hairy roots of *R. serpentina* and *R. vomitoria* and cell culture of *R. serpentina* were extracted three times for 10 min in 20 ml of methanol using a Sonifer-250 ultrasound device (Branson Ultrasonics, Danbury, CT, USA). The extracts were evaporated to dryness under vacuum and the residues were dissolved in 100 ml of 0.01 *M* HCl. The filtered solutions were adjusted to pH 6 with 0.01 *M* NaOH and analysed by HPTLC and RP-HPLC.

2.3. Chromatography

For HPTLC, Armsorb HPTLC-KSKG-PZ-UV-254 (high-purity silica, regular particles, 300 m^2/g, pore size 100 Å) and Armsorb HPTLC-Sil-10-PZ-UV-254 (industrial silica, regular particles, 250 m^2/g, pore size 40–80 Å) plates (5 × 10 cm) with a 100-μm silica coating and a preadsorbent layer (Armchrom, Armeniya, Yerevan) were used. The column used for HPLC was Armsorb-300-C_8 (25 × 0.4 cm I.D., particle size 10 μm). Chromatography was performed with a Beckman Model 110B pump, Beckman Model 160 UV detector, Altex Model 210A injector and Waters Model 740 integrator, with an element flow-rate of 0.8 ml/min. Detection was carried out at 280 nm. The indole alkaloids used were ajmaline, ajmalicine, reserpine, raucaffricine, serpentine and yohimbine, kindly provided by Professor J. Stöckigt (Institut für Pharmazie, Johannes Gutenberg-Universität, Mainz, Germany). The samples of alkaloids were dissolved in acetonitrile at a concentration of 1 mg/ml. Internal and external standard methods were selected for quantification. Procedures for the preparation and concentration of the standards were as described above. For HPLC of 1–10 μl of each alkaloid sample and 5–100 μl of each extract were injected. For HPTLC of 1–5 μl of each alkaloid sample and 5–100 μl of each extract were applied.

Chloroform, acetonitrile, methanol, water purified with a Milli-Q system (Waters, Milford, MA, USA), ammonia and trifluoroacetic acid (TFA) were used. Before chromatography, the eluents were filtered through nitrocellulose and

GVWP filters (pore diameter 0.45 μm; Millipore) and degassed for 20 min by means of vacuum. The samples were applied to the preadsorbent layer with 3–4 samples per plate as spots with diameter 5–6 mm. The spots of the samples are transformed into lines at the border of the preadsorbent and separation layers after crossing the preadsorbent layer. Erlich reagent was prepared according to Ref. [19]. After spraying with Erlich reagent the plates were heated at 100°C for 5 min. Cerium ammonium sulphate (CAS) was dissolved in 1% H_2SO_4.

3. Results and discussion

The metabolism of intact plants may differ from each other and from that of natural plants owing to the biotechnological ways in which they were obtained. For reliable analyses for indole alkaloids and their derivatives it is advantageous to use chromatographic methods such as HPLC and HPTLC.

3.1. High-performance thin-layer chromatography

The samples of indole alkaloids (ajmaline, ajmalicine, yohimbine, raucaffricine, reserpine and serpentine) were applied under different conditions to the Armsorb HPTLC-KSKG-PZ-UV-254 and Armsorb HPTLC-Sil-10-PZ-UV-254 plates. TLC was performed using different polar and pH systems. The time of analysis was 30 min. Detection of the spots and thin lines was performed by exposure to UV radiation (254 and 365 nm) and chemical treatment with Erlich reagent followed by heat treatment, and with CAS.

On exposure of the plates to UV radiation some thin and one dominant lines are registered. At 254 nm, when spots of the alkaloid specimens are absorbed on the fluorescent covering of silica, the spots of ajmaline, ajmalicine and yohimbine have a grey colour, serpentine bright blue, raucaffricine dark grey and reserpine grey-blue on the bright green background of the surface. A more effective detection is obtained at 366 nm. In this instance the spots of the specimens showed many-coloured lines: ajmaline grey-blue, ajmalicine pink-yellow, yohimbine yellow, raucaffricine invisible, reserpine yellow-green and serpentine bright blue. With Erlich reagent and subsequent heating, ajmaline appears as a blue spot, yohimbine and ajmalicine brown-red, reserpine light-brown and raucaffricine invisible. With CAS treatment ajmaline appears as a red spot and other alkaloids with different tinges of yellow-brown.

Mobile phase systems for alkaloid separation with high resolution were developed. With separation under acidic conditions the reserpine spot was mostly washed out and the resolution was low. The main systems for elution were chloroform–methanol–ammonia (9.5:0.5:0.01 and 9.8:0.2:0.01) (Table 1, Fig. 1). We used these two chromatographic systems in the analysis, because of the small difference between the R_F values of ajmalicine and reserpine in the first system and ajmaline and serpentine in the second system. The analyses of the extracts from cell cultures and hairy roots of *R. serpentina* and *R. vomitoria* were performed in the same systems. The results showed that *R. serpentina* cell culture extract contained mainly ajmaline and its derivatives (which with CAS treatment appeared as a red spot), reserpine and small impurities (Table 2, Fig. 1). Hairy root cultures of both *R. serpentina* and *R. vomitoria* contained mainly

Table 1
R_F values of indole alkaloids in HPTLC separation on Armsorb HPTLC-KSKG-PZ-UV-254 and Armsorb HPTLC-Sil-10-PZ-UV-254 plates (5 × 10 cm). Mobile phase: $CHCl_3$–MeOH–NH_3 [(A) 9.8:0.2:0.01 and (B) 9.5:0.5:0.01]. Application of 2–5 μl of each alkaloid

Indole alkaloid	R_F			
	KSKG (A)	Sil-10 (A)	KSKG (B)	Sil-10 (B)
Ajmaline	0.06	0.06	0.13	0.18
Ajmalicine	0.79	0.68	0.88	0.76
Yohimbine	0.19	0.10	0.46	0.38
Reserpine	0.53	0.31	0.85	0.75
Serpentine	0.01	0.01	0.03	0.03
Raucaffricine	0.01	0.01	0.01	0.01

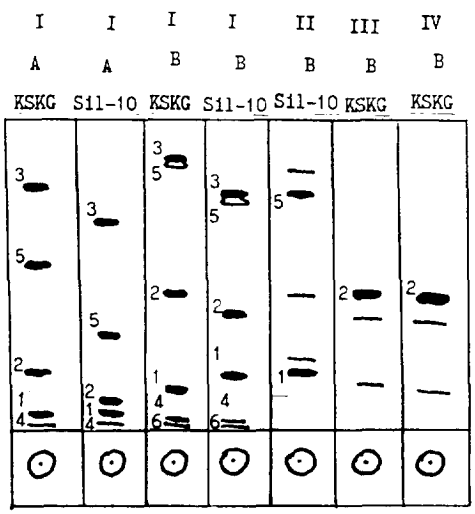

Fig. 1. Separation of (I) 5 μl each sample of indole alkaloids (1 = raucaffricine; 2 = ajmaline; 3 = yohimbine; 4 = ajmalicine; 5 = serpentine; 6 = reserpine), (II) methanol extract from cell culture of *R. serpentina*, (III) methanol extract from hairy roots of *R. serpentina* and (IV) methanol extract from hairy roots of *R. vomitoria*, by HPTLC on Armsorb HPTLC-KSKG-PZ-UV-254 (KSKG) and Armsorb HPTLC-Sil-10-PZ-UV-254 (Sil-10) plates (5 × 10 cm). Mobile phase: $CHCl_3$–MeOH–NH_3 [(A) 9.8:0.2:0.01 and (B) 9.5:0.5:0.01].

yohimbine on a background of small unidentified impurities (Table 3, Fig. 1).

3.2. Reversed-phase high-performance liquid chromatography

For a more effective alkaloid separation, ajmaline, ajmalicine, reserpine, raucaffricine, serpentine, yohimbine and their mixture were analysed by RP-HPLC on the Armsorb-300-C_8 column with different mobile phase systems. The best separation was achieved with acetonitrile–TFA (Fig. 2). The same analysis of extracts of cell cultures and hairy roots of *R. serpentina* and *R. vomitoria* was performed (Figs. 3 and 4). It can be seen from Table 2 and Fig. 3 that ajmaline is the main peak and reserpine and unidentified impurities are present in the *R. serpentina* cell culture. The peak of yohimbine is observed on a background of unidentified impurities in the hairy root cultures of both *R. serpentina* and *R. vomitoria*, as shown in Fig. 4. It is interesting that the amounts of yohimbine and other substances are the same in both hairy root cultures.

4. Conclusion

The separation of model mixtures of six indole alkaloids by RP-HPLC and HPTLC on the normal- and reversed-phase Armsorb supports, respectively, was studied. Procedure for the analysis of extracts from plant cell cultures and hairy roots of *R. serpentina* and *R. vomitoria* in the presence of these alkaloids and their derivatives using different chromatographic systems were elaborated.

It has been found that the alkaloid compositions of callus plant cell cultures and hairy roots of *R. serpentina* are different. The extract from callus cell culture of *R. serpentina* contains ajmaline as the main alkaloid and a small

Table 2
R_F values of the compounds extracted with methanol from *R. serpentina* cell culture in HPTLC separation on the Armsorb HPTLC-KSKG-PZ-UV-254 and Armsorb HPTLC-Sil-10-PZ-UV-254 plates (5 × 10 cm). Mobile phase: $CHCl_3$–MeOH–NH_3 [(A) 9.8:0.2:0.01 and (B) 9.5:0.5:0.01]. Application of 100 μl of the extract

Indole alkaloid	R_f			
	KSKG (A)	Sil-10 (A)	KSKG (B)	Sil-10 (B)
Ajmaline	0.06	0.06	0.14	0.17
Reserpine	0.54	0.32	0.84	0.76
Other substances	0.66	0.58	0.93	0.88
	0.24	0.22	0.42	0.40
	0.10	0.10	0.20	0.20

Table 3
R_F values of compounds extracted with methanol from *R. serpentina* and *R. vomitoria* hairy roots culture in HPTLC separation on Armsorb HPTLC-KSKG-PZ-UV-254 and Armsorb HPTLC-Sil-10-PZ-UV-254 plates (5 × 10 cm). Mobile phase: $CHCl_3$–MeOH–NH_3 [(A) 9.8:0.2:0.01 and (B) 9.5:0.5:0.01]. Application of 100 μl of the extract

Indole alkaloid	R_F			
	KSKG (A)	Sil-10 (A)	KSKG (B)	Sil-10 (B)
Yohimbine	0.18	0.11	0.45	0.39
Other substances	0.14	0.08	0.35	0.30
	0.05	0.05	0.10	0.09

amount of reserpine. The composition of *Rauwolfia* hairy root alkaloids is different to that of the roots of the native plant, but the alkaloid compositions of hairy roots of *R. serpentina* and *R. vomitoria* are identical.

More precise alkaloid separation and concentration determination in solution is achieved by

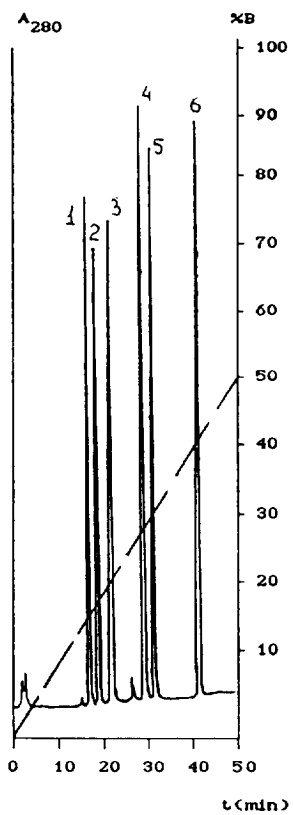

Fig. 2. Separation of indole alkaloids [1 = raucaffricine (5 μl); 2 = ajmaline (10 μl); 3 = yohimbine (3 μl); 4 = ajmalicine (2 μl); 5 = serpentine (8 μl); 6 = reserpine (5 μl)] by HPLC on the Armsorb-300-C_8 column (250 × 4 mm I.D.). Mobile phase: (A) 10% CH_3CN and 0.1% TFA in H_2O; (B) 0.1% TFA in CH_3CN. The percentage of eluent B in the mobile phase is shown by the dashed line.

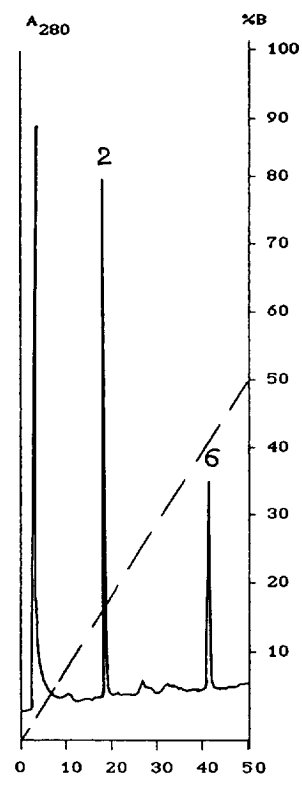

Fig. 3. Analysis of methanol extract from *R. serpentina* cell culture by HPLC on the Armsorb-300-C_8 column (250 × 4 mm I.D.). Mobile phase: (A) 10% CH_3CN and 0.1% TFA in H_2O; (B) 0.1% TFA in CH_3CN. The percentage of eluent B in the mobile phase is shown by the dashed line. Injection volume, 20 μl of the extract.

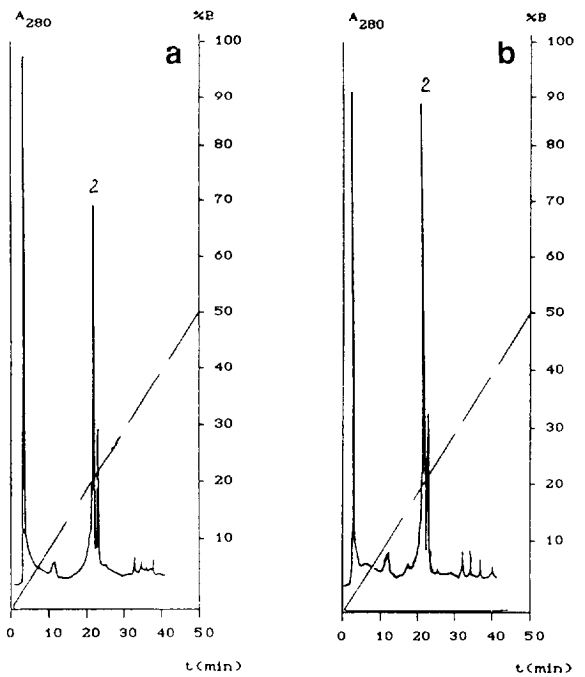

Fig. 4. Analysis of methanol extract from (a) *R. serpentina* and (b) *R. vomitoria* hairy roots by HPLC on the Armsorb-300-C_8 column (250 × 4 mm I.D.). Mobile phase: (A) 10% CH_3CN and 0.1% TFA in H_2O; (B) 0.1% TFA in CH_3CN. The percentage of eluent B in the mobile phase is shown by the dashed line. Injection volume, 20 μl of the extract.

RP-HPLC than HPTLC. With the help of HPTLC, alkaloid identification is achieved by colour treatment under visible and UV light. In addition, this method is simpler than HPLC. The combination of these two high-performance methods provides effective qualitative and quantitative analyses for indole alkaloids.

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