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Simple and reproducible HPLC–DAD–ESI-MS/MS analysis of alkaloids in *Catharanthus roseus* roots

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ABSTRACT

Catharanthus roseus is one of the most important medicinal plants worldwide. The leaves of this species are the only source of the indolomonoterpenic alkaloids vincristin (leurocristine) and vinblastin (vincaleucoblastine), whose anticancer activity represents powerful therapeutics to many diseases, such as Hodgkin lymphoma. Usually, the remaining plant parts go to waste. Here we describe a phytochemical study on this species roots. Alkaloids in aqueous extracts, the usual form of consumption of this matrix, were studied using HPLC–DAD–ESI-MS/MS, which allowed the identification of 19-*S*-vindolinine, vindolinine, ajmalicine and an ajmalicine isomer, tabersonine, catharanthine, serpentine and a serpentine isomer. Quantification of the identified compounds revealed that serpentine and its isomer were predominant (64.7%) over the other alkaloids, namely vindolinine and its isomer (23.9%), catharanthine (7.7%) and ajmalicine (3.8%). The used procedure revealed to be simple, sensitive and reproducible.

1. Introduction

Catharanthus roseus, formerly *Vinca rosea*, is a member of the Apocynaceae family, a taxonomical group that constitutes a vast source of pharmacologically active molecules. In particular, the leaves of *C. roseus* remain to this day the only natural source of the indolomonoterpenic alkaloids vincristine (leurocristine) and vinblastine (vincaleucoblastine), present only in the leaves [1]. These alkaloids have achieved a prominent role in modern cancer chemotherapy, presenting a wide spectrum of action, such as Hodgkin's and non-Hodgkins's lymphomas, acute lymphoblastic leukaemia, neuroblastoma, breast carcinoma, among others [2]. *In vivo*, these molecules are produced by the condensation of vindoline and catharanthine, both of which deriving from the terpenoid indole alkaloid (TIA) biosynthetic intermediate strictosidine through multiple steps. Vindoline is detected only in the green parts of the plant and not in the roots or cell suspension cultures, being biosynthesized from the branch-point intermediate tabersonine through the action of six enzymatic steps, a pathway initially proposed by De Luca et al. [3] and now almost fully elucidated using a combination of *C. roseus* plants and cell suspension cultures. Catharanthine has been reported to be present in the root tissue as well as in the aerial part of the plant [4,5].

The chemical complexity and unique bisindole alkaloid structure of the aforementioned *C. roseus* molecules have so far hindered their laboratorial synthesis. This partly derives from difficulties in achieving the correct conformation of stereogenic centers, which are, as with most natural products, critical for their activity. For example, in vincristine the configuration at the C-16′ stereogenic centre is *S* while at C-14′ it is *R* and *S* at C-20′. The inversion of C-16 configuration from *S* to *R* results in a complete loss of activity as does the C-14′ conversion from *R* to *S* [6].

The roots of *C. roseus* have been used worldwide for the treatment of several health disorders. In particular, the French pharmacopeia comprises an entry for *C. roseus* roots, given the fact that this matrix constitutes the start-point for the industrial extraction of ajmalicine [7]. Aqueous extracts (decocts or hot water extracts) of the roots are used worldwide for several conditions, including fever, malaria, diabetes, stomach problems, heart diseases, cancer, as antigalactagogue and cholagogue, among others [8].

Most of the existent bibliography on *C. roseus* concerns the determination of alkaloids on its leaves. The few works dealing with

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its roots report the use of methanolic extracts, some of them involving rather complex extractive procedures [9–12]. According to the folk use of this species referred above, in this work we describe a useful analytical procedure for the determination of alkaloids in water extracts of *C. roseus* roots. This procedure consists simply in boiling lyophilized biomass in water, without purification, followed by concentration and HPLC analysis.

2. Experimental

2.1. Standards and reagents

Serpentine and ajmalicine were from Sigma–Aldrich (St. Louis, MO, USA). Catharanthine sulfate was from Gedeon Richter Ltd. (Budapest, Hungary). Acetonitrile and acetic acid were from Merck (Darmstadt, Germany).

Water was treated in a Milli-Q (Millipore, Bedford, Massachusetts) water purification system.

2.2. Samples

Plants of *C. roseus* (L.) G. Don cv. Little Bright Eye were grown at 25 °C in a growth chamber, under a 16 h photoperiod, using white fluorescent light at a photon flux density of 70 μmol/m^2/s. Roots from three distinct plants were collected, frozen (−20 °C) and lyophilized. Dried material was powdered (910 μm) and kept in a desiccator at room temperature, in the dark, until the analysis. Voucher specimens were deposited at the Department of Pharmacognosy of the Faculty of Pharmacy of Porto University (CATROS-R0109).

2.3. Sample preparation

For HPLC–DAD–ESI-MS/MS analysis of alkaloids, lyophilized root of *C. roseus* were extracted with methanol:water (1:1). Initially the material was ultrasonicated for 1 h (0.1 g/ml), macerated for 24 h and then ultrasonicated again. After this, centrifugation was performed (12,000 rpm, 5 min) followed by filtration through a 0.2 μm size pore membrane.

For the quantification of alkaloids, an aqueous extract was prepared: 1.5 g of lyophilized material were extracted with 300 ml of boiling water for 20 min, with subsequent filtration. The resulting extract was then lyophilized and maintained in a desiccator at room temperature, in the dark.

2.4. HPLC–DAD–ESI-MS/MS analysis

Chromatographic separations were carried out on a 250 mm × 4.6 mm, 5 μm, Luna RP-C18 (2) (Phenomenex, United Kingdom) column at room temperature. Elution was performed with a flow rate of 1 ml/min. Solvents used were acetonitrile (A) and acetic acid 1% (B). Gradient was as follows: 12% A at 0 min, 20% A at 30 min, 50% A at 40 min, 50% A at 45 min, 12% A at 47 min, 12% A at 55 min and the injection volume 20 μl. The HPLC system was equipped with an Agilent 1100 Series diode array and a mass detector in series (Agilent Technologies, Waldbronn, Germany). It consisted of a G1312A binary pump, a G1313A autosampler, a G1322A degasser and a G1315B photo-diode array detector, controlled by ChemStation software (Agilent, v. 08.03).

Spectroscopic data from all peaks were accumulated in the range 240–400 nm, and chromatograms were recorded at 255, 290, 306 and 320 nm. The mass detector was a G2445A ion-trap mass spectrometer equipped with an electrospray ionisation (ESI) system and controlled by LCMSD software (Agilent, v. 4.1.). Nitrogen was used as nebulising gas at a pressure of 65 psi and the flow was adjusted to 11 l/min. The heated capillary and voltage were maintained at 350 °C and 4 kV, respectively. The full scan mass covered the range from m/z 100 to 1500. Collision-induced dissociation experiments were performed in the ion trap using helium as collision gas, with voltage ramping cycles from 0.3 up to 2 V. MS data were acquired in the positive ionization mode. The isolation width of the parent ions for following MS fragmentation events was set at ±0.5.

2.5. HPLC–DAD quantitative analysis of alkaloids

Twenty microlitres of the lyophilized aqueous extract were redissolved in water (100 mg/ml) and analyzed using an HPLC unit (Gilson) and a Luna RP-C18 (2) column (250 mm × 4.6 mm, 5 μm). Solvents used were acetonitrile (A) and acetic acid 1% (B). Elution was performed with a flow rate of 1 ml/min and gradient was as follows: 12% A at 0 min, 20% A at 30 min, 50% A at 40 min, 80% A at 45 min, 100% A at 50 min, 100% A at 52 min. Detection was achieved with a Gilson diode array detector. Spectral data from all peaks were accumulated in the range of 200–400 nm, and chromatograms were recorded at 282 nm. The data were processed on a Unipoint Software system (Gilson Medical Electronics, Villiers le Bel, France). Peak purity was checked by the software contrast facilities.

Quantification of alkaloids was achieved by comparison of their absorbance with that of external standards commercially available. 19-*S*-vindolinine, vindolinine and tabersonine were quantified as

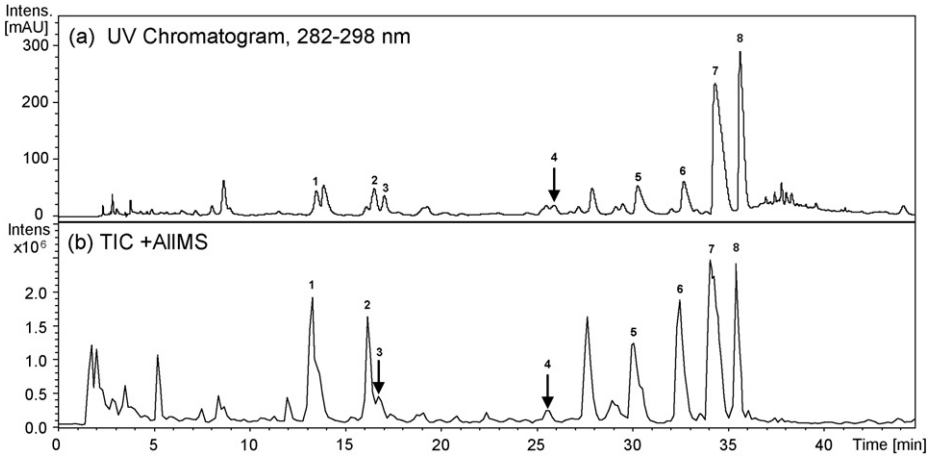

Fig. 1. HPLC–DAD–ESI-MS/MS of the alkaloids of hydromethanolic extract of *Catharanthus roseus* roots. (a) UV chromatogram at 282–298 nm; (b) Total ion current (TIC) chromatogram. (**1**) 19-*S*-vindolinine; (**2**) vindolinine; (**3**) ajmalicine (isomer); (**4**) tabersonine; (**5**) ajmalicine; (**6**) catharanthine; (**7**) serpentine; (**8**) serpentine (isomer).

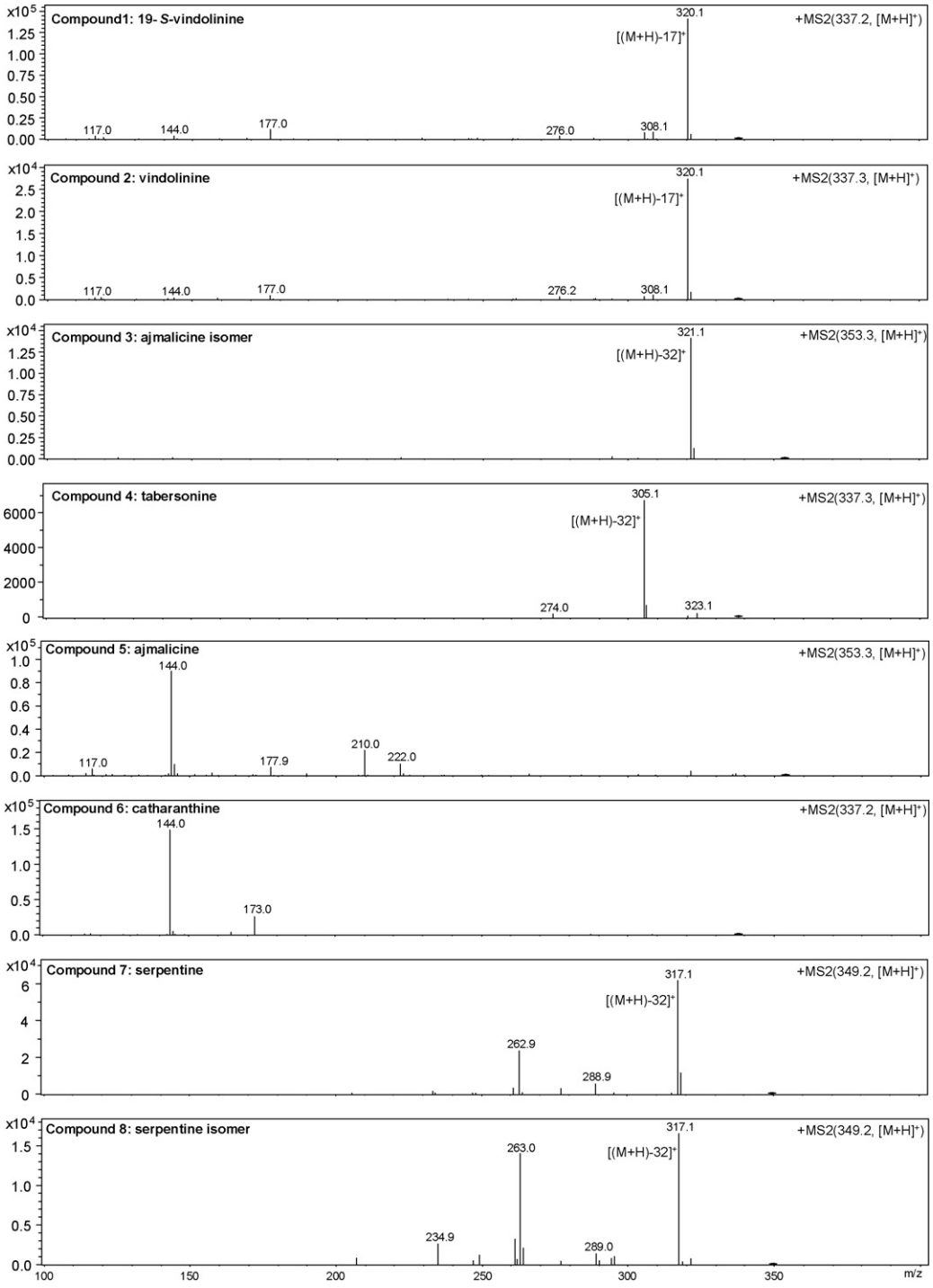

Fig. 2. MS spectra of the identified alkaloids.

catharanthine; the other compounds were quantified as themselves.

3. Results and discussion

3.1. HPLC–DAD–ESI-MS/MS analysis of alkaloids

About 130 alkaloids can be found in *C. roseus*. However, only few (~11) of this high number of chemical entities are frequently analyzed and even fewer (~8) are commercially available [9].

Separation of the compounds in a hydro-methanolic extract of *C. roseus* roots was performed by HPLC-RP and photodiode array detection, as well as by electrospray ionization tandem mass spectrometry ion trap in the positive mode (ESI-MS/MS), which proved to be suitable for the identification of the alkaloids (Fig. 1). The MS spectra of the identified compounds are displayed in Fig. 2.

Three compounds (**1**, **2** and **4**) with $[M+H]^+$ at 337 were noticed, being, therefore, catharanthine isomers. Compounds **1** (*R*t 13.4 min) and **2** (*R*t 16.4 min) shared the same UV spectrum (UV: 240, 294 nm) and their shape was similar to that of catharanthine, although the UV absorption bands presented a batochromic shift of about 13–14 nm when compared with catharanthine. Their +MS were also identical between each other (+MS: 337 $[M+H]^+$; +MS2(337): 320 (100, −17 Da = OH), 308 (6), 177 (8), 144 (2)) and

differed from that of catharanthine. This fragmentation is in line with that described by Zhou et al. [10] for 19-*S*-vindolinine and vindolinine in a commercial extract of *C. roseus*. As so, they putatively correspond to these compounds.

The other compound with $[M+H]^+$ at 337 (**4**) had an UV spectrum similar to that provided by Tikhomiroff and Jolicoeur [11] and Hisiger and Jolicoeur [9] for tabersonine (*R*t 25.8 min. UV: 228, 292, 330 nm. +MS: 337 $[M+H]^+$; +MS2(337): 305 (100, −32 Da = CH_3OH)).

One isomer of ajmalicine (353 $[M+H]^+$) was compound **3**, although its MS fragmentation and UV spectrum (*R*t 17.0 min. UV: 226, 260, 314, 340 nm. +MS: 353 $[M+H]^+$; +MS2(353): 321 (100, −32 Da = CH_3OH)) are not similar to those of the standard.

Other compounds equally identified by UV spectra, MS/MS and chromatographic behaviour comparison with those of authentic standards, were ajmalicine (**5**) (*R*t 30.2 min. UV: 226, 244sh, 278 nm. +MS: 353 $[M+H]^+$; +MS2(353): 222 (10), 210 (25), 178 (10), 144 (100), 117(6)) and catharanthine (**6**) (*R*t 32.6 min. UV: 226, 282 nm. +MS: 337 $[M+H]^+$; +MS2(337): 173 (17), 144 (100)).

Among the compounds found, two peaks were particularly abundant (compounds **7** and **8**) (Fig. 1). Chromatographic behaviour of compound **7** was in line with that of an authentic serpentine standard, in terms of *R*t, UV and MS spectra (*R*t 34.2 min. UV: 250, 306, 368 nm. +MS: 349 $[M+H]^+$; +MS2(349): 317 (100, −32 Da = CH_3OH), 263 (35)).

Compound **8** was expected to be an isomer of serpentine given its UV and MS/MS spectra practically identical to those of serpentine, presenting differences only in the relative amount of the *m/z* 263 fragment resulting from MS2 of the protonated molecular ion (*R*t 35.5 min. UV: 250, 308, 370 nm. +MS: 349 $[M+H]^+$; +MS2(349): 317 (100, −32 Da = CH_3OH), 263 (80)). For compounds **7** and **8** the peak at *m/z* 349 indicates M+ ion as the quaternary alkaloid base.

The presence of vinblastine and vincristine was equally checked by co-chromatographic study of these two standards with the root extract, with negative results. Even so, we have extracted from the MS chromatogram, extracted ion chromatogram (EIC), the molecular ions at *m/z* 811 and 825 corresponding to the protonated ions of the referred compounds, as well as *m/z* 427 and 457 for the confirmation of the possible presence of vindolidine and vindoline found by Zhou et al. [10], with equally negative results. As far as we know, no previous work described the occurrence of these compounds in *C. roseus* roots. In fact, given the biosynthetic pathway of the terpenoid alkaloids, the condensation of catharanthine and vindoline is an absolute requirement for the formation of vinblastin and, later, vincristine [11]. However, the fact that vindoline does not exist in the roots of *C. roseus*, but only in the green parts of the plant, renders that neither vincristine nor vinblastin can be found in the roots of this species. Tikhomiroff and Jolicoeur [11] used another solvent (methanol) to extract alkaloids from the hairy roots and did not found vincristine or vinblastin either.

Also, the precursor compounds tryptamine and tryptophan were searched by comparison with authentic standards, but none were found.

3.2. Quantitative analysis by HPLC–DAD

3.2.1. Validation of the method

Under the described assay conditions, a linear relationship between the concentration of ajmalicine, catharanthine and serpentine and the UV absorbance at 282 nm was obtained. The correlation coefficient for the standard curves invariably exceeded 0.99 (Table 1). The calibration curves were obtained by triplicate determinations of each of the calibration standards, and the peak area values (arbitrary units) were plotted as average values.

The limit of detection (LOD) was calculated based on the standard deviation (SD) of the response and the slope (S) of the calibration curves according to the formula [13]: LOD = 3.3(SD/S). The SD of the response was determined based on the *y*-intercepts of regression lines. Results are shown in Table 1.

Table 1
Equations for regression lines, correlation coefficients, limits of detection and quantification of the method for alkaloids.

	Equation		LOD[a] (μg/ml)	LOQ[b] (μg/ml)
Ajmalicine	$y = 3.0 \times 10^8 x - 1.1 \times 10^6$	$R^2 = 0.9965$	11.5	38.4
Catharanthine	$y = 3.0 \times 10^8 x - 1.4 \times 10^6$	$R^2 = 0.9932$	2.4	8.0
Serpentine	$y = 1.7 \times 10^8 x - 3.1 \times 10^5$	$R^2 = 0.9909$	3.1	10.2

[a] Detection limit.
[b] Quantification limit.

The limit of quantification (LOQ) was also calculated based on the SD of the response and the slope of the calibration curves, according to the formula [13]: LOQ = 10(SD/S). The values obtained are displayed in Table 1.

Repeatability was checked by analysing five times the same sample, by the same analyst, within the same day. Relative standard deviation (RSD) varying between 0.2% and 1.3% indicated that the repeatability of the procedure was good. Intermediate precision determined by different analysts on three separate days (three injections a day) was also found satisfactory (RSD ranged from 1.1% to 2.1%).

To study the recovery of the procedure the sample was added to known quantities of ajmalicine, catharanthine and serpentine. The sample was analyzed in triplicate before and after the additions. Recovery obtained for ajmalicine, catharanthine and serpentine were 79%, 85% and 56%, respectively. In fact, for ajmalicine this value is lower than that found before by Tikhomiroff and Jolicoeur [11], however, for catharanthine and serpentine the results obtained with our method are clearly higher.

3.2.2. Quantification of alkaloids

C. roseus roots aqueous extract, resembling the extract used for human consumption, exhibited a composition similar to that of the hydromethanolic one. Quantification of alkaloids in the lyophilized extract revealed that serpentine accounted for almost half of the identified compounds (ca. 46%), being its isomer the second most abundant one (ca. 18%) (Table 2).

Among the quantified compounds, ajmalicine was the one in lower quantity (ca. 4%). A likely explanation is that this compound is converted *in vivo* to serpentine [11], thus decreasing on behalf of this last, which was the predominant alkaloid in *C. roseus* roots (Table 2). Tabersonine was identified but only in non-quantifiable trace amounts, possibly due to its conversion to vindoline.

The number of works addressing the question of alkaloid quantification in the roots of *C. roseus* is rather scarce. Digvijay et al. [12] conducted studies on the simultaneous quantification of ter-

Table 2
Alkaloids in *Catharanthus roseus* aqueous extract of roots (mg/kg dry basis)[a].

	Alkaloid	Amounts	%[b]
1	19-*S*-vindolinine	5455 ± 84	12.8%
2	Vindolinine	4735 ± 645	11.1%
4	Tabersonine	nq	–
5	Ajmalicine	1613 ± 27	3.8%
6	Catharanthine	3271 ± 181	7.7%
7	Serpentine	19701 ± 2028	46.2%
8	Serpentine (isomer)	7910 ± 746	18.5%
	Total	42685	

[a] Results are expressed as means ± standard deviations of three determinations. nq, not quantified.
[b] Values represent relative percentage of the compound when compared with the sum of all quantified alkaloids.

penoid indole alkaloids and their precursors in both leaves and roots of different genotypes using reversed-phase liquid chromatography. The amounts of serpentine ranged from 0.0034% to 0.6490%, catharanthine from 0.003% to 0.0992%, ajmalicine from 0.0085% to 0.2910% (dry weight), with tabersonine not being quantified. The fact that the results were expressed as percentage renders direct comparison very difficult. However, in the referred study methanol/chloroform extraction was used, while in the work herein an aqueous extract was prepared, suggesting that the amounts of alkaloids extracted are very distinct.

To the best of our knowledge, no previous studies addressed the question of simultaneous quantification of 19-*S*-vindolinine, vindolinine, tabersonine, ajmalicine, catharanthine and serpentine in either an aqueous or methanolic extract. Our work provides a simple method, which allows the determination of a higher number of alkaloids in *C. roseus* roots. Additionally, the procedures found in literature involve continuous or multiple extractions [9,12], sonication, centrifugation [9,11] or fractionation [9]. On the other hand, those more or less complex extractive procedures start from methanol [9,11,12], which is not the solvent usually used in preparations for direct human consumption, as verified in several countries [8]. Thus, by the proposed method we can determine several components from an extract used in folk medicine. This kind of extraction and alkaloids analysis will render easier the execution and interpretation of biological assays, without the interference of solvents like methanol.

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