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# SEPARATION OF RAUWOLFIA ALKALOIDS BY THIN-LAYER CHROMATOGRAPHY

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## SUMMARY

The separation of twelve common Rauwolfia alkaloids for quantitative thin-layer chromatography can be effected using a minimum of five solvent systems on silica gel layers. The alkaloids can be identified by chromogenic reactions and ultraviolet and infrared spectrometry.

## INTRODUCTION

Thin-layer chromatography (TLC) has been recommended for the rapid separation of the structurally related indole alkaloids of Rauwolfia[1–3].

Court[4] concluded that silica gel layers permitted better separations than those obtained using other adsorbents. Several authors[5–8] have referred to the difficulties encountered in attempting to apply published methods quantitatively.

In an attempt to obtain better separations for quantitative work, we have investigated systems of separation using a mixture of twelve Rauwolfia alkaloids.

## METHODS

### *Materials*

Silica Gel G (Merck, Darmstadt, B.R.D.) and Kieselgel GF (Merck, Darmstadt, B.R.D.) were used for the thin layers. The plates (20 × 20 cm with layers 250 $\mu$m thick) were activated (60 min) at 110° and stored in a desiccator. Plates were used within 72 h of preparation.

### *Developing solvents*

The following solvent systems were used: (1) xylene–isooctane–ethyl acetate–diethyl ether (15:45:5:40); (2) *n*-butanol–ethyl acetate–ethylene dichloride (10:30:60); (3) acetone–petroleum ether (b.p. 40–60°)–carbon tetrachloride–isooctane (35:30:20:15); (4) methanol–methylethyl ketone–*n*-heptane (8.4:33.6:58)[9]; (5) acetone–*n*-butanol–isooctane (33.6:8.4:58)[6]; (6) acetone–petroleum ether (b.p. 40–60°)–glacial acetic acid (45:45:10); (7) acetone–methanol–glacial acetic acid (70:25:5); (8) *n*-butanol–glacial

acetic acid–water (4:1:1)[10]; (9) acetone–petroleum ether (b.p. 40–60°)–diethylamine (20:70:10); (10) acetone–methanol–diethylamine (70:20:10).

*Reference alkaloids*

Freshly prepared 0.1 % solutions of the marker alkaloids ajmalicine, ajmaline, aricine, deserpidine, rescinnamine, reserpiline, reserpine, reserpinine, serpentine, tetraphyllicine, yohimbine and α-yohimbine in acetone–methanol (1:1) were used. A reference mixture containing these alkaloids in the proportions stated in Table I was also prepared. Reference solutions must be stored in the dark and at low temperatures.

TABLE I

FLUORESCENCE COLOURS PRODUCED BY RAUWOLFIA ALKALOIDS VIEWED IN SCREENED UV LIGHT

| *Alkaloid* | *Proportion in test mixture (mg/10 ml)* | *Sorbent Kieselgel G Wavelength 350 nm* | *Sorbent Kieselgel $GF_{254}$ Wavelength 254 nm* |
|---|---|---|---|
| Ajmalicine | 1 | apple-green | blue |
| Ajmaline | 20 | violet | brown |
| Aricine | 10 | orange | brown |
| Deserpidine | 10 | blue-green | blue |
| Rescinnamine | 1 | blue-green | pale blue |
| Reserpiline | 20 | yellow | brown |
| Reserpine | 10 | apple-green | pale blue |
| Reserpinine | 10 | apple-green | pale blue |
| Serpentine | 20 | intense blue-violet | pale blue |
| Tetraphyllicine | 10 | violet | brown |
| Yohimbine | 10 | blue | blue-violet |
| α-Yohimbine | 10 | blue | blue-violet |

*Chromatographic technique and apparatus*

Glass developing tanks 20 × 24 × 9 cm were used. Test and reference solutions were applied to the TLC plates manually using an Agla micrometer syringe and automatically using a Burkard Autospot Applicator (Burkard Manufacturing Co. Ltd., Herts., Great Britain). All plates were developed by ascending technique, the solvent migration being 15 cm. To ensure adequate equilibration, tanks were lined with Whatman No. 1 filter paper and the angle of the plates with the vertical was arbitrarily controlled at 15°. The normal load applied was 4 μg of alkaloid (equivalent to 4 μl of solution) spotted as droplets of 0.25 μl at 5-sec intervals in order to minimise the size of the initial spot. Developed plates were dried at 100° for 1 h in a drying oven.

*Spectroscopy techniques*

*Ultraviolet spectrophotometry.* Spots, located by fluorescence colours or chromogenic reagents, were removed from the developed and dried plates using the micro-vacuum cleaner technique[11]. The removed adsorbent was extracted by shaking with methanol; the resultant suspension was filtered through sintered glass and the filtrate examined using a Unicam SP 800 recording spectrophotometer with a compensating blank prepared in a similar manner.

*Infrared spectrophotometry.* Microdiscs of potassium bromide and alkaloid

were prepared as described earlier[12]. Infrared (IR) spectrophotometric characteristics were determined using a Unicam SP 200 spectrophotometer with beam condenser and compensating attenuator. Spectrograms were compared with those prepared in a similar manner using authenticated alkaloids.

## RESULTS AND DISCUSSION

*Solvent systems*

The separation of feebly basic compounds on silica gel layers requires a solvent system with low elutive power. Several such solvents, *e.g.* diethyl ether and carbon tetrachloride, were investigated and found to be unsatisfactory. Mixtures of two solvents in varying proportions were also found to be unsatisfactory, but three or four component systems did produce satisfactory separations, the criteria for good separation being $R_F$ values and the compactness of the spots.

About 300 solvent systems were investigated and it was concluded that no single solvent system can adequately separate the available Rauwolfia alkaloids for quantitative determination.

For the separation of the difficult feebly basic alkaloid pairs reserpine/rescinnamine and ajmalicine/reserpinine solvent system 3 was found to yield better results. Reserpiline can be separated using the same solvent system but interference from yohimbine limits its usefulness in quantitative work.

Solvent system 2 was adequate for separating reserpiline, $\alpha$-yohimbine, yohimbine and ajmalicine but offered no advantages for the other alkaloids.

For the separation of ajmalicine and aricine from reserpinine, solvent system 1 was preferred.

Separation of the more polar compounds such as ajmaline and serpentine requires the use of strongly elutive solvents. Hence solvent systems 6 and 9 facilitated the separation of ajmaline and were preferred to solvent system 8 because they are quicker and more stable. Court *et al.*[8] stressed the importance of the effect of relative concentrations of components in extracts of natural products on TLC behaviour. Thus solvent system 2 should be employed for the separation of ajmaline if the reserpine/rescinnamine concentration is high and solvent system 9 if it is low. Furthermore solvent system 6 functions better with smaller loads (3 $\mu$g) whereas solvent system 9 can accommodate a greater load (10 $\mu$g).

The separation of serpentine and tetraphyllicine was best performed using solvent systems 7 or 10 although solvent system 7 is preferred as rounder spots were obtained, spots obtained with solvent system 10 being more elongate.

Table II summarises the $hR_F$ values obtained for these systems using a test mixture of twelve Rauwolfia alkaloids.

*Identification of the separated alkaloids*

The developed and dried plates were examined under screened ultraviolet (UV) light (wavelengths 254 and 350 nm). Using Kieselgel G and $GF_{254}$ plates and recording the resultant fluorescence colours, tentative identifications were possible (Table II).

Chromogenic tests[4] were applied to the twelve alkaloids and two additional reagents were found to be useful, *viz.* (i) 0.5% phosphomolybdic acid in 50% nitric acid and (ii) 1% ammonium vanadate in 50% nitric acid (Table III).

TABLE II

TLC $hR_F$ VALUES OF RAUWOLFIA ALKALOIDS USING MIXED SOLVENTS

The $hR_F$ values stated are the means of six determinations (mean ±1.2).

| *Alkaloid* | *Solvent system* | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | *1* | *2* | *3* | *4* | *5* | *6* | *7* | *8* | *9* | *10* |
| Ajmalicine | 13 | 74 | 55 | 60 | 66 | 12 | 54 | 56 | 91 | 96 |
| Ajmaline | 0 | 3 | 3 | 5 | 3 | 42 | 80 | 69 | 51 | 94 |
| Aricine | 30 | 83 | 71 | 64 | 74 | 12 | 74 | 53 | 92 | 96 |
| Deserpidine | 0 | 63 | 37 | 44 | 48 | 29 | 76 | 72 | 57 | 96 |
| Rescinnamine | 0 | 56 | 26 | 36 | 37 | 22 | 80 | 72 | 56 | 96 |
| Reserpiline | 0 | 30 | 20 | 28 | 30 | 6 | 42 | 50 | 62 | 93 |
| Reserpine | 0 | 58 | 32 | 38 | 40 | 22 | 80 | 72 | 60 | 96 |
| Reserpinine | 25 | 83 | 70 | 62 | 74 | 10 | 74 | 53 | 92 | 93 |
| Serpentine | 0 | 0 | 0 | 0 | 0 | 0 | 22 | 46 | 5 | 70 |
| Tetraphyllicine | 0 | 4 | 4 | 6 | 6 | 6 | 30 | 50 | 70 | 90 |
| Yohimbine | 0 | 18 | 22 | 30 | 31 | 0 | 34 | 45 | 68 | 94 |
| α-Yohimbine | 0 | 51 | 44 | 46 | 48 | 0 | 34 | 36 | 72 | 96 |

TABLE III

CHROMOGENIC REACTIONS OF SOME RAUWOLFIA ALKALOIDS

| *Alkaloid* | *1% ceric sulphate in 10% sulphuric acid* | *Sulphomolybdic acid (Frohde's reagent)* | *5% ferric chloride in 50% nitric acid* | *Iodoplatinate reagent* | *0.5% phosphomolybdic acid in 50% nitric acid* | *1% ammonium vanadate in 50% nitric acid* |
|---|---|---|---|---|---|---|
| Ajmalicine | grey | green | grey | pink | yellow | green |
| Ajmaline | crimson | red | deep red | pink | deep red | deep red |
| Aricine | brown | deep green | orange-brown | pink | pale green | pale green |
| Deserpidine | grey-brown | green | yellow-brown | pink | white | pale green |
| Rescinnamine | green-brown | yellow-green | yellow-brown | pink | yellow-green | yellow-green |
| Reserpiline | violet | pink | brown | brown | yellow-green | — |
| Reserpine | green-brown | yellow-green | yellow-green | pink | yellow-green | yellow-green |
| Reserpinine | yellow-brown | green | yellow | pink | yellow-green | yellow-green |
| Serpentine | — | — | — | brown | buff | buff |
| Tetraphyllicine | crimson | deep red | pink | pink | deep red | deep red |
| Yohimbine | grey | yellow-green | yellow | pink | yellow-green | grey |
| α-Yohimbine | grey | yellow | yellow | pink | yellow-green | grey |

Ajmaline and tetraphyllicine were the only alkaloids which could be identified positively by their chromogenic reactions; both were also readily located on fluorescent silica gel layers.

The UV absorption characteristics of the alkaloids recovered from the plates

agreed with the characteristics of the corresponding reference alkaloids (Table IV). Similarly, the IR spectra of the recovered alkaloids showed superimposable peaks when compared with the corresponding reference alkaloid spectra.

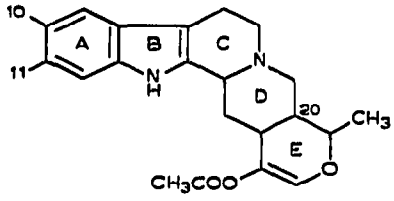
TABLE IV

UV ABSORPTION SPECTRA CHARACTERISTICS OF SOME RAUWOLFIA ALKALOIDS (IN METHANOL)

| *Alkaloid* | *Untreated reference alkaloid* | | *Recovered reference alkaloid* | |
|---|---|---|---|---|
| | $\lambda_{max.}$ *(nm)* | $\lambda_{min.}$ *(nm)* | $\lambda_{max.}$ *(nm)* | $\lambda_{min.}$ *(nm)* |
| Ajmalicine | 227, 282, 290 | 263 | 229, 280, 290 | 265 |
| Ajmaline | 214, 252, 290 | 226, 273 | 216, 247, 291 | 236, 270 |
| Aricine | 235, 280, 292 | 265 | 230, 279, 292 | 270 |
| Deserpidine | 227, 272, 290 | 244 | 223, 267, 290 | 245 |
| Rescinnamine | 229, 303 | 258 | 223, 304 | 260 |
| Reserpiline | 229, 300 | 277 | 226, 301 | 282 |
| Reserpine | 229, 267, 296 | 247 | 224, 265, 295 | 247 |
| Reserpinine | 235, 303 | 286 | 232, 298 | 284 |
| Serpentine | 254, 309, 360 | 282 | 251, 307, 361 | 280 |
| Tetraphyllicine | 214, 248, 290 | 226, 271 | 212, 248, 293 | 226, 271 |
| Yohimbine | 225, 282, 290 | 247 | 227, 281, 289 | 255 |
| α-Yohimbine | 225, 282, 290 | 247 | 226, 282, 291 | 250 |

*TLC behaviour of the alkaloids*

The ajmalicinoid alkaloids vary in the substitution at the C-10 and C-11 positions in the aromatic ring of the indole nucleus and also by *cis–trans* isomerism of the D/E rings.




| | *C-10* | *C-11* | *C-20* | *D/E conformation* |
|---|---|---|---|---|
| Ajmalicine | H | H | Hβ | *trans* |
| Reserpinine | H | $CH_3O$ | Hα | *cis* |
| Aricine | $CH_3O$ | H | Hα | *cis* |
| Reserpiline | $CH_3O$ | $CH_3O$ | Hα | *cis* |

The *trans* D/E ring junction alkaloid ajmalicine yields lower $hR_F$ values than the *cis* D/E ring junction alkaloids aricine and reserpinine (Fig. 1). Substitution at C-10 and C-11 is important. Although such substitution has little effect on $hR_F$ values, nevertheless the 10-methoxy variant (aricine) normally moves slightly further than the

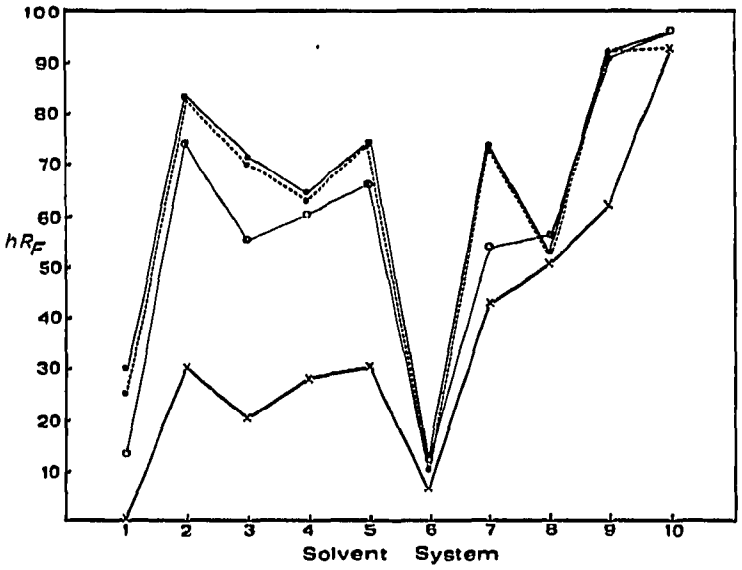



Fig. 1. $hR_F$ values of ajmalicine (○—○), aricine (●—●), reserpiline (×—×), and reserpinine (●---●).

11-methoxy variant (reserpinine) and useful separation was effected only with solvent system 1. The *cis* D/E ring junction, 10,11-dimethoxy alkaloid reserpiline yields a much reduced $hR_F$ value. These findings are in general agreement with the reports of Phillipson and Shellard[13,14].

The common weakly basic alkaloids of the yohimbinoid type include the ester alkaloids of pharmacological importance, deserpidine, reserpine and rescinnamine and yohimbine and its C-16 isomer, α-yohimbine (Fig. 2).

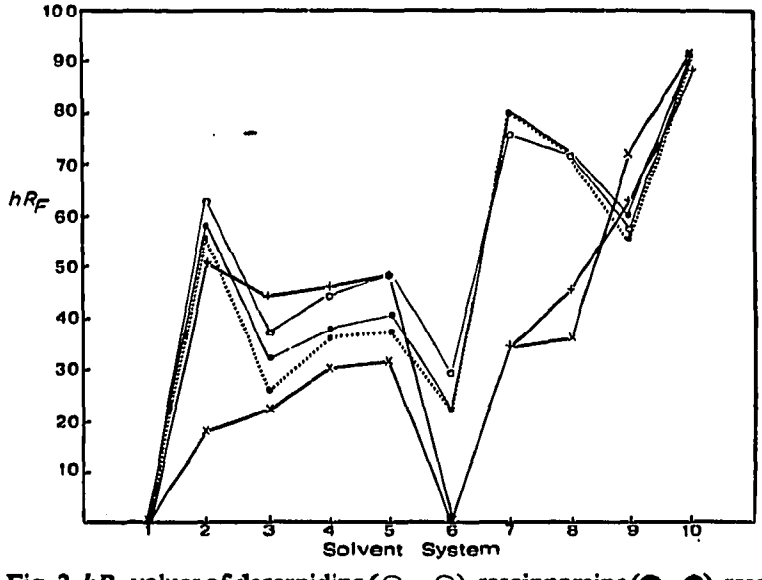
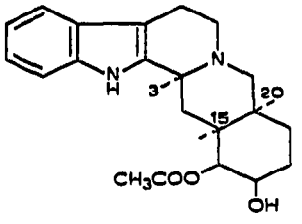
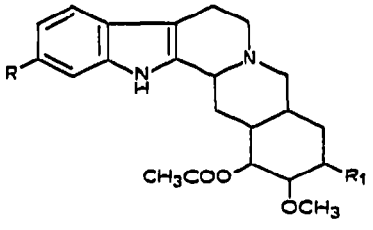



Fig. 2. $hR_F$ values of deserpidine (○—○), rescinnamine (●...●), reserpine (●—●), yohimbine (×—×) and α-yohimbine (+—+).

The ester alkaloids reserpine and rescinnamine possess a C-11 methoxy group and differ only in the ester group, trimethoxybenzoic (TMB) and trimethoxycinnamic (TMC), respectively. These alkaloids are very difficult to separate, the $hR_F$ value for reserpine normally exceeding that of rescinnamine, but usually only marginally; solvent system 3 is the most effective. Deserpidine, differing from reserpine only by the absence of the 11-methoxy group, generally yields a higher $R_F$ value.




| | | $R$ | $R_1$ |
|---|---|---|---|
| Yohimbine C-16 = $\alpha$ | Reserpine | $CH_3O$ | TMB |
| $\alpha$-Yohimbine C-16 = $\beta$ | Rescinnamine | $CH_3O$ | TMC |
| | Deserpidine | H | TMB |

Yohimbine yields a lower $hR_F$ value than the ester alkaloids except in solvent systems 9 and 10 involving diethylamine, where yohimbine and $\alpha$-yohimbine, both possessing C-17 hydroxyl groups move further than the ester alkaloids. As diethylamine will hydrogen bond to the hydroxyl groups of the adsorbent, the silica gel does not adsorb the alkaloids as efficiently and the $hR_F$ values are higher and the alkaloids less well separated.

Solvent system 3, which functions well with loads up to 10 $\mu$g, offers the best separation of the five test alkaloids deserpidine, reserpine, rescinnamine, yohimbine and $\alpha$-yohimbine, but its use is limited in the presence of reserpiline.

The main features of the chromatographic behaviour of the weakly basic alkaloids of Rauwolfia are:

(a) The principal adsorption due to the $N_b$ lone pair electrons hydrogen bonding to the silanol hydroxyl groups on the silica gel surface.

(b) The effect of C-10 or C-11 substitution, the unsubstituted forms, *e.g.* ajmalicine and deserpidine, showing higher $hR_F$ values.

(c) The effect of E ring substitution; thus C-16$\beta$ substitution yields higher $hR_F$ values than C-16$\alpha$ substitution in neutral systems and C-18 trimethoxybenzoic acid substitution yields higher $hR_F$ values than C-18 trimethoxycinnamic acid substitution.

(d) The effect of the D/E ring junction conformation, *cis* D/E conformation yielding higher $hR_F$ values than *trans* D/E conformation.

Overlap of these effects accounts for the difficulties in separating mixtures of the weakly basic alkaloids.

When neutral systems are employed, the stronger bases tend to remain near the baseline (solvent systems 1–5). In acid solvent systems 6–8 movement is dictated by the eluting power of the associated solvent; thus in solvent system 6 movement is restricted but markedly increased in solvent systems 7 and 8. In the diethylamine-

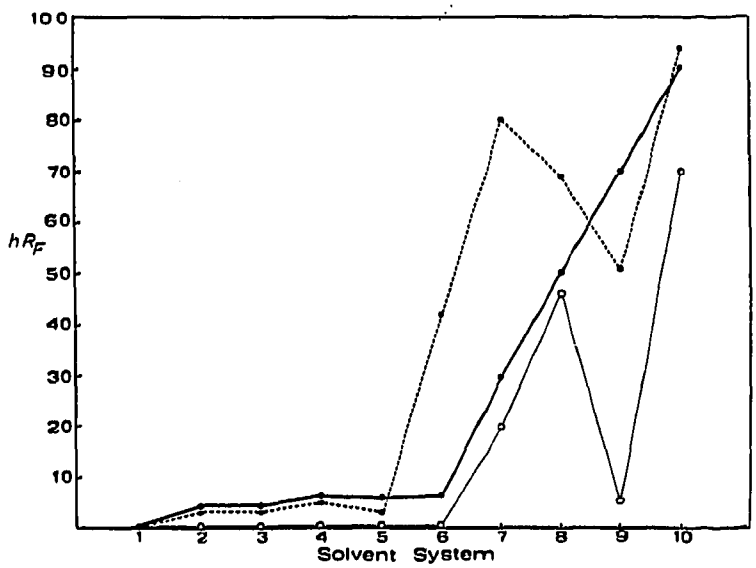
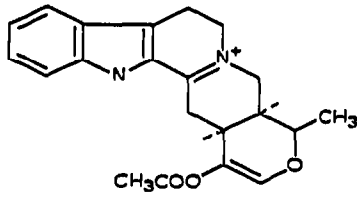
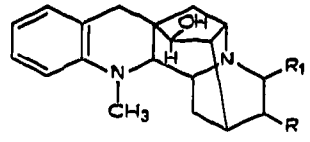



Fig. 3. $hR_F$ values of ajmaline (●---●), tetraphyllicine (●—●), and serpentine (○—○).

containing solvent systems 9 and 10, lowered adsorption on the silica gel causes greater elution of the alkaloids (Fig. 3).




Serpentine




| | $R$ | $R_1$ |
|---|---|---|
| Tetraphyllicine | $=CH-CH_3$ | H |
| Ajmaline | $-CH_2-CH_3$ | OH |

Ajmaline normally moves further than its congener tetraphyllicine, which lacks the C-21 hydroxy group and possesses a C-18 ethylidene group instead of an ethyl group.

Serpentine, which is more strongly basic, can only be moved significantly from the baseline in acid solvent systems (7 and 8) or in alkaline solvent systems (10) where the adsorptive power of the silica gel is reduced by competition between the alkaloid and diethylamine molecules for the exposed silanol hydroxyl groups.

## CONCLUSIONS

The separation of twelve Rauwolfia alkaloids for quantitative TLC assay was satisfactorily achieved using a minimum of five solvent systems.

The alkaloids were therefore separated into five groups, *viz.*: (i) reserpine, rescinnamine and deserpidine (solvent systems 3 and 4), (ii) reserpiline, yohimbine and α-yohimbine (solvent system 2), (iii) reserpinine, aricine and ajmalicine (solvent system 1), (iv) ajmaline (solvent systems 6, 8, and 9), and (v) serpentine and tetraphyllicine (solvent systems 7–10).

Manipulative losses are minimised by this technique, which is preferred to the earlier fractionation into weakly and strongly basic fractions prior to chromatography.

Ajmaline and tetraphyllicine were the only alkaloids identified positively by their chromogenic reactions and location on fluorescent silica gel layers but the identities of all the alkaloids used were confirmed by UV and IR spectrophotometry.

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