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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF THE AJMALICINE DISTRIBUTION IN ROOTS OF *CATHARANTHUS ROSEUS* LINES WITH DIFFERENT FLOWER COLOURS

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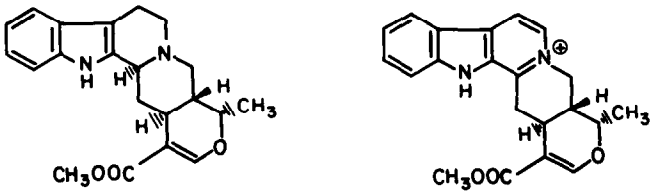
SUMMARY

The distribution and yield of ajmalicine in roots were studied in three pure lines of *Catharanthus roseus* plant with different flower colours, by means of high-performance liquid chromatography. The highest levels of ajmalicine were found in the line with red-eyed flowers. In all lines, the tap root contained higher amounts of ajmalicine than the lateral roots. The implications of these findings for plant breeding and yield optimization are evaluated.

INTRODUCTION

The indole alkaloids ajmalicine (1) and serpentine (2) (Fig. 1) have been found in roots of several *Catharanthus* and *Rauwolfia* plant species[1]. The minor component



1 2

Fig. 1. The structures of ajmalicine (1) and serpentine (2).

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1 is employed as a hypotensive and tranquilizing agent, whereas the major product 2, which is pharmacologically inactive, can be readily converted into 1 by reduction[1]. An additional source of these derivatives might be provided by cell suspension cultures[1].

As part of a study on the production of compound 1 from *Catharanthus roseus* (L.) G. Don (Apocynaceae) roots, analytical methods were considered for the determination of 1 and 2. Previously reported procedures include thin-layer chromatography (TLC)–spectrophotometry[2,3], TLC–fluorimetry[4,5,16], densitometric TLC[3] and radioimmunoassay[4]. High-performance liquid chromatography (HPLC) has also been applied to determination of compound 1 in mixtures of *C. roseus* alkaloids[6], and in *C. roseus* roots[3,7,16] and cell cultures[8–13,17].

The aim of this work was to study the distribution and yield of compound 1 in roots of *C. roseus* plants, in three pure lines with different flower colours. We report the use of reversed-phase HPLC for the estimation of this compound in the tap and lateral roots of *C. roseus* plants with white, pink and red-eyed flowers, respectively.

EXPERIMENTAL

Reference compounds and solvents

The ajmalicine used as a standard for the preparation of the calibration graph was isolated from *C. roseus*, purified and identified by comparison with an authentic sample kindly supplied by Dr. G. G. Marconi, Lilly Research Laboratories, Indianapolis, IN, U.S.A. Methanol was of LiChrosolv grade (Merck, Darmstadt, F.R.G). A solution of compound 1 in methanol (0.2 mg/ml) was used as the calibration standard.

Apparatus

Chromatographic separations were done on a Tracor 985 liquid chromatograph equipped with a Model 970 A variable-wavelength detector and a Model 951 pump, connected to an Hewlett-Packard recorder, Model 7131 A. A reversed-phase column (Alltech C_{18}, 25 cm × 4.6 mm I.D., 10 μm) was used at ambient temperature, the chromatograms being monitored at 254 nm. The mobile phase was methanol–water (8:2) containing 0.5 ml concentrated ammonium hydroxide solution per litre, the flow-rate 0.6 ml/min, the pressure 2500 p.s.i. and the recorder chart speed 0.25 cm/min.

Plant material and growth conditions

Three true breeding, unrelated pure lines, representing the three common flower types of the species were developed by two successive generations of artificial selfing of single plants with a different corolla colour: pink, white and white with a red eye in the centre[14]. Seeds of each line were sown separately in a greenhouse and transplanted to the field 3 weeks later. Five plants randomly sampled from each line were dug out at the end of the growing season, about 5 months after transplantation. The fresh and dry weights of the roots of each plant (drying in an oven at 60°C for 4 days) were determined, and its tap root and lateral roots were separately ground and subjected to extraction.

Extraction procedure

Dried and finely powdered plant samples (1 g) were extracted with methanol (20 ml) for 48 h at room temperature. The suspensions were filtered quantitatively and the filtrates were made up to 25 ml with methanol in volumetric flasks. Aliquots (2 ml) of the extracts were treated with sodium borohydride (*ca.* 30 mg) and kept at room temperature for 1 h for complete reduction of compound 2 to 1 (TLC evidence). The reaction mixtures were filtered quantitatively into 2-ml measuring flasks and made up to volume with methanol.

HPLC determination

Aliquots (5 μl) of the borohydride-treated extracts were injected into the chromatograph using a sample clarification kit (Schleicher and Schüll, Dassel, F.R.G.) including a filter holder and a membrane filter disc (diameter 13 mm, porosity 0.45 μm). The mobile phase was filtered through a membrane filter disc (diameter 47 mm, porosity 0.45 μm; Schleicher and Schüll). The column was regenerated by washing with methanol after analyses.

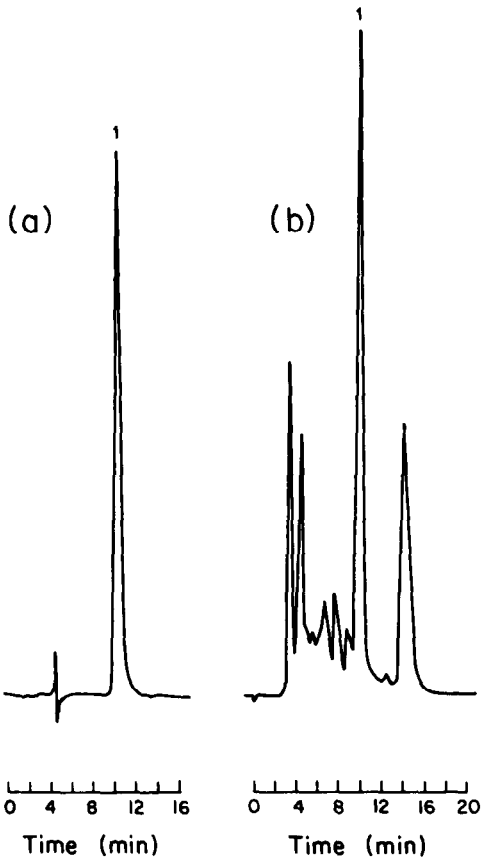

Fig. 2. Typical chromatograms of (a) standard ajmalicine (1 μg), (b) *C. roseus* extract (100 μg dry plant). Peak 1 = ajmalicine.

TLC–spectrophotometric determination

The assay was performed according to a published procedure[2], except that borohydride-treated samples prepared as described above were processed, rather than crude methanolic extracts. Following analytical TLC, the absorbances of the methanol-eluted samples were recorded at 283 nm, and their contents of compound 1 estimated with reference to a standard curve.

RESULTS AND DISCUSSION

Reversed-phase chromatography provided satisfactory resolution of compound 1 contained in borohydride-treated methanolic extracts of *C. roseus* roots, as no interfering peaks appear at a similar retention time in the chromatograms (Fig. 2). The addition of a few drops of ammonia to the mobile phase greatly improved the peak shapes, and stable baselines were obtained using isocratic elution. Virtually no differences in retention time between various batches were observed under these conditions. A linear plot was obtained of the peak height against concentration ($r = 0.995$, $y = 0.465\ x + 0.353$, in the range of 0.8–1.6 μg of compound 1). The recovery (mean ± S.D.) of compound 1 from ajmalicine-fortified plant extracts was 96.6 ± 4.0% (Table I). Assays done on 1-g samples of dried plant material were found to be convenient, accurate and reproducible. The reproducibility of analyses was estimated by the coefficient of variation of a standard solution of compound 1 within four successive assays, and between fourteen assays at various time intervals (1–100 days); the coefficients were 7.66 and 19.60% within and between assays, respectively. Slightly lower recoveries were recorded when 100–500 mg of plant material were analyzed.

Previously reported methods for the determination of compound 1 in *C. roseus* roots involve the addition of water to the methanolic solution obtained following reduction with sodium borohydride, subsequent extraction of the resulting mixture with dichloromethane, and concentration of the extract to a suitable volume prior to HPLC analysis[3,7]. Our approach suggests that the borohydride-reduced plant extracts may be injected directly into the HPLC system, thus avoiding further operations and transfers.

For comparison, several *C. roseus* roots samples were concurrently assayed by

TABLE I

RECOVERY OF AJMALICINE FROM SAMPLES OF *CATHARANTHUS ROSEUS* BY HPLC

Ajmalicine (mg)				*Recovery (%)*
Determined in sample	*Added*	*Calculated*	*Found*	
2.5	5.0	7.5	7.6	101.3
3.2	5.0	8.2	8.3	101.2
4.3	5.0	9.3	8.4	90.3
5.3	5.0	10.3	10.1	98.1
6.2	5.0	11.2	10.8	96.4
7.1	5.0	12.1	11.3	93.4
12.0	5.0	17.0	16.3	95.9

TABLE II

AJMALICINE YIELDS IN THREE LINES OF *CATHARANTHUS ROSEUS* PLANTS WITH DIFFERENT FLOWER COLOURS

Values represent means ± standard errors of five replications.

Flower colour	*Root weight (g per plant)*		*Ajmalicine level (% of dry weight)*	
	Fresh	*Dry*	*Tap root*	*Lateral roots*
Pink	81.9 ± 9.6*	23.1 ± 2.7	0.63 ± 0.04	0.45 ± 0.04
White	97.8 ± 4.5	27.9 ± 1.1	0.46 ± 0.03	0.32 ± 0.02
Red-eyed	109.3 ± 3.3	31.3 ± 2.0	0.77 ± 0.03	0.64 ± 0.05

the present HPLC method and by a TLC–spectrophotometric procedure[2], slightly modified by us for convenience and reproducibility. The values obtained by both methods are significantly correlated [$r = 0.95$; the regression line for the TLC–spectrophotometry (y) and HPLC (x) values is $y = 1.68\ x - 0.19$], however, the results of the TLC–spectrophotometric assays were generally higher. Admittedly, the inclusion of TLC before the spectrophotometric analysis leads to a significant increase in the errors, as well as in the assay time. The requirement of fine grinding in the sample preparation in order to ensure complete extraction is substantiated by both methods, the finely powdered samples consistently showing higher levels of compound 1 than their coarse-grained counterparts (from 1.4:1 to as much as 4:1).

The use of the present HPLC procedure pointed to significant differences between the three breeding lines in the yield of total 1, *i.e.*, naturally occurring ajmalicine and ajmalicine resulting from reduction of serpentine (Table II). The line with red-eyed flowers had the highest weights of fresh and dry roots per plant, and the highest levels of total 1 in the tap (0.77%) and in the lateral roots (0.64%). The roots of the white-flowered line displayed the lowest content of total 1, however, their fresh and dry weights were higher than those of the pink-flowered line.

The amount of total 1 in the tap root was higher than that in the lateral roots in all lines. A significant ($p < 0.01$) correlation was found between the contents of compound 1 in the two parts of the root ($r = 0.86$). Since the tap root also contributes most of the dry biomass (about 70%), the yield of total 1 in the plant is chiefly produced by this part. These results indicate that breeding and culture practices aimed at increasing the content and yield of total 1 in the species should be directed towards increasing the tap root biomass rather than that of the lateral roots.

The differences in levels of total 1 between lines with different flower colours suggest that this character might be an ideal marker for breeding cultivars of *C. roseus* with a high concentration of total 1[15]. Nonetheless, the linkage between the flower colour and the content of total 1 should be ascertained by genetic analysis before screening segregating populations on the flower phenotype.

In conclusion, we propose that the HPLC procedure described can be reliably applied to large screening programmes and plant selection and breeding, as well as in cell culture studies. The method involves essentially *in situ* reduction of compound 2 to 1 in crude methanolic plants extracts, which are further made up to the measuring range and directly assayed by HPLC, without prior purification steps. The assay

might also be useful in monitoring the extraction, isolation and purification of compound 1 from the plant material.

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