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Determination of *Catharanthus roseus* alkaloids by high-performance liquid chromatography–isotope dilution thermospray-mass spectrometry

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ABSTRACT

This paper describes a high-performance liquid chromatography–isotope dilution thermospray-mass spectrometry method for the determination of catharanthine, tabersonine, yohimbine and ajmalicine in cell culture samples of *Catharanthus roseus*. The use of deuterated analogues of the alkaloids as internal standards remarkably improved the precision of the analysis. The standard deviation between six repeated assays was close to or below 10% when the reference compounds were analysed, but was about 10% higher, in most instances, when vincamine was used as the internal standard. The precision of this method was about 20% for biological extracts.

INTRODUCTION

The Madagascan periwinkle *Catharanthus roseus* produces a wide range of medicinally active alkaloids [1]. Thermospray high-performance liquid chromatography–mass spectrometry (HPLC–TSP-MS) has been effectively used in the determination of these alkaloids in plant and plant cell culture samples [2–4]. Careful optimization of the TSP conditions is always necessary to obtain a good sensitivity and reproducibility [5]. In spite of this, the precision of the methods tends to be rather poor. The precision in the quantitation of several compounds with HPLC–TSP-MS has been improved by using internal standards labelled with stable isotopes [6–9]. For example in the HPLC–MS analysis of sumatriptan in plasma, the accuracy and precision were reduced to less than 10% with stable isotopes, whereas they exceeded 20% when a homologue was used as the internal standard [9]. In this study the same approach was used and ajmalicine, yohimbine, catharanthine and tabersonine were determined using HPLC–TSP-MS with trideuterated analogues as the internal standards.

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EXPERIMENTAL

Chemicals and sample preparation

Catharantine was purified from *C. roseus* leaves and identified as described previously [10]. Tabersonine hydrochloride was generously provided by Professor W. G. W. Kurz (Plant Biotechnology Institute, National Research Council, Saskatoon, Canada). Ajmalicine hydrochloride was purchased from Sigma (St. Louis, MO, USA) and yohimbine was a gift from Torkel Berglund (Tekniska Högskolan, Stockholm, Sweden). The [methyl-^{2}H]-labelled internal standards were synthesized by trans esterification in perdeuterated methanol and the labelled analogues were determined to contain less than 2% of the original compound [11].

The cultured plant cells were freeze-dried and samples of about 30 mg were pre-purified with Bond-Elut C_{18} cartridges (Analytichem, Harbor City, CA, USA) as described by Morris *et al.* [12]; the method was modifed by using ethanol as the solvent instead of methanol, and deuterated internal standards were added prior to extraction.

High-performance liquid chromatographic conditions

The HPLC system consisted of a Spectra-Physics SP8810 pump and a Rheodyne 7125 injector (Rheodyne, Cotati, CA, USA) with a 20-μl loop. The HPLC column was a μBondapak C_{18} (Waters Assoc. Milford, MA, USA, 300 × 3.9 mm, 10 μm). The isocratic eluent system consisted of 0.1 *M* ammonium acetate (pH 7.2)–acetonitrile (45:55). The flow-rate was 1.1 ml/min.

Mass spectrometry

The HPLC–MS system used was a VG thermospray–plasmaspray probe coupled to a VG Trio-2 quadrupole mass spectrometer (VG Masslab, Manchester, UK). The measurements were carried out using the thermospray mode. The ion source temperature was 150°C, the vaporizer tip temperature 200°C and the repeller potential 180 V. The thermospray spectra were recorded by injecting 2–10 μg of the alkaloids chromatographed as described in the preceding section. Selected ion recording was based on the protonated molecular ions: *m/z* 337 for catharanthine and tabersonine, *m/z* 353 for ajmalicine and *m/z* 355 for yohimbine (*m/z* 340, 356 and 358 for the trideuterated internal standards, respectively).

Calibration and quality control

Quantitation of the alkaloids was based on the internal standard method. Four-point calibration graphs (triplicate injections) were created for the range 2–200 ng per injection (10–200 ng for catharanthine) by plotting the ratios of analyte and internal standard *versus* the amounts of analyte. The amount of labelled standard was 10 ng per injection (50 ng for catharanthine). Linear regression analysis was used to calculate the curve parameters. Relative standard deviations were determined using 2 and 200 ng of the alkaloids (10 and 200 ng for catharanthine). The precision of the whole analytical procedure was tested by adding deuterated internal standards to six replicate plant cell culture samples that were extracted and analysed as described.

RESULTS AND DISCUSSION

The TSP mass spectra of the [methyl^{2}H]-labelled compounds (Fig. 1) are comparable to the mass spectra of non-labelled indole alkaloids which also show protonated molecular ions as the base peaks [4]. The mass spectrum of trideuterated catharanthine shows a fragment ion at m/z 338, which is formed by the loss of two protons. The corresponding ion at m/z 335 is also present in the spectrum of the original compound [4].

Selected ion recording of the protonated molecular ions of ajmalicine, yohimbine, tabersonine, catharanthine and the corresponding trideuterated standards was used for the quantitation of the compounds. Good linearity was observed ($r > 0.982$ in all instances) for the range 2–200 ng per injection (10–200 ng for catharanthine) when the analyte and internal standard peak-area ratios were plotted against the amount of analyte. The relative standard deviation (R.S.D.) for parallel samples (Table I) were markedly better at low sample concentrations than those (around 20%) of the method using vincamine as the internal standard [4].

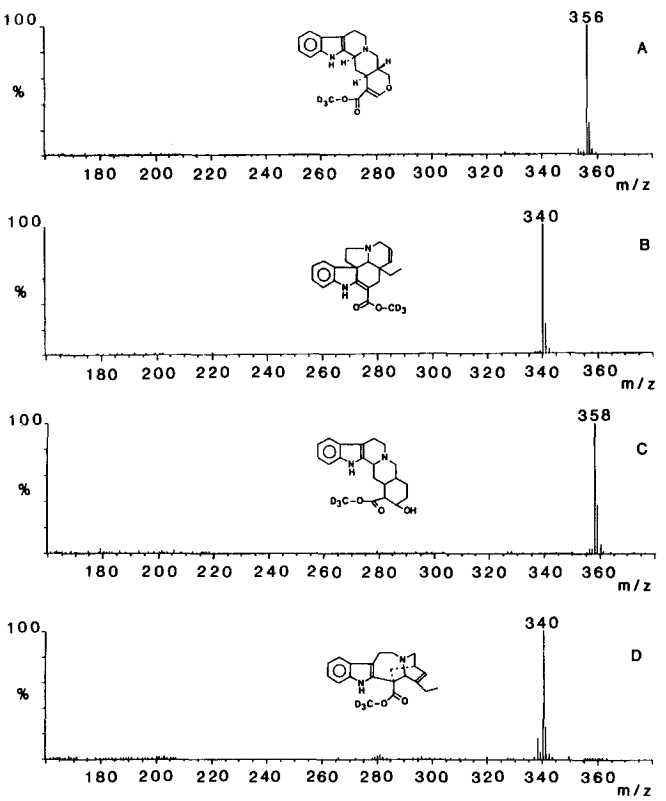

Fig. 1. Thermospray mass spectra of (A) [$^2H_3$]ajmalicine, (B) [$^2H_3$]tabersonine, (C) [$^2H_3$]yohimbine and (D) [$^2H_3$]catharanthine. Conditions: 0.1 *M* ammonium acetate–actonitrile (45 + 55); flow-rate, 1.1 ml/min; source temperature, 150°C; vaporizer temperature, 200°C; repeller 180 V. A 2–10 μg mass of the compounds was injected via a μBondapak C_{18} column (300 × 3.9 mm, 10 μm). No discharge or filament were used.

TABLE I

QUALITY PARAMETERS OF THE HPLC–TSP-MS ASSAY OF INDOLE ALKALOIDS

r = coefficient of correlation; R.S.D. = relative standard deviation (n = 6); x = amount of analyte in ng; y = peak-area ratio of analyte and internal standard.

Compound	Curve equation	r	R.S.D. (%)	
			2 ng	200 ng
Ajmalicine	$y = 3.0052x + 0.5291$	0.992	5.7	9.9
Yohimbine	$y = 2.8181x + 1.0528$	0.989	5.6	7.5
Tabersonine	$y = 3.1732x + 0.8321$	0.993	11.5	6.4
Catharanthine	$y = 5.0974x + 0.8508$	0.982	13.9 (10 ng)	10.8

The plant cell culture samples were chromatographed in 18 min using the isocratic solvent system. The trideuterated internal standards eluted a few seconds earlier than the non-labelled compounds. As a result of the complex matrix, baseline separation was not achieved for all of the compounds (Fig. 2).

The precision of the whole assay method was determined by analysing six replicate cell culture samples. The labelled standards were added before the extraction procedure. The precision is dependent on the varying chromatographic separation of the analytes and the amount of the compounds present in the cell samples. The R.S.D. values were 9.5% for yohimbine (approximately 18 ng per injection), 16% for

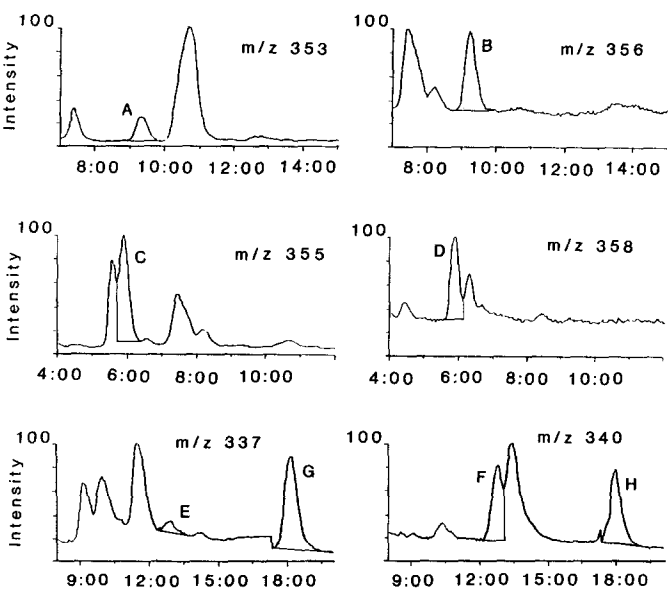

Fig. 2. Selected ion chromatograms of a cell culture sample of *C. roseus*. The intensities of the chromatograms were independently normalized. Compounds monitored: (A) ajmalicine, *m/z* 353; (B) [$^2H_3$]ajmalicine, *m/z* 356; (C) yohimbine, *m/z* 355; (D) [$^2H_3$]yohimbine, *m/z* 358; (E) catharanthine and (G) tabersonine *m/z* 337; (F) [$^2H_3$]catharanthine and (H) [$^2H_3$]tabersonine, *m/z* 340. Conditions as in Fig. 1.

tabersonine (14 ng), 21% for ajmalicine (3 ng) and 24% for catharanthine (less than 10 ng).

As seen in Fig. 2, the chromatograms for the ions at m/z 337 and m/z 340 show a sudden drop in the baseline at 17 min. This indicates some undefined change in the TSP source or probe conditions. The general and inevitable risk of this type of change in TSP analyses with the instruments currently available emphasizes the usefulness of stable isotope labelled internal standards.

CONCLUSIONS

HPLC–TSP-MS and selected ion monitoring of the MH^+ ions is a suitable method for the determination of various indole alkaloids in cell culture samples of *C. roseus*. The wide linear range of the assay is useful as the concentration of alkaloids varies greatly between different cell lines. The use of deuterium-labelled standards markedly improves the precision and general reliability of the analytical procedure.

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