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APPLICATION OF DROPLET COUNTER-CURRENT CHROMATOGRAPHY TO THE ISOLATION OF NATURAL PRODUCTS

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SUMMARY

Droplet counter-current chromatography (DCC), a recently developed all-liquid separation technique, has been applied to the preparative separation of various classes of natural products. From a crude extract of *Ajuga pyramidalis* (Labiatae), several hundred milligrams of pure iridoid glycosides could be obtained within 8 h. The volume of mobile phase used for the elution was less than 150 ml. Another example dealing with iridoid glycosides is given by the isolation of several constituents of a *Castilleja* species (Scrophulariaceae). DCC has also been used to separate xanthone-O-glycosides from a North-American *Gentiana* species (Gentianaceae) and the main alkaloids of an opium extract. Thin-layer chromatography (TLC) was found to be an ideal and quick method for the selection of solvent systems.

INTRODUCTION

Among the various availabie separation techniques for natural products, droplet counter-current chromatography (DCC) is becoming increasingly popular. DCC is a form of partition chromatography which is carried out by passing droplets of a mobile phase through a column of surrounding stationary phase[1]. Initially developed by Tanimura *et al.*[1], it has been used by Ogihara *et al.*[2] for the separation and purification of saponins and sugars. Recently, it has been employed extensively for the determination and separation of ginsenosides in the roots and leaves of different *Panax* species[3–7] (Araliaceae). DCC has also been applied to the separation of saikosaponins of the roots of *Bupleurum falcatum* L. (Umbelliferae) by Otsuka *et al.*[8]. In connection with systematic isolation and structural studies on biologically active compounds from medicinal plants, preparative separations of molluscicidal saponins were carried out by DCC. Thus, two sarsapogenin glycosides from *Cornus florida* L. (Cornaceae)[9] and four hederagenin glycosides from a crude extract of fresh common ivy berries (*Hedera helix* L.) have been isolated. DCC has proved to be an ideal method for the separation of various types of saponins. In a recent paper, we demonstrated that DCC is also suitable for the separation of phenolic compounds such as catechins and flavone glycosides[10].

This paper is an extension of the previous study[10] and deals with the prepara-

tive separation of other classes of natural products such as xanthone-O-glycosides, iridoid glycosides and alkaloids. We also propose a quick and simple method based on thin-layer chromatography (TLC) for selecting the solvent system.

EXPERIMENTAL

All separations were carried out on a Model A DCC apparatus (Tokyo Rikakikai, Tokyo, Japan). The apparatus consists of a number of glass tubes (length 400 mm, I.D. 2 mm) interconnected in series by capillary PTFE tubes. In the present studies, 300 tubes were used. The samples were dissolved in a mixture of both mobile and stationary phases and injected into the apparatus using a 5- or 10-ml sample chamber. The flow-rate was 10–15 ml/h, depending on the solvent system, and the eluates were collected in 1–3-ml fractions. The fractions were monitored by TLC on pre-coated silica gel plates (E. Merck, Darmstadt, G.F.R.). The solvent systems were a lower layer of chloroform–methanol–*n*-propanol–water (9:12:1:8), chloroform–methanol–water (43:37:20) or chloroform–methanol–water (13:7:8); the compounds were detected with cerium(IV) sulphate in sulphuric acid or with Godin reagent[11]. In the separation of xanthone glycosides, the fractions were monitored by UV spectroscopy at 300 nm.

RESULTS

Choice of solvent system and separation of xanthone-O-glycosides

Solvent systems that form two immiscible layers are generally suitable for DCC. However, as the droplet formation depends on various factors, there are some limitations[10]. A list of solvent systems suitable for DCC has been published by Ogihara *et al.*[2]. We obtained the best results by using chloroform–methanol–water or chloroform–methanol–*n*-propanol–water mixtures in different proportions. Modification of the amount of methanol in a given mixture leads to a great change in the polarity of the system. A quick way of selecting a solvent system consists in checking the sample by TLC on silica gel using the water-saturated organic layer as eluent[10]. Empirically, we found that if the R_F values of the compounds to be separated are higher than about 0.40 (less polar solutes), the less polar phase is suitable for use as the mobile phase. With more polar substrates ($R_F < 0.40$), the more polar phase should be used as the mobile phase. If the R_F values are in the range 0.4–0.6, the separation can be achieved by using either the more polar layer or the less polar layer as the mobile phase.

This is illustrated by the separation of a fraction (60 mg) of a crude extract of a Rocky Mountains *Gentiana* species (*Gentiana strictiflora* (Rydb.) (A. Nels) obtained after column chromatography on Sephadex LH-20. On silica gel TLC with the solvent system chloroform–methanol–*n*-propanol–water (9:12:1:8) (lower layer), this fraction showed two UV-active spots at $R_F = 0.35$ and 0.41. DCC separation by using the more polar upper layer as the mobile phase yielded two pure compounds, 1 (32 mg) and 2 (26 mg), identified as 1,3,5-trihydroxy-xanthone-8-O-β-D-glucoside and 1,5-dihydroxy-3-methoxyxanthone-8-O-β-D-glucoside, respectively, by comparison with authentic samples[12] (see Fig. 1, top elution curve).

It is noteworthy that both glycosides were separated with a total volume of

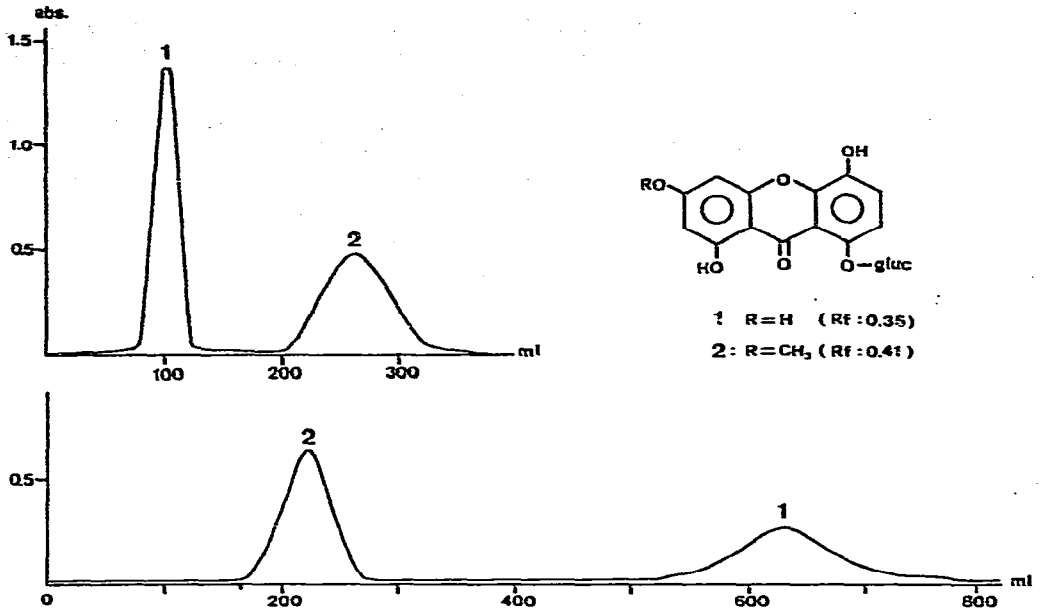

Fig. 1. DCC separation of xanthone-O-glycosides with chloroform–methanol–*n*-propanol–water (9:12:1:8). Top elution curve: the mobile phase was the more polar upper layer. Bottom elution curve: the mobile phase was the less polar lower layer. Detection: UV at 300 nm.

about 350 ml of mobile phase. As expected, the use of the more polar phase as the mobile phase resulted in earlier elution of the more polar compound 1. For comparison purposes, the same mixture of 1 and 2 was submitted to DCC and eluted with the less polar lower layer. The total volume of mobile phase used for eluting both compounds was 800 ml (see Fig. 1, bottom elution curve); however, the resolution was better than in the previous run. As expected, the less polar glycoside 2 was eluted first.

These comparative experiments suggest that in difficult separations, it could be advantageous to use the less polar layer as the mobile phase for eluting compounds with relatively low R_F values. In a similar manner, the more polar layer can be used for eluting compounds with relatively high R_F values. A better resolution will be obtained, but the separation time will be much longer. However, if the R_F values of the compounds to be separated are too low (< 0.3–0.2) or too high (>0.8–0.7), no elution will take place in a reasonable time, but the compounds can be recovered from the remaining stationary phase.

Isolation of iridoid glycosides

The various methods for the isolation of iridoid glycosides have recently been reviewed[13,14]. Among them, preparative liquid chromatography on C_{18} chemically bonded silica gel used with different methanol–water mixtures proved to be very efficient[14,15]. The resolution is high and the entire separation can be achieved in less than 1 h. However, owing to the complexity of a crude plant extract, cleaning of the sample prior to injection is necessary in order to avoid column contamination. As

there is no solid packing material in DCC that might cause irreversible adsorption, the sample can be recovered quantitatively. Major plant constituents can be obtained directly in a pure form from crude plant extracts.

In connection with our systematic isolation and structural studies on iridoid glycosides from various medicinal plants, we undertook the investigation of *Ajuga pyramidalis* L. (Labiatae). The dried aerial parts of the plant (120 g) were extracted successively with chloroform and methanol. After concentration, the methanolic extract was partitioned between *n*-butanol and water. The *n*-butanol extract (1.4 g) was submitted to DCC using the upper layer (more polar) of the system chloroform–methanol–water (43:37:20) as the mobile phase and the fractions were monitored by TLC. This gave three major fractions as shown in Fig. 2.

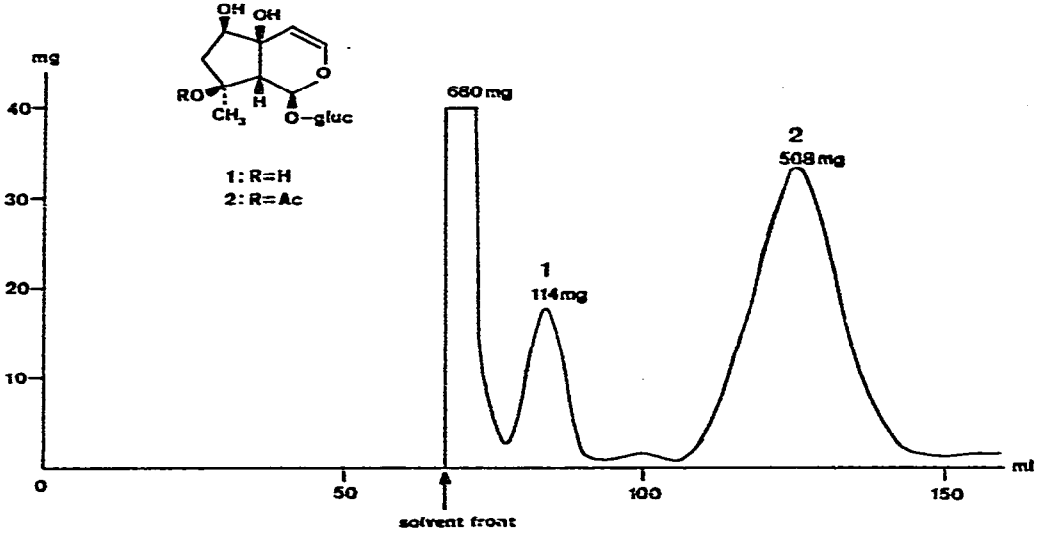

Fig. 2. DCC of a crude extract of the aerial parts of *Ajuga pyramidalis* L. (1.4 g) with chloroform–methanol–water (43:37:20). Ascending mode; mobile phase, upper layer (more polar).

The fraction eluted with the solvent front (680 mg) was a mixture of the most polar constituents, whereas peaks 1 (114 mg) and 2 (508 mg) showed single spots on TLC. The pure compounds 1 and 2 have been identified, by means of UV and ^{13}C NMR spectroscopy and by comparison with authentic samples, to be harpagide and 8-O-acetylharpagide[16], respectively. The entire isolation process required only about 8 h and the total amount of solvent used for the elution was less than 150 ml. From the remaining stationary phase, 94 mg of a mixture of minor constituents could be recovered.

The solvent system used in the above experiment is suitable for the separation of other types of iridoid glycosides. From a *Castilleja* species (Scrophulariaceae) collected in Colorado, we obtained after column chromatography on Sephadex LH-20 a fraction containing minor iridoid constituents. This fraction, when submitted to DCC, gave four nearly pure glycosides. Final purification was achieved by semi-preparative high-performance liquid chromatography on a C_{18} chemically bonded

silica gel with methanol–water (3:7). Spectroscopic studies on the pure compounds indicate that they are closely related to loganin[16] and dihydrocornin[17], some of them being isomers. The structure determination is currently under investigation and will be published shortly.

Separation of the main alkaloids of Papaver somniferum L. (Papaveraceae)

In order to establish if DCC can be apllied to the separation of alkaloids, we undertook a TLC examination of an opium extract with appropiate solvent systems (see above). The lower layer of the system chloroform–methanol–water (13:7:8) gave a clear separation of the main constituents, namely (in order of increasing R_F) morphine, narceine, codeine, thebaine, papaverine and narcotine (see Fig. 3).

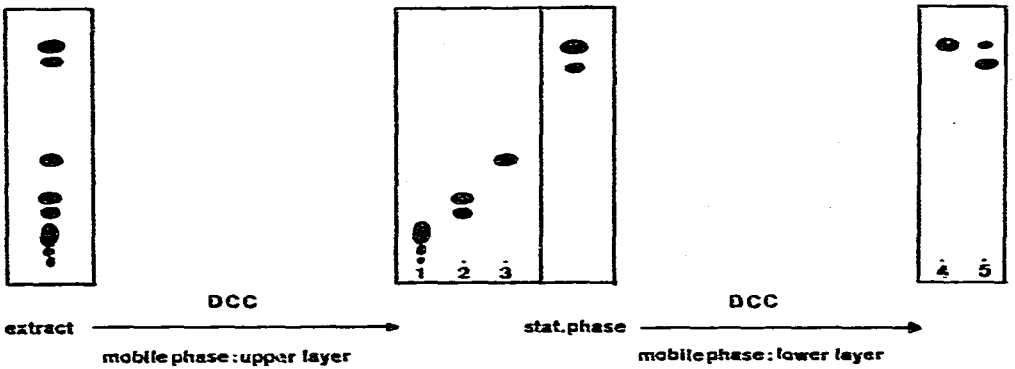

Fig. 3. TLC analysis of fractions collected from DCC separation of an opium extract (*Papaver somniferum* L.) on silica with chloroform–methanol–water (13:7:8) (lower layer). 1 = Crude morphine; 2 = mixture of narceine and codeine; 3 = thebaine; 4 = narcotine; 5 = papaverine with traces of narcotine.

As the extract consisted of components with a wide range of polarity, the separation had to be achieved in two steps. The crude extract was submitted to DCC and a first run with the more polar upper layer as the mobile phase afforded fractions 1–3, consisting of crude morphine, a mixture of narceine and codeine, and pure thebaine, respectively. After elution of fraction 3, the remaining stationary phase, consisting of the less polar benzylisoquinoline alkaloids was recovered. A second run with the less polar lower layer as the mobile phase resulted in the separation of two fractions, 4 (narcotine) and 5 (papaverine with traces of narcotine). The total volume of mobile phase used in the first run was 92 ml, whereas 77 ml of mobile phase were necessary for the elution of fractions 4 and 5. Although not all opium constituents have been separated in this preliminary experiment, it indicates that DCC could be used for further alkaloid isolations.

CONCLUSION

After being applied successfully to the separation and purification of various saponins, DCC has proved to be a suitable method for the isolation of xanthone-O-glycosides, iridoid glycosides and alkaloids. In the future, DCC will certainly be

applied to many other natural products. The method is simple and reproducible and the consumption of solvent is very small. Sample amounts from several milligrams up to grams can easily be handled. There is no irreversible adsorption and no cleaning of the sample prior to injection is necessary. However, as water is one of the components of the solvent system, separations are restricted to polar compounds. We are currently attempting to extend the application of DCC to non-polar and water-sensitive substances.

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