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Note

Separation and quantitation of cinchona major alkaloids by high-performance liquid chromatography

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Various papers[1–5] have been published on the high-performance liquid chromatographic (HPLC) analysis of cinchona alkaloids. The HPLC systems used did not provide complete separation and reliable quantitation of the four cinchona alkaloids (cinchonine, cinchonidine, quinidine and quinine) from their dihydro derivatives. Berlex labs, developed solvent systems S1 and S2 (ref. 6) that were a modification of those used by Pound and Sears[3] which could separate most of the major cinchona alkaloids. Recently, McCalley[7,8] developed HPLC systems that separated the four major cinchona alkaloids from their dihydro derivatives with the exception of dihydrocinchonine.

We now report a dual column system which, with S2, separated and quantitated the major cinchona alkaloids from partially purified biological samples. An alternative mobile solvent system (S3) is also reported which separated the major cinchona alkaloids, except cinchonine–cinchonidine, from a single column. The dual-column system developed has a very high reproducibility of retention times and peak areas. The problem of reproducibility resulting from the presence of water as a polar modifier, suggested by McCalley[7], did not occur.

EXPERIMENTAL

Dual-column system

A Beckman Model 110A pump, an Altex injector and a Beckman Model 153 UV detector (operated at 254 nm) (Beckman/Altex, Berkeley, CA, U.S.A.) were used. Two columns in series, an Altex Ultrasphere-Si (5 μm, 250 × 4.6 mm I.D.) joined to a Whatman Partisil (PXS 10/25; 250 × 4.5 mm I.D.) (Whatman, Clifton, NJ, U.S.A.) column were used as the stationary phase. The mobile phases used at a flow-rate of 2 ml/min were tetrahydrofuran (THF)–*n*-butyl chloride (*n*-BuCl)–aqueous ammonia [60:40:0.5 (S1)] and [60:40:0.25 (S2)].

Single-column system

All HPLC components used were the same as in the dual-column system except that an Altex Ultrasphere-Si column was used with dioxane–hexane–aqueous ammonia [80:20:0.21 (S3)] at a flow-rate of 2.1 ml/min.

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Solvents and chemicals

Organic solvents were obtained from Burdick and Jackson (Muskegon, MI, U.S.A.) and aqueous ammonia from MCB Manufacturing Chemist (Curtin Matheson, Minneapolis, MN, U.S.A.). Cinchonine, cinchonidine, quinidine, quinine and anthraquinone were obtained from Aldrich (Milwaukee, WI, U.S.A.). Dihydrocinchonidine, dihydroquinidine and dihydroquinine were prepared by reduction of the respective alkaloids using platinum(IV)oxide (Adam's catalyst, Aldrich) and hydrogen, according to the procedure of Leete[9]. Cinchoninone–cinchonidinone and quininone–quinidinone were prepared by oxidation of the four major parental cinchona alkaloids using benzophenone and potassium *tert.*-butoxide (Aldrich) in anhydrous benzene according to the procedure of Woodward[10]. The name used on the individual ketone only reflects the origin of the parental alkaloid used as the starting material. The optical purity of the ketone pairs is indiscriminatory.

All mobile solvent combinations were mixed and used immediately. Samples were dissolved in either THF–*n*-BuCl (60:40) or dioxane–hexane (80:20) and their solutions passed through 0.2 μm Millipore filters (Bedford, MA, U.S.A.).

Preparations of biological samples for HPLC analysis

Crude alkaloid extracts. Cinchona tissue culture materials[11] were macerated in a Waring Blendor with chloroform–aqueous ammonia [1:1 (S4)], diluted with S4, transfered to capped bottles and shaken at 4°C for 24 h. The mixtures were strained through cheese cloth and the organic layers separated and filtered. After drying with anhydrous magnesium sulfate, the chloroform was removed and the crude alkaloid extracts were dried under high *vacuo* for 24 h. Crude alkaloid extracts were obtained from Eucadorian cinchona bark powder* as described but without blendor maceration.

Acid–base partially purified alkaloid extracts. Crude alkaloid extracts were treated with 3 *N* hydrochloric acid and the acidic solutions were filtered and made basic to pH 12 with aqueous ammonia. The basic solutions were extracted with chloroform and the organic layers were separated and removed under *vacuo*. After repeating the procedure three times, the final chloroform layers were dried with anhydrous magnesium sulfate and the chloroform removed and were dried under high *vacuo*.

Silica gel micro-column partially purified alkaloid extracts. Acid–base partially purified alkaloid extracts (80–100 mg) were further purified as 0.3-ml fractions by micro-column chromatography (125 × 5 mm I.D. glass column packed with 2.0 g Kieselgel 60, 230–400 mesh ASTM) (E. Merck, Darmstadt, F.R.G.) and eluted with chloroform–methanol [3.5:1.0 (S5)]. The various fractions were spotted on Si 250 μm GF thin layer plates (Analtex, Newark, DE, U.S.A.) and developed with S5. The spots were visualized under long- and short-wavelength UV and by iodine vapor treatment. Column fractions were combined as follows: blank (1–11), I(12–15), II(16–19), III(20–25), IV(26–34), V(35–37) and VI(38–46). The eluent was removed from the combined fractions and dried under high *vacuo* for 24 h.

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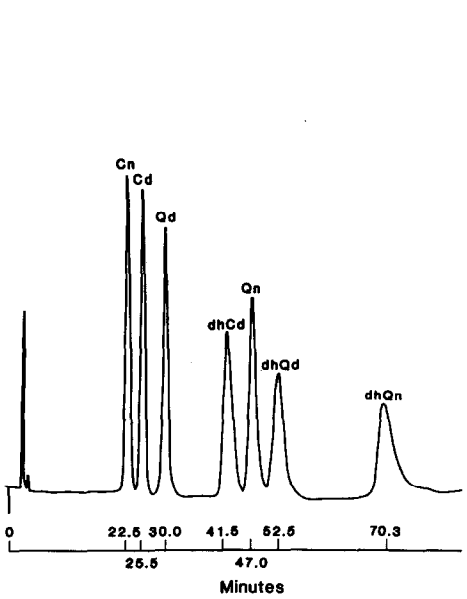
* Ecuadorian bark of unknown age obtained from Berlex Labs., Wayne, NJ, U.S.A.

RESULTS AND DISCUSSIONS

Seven cinchona alkaloid standards were separated in approximately 71 min on a dual column system using S2 (Fig. 1). A more rapid analysis can be achieved in approximately 40 min using S1; however, without dihydroquinidine separating from quinine.

The single column system using S3 enable reasonable alkaloid separation in approximately 35 min (Fig. 2). Complete separation of cinchonine and cinchonidine was not achieved.



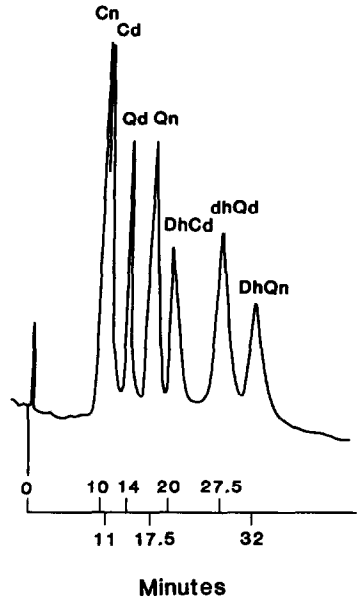


Fig. 1. HPLC analysis of cinchona alkaloid standards. Peaks: Cn = cinchonine; Cd = cinchonidine; Qd = quinidine; Qn = quinine; dhCd = dihydrocinchonidine; dhQd = dihydroquinidine; dhQn = dihydroquinine.

Fig. 2. HPLC analysis of cinchona alkaloid standards using a single Altex Ultrasphere-Si 5-μm column and S3. Peaks as in Fig. 1.

A significant problem with the HPLC analysis of *C. ledgeriana* tissue culture extracts is the potential of non-resolution of the major alkaloids from the minor alkaloids and other compounds having similar retention times. Approximately 24 extractable cinchona alkaloids from plant materials are reported[12,13]. The majority of the unidentified minor alkaloids are eluted between retention times 3 to 18 min. Depending on the concentration of the sample injected, these minor alkaloids can have substantial interference on the base line at the time cinchonine and cinchonidine are eluted. Silica gel micro-column purification is necessary for leaf-shoot organ culture extracts in which the alkaloid pattern is complex and for root organ and unorganized callus culture extracts in which anthraquinone type compounds predominate. The thin-layer chromatographic (TLC) analysis of leaf-shoot organ crude extracts using S5 showed 22 or more distinguishable blue, purple and dull orange spots when visualized under a combination of long- and short-wavelength UV[14]. When

compared to standards the four major cinchona alkaloids and their dihydro derivatives overlap between R_F 0.18 to R_F 0.37. The dull orange spots near R_F 0.78 may be the anthraquinone type compounds reported by Verpoorte[15]. Quininone–quinidinone alkaloid at R_F 0.61 is visualized as a bright greenish-yellow spot under short-wavelength UV and cinchoninone–cinchonidinone alkaloid at the same R_F as a dark purple spot.

The dual-column system can be used directly to analyze the acid–base partially purified *C. ledgeriana* leaf-shoot organ culture extracts (Fig. 3A) and the crude extracts of Ecuadorian cinchona bark (Fig. 3B).

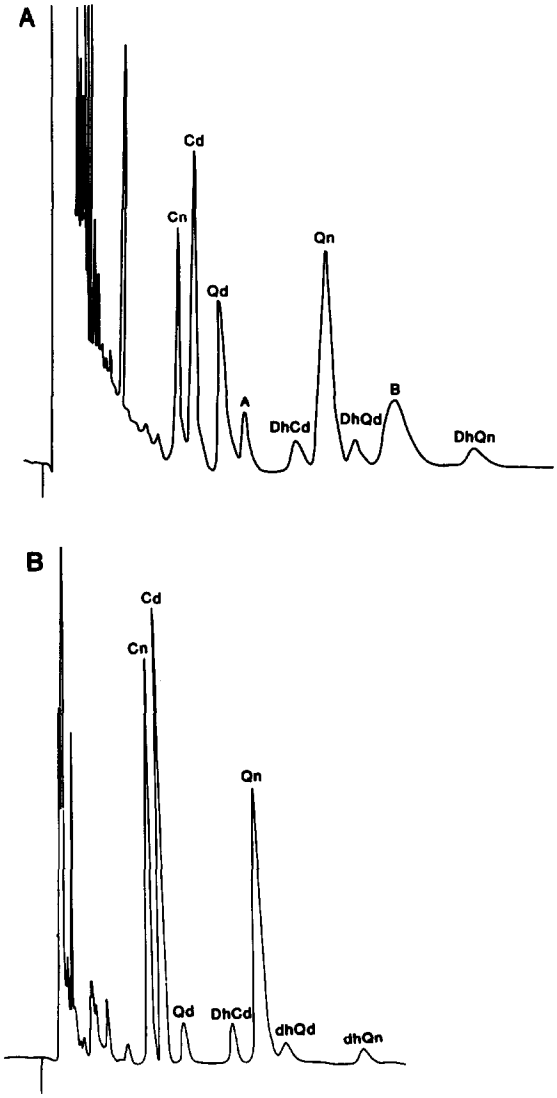


Fig. 3. (A) HPLC analysis of acid–base partially purified sample of *C. ledgeriana* leaf-shoot organ culture extract. (B) HPLC analysis of crude extract of Ecuadorian cinchona bark powder. as in Fig. 1; A and B are unknown components.

TABLE I

ALKALOID ANALYSIS OF *CINCHONA LEDGERIANA* TISSUE CULTURES AND ECUADORIAN CINCHONA BARK

Tissues analyzed were six weeks old. Cultures were approximately two years old and were subcultured approximately every four weeks. Alkaloid abbreviations as in Fig. 1. Asterisk indicates undetectable alkaloids.

Tissue cultures	*Percent dry weight* × 10^4						
	Cn	*Cd*	*Qd*	*dhCd*	*Qn*	*dhQd*	*dhQn*
Leaf-shoot organ	690 ± 180	830 ± 68	280 ± 190	430 ± 84	570 ± 230	20 ± 14	20 ± 14
Root organ	40 ± 28	20 ± 14	80 ± 42	10 ± 2	60 ± 42	40 ± 14	10 ± 5
Callus	3.6 ± 1.5	3.0 ± 1.4	3.7 ± 1.5	*	130 ± 45	29 ± 8	*
Plant material							
Ecuadorian bark	1.68 ± 0.45	2.43 ± 0.53	0.092 ± 0.011	0.089 ± 0.027	2.57 ± 0.35	0.038 ± 0.015	0.045 ± 0.021

When employing the dual column system for the analysis of the leaf-shoot organ culture extracts that were partially purified with silica gel micro-column chromatography, unidentified peaks with retention times between 3 to 20 min persist throughout fractions I and II. In fractions III to VI the unidentified peaks persist from 3 to 10 min but are less interfering in the separation of the major alkaloids. Standards quininone–quinidinone and cinchoninone–cinchonidinone all have a retention time of 5.5 min and anthraquinone itself has a retention time of 3.0 min. The quininone and anthraquinone types of compounds cannot be analyzed together with analysis of major cinchona alkaloids. Quinine and quinidine alkaloids constitue the major components in fraction III with trace of cinchonine, cinchonidine and dihydroquinidine. Cinchonine, cinchonidine, quinidine, dihydrocinchonidine, quinine and dihydroquinine can all be found in fraction IV. Cinchonine and cinchonidine are the major components in fraction V together with an unknown peak A, and traces of quinine and dihydroquinidine. Fraction VI constitutes the majority of an unknown component B. The HPLC analysis of micro-column purified fractions from root organ and unorganized callus organ extracts had similar results as described. Analytical data for alkaloids is given for *C. ledgeriana* leaf-shoot organ, root organ and unorganized callus samples by the analysis of fractions I–VI and for Ecuadorian cinchona bark powder crude extracts (Table I).

ACKNOWLEDGEMENT

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