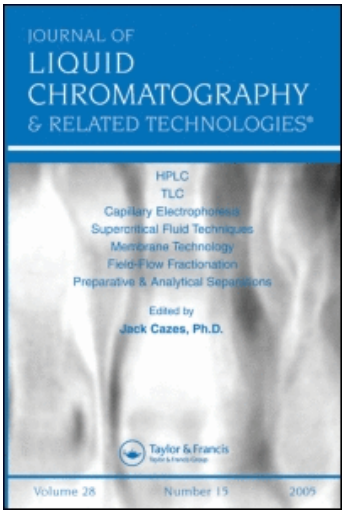

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HPLC SEPARATION OF CEPHALOTAXINE, HARRINGTONINE AND HOMO-HARRINGTONINE FROM CALLUS AND ROOT CULTURES OF *CEPHALOTAXUS HARRINGTONIA*

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ABSTRACT

A simple extraction and analytical protocol was developed to assay cephalotaxine, harringtonine, and homoharringtonine from callus and root cultures of *Cephalotaxus harringtonia*. The process involves extraction by methanol followed by partitioning between 0.5% ammonium hydroxide and chloroform. The chloroform fraction recovered greater than 90% of all three alkaloids. This fraction was concentrated to dryness, resuspended in methanol and analyzed by high pressure liquid chromatography using a UV detector.

Peaks corresponding to all three alkaloids were identified by comparing their retention times with authentic standards and their identity confirmed by fast atom bombardment spectrometry. The root cultures contained higher levels of harringtonine and

homoharringtonine (2.4 and 3.9 mg/kg dry matter, respectively) compared to the callus cultures; however, the levels of cephalotaxine were comparable (10.2 mg/kg dry matter).

INTRODUCTION

Harringtonine and homoharringtonine are two esters of the alkaloid cephalotaxine, isolated from the *Cephalotaxus* species.[1] Both compounds were selected for preclinical development as new anticancer agents[2] and, to date, have been used in the treatment of different types of leukemia,[3,4] carcinomas,[5,6] and chloroquinine-resistant malaria.[7]

Several studies on the synthesis of cephalotaxine and its structural analogs have been reported recently.[8] However, to date, extraction from plant sources has been the major source of these important alkaloids. The use of callus cultures derived from *C. harringtonia* as an alternative source of these alkaloids has also been reported.[9] Recently, we reported the establishment of fast-growing callus and root cultures of *C. harringtonia*;[10] however, the alkaloid contents of these cultures were not evaluated.

Prior to this report, all the protocols used in the extraction and isolation of these alkaloids from *Cephalotaxus* plant extracts included two or more partitioning steps followed by separation and analysis using counter-current chromatography and/or gas chromatography-mass spectrometry (GC-MS).[1,11,12,13] The analysis of callus cultures reported by Delfel and Rothfus[9] was also performed using the two-step partitioning protocol (chloroform with 2.5% aqueous tartaric acid followed by basification of the aqueous phase with ammonium hydroxide and re-extraction with chloroform) followed by GC-MS analysis, as described above.[1,11] However, a simple, yet efficient extraction and analytical protocol (for separation and identification) that would facilitate the screening of large numbers of plant tissue culture samples has to be developed or modified from existing protocols.[14]

In this report, we present a simple extraction and analytical protocol for characterizing cephalotaxine, harringtonine, and homoharringtonine from callus and root cultures of *C. harringtonia*, via a single-step partitioning process (partitioning between chloroform and 0.5% ammonium hydroxide) followed by high pressure liquid chromatography equipped with a UV detector.

MATERIALS AND METHODS

Plant Tissue Cultures

Callus and root cultures were established as described by us previously.[10] The callus cultures were grown in Magenta vessels and and root cultures were grown in 250 mL flasks. Callus cultures and root cultures were harvested during the log growth phase (four to five weeks and three to four weeks, respectively) and after being left to "age" without being subcultured (ten to twelve weeks and eight to ten weeks old, respectively). They were lyophilized and stored in a desiccator at room temperature until the time of analysis. All samples ranged between one to five grams, on a dry weight basis.

UV Spectra

Cephalotaxine, harringtonine, and homoharringtonine were purchased from Sigma Chemical Company (St. Louis, Missouri, USA) for use as authentic standards, and were resuspended in acetonitrile at a concentration of 250 µg/mL. The UV spectra for all three alkaloids were scanned on a Shimadzu (Model 160U-UV) UV-visual recording spectrophotometer (Shimadzu Corp., Kyoto, Japan).

Extraction

Samples of callus and roots (1 to 5 g) were extracted in methanol (50 to 250 mL) by homogenizing in an Omni-mix homogenizer (Omni International, Waterbury, Connecticut, USA) followed by sonication for five minutes. The extract was concentrated to dryness *in vacuo*, resuspended in 0.5% (v/v) ammonium hydroxide (at a ratio of 10 mL/g of callus or roots), and partitioned with an equal volume of chloroform. For larger volumes, the samples were allowed to stand overnight at 4°C for separation, while smaller volumes (less than 40 mL) were separated by centrifugation (1000*g*). The chloroform fraction was carefully separated and re-partitioned twice with additional 0.5% ammonium hydroxide. Finally, the chloroform fraction was concentrated to dryness under a stream of nitrogen and the residue resuspended in methanol (at the ratio of 1 mL /g of callus or roots).

During the process of developing the extraction protocol, samples fortified with authentic standards were partitioned with either 0.1, 0.5, or 2.0% (v/v) ammonium hydroxide or Milli-Q UF Plus water (Millipore Corp., Bedford,

Massachusetts, USA) and chloroform. This extraction was also compared with the two-step partitioning protocol (chloroform with 2.5% aqueous tartaric acid followed by basification of the aqueous phase with ammonium hydroxide and re-extraction with chloroform) described by Powell et al.[1]

Analytical High Pressure Liquid Chromatography (HPLC)

Instrumentation consisted of a HPLC system equipped with a Waters 600E multisolvent delivery system, a Waters model 700 satellite WISP, and a Waters 484 tunable absorbance detector (all from Millipore Corp., Milford, Massachusetts, USA). Peak areas were calculated with a HP 3394A integrator (Hewlett-Packard Co., Avondale, Pennsylvania, USA).

Separation was performed on a Dynamax 60 Å 8 μm phenyl column (4.6 mm x 250 mm) with a phenyl guard module (Rainin Instrument Co. Inc., Woburn, Massachusetts, USA). A linear gradient was used, starting from 70:15:15 (10 mM ammonium acetate buffer [pH = 4.0] : acetonitrile : methanol) and ending at 55:30:15, in 30 minutes. This ratio was then maintained for the next 30 minutes. The flow rate was held constant at 1.2 mL/min for the entire run. Cephalotaxine, harringtonine, and homoharringtonine were detected by monitoring the absorbance at 291 nm.

The final methanol extract from callus and root samples was filtered through a 0.2 micron nylon filter and used for HPLC analysis.

Identification and Confirmation of the Presence of Cephalotaxine, Harringtonine, and Homoharringtonine

The alkaloids were identified by comparing their HPLC retention times with authentic standards, and also by spiking.

Fractions corresponding to the cephalotaxine, harringtonine, and homoharringtonine peaks were collected from root extracts, concentrated *in vacuo* to remove the organic solvents, and lyophilized to yield a trace residue. This residue was then subjected to fast atom bombardment mass spectrometry along with samples of authentic cephalotaxine, harringtonine, and homoharringtonine, according to a previously published protocol,[15] at the mass spectrometry facility in the Department of Chemistry, The Pennsylvania State University, University Park, Pennsylvania, USA.

RESULTS AND DISCUSSION

Cephalotaxine, harringtonine, and homoharringtonine exhibited UV absorbance spectra consisting of a single absorbance peak between 260 and 312 nm, with an absorbance maxima at 289.5, 290.8, and 290.6 nm, respectively. Therefore, the wavelength 291 nm was selected to monitor peaks during HPLC analysis.

Separation of cephalotaxine, harringtonine, and homoharringtonine was achieved, with the peaks eluting after 15.5, 26.1 and 31.9 minutes, respectively (Figure 1). The HPLC protocol was capable of detecting levels of cephalotaxine, harringtonine, and homoharringtonine at amounts as low as five nanograms per injection.

A single partitioning step (partitioning between chloroform and 0.5% ammonium hydroxide) was sufficient to provide a semi-crude extract which contained cephalotaxine, harringtonine, and homoharringtonine, as determined by HPLC (Figure 1). Partitioning with 0.5% (v/v) ammonium hydroxide gave the best recovery of all three alkaloids (greater than 90%), compared to partitioning in 0.1% or 2.0% ammonium hydroxide or Milli-Q water (Table 1). Similar recovery rates were also observed when methylene chloride was substituted for chloroform (data not shown).

Table 1

*** Recovery of Cephalotaxine (CT), Harringtonine (HT), and Homoharringtonine (HHT) following Partitioning with Chloroform and Different Aqueous Phases+**

Aqueous phase	CT (%)	HT (%)	HHT (%)
Milli-Q water	34 ± 2	66 ± 3	68 ± 3
0.1% NH_4OH	64 ± 4	105 ± 5	100 ± 7
0.5% NH_4OH	94 ± 1	108 ± 5	106 ± 3
2.0% NH_4OH	92 ± 2	97 ± 4	95 ± 4

* Recovery is based on the amount of authentic standards that were used to fortify each callus/root sample. Data represents the mean ± SD from three replications.

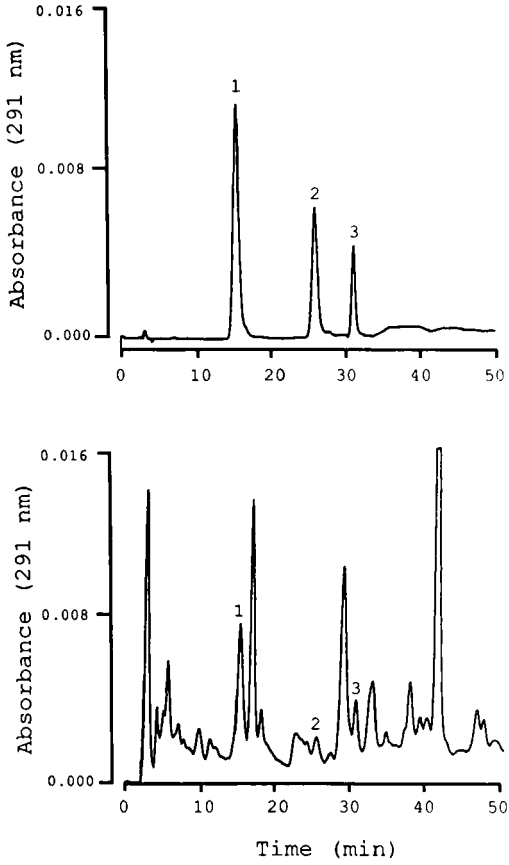

Figure 1. HPLC separation of authentic standards (TOP) and a semi-crude root culture extract following partitioning with 0.5% ammonium hydroxide and chloroform (BOTTOM). Peak number 1 = cephalotaxine, 2 = harringtonine, and 3 = homoharringtonine. Separation was performed on a Dynamax 60 Å 8 μm phenyl column and peaks detected by monitoring absorbance at 291 nm. A linear gradient was used, starting from 70:15:15 (10 mM ammonium acetate buffer [pH = 4.0] : acetonitrile : methanol) and ending at 55:30:15, in 30 minutes. This ratio was then maintained for the next 30 minutes. The flow rate was held constant at 1.2 mL/min during the entire run.

We found that the two-step partitioning protocol (chloroform with 2.5% aqueous tartaric acid, followed by basification of the aqueous phase with ammonium hydroxide and re-extraction with chloroform) described by Powell

et al.[1] gave a recovery of 72% cephalotaxine, 86% harringtonine, and 93% homoharringtonine. Therefore, the single-step partitioning protocol (partitioning between chloroform and 0.5% ammonium hydroxide) described here gave superior recovery of all three alkaloids, compared to the two-step partitioning protocol of Powell et al.[1]

The root cultures and callus cultures that were allowed to "age" contained similar amounts of cephalotaxine (10.2 mg/kg on a dry weight basis). However, the "aged" root cultures had at least two- to three-times more harringtonine and homoharringtonine (2.4 and 3.9 mg/kg on a dry weight basis, respectively) compared to the "aged" callus cultures. These values are two- to five-fold higher than the concentrations found in callus cultures reported initially by Delfel and Rothfus,[9] but comparable with their 1979 report.[16] The young actively growing callus and root cultures contained less than one-tenth the amount of cephalotaxine, harringtonine and homoharringtonine compared to the "aged" cultures (data not shown).

The HPLC peaks corresponding to cephalotaxine, harringtonine, and homoharringtonine exhibited molecular ions $(M + H)^+$ with mass-to-charge ratios of 316, 532, and 546, respectively. These values corresponded to the values of the molecular ions observed from authentic standards of cephalotaxine, harringtonine, and homoharringtonine.

Callus and organ cultures in general, accumulate only a fraction of the levels of the secondary products found in field-grown plants, and therefore, in most cases, the protocols developed for the analysis of whole plants and plant parts cannot be extrapolated to analyze callus and organ cultures.[17] In addition, the metabolites may be stored in different tissues and/or compartments than found in the whole plant, or bound in such a way that they may not be retrieved by conventional extraction methods used with field-grown plants. Therefore, in order to detect the presence of such metabolites, more efficient extraction protocols are needed. Existing extraction protocols could also be simplified due to the absence of very hydrophobic compounds such as oils, waxes, and other complexed cuticular components in callus and organ cultures, otherwise usually associated with plant extracts.

The protocol described in this paper sufficed as the only partitioning step needed to yield a semi-crude extract rich in cephalotaxine, harringtonine, and homoharringtonine from callus and root cultures.

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