HPLC Analysis of Alkaloids in Extracts of Callus Cultures of *Cinchona* Species

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The quantitative analysis of the alkaloids in *Cinchona* tissue cultures by means of a reversed-phase ion pair HPLC method is reported.

In connection with our studies on the alkaloid production in cell- and tissue-cultures of *Cinchona* species [1–4] a method for the quantitative analysis of the quinoline alkaloids was developed. In a previous report we described the analysis of the *Cinchona* alkaloids by means of normal-phase HPLC [5]. However, not one of the systems was able to separate all major, naturally occurring quinoline alkaloids: cinchonine, cinchonidine, quinine, quinidine and their dihydro derivatives (Figs. 1, 2). Various reversed phase HPLC separations have been reported in the literature for the analysis of *Cinchona* alkaloids [6], however, these systems all suffered from poor peakshape or poor separation of all eight quinoline alkaloids. Finally we have obtained good separations by means of a reversed phase ion-pair HPLC separation on columns impregnated with dodecylsulfonic acid and cetrimide [7].

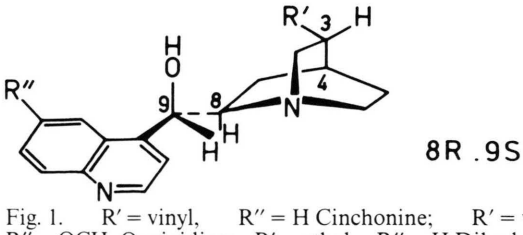

Fig. 1. R′ = vinyl, R″ = H Cinchonine; R′ = vinyl, R″ = OCH_3 Quninidine; R′ = ethyl, R″ = H Dihydrocinchonine; R′ = ethyl, R″ = OCH_3 Dihydroquinidine.

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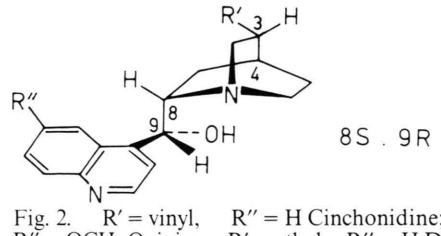

Fig. 2. R′ = vinyl, R″ = H Cinchonidine; R′ = vinyl, R″ = OCH_3 Quinine; R′ = ethyl, R″ = H Dihydrocinchonidine; R′ = ethyl, R″ = OCH_3 Dihydroquinine.

As the percentage of organic solvent, pH and counterion were found to be important for the separation, the influence of these parameters was further studied in order to optimize the separation. For the quinoline alkaloids it was found that an aqueous mobile phase containing 1–2% dioxane or tetrahydrofuran gave the best results. As counterions camforsulfonic acid, butanesulfonic acid and methanesulfonic acid were compared as to their influence on the separation. In a concentration of 0.02 M all three counterions gave similar results, although with the camforsulfonic acid containing mobile phase the k'-values of the quinoline alkaloids were smaller than with the other two counterions.

Although the butanesulfonic acid containing mobile phase gave a slightly better resolution than the methanesulfonic acid containing eluent, the latter mobile phase was used for the routine analyses because methanesulfonic acid is much cheaper.

Optimum pH was found to be ca. 3.5, above pH 4 peaks showed tailing, which increased with the pH. The pH can be adjusted by means of an ammonium acetate buffer (0.4 M), a McIlvaine buffer (0.4 M) or by addition of sulfuric acid, followed by adjustment of the pH with sodium hydroxide.

In Fig. 3 the separation of the eight quinoline alkaloids by such a mobile phase is shown. Using this HPLC system a series of extracts obtained from various cell lines of *Cinchona* callus cultures and previously studied for their alkaloid content [3, 4] was analyzed. The results are shown in Table I.

As the amounts available of cinchonamine, 10-methoxycinchonamine (Fig. 4) and quinamine (Fig. 5) were too small to make standard curves for these alkaloids, quantitative analysis could not be performed. However, considering that at the wavelength of detection (280 nm) cinchonamine and 10-methoxycinchonamine have a UV absorption which is about twice that of quinine and quinidine,

it could be concluded from the peaks observed for these indole alkaloids, that considerable amounts of these alkaloids are present. In fact they are the major constituents in the extracts of the *C. pubescens* cell lines. The results of the analyses give further support to our previous conclusions, drawn from the amounts of individual alkaloids isolated from these extracts [3, 4].

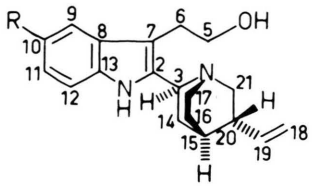

Fig. 4. R=H Cinchonamine; R=OCH_3 10-methoxycinchonamine.

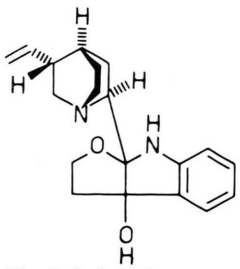

Fig. 5. Quinamine.

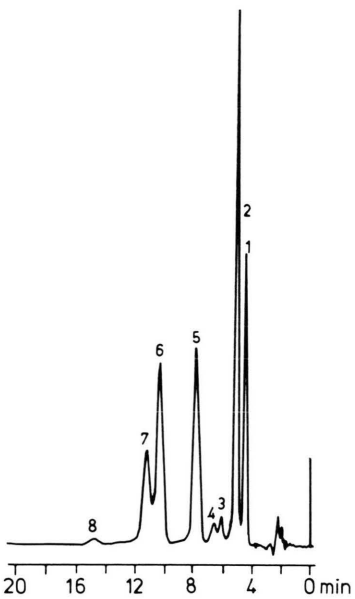

Fig. 3. HPLC separation of standard mixture of *Cinchona* alkaloids. Column 300 × 4.6 mm I.D. stainless steel, filled with Lichrosorb RP 18, 5 µm, loaded with 0.01 M dodecylsulfonic acid in water-methanol (1 : 1), followed by 0.02 M cetrimide in water. Mobile phase was 0.02 M methanesulfonic acid in water-dioxane-sulfuric acid (98.5 : 1 : 0.5) (pH = 3.5 with 10 M sodium hydroxide), flow rate 1.5 ml/min. Detection UV 280 nm. 1 = cinchonine, 2 = cinchonidine, 3 = dihydrocinchonine, 4 = dihydrocinchonidine, 5 = quinidine, 6 = quinine, 7 = dihydroquinidine, 8 = dihydroquinine.

Table I. Amount of alkaloid (µg)[a] found in 25 g calli (dry weight).

Cell line	Medium[b]	Light/dark[b]	Alkaloids							
			C	HC	Cd	HCd	Qd	HQd	Q	HQ
C. ledgeriana										
Root 1 L 28-11	H	L	1365	372	399	+[c]	26	+	+	+
Leaves 2 L 22-6 IV	H	L	92	73	11	8	46	−[d]	73	−
Seeds L 28-11 (1)	H	L	132	32	99	20	+	+	+	+
Seeds L 28-11 (2)	H	L	562	130	164	7	+	−	+	−
C. pubescens										
Stem S 20-6D	H	L	924	513	300	187	72	+	+	+
Stem S 20-6D	H	D	246	112	122	+	10	55	+	+
Stem S 20-6D	3	L	179	84	+	33	14	32	51	+
Leaves S 20-6A	H	L	1767	+	781	+	301	+	+	+
Leaves S 20-6A	H	D	358	+	408	+	36	51	40	+
Leaves S 20-6A	3	L	167	122	+	91	18	49	19	+

[a] Minimum detectable amount at 280 nm ca. 100 ng.
[b] For culture conditions see reference [4] and references cited therein.
[c] + Present but not possible to qualify due to low concentration or interfering peaks.
[d] − Not detected.

Particularly the positive influence of light on the alkaloid production is clearly shown if the amounts of alkaloid in the cell lines of *C. pubescens* grown on medium H in the dark are compared with those grown in a 12 h light-12 h dark cyclus. Also the previous conclusion that medium H is a better production medium than medium 3 [2] is further supported by the quantitative analysis reported here. Compared with the results of previous analyses by means of fluorodensitometry for the quinine and quinidine content [2] it can be noted that the amount of alkaloid present in the stem callus cell line of *C. pubescens* has declined during the years of subculturing.

Experimental

For details of the tissue culture methods see references [1–4]. The extracts used for the quantitative analysis were the same as used for the isolation and identification of the alkaloids [3, 4]. These extracts were made by grinding 25 g freeze dried calli, mixing this with 50 ml 10% aqueous sodium bicarbonate solution, followed by extraction with 125 ml chloroform (three times). Extraction was performed in the dark, under nitrogen for 16 hrs. After collection of the chloroform extract it was taken to dryness under reduced pressure. The combined chloroform extracts were extracted three times with 100 ml 1% aqueous acetic acid. The acetic acid extracts were combined and concentrated to 100 ml under reduced pressure, basified with sodium bicarbonate to pH = 8 and subsequently extracted three times with 100 ml chloroform. The combined chloroform extracts were taken to dryness under reduced pressure and weighted. One tenth of these extracts was saved and has been used for the quantitative analysis as reported here.

The HPLC analyses were carried out on an apparatus consisting of a Waters Model U6K injector, a Waters Model 6000 A pump and a Schoeffel Model SF 770 variable wavelength detector operating at 280 nm or a Waters Model 440 UV detector also operating at 280 nm. The column was a 300 × 4.6 mm I.D. stainless steel column filled with Lichrosorb RP 18, 5 µm, loaded with 0.01 M dodecylsulfonic acid in methanol-water (1 : 1), followed by 0.02 M cetrimide in water as reported recently [7]. As mobile phase for the quantitative analysis 0.02 M methanesulfonic acid in water-dioxane-sulfuric acid (98.5 : 1 : 0.5) (pH = 3.5 with 10 M sodium hydroxide) (see Fig. 1) was used, flow rate 1.5 ml/min. For the quantitative analysis the extracts were dissolved in 0.5 or 1 ml of the mobile phase and 10–20 µl of the solution was injected. Of the eight quinoline alkaloids cinchonine, cinchonidine, quinidine, quinine and their dihydro derivatives, standard curves were made, from which the amounts of alkaloids in the various extracts could be calculated. For the quantitative analysis a Packard model 603 integrator was used.

[1] Th. Mulder-Krieger, R. Verpoorte, and A. Baerheim Svendsen, Planta Med. **44,** 237 (1982).
[2] Th. Mulder-Krieger, R. Verpoorte, Y. P. de Graaf, M. van der Kreek, and A. Baerheim Svendsen, Planta Med. **46,** 15 (1982).
[3] Th. Mulder-Krieger, R. Verpoorte, A. de Water, M. van Gessel, B. C. J. A. van Oeveren, and A. Baerheim Svendsen, Planta Med. **46,** 19 (1982).
[4] Th. Mulder-Krieger, R. Verpoorte, M. van der Kreek, and A. Baerheim Svendsen, Planta Med. **17** (1984).
[5] R. Verpoorte, Th. Mulder-Krieger, M. J. Verzijl, J. M. Verzijl, and A. Baerheim Svendsen, J. Chromatogr. **261,** 172 (1983).
[6] R. Verpoorte and A. Baerheim Svendsen, Chromatography of Alkaloids, part B; GLC and HPLC. Chromatograph. Library, **Vol. 23 B,** Elsevier, Amsterdam, in press.
[7] R. Verpoorte, J. M. Verzijl, and A. Baerheim Svendsen, J. Chromatogr. **283,** 401 (1984).