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# Short Communication

# High-performance liquid chromatography of the alkaloid perivine from *Catharanthus roseus* after derivatisation with dansyl chloride

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ABSTRACT

The Madagascar periwinkle *Catharanthus roseus* produces an unusually large number of alkaloids which are often difficult to analyse because of their similar physical properties and because of their multiplicity. The majority of these "Vinca alkaloids" contain only indolyl and tertiary nitrogen substituents which are not amenable to derivatisation. However, we found that the 2-acylindole alkaloid perivine does react with dansyl chloride to give a stable, fluorescent derivative with good chromatographic properties. Here we report a reversed-phase high-performance liquid chromatography procedure to selectively detect perivine as its dansyl derivative in the presence of all other co-extractable Vinca alkaloids. The method is applied to detect perivine in different tissues of *C. roseus*, and in *Agrobacterium*-transformed cultures of *C. roseus*.

## INTRODUCTION

*Catharanthus roseus* G. Don. (Madagascar periwinkle) produces more than one hundred

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different indole alkaloids (reviewed in ref. 1) the so-called Vinca alkaloids, two of which, vincristine and vinblastine, are important in the treatment of leukemia [2]. Analysis of these alkaloids from plant extracts can be a formidable problem because of their large number and the very different amounts of each component [3,4]. Thin-layer chromatography utilizing ceric ammonium sulphate spray reagent and UV detection has been used with some success [3], as has reversed-phase HPLC with UV [4–6] or MS [7] detection, and supercritical fluid chromatography with MS detection [8]. However, none of these methods are selective, and the last at least requires rather specialised equipment.

The reaction of dansyl chloride with amino compounds gives highly fluorescent derivatives with good chromatographic properties [9] which are stable enough to allow subsequent spectroscopic analysis. This has been used previously to isolate a novel alkaloid, hydroxynorcytisine, from *Laburnum anagyroides* [10], and to assay cephaeline, emetine, ephedrine and morphine [11,12]. However, many Vinca alkaloids contain only tertiary amino groups which do not react with dansyl chloride. An exception is perivine [13], a Vinca alkaloid which contains an imino group that can be derivatised (Fig. 1). Pre-column dansylation of crude methanolic extracts from *C. roseus* and subsequent separation by reversed-phase high-performance liquid chromatography (HPLC) allowed selective detection of perivine by fluorescence in the presence of all co-extracted alkaloids.

Unlike most other alkaloids from *Catharanthus roseus*, perivine is localised in the leaf tissue [1]. Here, a further study of the tissue-specific location of perivine has been made using the described dansylation method. Furthermore, various *Agrobacterium*-transformed cultures derived from *C. roseus* leaf tissue were established, from a variety of diverse *Agrobacterium* strains, and an application of the method to determine their perivine content is described.

## EXPERIMENTAL

### *Materials*

Reagent grade chemicals were from Sigma (Poole, UK). HPLC-grade methanol (Romil, Leicester, UK) was used. Glass-distilled water was used throughout. An authentic sample of perivine was a gift from Professor N.R. Farnsworth.

### *Sources of plant material*

Seeds of *C. roseus* cv. Magic Carpet were obtained from Thompson and Morgan (Ipswich,

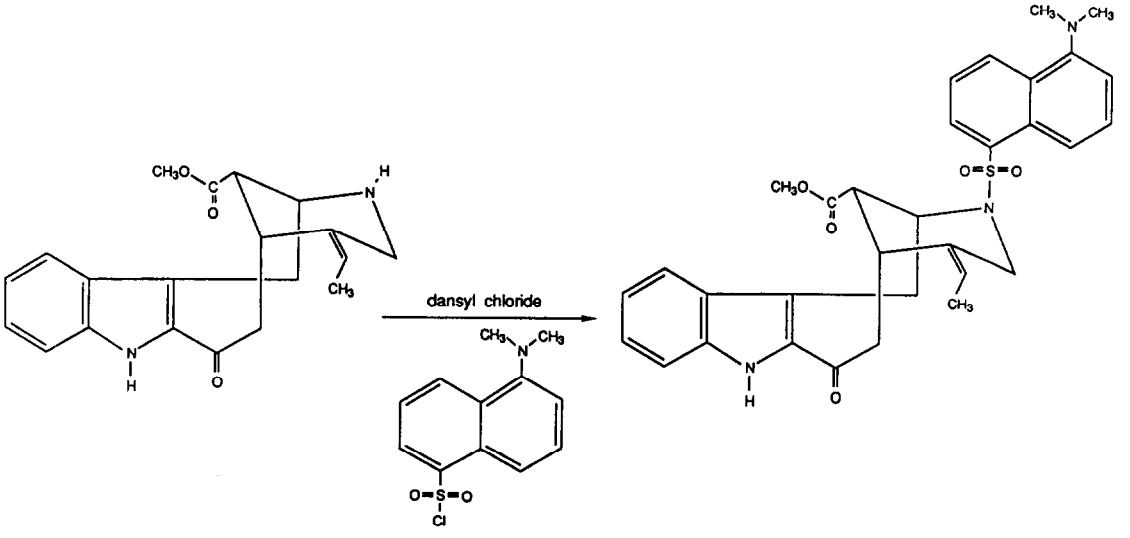


Fig. 1. Reaction scheme for the N-derivatisation of the Vinca alkaloid perivine with dansyl chloride.

UK) and mature plants were grown under conventional greenhouse conditions. Untransformed callus tissue was cultured on Murashige and Skoog [14] medium solidified with 0.7% agar (M–S agar) and supplemented with indole acetic acid (10 μg/ml) and kinetin (0.1 μg/ml). Mature plants were infected with different strains of *Agrobacterium tumefaciens* by wounding with an infected needle. Crown gall tumours developed after four weeks and were excised. Bacteria-free callus cultures were established on phytohormone-free M–S agar containing vancomycin (100 μg/ml) and carbenicillin (100 μg/ml). Phytohormone-free growth and opine production were taken as evidence of transformation [15]. Bacteria-free *Agrobacterium rhizogenes* strain LBA 9402 transformed root cultures of *C. roseus* were maintained in liquid culture in half strength Gamborg B5 medium [16].

*Extraction and derivatisation of the basic nitrogenous fraction*

The basic nitrogenous fraction was recovered from fresh tissue (<2 g fresh mass) by extraction with methanol–aqueous 0.3 *M* HCl (70:30, v/v; 5 ml) in a Potter homogeniser. After filtration, the supernatant was concentrated by evaporation under vacuum. Aliquots equivalent to 0.1 g fresh mass of tissue were redissolved in water (0.2 ml) and mixed with a solution of dansyl chloride in acetone (0.8 ml, 5 mg/ml). After saturating with solid sodium hydrogencarbonate, the mixture was left to react in the dark at room temperature for 15 h. Excess acetone was removed by warming to 60°C for 10 min, and the mixture was diluted to 1 ml with water. Dansylated derivatives were extracted by vortex mixing with toluene (20 s; 3 × 2 ml). The phases were separated by centrifugation (2700 g, 10 min) and the toluene fractions carefully removed, combined, and evaporated to dryness under a stream of air. At all stages exposure to light was kept to a minimum. Amine standards (20 nmoles) were derivatised by the same procedure. Dansylated residues were redissolved in methanol (500 μl) and centrifuged (9000 g; 4 min) prior to HPLC analysis. The HPLC autoinjector made 30 μl injections of this dilution, equivalent to 60 mg fresh mass of plant tissue.

*High-performance liquid chromatography*

The HPLC procedure was essentially as described previously [17]. The instrumentation consisted of a Gilson 401 diluter and 231 autoinjector (Gilson, Villiers-le-Bel, France), a Rheodyne Model 7010 injection valve fitted with a 50-μl loop (Rheodyne, Cotati, CA, USA), and two LDC Constametric IIIG pumps (Milton Roy, Riviera Beach, FL, USA). A Brownlee Spheri-5 RP18 reversed-phase column (250 × 4.6 mm, 5 μm particle size) was used (Brownlee Labs., Santa Clara, CA, USA), protected by a Brownlee RP18 guard column (30 × 4.6 mm). Detection was accomplished with an inline Perkin-Elmer (Beaconsfield, UK) LS4 fluorescence spectrophotometer (3-μl cell) set at 340 nm excitation and 540 nm emission. A BBC Goerz Metrawatt SE130 dual pen recorder set at 0.5 cm/min monitored output.

A two step methanol–water solvent gradient was used, consisting of linear sections 60–67% methanol (15 min) and 67–95% methanol (39 min). A 10-min period was allowed to re-equilibrate the system to 60% methanol. Flow-rate was kept constant at 1.0 ml/min.

## RESULTS AND DISCUSSION

Acidified methanolic extracts from *C. roseus* leaf tissue were derivatised with dansyl chloride to screen for alkaloids containing primary or secondary amino functions. Non-polar derivatives were separated from the hydrolysis product sodium dansyl sulphonate by toluene extraction and analysed by reversed-phase HPLC (Fig. 2). By this procedure it was possible to detect specifically just one alkaloid, perivine, as its dansylated derivative. A number of biogenic amine derivatives also extracted under these conditions, but in spiking experiments dansyl-perivine did not co-chromatograph with any of forty dansylated amine standards used for comparison [17,18], either by HPLC or by two-dimensional TLC.

An authenticated sample of pure perivine was also dansylated and separated by reversed-phase HPLC and gave a major peak attributable to dansyl-perivine ($t_R = 42$ min) plus minor peaks representing dansyl-ammonia, dansyl-methyl-

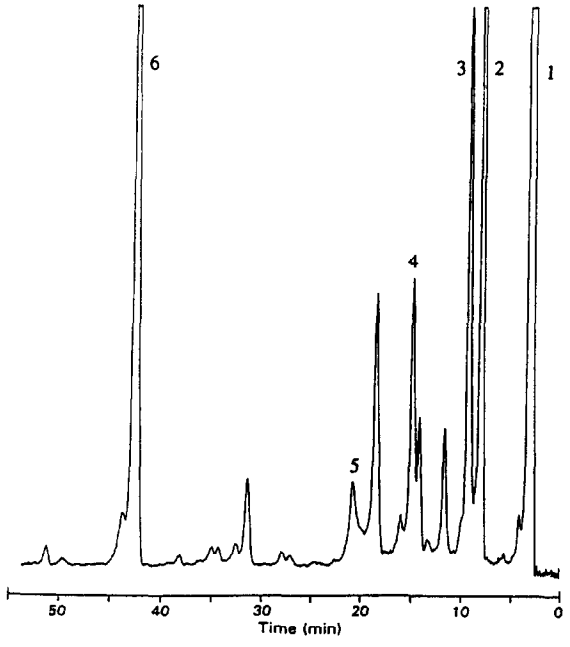


Fig. 2. Reversed-phase HPLC profile of dansylated derivatives recovered from leaf tissue of *Catharanthus roseus*. Injection volume (30 μl) was equivalent to 60 mg fresh mass. Extraction, derivatisation and HPLC conditions are described in the Experimental section. Peaks: 1 = dansyl sulphonate (3.0 min); 2 = dansyl-ammonia (7.9 min); 3 = dansyl-ethanolamine (9.3 min); 4 = dansyl-methylamine (15.0 min); 5 = dansyl-dimethylamine (20.2 min); 6 = dansyl-perivine (42.5 min).

amine, dansyl-dimethylamine, dansyl sulphonate, and excess dansyl chloride. All these minor components arise as by-products of the dansylation reaction [9], and none obscured the dansyl-perivine peak. Only one peak was observed for dansyl-perivine itself indicating that only one major derivative is formed (Fig. 1). At the chosen reaction conditions dansylation does not occur on hydroxy groups or on the indolic nitrogen [17]. This suggested that the perivine was mono-N-dansylated, and this was confirmed by NMR spectroscopy (data not shown).

Phenolics and thiols can also interfere with the dansylation reaction but the derivatives tend to fluoresce at longer wavelengths [9]. On TLC plates dansylated phenolic spots appear orange under 265 nm UV light, whereas dansylated amines and the dansyl-perivine derivative exhibited green fluorescence. Thus, further selectivity was achieved by using a HPLC fluorescence detector optimised to detect dansylated amino derivatives [18].

The major alkaloids in *C. roseus* leaf tissue are catharanthine, vindoline, and 3′,4′-anhydrovinblastine [8], while perivine is present at only about 3–4% of the total alkaloids [16]. Dansylation of perivine renders it less polar, allowing selective isolation from other, underivatised alkaloids by solvent extraction into toluene. HPLC analysis of the spent aqueous phase after solvent extraction suggested that the recovery was better than 90% for this step. Large-scale extraction of perivine from leaf tissue affords recoveries of about 80 μg/g fresh mass [19]. Assuming that the recovery of dansyl-perivine is similar to that of dansylated amines, *i.e.* generally more than 90% [17], this indicates that dansyl perivine can easily be detected in the low μg range. The injection in Fig. 2 (=60 mg fresh mass of leaf tissue), for example, was equivalent to about 5 μg of dansyl-perivine, and suggests that much greater sensitivity is possible. This compares favourably with detection limits of Vinca alkaloids by the ceric ammonium sulphate method [3]. Furthermore, 2-acylindole alkaloids such as perivine and periformyline do not react at all with this reagent [20]. Other workers [4–6] have not reported minimum detection limits.

*Location of perivine in C. roseus tissues*

Perivine is generally considered to be confined to leaves of *C. roseus* and our method confirmed that the highest concentrations were indeed in leaf tissue. However, we also analysed isolated roots, stems, and flower petals by the same methodology and detected perivine in all three tissue types (Fig. 3), albeit at lower concentrations. Assuming similar recoveries for the different tissues the results indicate that perivine concentration is approximately 10-fold higher in leaves than in other parts of the plant.

*Agrobacterium* species are plant pathogenic bacteria and convenient tools for plant genetic engineering. Wild type *A. tumefaciens* induces crown galls and *A. rhizogenes* adventitious hairy roots on infected plants [21], and they often have a profound effect on plant secondary metabolites [16]. HPLC–MS analysis of pharmaceutically

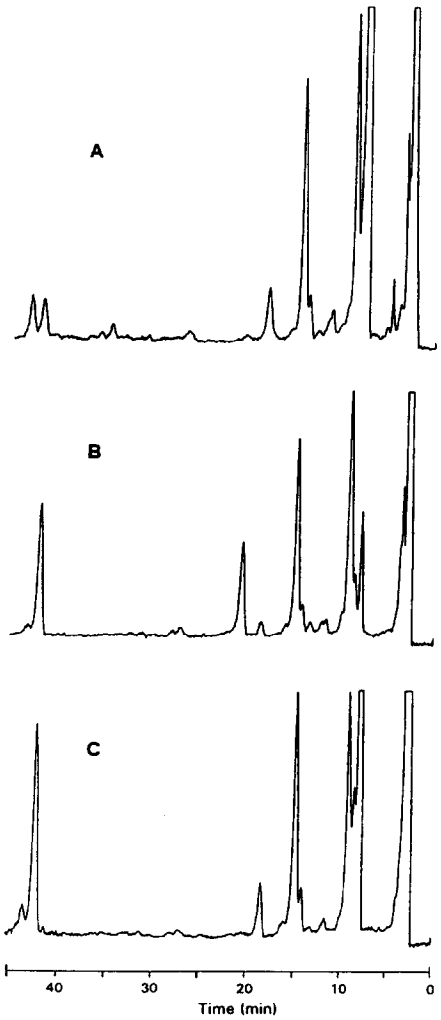


Fig. 3. Location of perivine in *C. roseus* tissues by HPLC after dansylation. (A) Root tissue; (B) flower petals; (C) stem tissue. In each case the injection volume was equivalent to 60 mg fresh mass. The peak attributable to dansyl-perivine is evident at 42 min. Other peaks were assigned as in Fig. 2.

active alkaloids from *Agrobacterium*-transformed *C. roseus* has been investigated previously [7,16] and, as found with other secondary metabolites [22] transformation was shown to generally increase alkaloid biosynthesis.

To investigate further the effect of tissue morphology on endogenous perivine levels the dansylation method was used to detect perivine in hairy root or crown gall cultures originally derived from *C. roseus* leaf tissue. The HPLC trace of the dansylated extract from a *C. roseus*–*A. rhizogenes* LBA 9402 hairy root culture is shown as an example (Fig. 4).

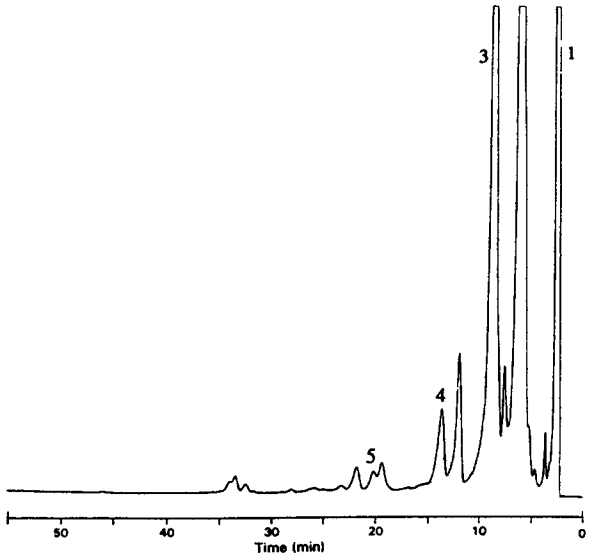


Fig. 4. HPLC chromatograph of the dansylated extract from a hairy root culture (injection = 60 mg fresh mass) incited on *C. roseus* leaf tissue by *Agrobacterium rhizogenes* LBA 9402. Experimental conditions are as described in the Experimental section, and peak assignments as in Fig. 2.

Although co-extractable biogenic amines were present, no dansyl-perivine was detected in either this culture or in *C. roseus*–*A. tumefaciens* (pTiT37), *C. roseus*–*A. tumefaciens* (pTiC58), *C. roseus*–*A. tumefaciens* (pTiAch5), or *C. roseus*–*A. tumefaciens* (pTiBo542) crown gall cultures, despite the fact that all were derived from transformed leaf tissue. This suggests not only that the majority of perivine is localised in *C. roseus* leaf tissue, but also that the leaf morphology is a prerequisite for perivine synthesis or storage. In contrast, by themospray LC–MS Mellon [7] found more than 20 alkaloids in a *C. roseus* transformed root extract, one of which was assigned as perivine. However, this assignment was made on molecular mass alone ($m/z$ 341) without comparison with an authentic perivine standard. Furthermore, the quantity detected was rather low (>200 ng) and this may represent differences in culturing conditions and/or age of the culture.

More than one hundred different alkaloids have been isolated from *C. roseus* (L.) G. Don, most with similar physical properties, and this

complexity hinders the analysis of individual alkaloids. Pre-column dansylation of *C. roseus* extracts allows selective detection of the imino alkaloid perivine, even in the presence of complicated mixtures of other Vinca alkaloids. In principle, this approach should be equally applicable to the detection of minor amino or imino type alkaloids in other plants which may have been overlooked by more classical methods of alkaloid analysis.

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