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High-performance liquid chromatographic–mass spectrometric assay of high-value compounds for pharmaceutical use from plant cell tissue culture: *Cinchona* alkaloids

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ABSTRACT

The use of high-performance liquid chromatography (HPLC) interfaced with thermospray (TSP) mass spectrometry is described for the separation and identification of various alkaloids from *Cinchona ledgeriana* extracts. The use of water–acetonitrile–acetic acid (71:25:4) with 0.01 *M* ammonium acetate (pH 3.0) as the mobile phase gave good HPLC separation and good TSP sensitivity. The specificity obtained by single-ion monitoring allowed the analysis of commercially important alkaloids such as quinine and quinidine in plant material, transformed roots and in cells from tissue culture, with relatively simple extraction and work-up procedures. TSP gave protonated species with few fragment ions but collision-induced dissociation offers the promise of increased analytically specificity from the fragment ion data. This work has important implications for the biotechnological production of pharmaceuticals normally obtained from plant sources.

INTRODUCTION

Plants are the most economically viable source of many commercially important chemicals produced as secondary metabolites, including many pharmaceuticals, some 25% of prescription drugs being derived from plants [1]. Whilst still using plant material, plant tissue cultures have the ability to produce many secondary metabolites of pharmaceutical interest including a range of different alkaloids [2]. Biotechnology offers the promise of transferring the production of these chemicals to the more controlled conditions of the laboratory or factory environment through the use of isolated cell tissue culture techniques or the development of so-called "hairy roots", transformed by infection with *Agrobacterium rhizogenes*. Biotechnology can improve yields, lower unit costs and reduce the

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risks associated with dependence on climatic and political factors. One potential target is the *Cinchona* species and their quinoline-quinuclidine alkaloids. The anti-malarial alkaloid quinine and the cardiac antiarrhythmic quinidine have been produced, in our laboratory, from root organ cultures of *Cinchona ledgeriana* [3,4]. As an example of the production of alkaloids by hairy root cultures, which offer the particular advantage over isolated cells of biochemical and genetic stability, we have established hairy root cultures of a *Datura candida* hybrid which produces high yields of tropane alkaloids, including the clinically useful scopolamine [5].

Analytical chemistry has an important role to play in the evaluation and selection of high yielding plant strains and cell lines and in the optimisation of the culture proces for the production of the target metabolites. The best prospect for rapid assays of the culture medium or extracts of the cultured material with minimal separation and work-up lies with the use of chromatography–mass spectrometry. The tropane alkaloids are sufficiently volatile for analysis by gas chromatography–mass spectrometry (GC–MS) but the *Cinchona* alkaloids are less volatile and are more readily analysed by high-performance liquid chromatography–mass spectrometry (HPLC–MS) which is now a routine analytical technique in areas such as drug metabolism. We have produced suspension cultures of *C. ledgeriana* cells and have developed assays for the alkaloids using thermospray (TSP) HPLC–MS [6,7], which will be described in this paper.

EXPERIMENTAL

Cell cultures of *C. ledgeriana* were produced in our laboratory as described previously [4]. Extraction of the alkaloids was by a modified version of a procedure described by Robins *et al.* [8]. Freeze-dried cells (3 g) were ground, homogenised and sonicated in 36 ml of 0.2 *M* H_2SO_4–$CHCl_3$ (1:1). After standing, the cell debris was filtered off and washed with 9 ml of 0.2 *M* H_2SO_4, which was combined with the extract. The $CHCl_3$ was decanted and the aqueous phase was made alkaline with 10 *M* NH_4OH to pH 9.5–10 and poured onto an Extrelut column (Merck, Darmstadt, F.R.G.). The lipophilic components were eluted with $CHCl_3$, which was then evaporated to dryness. The residue was dissolved in 1 ml of CH_3OH and passed through a C_{18} Sep-Pak (Waters Assoc.), washing with a further 2 ml of CH_3OH. The final concentration corresponded to 1 g cells per 1 ml of methanol.

HPLC was carried out on a Du Pont Model 870 under isocratic conditions using a C_{18} Waters μBondapak 10-μm, 25 cm × 4.6 mm I.D. column with water–methanol–acetic acid (71:25:4) at a flow-rate of 1 ml/min. Sample loading was via a Rheodyne injector (10-μl loop). Detection was with a Kratos FS970 fluorimeter (excitation 250 nm, emission 418 nm). HPLC–MS was carried out on a VG MassLab 12/250 quadrupole mass spectrometer fitted with a commercial TSP source and VG 11-73 data system. The TSP ion source conditions were

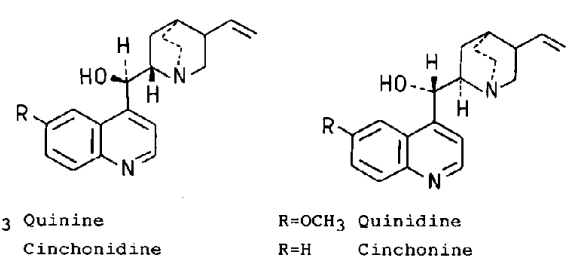
optimised with approximate temperatures: source, 200°C; nozzle, 190°C; chamber, 230°C. Chromatographic conditions were unchanged except that the mobile phase was buffered with 0.01 *M* ammonium acetate. The *Cinchona* alkaloids were monitored at *m/z* 295 and 325 and the internal standard [^{2}H]19-methoxydihydroquinine at *m/z* 330.

Tandem MS was carried out on a VG ZAB-2F double-focusing mass spectrometer with VG 11-253 data system. Fast atom bombardment (FAB) ionisation was achieved by bombardment with 8-keV xenon atoms from a saddle-field gun (Ion Tech), with the sample dissolved in a glycerol–thioglycerol matrix. The mass-selected protonated molecules were subjected to collisions with helium in the second field-free region, the helium pressure being adjusted to give 50% transmission of the parent ions. The spectra were obtained by scanning the electric sector field.

RESULTS AND DISCUSSION

The most important *Cinchona* alkaloids are the amino alcohols consisting of quinoline and quinuclidine moieties linked by a carbinol group, and their corresponding dihydro derivatives. Four asymmetric centres give sixteen possible stereoisomers, but only two pairs of stereoisomers occur as major components in the tree bark. The stereoisomers quinine and quinidine differ in configuration at C-8 of the quinuclidine ring and the carbinol C-9, quinine being 8(*S*),9(*R*) and quinidine 8(*R*),9(*S*). Cinchonidine and cinchonine are stereochemically analogous but they lack the quinoline 19-methoxy group. These four *erythro*-alkaloids are all active as antimalarial agents whereas their *threo*-epimers are inactive (Fig. 1).

The electron-impact (EI) mass spectra of quinine and quinidine have been reported. They readily undergo fragmentation to give several abundant fragment ions. The observation that the molecular ion (*m/z* 324) is more stable for quinidine (12%) than for quinine (< 1%) has been rationalised by the close proximity of the vinyl double bond to the 9-OH in quinidine shown by ^{13}C nuclear magnetic resonance (NMR) spectroscopy [9] allowing hydrogen bonding to stabilise the



R=OCH$_3$ Quinine R=OCH$_3$ Quinidine
R=H Cinchonidine R=H Cinchonine

Fig. 1. Structures of quinine and cinchonidine.

ion [10]. We have modelled these epimers by molecular graphics using the program QUANTA (Polygen), running on a Silicon Graphics IRIS 4D/20 personal workstation, and have confirmed that H-bonding is possible in quinidine but not in quinine. Cinchonine and cinchonidine exhibit similar behaviour.

HPLC and HPLC–MS

The rather weak molecular ions and their widely differing intensities means that EI ionisation is not ideal for detecting or assaying these alkaloids in complex mixtures, although the use of a moving belt interface with EI ionisation has been described [11]. A soft ionisation method such as TSP is preferable as the protonated molecular ion is likely to be the base peak and TSP is directly compatible with HPLC, allowing selected-ion monitoring (SIM) to give the optimum selectivity and sensitivity. TSP analysis of *Cinchona* alkaloids has been described previously [12]. As anticipated, direct injection of solutions of the authentic alkaloids into the TSP source of a quadrupole mass spectrometer showed the $[M+H]^+$ ions of m/z 325 and 295 to be the predominant ionic species. Even with a repeller electrode in the TSP source there was no fragmentation [13], and the NH_4^+ adduct ions were weak and variable in intensity [14].

HPLC separations of alkaloids are hampered by their basic properties, causing interactions with any weakly acidic silanol groups exposed on the stationary phase, leading to tailing of the peaks. This effect is minimised by operating at lower pH than pertains in the 20% CH_3OH–0.1 *M* ammonium acetate buffered solutions normally used in TSP. Both normal-phase and reversed-phase separations have been reported [15–19]. Literature methods employ water–acetonitrile–acetic acid–tetrahydrofuran as the mobile phase for separation of these alkaloids at pH 2.9, which is not ideal for TSP, although it has been used before [12]. Using a C_{18} stationary phase, we found that the optimum separation and TSP ionisation for quinine and quinidine monitored at m/z 325 and cinchonine and cinchonidine at m/z 295 was obtained with water–methanol–acetic acid (71:25:4) with 0.01 *M* ammonium acetate (pH 3.0). This is illustrated in the SIM chromatograms in Fig. 2A for a mixture of 250 ng each of the authentic alkaloids and in Fig. 2B for a sample extracted from cultured cells equivalent to 10 mg of the original dry cells.

The peaks illustrated in Fig. 2 show some tailing but they are sufficiently well resolved for their areas to be evaluated. Quinidine and cinchonidine both elute close to 13 min, and they would be unresolved in a total-ion current chromatogram, but as they have different molecular masses the use of SIM separates them completely. The ammonium acetate buffer degrades the resolution slightly and extends the retention times, as can be seen by comparing the TSP data with that obtained by fluorescence detection on the same cultured cell extract in the absence of ammonium acetate (Fig. 3), but this effect is lower with 0.01 *M* ammonium acetate rather than 0.1 *M*. Fluorescence detection gives a superior signal-to-noise ratio but it detects a fifth major component which is clearly not one of the

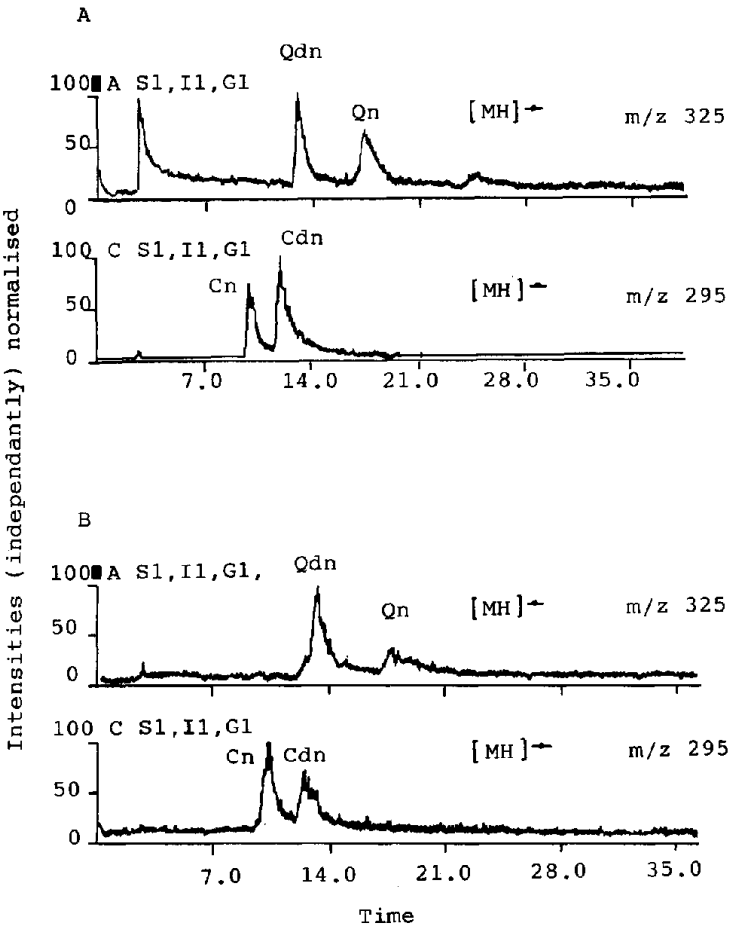


Fig. 2. HPLC SIM TSP profiles of (A) authentic *Cinchona* alkaloids and (B) alkaloids extracted from a *C. ledgeriana* cell culture.

target metabolites. Furthermore, in the absence of other analytical information, it is impossible to assign the peaks to the individual alkaloids.

Quantitative analysis

The similarity of the four alkaloids under study makes it likely that they would all give a similar response by TSP. This is broadly supported by the measured peak areas from Fig. 2A for the equimolar mixture which for quinine, quinidine, cinchonine and cinchonidine are in the ratio 1:1:1.5:1.7. The fact that these are not equal may be due in some measure to differences in the purities of the standards used and also to the tailing of the peaks introducing errors into the mea-

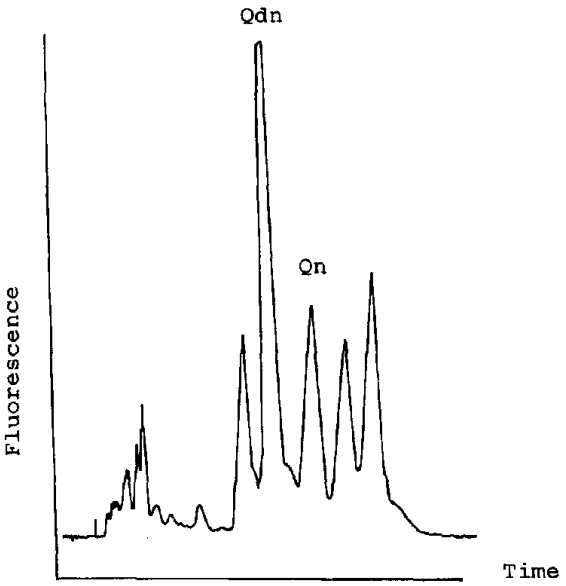


Fig. 3. Fluorescence chromatogram of alkaloids extracted from a *C. ledgeriana* cell culture. Peaks: Qn = quinine; Qdn = quinidine.

surement of the peak areas. There is also some variability in the sensitivity of response in TSP. Fig. 4 shows a series of replicate injections of 10 pg of quinidine directly into the TSP source without HPLC separation. The signal-to-noise ratio is about 10:1, except that after each injection the main peak is followed by a series of ions that have the effect of increasing the noise. The standard deviation in the signal intensity is approximately 20%, which may be partly attributable to injection technique and partly due to pulsing of the flow and variations in ionisation efficiency. These latter variations may be worse for the narrow peaks

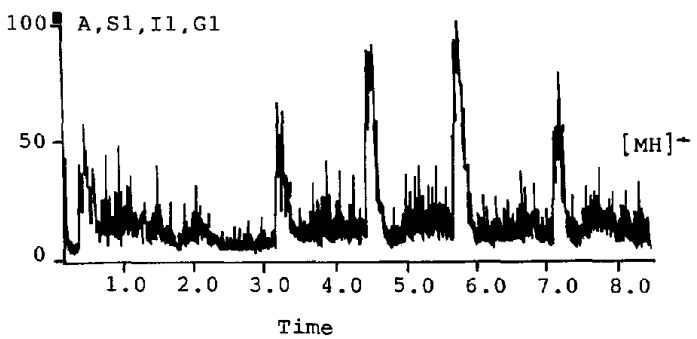


Fig. 4. TSP response for a series of replicate injections of 1 ng/ml quinidine.

arising from direct injection without HPLC (approximately 10 s) than for the wider HPLC peaks (approximately 1 min), for which the fluctuations may average out.

Assuming that the individual alkaloids do give equal responses, the peak areas in Fig. 2B show quinine, quinidine, cinchonine and cinchonidine in the cell extract to be present in the approximate ratio 1:7:2:1. For true quantitation it is necessary to have an appropriate internal standard. Starting from quinine we have synthesised [^{2}H]19-methoxydihydroquinine (M_r 329) by low-temperature catalytic hydrogenation of the vinyl bond, demethylation of the 19-methoxy with HBr, formation of the sodium quinolinoxide salt and remethylation with C^2H_3I. The use of dihydroquinine without deuteration would have been unsatisfactory as it can occur naturally, and the mass difference of only two mass units is barely sufficient. It seems unlikely that TSP ionisation efficiency will be affected by reduction of the vinyl group as protonation should occur at one or other of the nitrogen atoms.

Tandem mass spectrometry

An earlier study used EI ionisation without chromatographic separation but with tandem methods (unimolecular metastable fragmentation monitored by B/E-linked scans) in an attempt to monitor plant cell tissue culture of *Cinchona* alkaloids [20].

For the analysis of individual components in complex mixtures, the reliance on the observation of a single peak under SIM conditions may be considered inadequate for compound characterisation. For the *Cinchona* alkaloids, TSP gives only

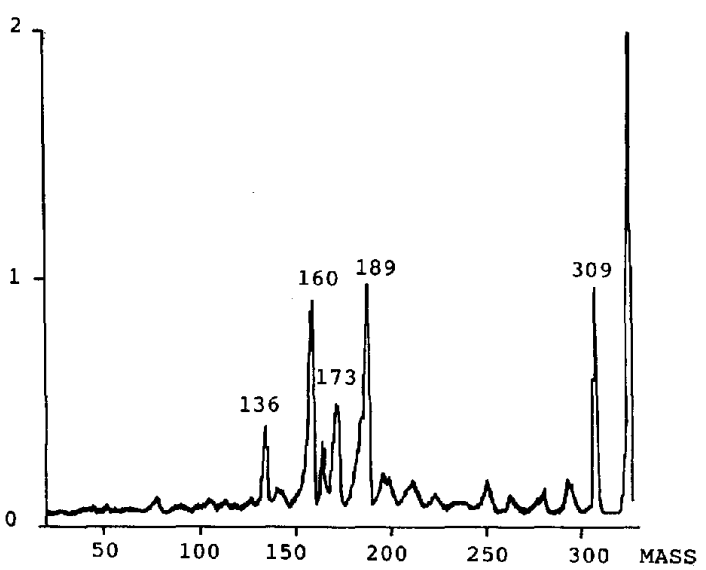


Fig. 5. CID–MIKES spectrum of protonates quinine (*m/z* 325) obtained under FAB conditions.

protonated molecular ions, even with a repeller electrode, and it may be desirable to carry out collision-induced dissociation (CID) in a tandem mass spectrometer. Initial experiments were carried out on the protonated molecular ions formed by FAB and subjected to CID in the second field-free region of a double-focusing mass spectrometer, *i.e.* CID–mass-analysed ion kinetic energy mass spectrometry (CID–MIKES). The spectrum of quinine given in Fig. 5 shows major fragment ions at *m/z* 309, 189, 173, 160 and 136, corresponding to the main CIDs of the molecule. It is very probable that the same peaks would be obtained by TSP CID–MIKES, which would allow multiple ion monitoring, *e.g.* with a triple quadrupole mass spectrometer, thereby increasing the analytical reliability of the data.

CONCLUSION

The use of TSP LC–MS has been shown to provide a rapid, sensitive and selective assay for commercially important secondary metabolites of plant cells. The use of tissue culture techniques to improve the costs effectiveness of the production of pharmaceutically important alkaloids may become an increasingly attractive option. The LC–MS techniques presented here will have a useful role in defining the optimum tissue culture conditions for the production of the compounds of interest.

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