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Note

Analytical and preparative reversed-phase liquid chromatography of secoiridoid glycosides

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Secoiridoid glycosides are typical constituents of Gentianaceae, such as *Gentiana* and *Swertia*. They have a bitter taste and show antimicrobial properties after enzymatic hydrolysis[1,2]. Swertiamarin (1), gentiopicrin (2) and sweroside (3) are widespread, whereas their esters with hydroxybenzoic or diphenylcarbonic acids, which are among the most bitter natural products known[3,4], are less common. Previously[5], we reported on the desorption/chemical ionization mass spectrometry (D/CI MS) of the underivatized glycosides, a useful method for structural analysis of these rather unstable compounds.

Analytical high-performance liquid chromatography (HPLC) has been proposed for the separation and quantitation of secoiridoid glycosides in medicinal plant extracts[6–8]. The use of this technique together with the recently developed photodiode array detector[9,10] represents an important advance in HPLC. Any separated compound can be characterized by its complete UV–VIS spectrum.

Preparative isolation techniques are, at the same time, important since bioassays or structural elucidations need a certain quantity of a pure compound. Preparative liquid chromatography has been carried out on silica gel in order to isolate secoiridoid glycosides from *Lomatogonium carinthiacum*[11] and *Centaurium spicatum*[12], using dichloromethane–methanol (82.5:17.5) and ethylacetate–methanol (95:5) as mobile phases respectively. However, very complex mixtures would not have been separated under these conditions. Sakamoto *et al.*[13] isolated the glycosides 1–3 from *Swertia* species by semi-preparative reversed-phase (RP) HPLC with acetonitrile–water (20:80). This system is not suitable for a larger preparative scale, due to its expense and the toxicity of the organic solvent.

In the present paper we show the advantages of a new method for the isolation of secoiridoid glycosides. Preparative separations are carried out on a reversed phase (RP-8) with methanol–water as eluent, using a system which is generally described as medium-pressure liquid chromatography (MPLC)[14,15].

EXPERIMENTAL

Plant material and extracts

Gentiana lactea PHIL. and *Coutoubea spicata* AUBL. were collected in Chile

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and in Panama, respectively. The dried plant material (aerial parts) was extracted with solvents of increasing polarities: light petroleum (b.p. 80–95°C), chloroform and methanol. The secoiridoid fractions were obtained by column chromatography of the crude methanolic extracts on Polyamide SC 6 (Macherey Nagel, Düren, F.R.G.) with 50% (*G. lactea*) or 20% aqueous methanol (*C. spicata*).

HPLC

HPLC was carried out on a Hypersil column RP-8, 5 μm (10 cm × 4.6 mm I.D., Hewlett-Packard), with isocratic elution with 20% and 30% aqueous methanol (8 min after injection). The mobile phase was delivered at a flow-rate of 1.5 ml/min by a SP 8700/SP 8750 pump (Spectra Physics, San José, U.S.A.). The chromatogram at 254 nm and the UV/VIS spectra were recorded with a photodiode array detector HP 1040 A, coupled with a HP-85 personal computer (Hewlett-Packard). Details of the post-column derivatization are given in ref. 16.

Preparative liquid chromatography

Medium-pressure system. Separations were carried out with an MPLC liquid chromatograph B-680 and an UV detector B-638 (Büchi Laboratoriumstechnik, Flawil, Switzerland). A 46 cm × 25 mm I.D. glass column, connected to a 11.5 cm × 10 mm I.D. precolumn (Büchi), was packed with LiChroprep RP-8, 15–25 μm (E. Merck, Darmstadt, F.R.G.). The mobile phase, 20 and 30% aqueous methanol (90 min after sample introduction), was delivered at a flow-rate of 18 ml/min and under a maximum pressure of 36 bar. Samples were dissolved in 3–5 ml of mobile phase and introduced into the precolumn. The secoiridoid-containing fractions were collected and lyophilized.

Low-pressure system. Prepared Lobar® columns of LiChroprep RP-8, 40–63 μm (31 cm × 25 mm I.D.), were purchased from E. Merck. The mobile phase of 20 and 30% aqueous methanol (100 min after sample introduction) was delivered by a Duramat pump (Chemie und Filter, Heidelberg, F.R.G.) at 9 ml/min and a maximum pressure of 4 bar.

RESULTS AND DISCUSSION

Fig. 1 shows the RP-HPLC separation of a secoiridoid fraction from *Gentiana lactea*. Compounds 1–4 could be separated in less than 15 min and their corresponding UV spectra recorded on-line by a photodiode array detector (LC–UV). Gentiopicrin (2) and the only secoiridoid ester detected, desacetylcentapicrin (4), are clearly distinguished from the glycosides 1, 3 and also from each other by their characteristic spectra. This example illustrates how the use of a photodiode array detector can improve the peak identification and also afford structural information about the separated compounds.

The HPLC separation of the secoiridoid fraction was repeated and an aqueous solution of 0.3 *M* potassium hydroxide added by means of a post-column derivatization system. The modified UV spectrum of compound 4 was recorded and compared with the spectrum obtained in pure eluent (Fig. 2). The bathochromic shift of the absorption maximum from 300 to 333 nm indicated a free phenolic group, which was deprotonated by the strong base. This technique of LC-UV and post-column

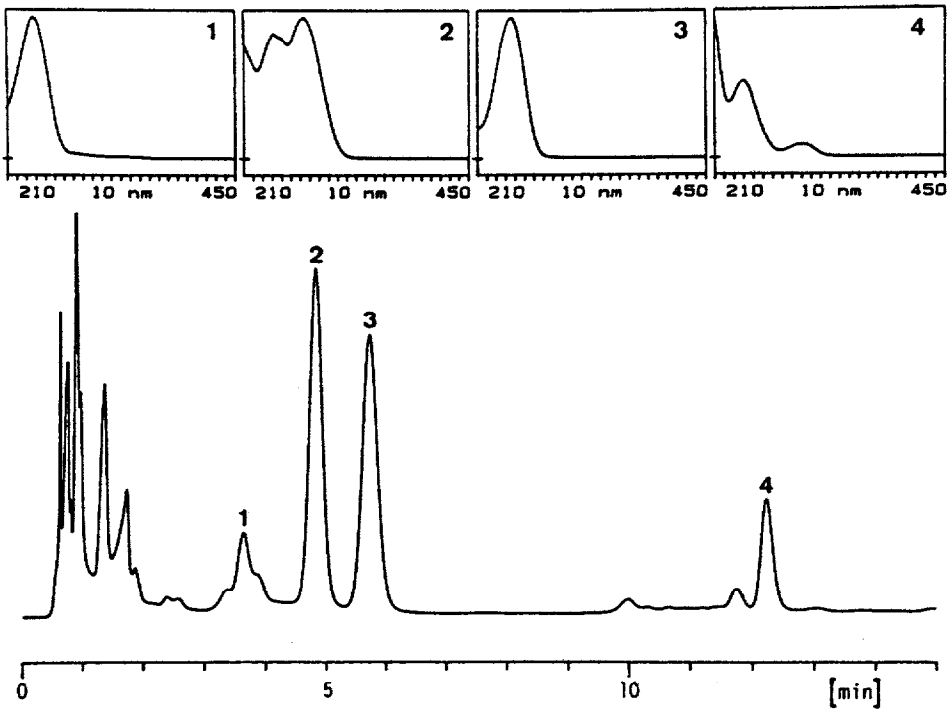

Fig. 1. RP-HPLC separation with photodiode array detection of secoiridoid glycosides from *Gentiania lactea*. Peaks: 1 = swertiamarin; 2 = gentiopicrin; 3 = sweroside; 4 = desacetylcentapicrin. Column: Hypersil RP-8, 5 μm (10 cm × 4.6 mm I.D.). Eluent: 20 and 30 % aqueous methanol; flow-rate 1.5 ml/min. Detection: 254 nm, spectra from 210 to 450 nm.

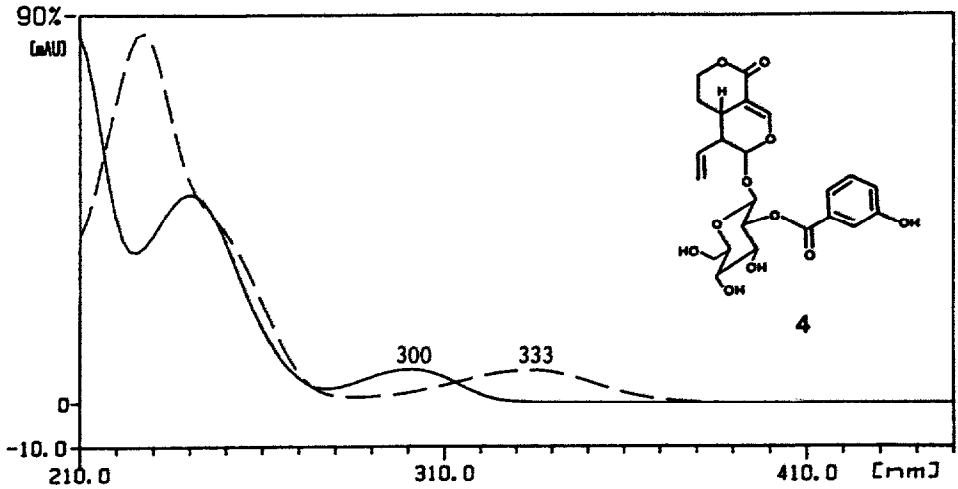

Fig. 2. HPLC of secoiridoid glycosides: UV spectra of desacetylcentapicrin 4 recorded on-line in pure eluent (aqueous methanol, ——) and with potassium hydroxide (----) added by a post-column derivatization system.

derivatization has recently been discussed for polyphenolic compounds from gentians[16]. A systematic application to secoiridoid glycosides is currently underway.

The HPLC conditions were directly transposed to preparative liquid chromatography. Separations were carried out on reversed-phase material (RP-8, 15–25 μm) with 20 and 30% aqueous methanol at a maximum pressure of 40 bar. The system of MPLC used is described in Experimental. A crude secoiridoid fraction (1.5 g) from *G. lactea* was directly separated by MPLC on the reversed phase. The chromatogram is shown in Fig. 3. The resolution of this preparative separation is surprisingly high and can be compared with that of analytical HPLC (Fig. 1). The four bitter principles, 1 (8 mg), 2 (43 mg), 3 (37 mg) and 4 (14 mg), were isolated in pure form and in one separation step of less than 3 h. The same fraction was submitted to preparative liquid chromatography using the low-pressure system Lobar® (E. Merck). No baseline separation of the two major compounds, 2 and 3, could be obtained, although relatively small amounts of the secoiridoid fraction (300 mg) had been introduced.

As a further application of MPLC, the bitter principles of a tropical Gentianaceae, *Coutoubea spicata*, were separated under the same conditions as described in Fig. 3. The major bitter constituent, swertiamarin 1 (161 mg), and gentiopicrin 2 (4 mg) could be isolated within 1 h from 500 mg of a crude secoiridoid fraction.

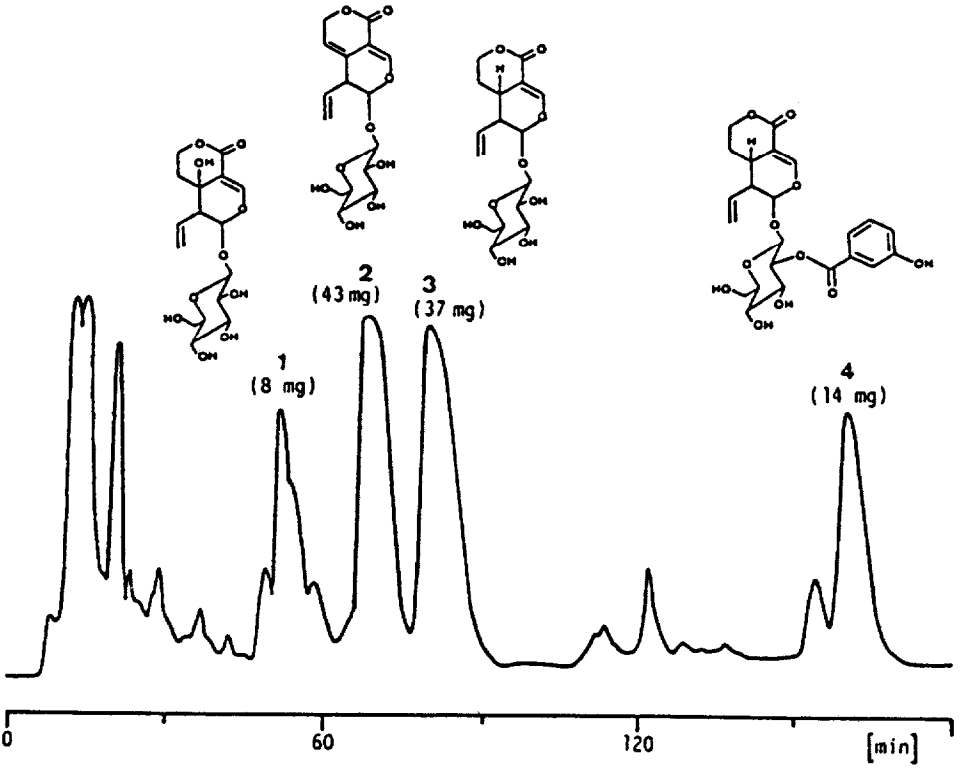

Fig. 3. Preparative MPLC of secoiridoid glycosides from *Gentiana lactea*. Column: LiChroprep RP-8, 15–25 μm (46 cm × 25 mm I.D.). Eluent: 20 and 30% aqueous methanol; flow-rate 18 ml/min. Detection: 254 nm. Sample: 1500 mg of secoiridoid fraction.

CONCLUSION

High-performance liquid chromatography, coupled with a photodiode array detector (LC–UV), is of great interest for phytochemical investigations. Constituents, including secoiridoid glycosides, can be detected and characterized in complex mixtures such as plant extracts. If the same packing material is used for preparative liquid chromatography and HPLC, the separation conditions can easily be selected by LC–UV. A prepurification of crude extracts is recommended and simplifies the regeneration of the reversed-phase material. Interfering polyphenolics, *e.g.*, flavonoids, can be removed by chromatography over polyamide.

The medium-pressure system of preparative liquid chromatography described has the advantage that mobile phases containing large proportions of water can be used with reversed-phase packing materials of small particle size. Crude and complex samples of more than 1 g can be separated in this system within 1–3 h and remarkable resolution can be obtained.

Medium-pressure liquid chromatography (MPLC) on a reversed phase is consequently a very promising technique for the isolation of secoiridoid glycosides and related natural compounds.

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