CHROM. 11,464

DROPLET COUNTER-CURRENT CHROMATOGRAPHY FOR THE PRE-PARATIVE ISOLATION OF VARIOUS GLYCOSIDES

KURT HOSTETTMANN, MARYSE HOSTETTMANN-KALDAS and KOJI NAKANISHI

Department of Chemistry, Columbia University, New York, N.Y. 10027 (U.S.A.)

(Received September 11th, 1978)

SUMMARY

Droplet counter-current chromatography has proven to be an efficient method for separating *polar* compounds in the range of a few mg to gram quantities, and in this respect is complementary to the popular high-performance liquid chromatography. This is exemplified in the following by the direct obtention of four bioactive saponins in pure form from the crude extract of *Hedera helix* berries, isolation of an extremely unstable catechin glycoside from *Dalbergia nitidula* and the separation of flavone glycosides. A rapid thin-layer chromatography method for selecting the solvent system is also described.

INTRODUCTION

Droplet counter-current chromatography (DCC) is a recently developed all-liquid separation technique based on the partitioning of solutes between a steady stream of droplets of mobile phase and a column of surrounding stationary phase. It has been initially constructed by Tanimura *et al.*[1] for the separation of 2,4-dinitrophenyl (DNP) amino acids. An application of DCC to the separation and purification of natural products has been reported by Ogihara *et al.*[2]. These authors separated various saponins as well as monosaccharides on a home-made apparatus using chloroform–methanol–water mixtures as the solvents. Otsuka *et al.*[3,4] used DCC for the estimation of ginsenosides in the roots of *Panax ginseng* and for the separation and determination of saponins of *Bupleuri radix*. Recently, we isolated two molluscicidal saponins from the bark of *Cornus florida* (Cornaceae) using an apparatus which has recently become commercially available[5].

DCC seems to be an ideal method for the separation of polar compounds since there is no solid packing material, and thus is devoid of any irreversible adsorption. We had previously reported the direct isolation of less polar compounds from the crude hexane extract of plants by preparative liquid chromatography using a two-step solvent elution process[6]. In contrast, in the following, we wish to describe the isolation of the polar constituents, *e.g.*, glycosides, by DCC; these glycosides could be separated far more readily than by conventional liquid chromatography methods. An application of DCC to separation of naturally occurring glycosides other than saponins is also

presented. Some advantages and the potential use of DCC in natural products isolation are discussed.

EXPERIMENTAL

Choice of the solvents

DCC is carried out by passing droplets of a mobile phase through a column of stationary phase. The mobile phase may be either heavier or lighter than the stationary phase. When heavier, the mobile phase is delivered at the top of the column (descending mode), and, when lighter, through the bottom (ascending mode). When a droplet reaches the top (or bottom) of the column, it is delivered to the bottom (or top) of the next column through narrow-bore PTFE tubings, which under suitable conditions only allow the mobile phase to flow[1]. Solvent systems that form two immiscible layers are usually suitable for DCC. However, there are necessarily some limitations to the solvent systems. For example, the formation of droplets having suitable sizes and mobility are governed by factors such as the difference in specific gravities of the two liquid phases, the viscosity of solvents, the flow-rate of the mobile phase, the diameter of the column and other unknown factors. The solvent system of methanol–hexane–water which is frequently used in the Craig counter-current distribution[7] produces flow plugs which results in the displacement of the stationary phase by the mobile phase. Another unsuitable solvent system was n-butanol–pyridine–water which gave no droplets due to its high viscosity. A list of solvent systems suitable for DCC has been published by Shibata and co-workers[2,3]. Most of them are tertiary or quaternary systems, one of the components being water.

A quick way to select a solvent system consists in checking the sample by thin-layer chromatography (TLC) on silica or cellulose using the organic layer as eluent. Empirically, we find that if the R_F values of the compounds to be separated are higher than 0.40, i.e., less polar solutes, the less polar phase is suited for usage as the mobile phase. In case of more polar substrates ($R_F < 0.40$), the more polar phase should be used as the mobile phase. When the sample consists of many compounds with a wide range of polarity, e.g., crude plant extracts, the separation can be achieved in two steps: a first run with the less polar phase as mobile phase, and after recovery of the stationary phase, a second run with the more polar phase as mobile phase. However, since DCC depends solely on the partition coefficient, separation cannot always be preducted from the TLC behaviour.

Apparatus

All separations were carried out on a Model DCC-A apparatus (Tokyo Rikakikai, Tokyo, Japan) equipped with 300 standard glass tubes (40 cm × 2.0 mm I.D.); the number of tubes can be increased up to about 600. The samples were dissolved in a 1:1 mixture of both mobile and stationary phases and injected into the apparatus using a 5-ml or a 10-ml sample chamber. The flow-rate was 10–15 ml/h, depending on the solvent system, and the elutants were collected in 1–4-ml fractions. The monitoring of respective fractions is carried out by TLC or silica-precoated aluminium sheets (E. Merck, Darmstadt, G.F.R.) with the general solvent system chloroform–methanol–water (65:35:10) (lower layer) or with n-butanol–acetic acid–water (4:1:5) (upper layer); the compounds are detected with ceric sulfate in sulfuric acid.

In the case of the separation of flavone glycosides, the fractions were monitored by UV at 330 nm.

RESULTS AND DISCUSSION

Direct obtention of pure triterpenoid glycosides from a crude extract of Hedera helix berries

During the course of our systematic isolation and structural studies on biologically active compounds from medicinal plants[8,9], we found that the crude ethanol extract of the berries of the common ivy, *Hedera helix* (Araliaceae), possessed strong molluscicidal[5,10] and antifungal activities. The fresh berries (150 g) were extracted with 70% ethanol. After concentration, this extract was partioned between *n*-butanol. The *n*-butanol layer exhibiting the biological activity was submitted to DCC using chloroform–methanol–water (7:31:8) as solvent (mobile phase: the less polar lower layer) and TLC (see above) for monitoring. This gave six fractions as shown in Figs. 1 and 2. The crude extract (1.2 g) afforded four pure compounds: 2 (89 mg), 3 (6 mg), 4 (17 mg) and 5 (q15 mg) within 20 h in order of increasing polarity. Fraction 1 which was eluted with the solvent front is still a mixture of the least polar components (*i.e.*, fatty acids, chlorophylls, etc.) of the crude extract. The main fraction obtained (252 mg, unshaded area in Fig. 1) consisted of a mixture of compounds 3 and 4. This fraction was recycled to afford an additional 15 mg of 3 and 34 mg of 4; no baseline separation could be achieved with these two compounds (although they could be separated on TLC, Fig. 2). After elution of compound 5, about 670 mg of the most polar components of the extract were recovered from the stationary phase of the apparatus (Fig. 2). Since this fraction was inactive, it was not further examined. It should be noted that all of the biologically active constituents were eluted with less than a total of 300 ml of solvent (Fig. 1).

Acid hydrolysis of the pure compounds 2–5 afforded the same aglycone
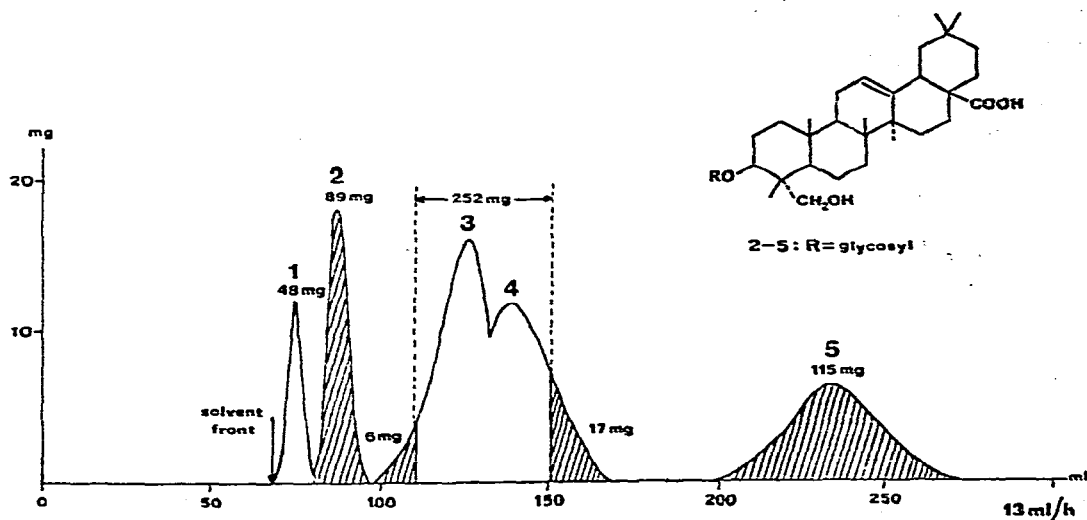

Fig. 1. DCC of a crude extract of *Hedera helix* berries (1.2 g) with chloroform–methanol–water (7:13:8); mobile phase, lower layer.
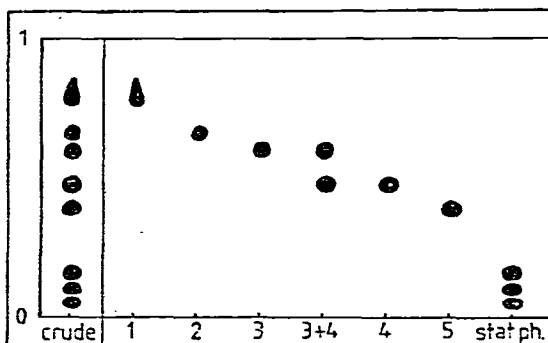
Fig. 2. TLC analysis of various fractions obtained from DCC separation of a crude extract of *Hedera helix* berries (see Fig. 1) on silica with chloroform–methanol–water (65:35:10), lower layer; detection with ceric sulfate in sulfuric acid.

(M^+ = 472, $C_{30}H_{48}O_4$) identified as hederagenin[11] by ^{1}H nuclear magnetic resonance (NMR) and ^{13}C NMR. The structure determination of the triterpenoid saponins is currently in progress and will be published along with the results of the biological tests[12].

Isolation of a catechin glycoside from Dalbergia nitidula (Leguminosae)

The dried bark (180 g) of this African medicinal plant was extracted with solvents of increasing polarity (hexane, diethyl ether, methanol). The methanol extract showed antifeeding[8,9] activity against the Mexican bean beetle *Epilachna varivestis* and was separated into ten fractions by Sephadex LH-20 column chromatography using methanol as solvent. The biologically active fraction showed several spots on silica gel TLC with chloroform–methanol–water (7:13:8). Since the R_F values were relatively low, we decided to submit this fraction (700 mg) to DCC using the more polar upper layer as the mobile phase. The results are summarized in Fig. 3 which shows a TLC analysis of the five different fractions obtained. Very polar and non-active constituents were eluted first and grouped into fractions 1–3. Fraction 4 (50 mg) shows one single spot on TLC and was responsible for the bioactivity. However, this compound (white powder) is extremely unstable and decomposes immediately to more polar brown degradation products upon removal of solvent. Acid hydrolysis
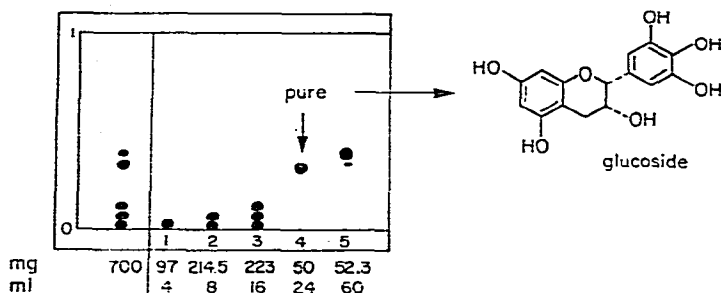

Fig. 3. TLC analysis of fractions collected from DCC separation of an extract of *Dalbergia nitidula*. Same conditions as in Fig. 2. The "mg" and "ml" denote respectively the quantities of elutants in each fraction and the volume of mobile phase used for eluting each fraction. Note that 60 ml of the mobile phase elutes a total of *ca.* 640 mg of solute.

afforded glycose. Upon acetylation, a stable hexaacetate ($M^+ = 558$, $C_{27}H_{26}O_3$) of the aglycone was obtained, the mass spectral (MS) and ^1H NMR data of which established it to be hexaacetyl-(—)-epigallocatechin[13].

Separation of flavone glycosides

A large variety of flavone glycosides have been encountered in nature[14] and numerous papers dealing with their separation have been published. For a preparative scale isolation, polyamide or cellulose column chromatography is generally used[15]. However, this technique is often very tedious, not very reproducible and time-consuming, especially when solvent gradients have to be employed (which is frequently the case). Also, in some cases, the polyphenolic compounds remain adsorbed onto the packing material. Recently, high-performance liquid chromatography (HPLC) has been used for the analytical separation of some glycosides[16,17], but since chemically bonded phases are required, this technique is not yet in common practice for the preparative scale isolation.

The solvent system n-butanol–acetic acid–water (4:1:5) proved to be suited for the DCC separation of very polar glycosides using either the lower or the upper layer as the mobile phase. For the separation of isoorientine-3'-O-glucoside (1), rutine (2) and isoorientine (3), we used the aqueous layer (more polar) as mobile phase in the descending mode as illustrated in Fig. 4. Injection of 90 mg of the artificial mixture gave quantitative recovery of pure compounds. As expected, usage of the more polar phase as mobile resulted in earlier elution of disaccharides 1 and 2 than the monosaccharide 3. For less polar flavones (e.g., monosaccharides, aglycones possessing several free hydroxyl groups), the solvent system chloroform–methanol–water (7:13:8) gave good and rapid separations. For example, from a crude fraction (130 mg) of Tecoma stans (Bignoniaceae), a Mexican medicinal plant, we were able to isolate within 6 h, 27 mg of a pure glycoside identified as isoquercitrine[18] (quercetin-3-O-glucoside) using the upper layer as mobile phase.
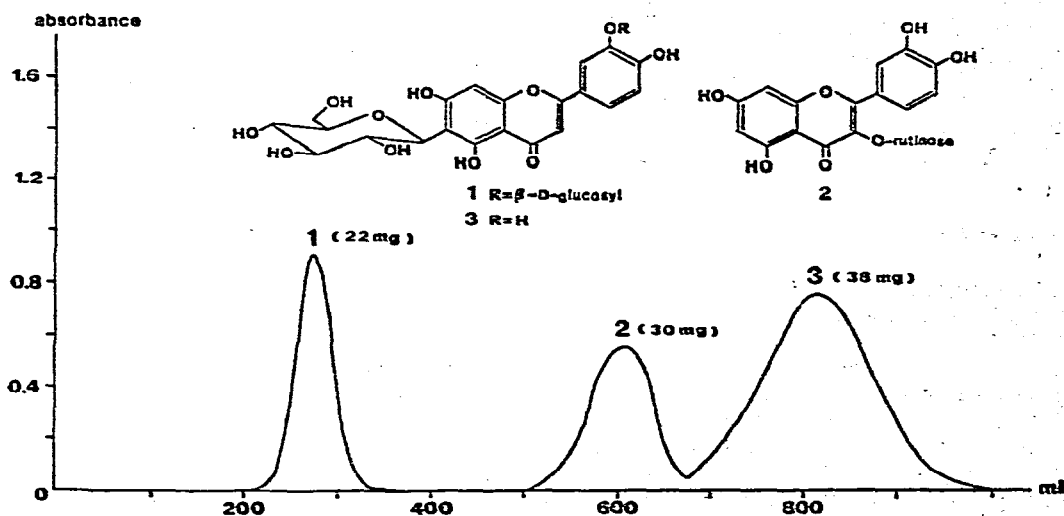

Fig. 4. Separation of flavonoid glycosides by DCC with n-butanol–acetic acid–water (4:1:5); mobile phase, lower layer. Detection by UV at 330 nm.

CONCLUSION

Although DCC has as yet not been used as extensively as HPLC, it has proven to be a very suitable and reproducible technique for the preparative separation of polar natural products, in particular glycosides. Quantities ranging from about 1 mg up to 2 g can easily be handled.

Since there is no solid support which might cause irreversible adsorption, the sample is recovered quantitatively. This is of particular interest when isolating biologically active compounds because the activity is frequently lost during the time-consuming column chromatography process. In contrast to the conventional counter-current distribution (CCD) method, no shaking is involved and hence there is no formation of foams; furthermore, since there is no space for atmospheric oxygen in the apparatus, aerial oxidation or sensitive compounds can be avoided. However, some of the solvent systems suited for use in CCD are not applicable to DCC as mentioned above (see Experimental).

Since a given solvent system can be used either in the ascending or in the descending mode, compounds with a large range of polarity can be separated. For example, the system chloroform–methanol–water (7:13:8) is very suitable for the less polar saponins when the less polar layer is ysed as mobile phase (Fig. 1); on the other hand, it is also suited for the separation of polar polyphenolic glycosides when the more polar layer is employed as mobile phase (Fig. 3). Preliminary experiments have shown that DCC is also applicable to the separation of other classes of glycosides, such as iridoid glycosides.

ACKNOWLEDGEMENT

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