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EXTRACTION OF 3',4'-ANHYDROVINBLASTINE FROM *CATHARANTHUS ROSEUS*

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Key Word Index—*Catharanthus roseus*; Apocynaceae; Madagascan periwinkle; plant chemistry; bis-indole alkaloids; 3',4'-anhydrovinblastine

Abstract—High levels of 3',4'-anhydrovinblastine were extracted from freshly-picked leaves of *Catharanthus roseus* using an acidic aqueous medium. Enzymic digestion of leaf material, followed by acidification, improved the recovery. Yields were enhanced when the aqueous extract was treated with sodium borohydride, suggesting that an iminium intermediate (produced from the enzymic coupling of catharanthine and vindoline) also exists in the extract. The maximum yield obtained was 0.23% of the dry weight.

INTRODUCTION

Catharanthus roseus (L.) G. Don produces a large number of indole alkaloids, including the class of bis-indole alkaloids, renowned for their anti-tumour properties. Two of the most valuable drugs in cancer chemotherapy, vinblastine (VLB) and vincristine (VCR), belong to this class of dimeric alkaloids and are obtained in low yields from the plant by a lengthy differential extraction process [1]. They are synthesized *in vivo* from coupling of the monomeric alkaloids catharanthine and vindoline [2–4], which occur at high concentrations in the plant. A key intermediate in this biosynthetic pathway is though to be 3',4'-anhydrovinblastine (AVLB) which was observed as a product when labelled monomers were fed *in vivo* to *C. roseus* seedlings [3]. The conversion of AVLB to VLB has been demonstrated with cell-free extracts of both leaves [4, 5] and suspension cultures [6, 7].

Recent work has demonstrated that efficient coupling of catharanthine and vindoline to form AVLB can be performed with peroxidase isozymes isolated from *C. roseus* suspension cultures [8] or with commercially available horseradish peroxidase [9]. The enzymic coupling mechanism is thought to be similar to the modified Polonovski chemical coupling process whereby catharanthine is oxidized to a reactive species, which is then coupled with vindoline to form an iminium ion. This can be converted to AVLB by 1,2-reduction with sodium borohydride [10, 11]. Despite the belief that AVLB is a natural product of *C. roseus* it has not been commonly reported as a product from extractions of the plant. However, Jovanovics *et al.* [12] obtained 0.017% of the dry weight as AVLB from dried leaves. Since aqueous acids have proved useful in extracting alkaloids from *C. roseus* [e.g. 13], we have examined this as a means of recovering AVLB from freshly picked leaves.

RESULTS AND DISCUSSION

When leaves of *C. roseus* were ground to a powder with liquid nitrogen and extracted with water, AVLB was recovered as 0.041% of the dry weight. An enhanced yield was achieved by the addition of sodium chloride over a range of concentrations, with a maximum of 0.066% at 2.3 M NaCl, above which there was a marked decline (Fig. 1). The increased recoveries may result from an inhibition of putative interactions between AVLB and cellular macromolecules (e.g. polyphenols, polysaccharides and proteins), due to the elevated ionic strength of the medium. The drop in yields at higher salt concentrations could be the result of AVLB precipitation which would

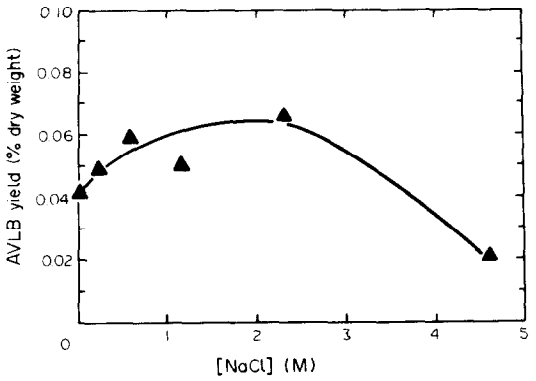

Fig. 1. The effect of sodium chloride concentration on alkaloid extraction: 2.5 g (fr.wt) ground leaves were mixed in 7.5 ml water, containing sodium chloride, which was vortexed then sonicated for 15 min. Each value is mean of duplicate extractions, analysed by HPLC.

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thus be lost during the centrifugation step that removes leaf debris.

When an acidic aqueous extraction medium (pH 3) was used, extractable AVLB levels rose to almost 0.1% of the dry weight (Table 1), whilst catharanthine and vindoline levels were unchanged. When a basic medium (pH 9) was used, no AVLB and only small quantities of the monomers were observed. The basic pH neutralizes the alkaloids, reducing their water solubility and this may therefore decrease their recovery following centrifugation. Inclusion of sodium chloride in the acidic medium did not enhance the yield of AVLB, catharanthine or vindoline, and at higher concentrations it again caused a decline in recoveries of the alkaloids (results not shown).

When the effect of HCl concentration on AVLB recovery was examined, a maximum yield was attained with 0.025 M HCl (Table 2). The final pH of the medium (following centrifugation) was 4.5—a rise of 1.5 units. Comparable yields of AVLB were obtained with equivalent concentrations of hydrochloric, sulphuric and acetic acids (results not shown). When the pH of the supernatant (following centrifugation) was raised to 7.3 or 9.0, AVLB yields dropped in comparison with controls at pH 4.5. At pH 9.0 the decrease in AVLB was between 30 and 40% of the control value, whereas catharanthine and vindoline recoveries were increased by 13 and 25% respectively. An acidic pH will enhance both the solubility and stability of AVLB in an aqueous medium (Choi, L., personal communication) and these results show that it is necessary to maintain a low pH throughout the extraction procedure in order to attain maximum recoveries.

Since AVLB can easily be oxidized [14], ascorbate (at concentrations from 0.19 to 1.14 mM) was added to the aqueous extraction medium as an anti-oxidant. However, no improvement in recoveries was detected, which suggests that AVLB is relatively stable under the conditions employed.

Routine identification of AVLB was made on the basis of HPLC and TLC as described in the Experimental. These results were confirmed by high resolution MS analysis of a purified sample, which showed a 0.9 millimass unit deviation from the theoretical value for the accurate molecular mass. The fragmentation pattern closely matched that of an authentic standard. The two monomeric components of bis-indole alkaloids are linked at a chiral centre (C'_{18}), but only the α configuration imparts anti-mitotic activity [1]. CD analysis of the purified sample revealed Cotton effects at 225 nm (positive) and 212 nm (negative), both of which were also observed with an authentic standard. Similar Cotton effects have also been reported for other related bis-indole alkaloids and they differ markedly from those of the β-stereoisomer [15].

An extraction of 100 g (fr.wt) of leaves also enabled the isolation of a product believed (from HPLC and TLC analysis) to be VLB. The yield was calculated to be roughly 3.5×10^{-4}% of the dry weight. High resolution MS data upheld its identification, showing a 0.8 millimass unit deviation from the theoretical value for VLB.

Peroxidase-mediated coupling is believed to produce a

Table 1. The effect of pH on the recovery of AVLB, catharanthine and vindoline from leaves

	Alkaloid yields (% Dry Weight)		
pH of medium	AVLB	Catharanthine	Vindoline
3.3	0.099	0.11	0.13
6.2	0.066	0.11	0.14
9	0	0.01	0.03

2.5 g (fr.wt) ground leaves were mixed in 7.5 ml aqueous solution of 2.3 M sodium chloride with the pH adjusted as shown above. This was vortexed, then sonicated for 15 min, after which the cell debris was removed by centrifugation. Alkaloid yields are the mean of duplicate extractions, measured by HPLC analysis.

Table 2. HCl concentration and AVLB recovery

HCl concentration (M)	pH of HCl*	pH of Supernatant†	AVLB yield (% of dry weight)
0.001	3.2	6.0	0.06
0.005	2.6	5.6	0.08
0.010	2.4	5.2	0.10
0.025	2.0	4.5	0.12
0.050	1.5	3.6	0.04
0.100	1.3	2.3	0

1 g (fr.wt) of ground leaves was mixed in 3 ml of acid. Values quoted are means of duplicate extractions, analysed by TLC.

*Prior to addition of leaves.

†Following centrifugation to remove cell debris.

dimeric iminium ion, which can subsequently be reduced with sodium borohydride to AVLB, and this is the coupling product normally isolated [8, 9]. In the AVLB extraction process, addition of sodium borohydride to the acidic aqueous extract increased the AVLB yields by as much as 100% (Fig. 2). It was necessary to add the sodium borohydride to the supernatant following centrifugation; if it was added prior to this, no AVLB was recovered. The iminium ion may therefore exist as a natural product in the plant, although alternatively it could be formed through oxidation of AVLB during the extraction process. The latter suggestion is unlikely since, as described above, an anti-oxidant did not alter the recovery of AVLB. It is also possible that the iminium is synthesized from catharanthine and vindoline *in vitro* during the extraction process, but, this is also considered improbable because the levels of AVLB were not altered in aqueous extracts incubated over various time intervals.

The high yields of AVLB attained using aqueous acid as the extraction medium can probably be attributed to an improved solubility and stability of the alkaloid at low pH. In addition, the use of freshly-picked leaves may be important since AVLB is relatively unstable and might be degraded if the leaves are dried. Thus, conventional extraction processes employed for the production of other *C. roseus* alkaloids may result in low AVLB recoveries due to the common practice of using dried leaves and the generation of basic conditions at certain stages. The other bis-indole alkaloids (notably VLB and VCR) found in *C. roseus* exist at levels far lower than that of AVLB and may conceivably be produced as minor by-products of AVLB. It is also possible that they are synthesized during extraction procedures, since Langlois and Potier [16] found that AVLB in organic solution was converted to a number of other dimers, including VLB and leurosine. The high levels of extractable AVLB were retained when leaves were frozen at −20°C for up to six weeks and the acidic aqueous extract can be frozen for at least five months without any significant loss of AVLB. Comparable yields of AVLB were obtained when leaves were homogenized in acid with a Waring blender as opposed to grinding in liquid nitrogen.

Enzymes that digest the plant cell wall have been found to enhance yields of secondary products in cultured cells [17]. Two such enzyme preparations: Macerozyme (crude pectinase) and Driselase (laminarase, xylanase and cellulase), as well as β-glucosidase were tested for their effect on AVLB extraction. Macerozyme treatment brought about a marked enhancement (67%) of AVLB yields (Table 3). This suggests that some AVLB may be localized in the cell wall and that enzymic digestion helps release it into the aqueous medium. The acidification (to pH 4.5) of an enzymic digestion of leaves caused a further increase in the level of AVLB extracted (Table 4), and when this was followed by borohydride treatment the level of AVLB was increased even more. This combination of treatments produced a maximum AVLB recovery of about 0.23% of the dry weight.

The existence of AVLB as the major bis-indole alkaloid in *C. roseus* leaves raises many questions as to how and where it is stored such that it is not toxic to the plant. Idioblasts within the mesophyll of *C. roseus* leaves that are enriched in catharanthine and vindoline [18] might also accumulate AVLB. The most obvious subcellular

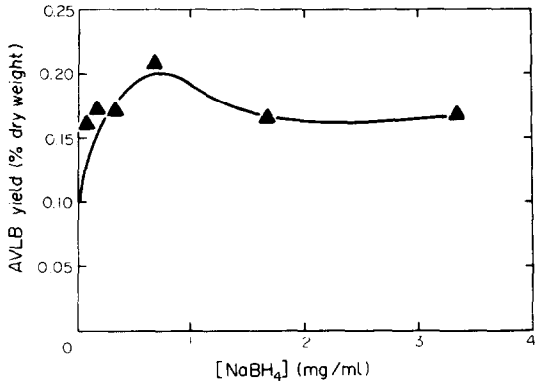

Fig. 2. The effect of sodium borohydride on AVLB extraction: 1 g (fr.wt) of ground leaves was mixed in 3 ml 0.025 M HCl, containing 0.1 M magnesium chloride and 2.0 mM hydrogen peroxide. Each value is the mean of duplicate extractions analysed by HPLC.

Table 3. Enzyme digestion and alkaloid recovery

	Final pH*	Yield (% of dry weight) AVLB	Catharanthine	Vindoline
Control (no enzyme)	6.1	0.06	0.14	0.21
Macerozyme (0.4%)†	5.8	0.10	0.15	0.29
Driselase (4.0%)†	5.5	0.07	0.08	0.20
β-Glucosidase (50U)†	6.0	0.04	0.07	0.17

1 g (fr.wt) of ground leaves were mixed with 3 ml pH 5.5 MES buffer (0.1 M) and incubated for 1 hr at room temp. Alkaloids were quantified using HPLC and each value is the mean of duplicate extractions.

*pH of supernatant after centrifugation to remove cell debris.

†W/v.

‡One unit will liberate 1.0 μmol of glucose/min from salicin at pH 5.0 and 37°.

Table 4. Enzyme digestion and acidification

	Final* pH	AVLB (μg)
Macerozyme (0.4%)†	5.8	93.2
HCl	4.5	90.6
Macerozyme (0.4%)† + HCl	4.5	132.8

1 g (fr.wt) ground leaves was mixed in 3 ml water and treated either with macerozyme (for 1 hr at room temp.), acid (1 M HCl added to bring final pH to 4.5) or macerozyme incubation followed by acidification to pH 4.5. AVLB was quantitated by HPLC and each value is the mean of duplicate extractions.

*pH of supernatant after centrifugation to remove cell debris.

†W/v.

compartment is the vacuole which has an acidic environment which would enhance the stability of the alkaloid.

The high levels of AVLB (and its iminium ion) in *C. roseus* suggest that it may have a physiological role in the plant and, considering the toxic nature of bis-indole alkaloids (including AVLB), it could be involved in chemical defence. For example, *C. roseus* leaves and leaf extracts were found to induce phagodeterrency in the larvae of *Spodoptera littoralis* and VLB, structurally similar to AVLB, was shown to be a particularly strong deterrent [19]. It could also have an anti-microbial role, since peroxidases are induced in response to microbial invasion in *C. roseus* [20] and are known to perform the coupling reaction [8, 9]. Thus, it would be possible to increase levels of AVLB during infection. Another form of stress–apical wounding–was found to cause enhanced metabolism of catharanthine and vindoline in *C. roseus* [21]. Peroxidase activity can also be enhanced by wounding [22], and such a response might enable increased coupling of the monomers, resulting in ultimate formation of AVLB.

EXPERIMENTAL

Plants. Catharanthus roseus (cv. Little Blanch) plants were kept in a greenhouse at 25–30° with 10 hr illumination each day. They were grown in a peat-based soil-less mix and fertilized with 150 ppm N (60% nitrate and 40% ammonium) per day.

Alkaloid extraction. Freshly-picked leaves were ground to a powder in liquid N_2. 1 g portions were mixed in 3 ml 0.025 M HCl, vortexed and spun at 23000 g for 30 min. The supernatant was removed and extracted x2 with equal vols of EtOAc. The pooled extracts were evapd to dryness under vacuum and the residue was dissolved in MeOH and filtered prior to analysis.

Alkaloid analysis. HPLC was routinely employed for analysis, of the alkaloids using a method described previously [9]. TLC was performed on silica plates with a mobile phase of either toluene–Me_2CO–MeOH–28% NH_4OH (28:10:2:0.5, or Et_2O–$CHCl_3$–MeOH (10:7:4). Plates were scanned at 226 nm, and alkaloids were identified on the basis of UV spectra, R_f values and colour reactions with ceric ammonium sulphate spray [23]. Alkaloids were isolated by prep. TLC on silica plates. Separation was performed initially with the toluene–Me_2CO–MeOH–NH_4OH mobile phase and semi-purified samples were extracted and separated a second time using the Et_2O–$CHCl_3$: MeOH mobile phase. Alkaloids were removed from silica by extraction (×3) with MeOH–CH_2Cl_2 (2:1) containing 1.5% (v/v) NEt_3 and the pooled extracts were evapd to dryness under vacuum. The residue was dissolved in CH_2Cl_2, filtered and dried again. Purified samples were analysed with desorption electron impact mass spectrometry (70 eV, 270°) and a spectropolarimeter.

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