214. Preparative Scale Separation of Xanthones and Iridoid Glycosides by Droplet Counter-Current Chromatography[1])

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Summary

Droplet counter-current chromatography (DCCC.) has been used for the preparative scale separation of pure constituents from crude medicinal plant extracts. Xanthones and the bitter principles have been isolated from North-American *Gentiana* species. From a crude extract of *Ajuga pyramidalis* L. *(Labiatae),* several hundred mg of iridoid glycosides were obtained within eight hours. The total volume of mobile phase employed for the elution was less than 150 ml. All the separations were achieved far more readily than by conventional chromatography. In addition, some general rules for solvent system selection are proposed.

Introduction. – Droplet counter-current chromatography (DCCC.) is a recently developed form of partition chromatography and is carried out by passing droplets of a mobile phase through a column of surrounding stationary phase [1]. It is a modification of the well-known counter-current distribution technique [2], but is less cumbersome and has a higher resolution power. As no formation of emulsion takes place, DCCC. has been extensively used for the purification and separation of various types of saponins [3–6], including ginsenosides [7]. It has also been successfully applied to the separation of phenolic compounds, namely catechin and flavone glycosides [6]. Buffer solutions can be used as the mobile phase; thus DCCC. is an ideal method for the isolation of alkaloids. A systematic study on the separation of the alkaloids of Papaveraceous plants has recently been published by *Tani et al.* [8], who employed pH gradients for the isolation of numerous tertiary and quaternary bases from *Corydalis* species. Water being a component of the solvent systems suitable for DCCC., this technique has been applied mainly to polar compounds. However, *Hayashi et al.* [9] separated non polar diterpene dilactones with the lower layer of a $CHCl_3$/MeOH/H_2O mixture as mobile phase.

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In the present study, constituents of medicinal plants have been obtained directly in pure form by DCCC. from crude extracts, without purification of the sample prior to injection. Xanthone aglycones and their corresponding glycosides, as well as the bitter principles have been isolated from North-American *Gentiana* species *(Gentianaceae)*. A crude extract of *Ajuga pyramidalis* L. *(Labiatae)* afforded two glycosides identified as harpagide and 8-*O*-acetylharpagide [10] within 8 h. In addition, a quick and simple method, based on thin-layer chromatography (TLC.), is proposed for selection of the solvent system.

Results. - *Choice of solvent system and separation of xanthone-O-glycosides.* Solvent systems that form two immiscible layers are generally suitable for DCCC. However, as the droplet formation depends on various factors, there are some limitations [6]. We obtained the best results by using $CHCl_3/MeOH/H_2O$ or $CHCl_3/MeOH/PrOH/H_2O$ mixtures in different proportions. Modification of the amount of MeOH in a given mixture leads to a large 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 with the water-saturated organic layer [6]. Empirically, it was 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 layer is suitable for use as the mobile phase. With more polar substrates ($R_f < 0.40$), the more polar layer should be used as the mobile phase. If the R_f values are in the range 0.40-0.60, the separation can be achieved by using either the more polar layer or the less polar layer as the mobile phase.

This is illustrated in the following by the separation of a fraction (60 mg) of a crude extract of a Rocky Mountains *Gentiana* species (*Gentiana strictiflora* (Rydb.)

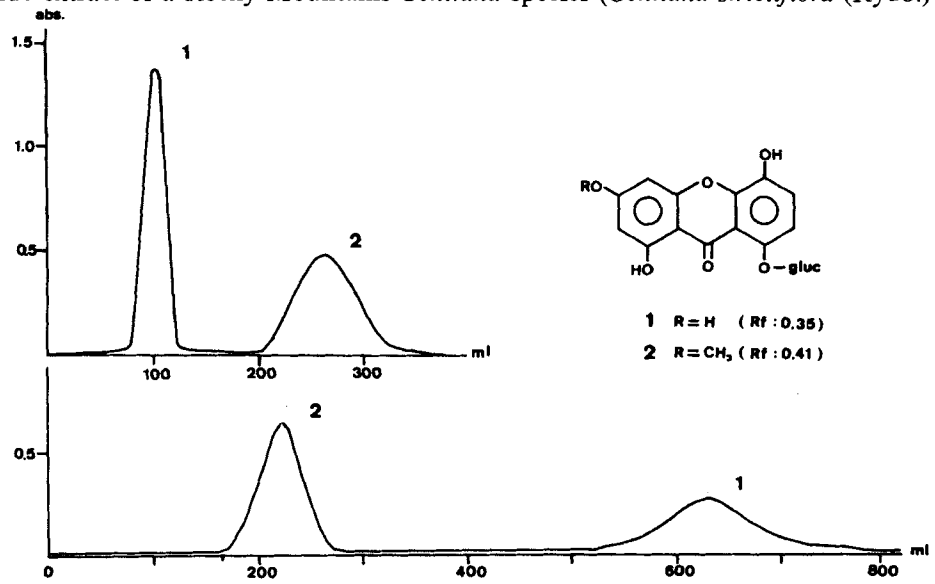

Fig. 1. DCCC. separation of xanthone-O-glycosides with $CHCl_3/MeOH/PrOH/H_2O$ 45:60:5:40. Top elution curve: the mobile phase was the more polar upper layer. Bottom elution curve: the mobile phase was the less polar lower layer.

A. NELS) obtained after column chromatography on *Sephadex* LH-20. On silica gel TLC. with $CHCl_3$/MeOH/PrOH/H_2O 45:60:5:40, lower layer, this fraction showed two UV. active spots at R_f=0.35 and 0.41. DCCC. separation by using the more polar upper layer as mobile phase yielded two pure compounds **1** (32 mg) and **2** (26 mg), identified as 1,3,5-trihydroxyxanthone-8-*O*-*β*-D-glucoside and as 1,5-dihydroxy-3-methoxyxanthone-8-*O*-*β*-D-glucoside by comparison with authentic samples [11] (see *Fig. 1*, top elution curve). Both glycosides were separated with a total volume of about 350 ml of mobile phase. As expected, the use of the more polar layer as the mobile phase resulted in earlier elution of the more polar compound **1**. For comparison, the same mixture of **1** and **2** was submitted to DCCC. 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 the case of difficult separations, it could be better to use the *less polar* layer as mobile phase for eluting compounds with relatively low R_f values, and the *more polar* layer 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 either too low (<0.3–0.2), or too high (>0.8–0.7), no elution will take place in a reasonable time, although the compounds can be recovered quantitatively from the remaining stationary phase.

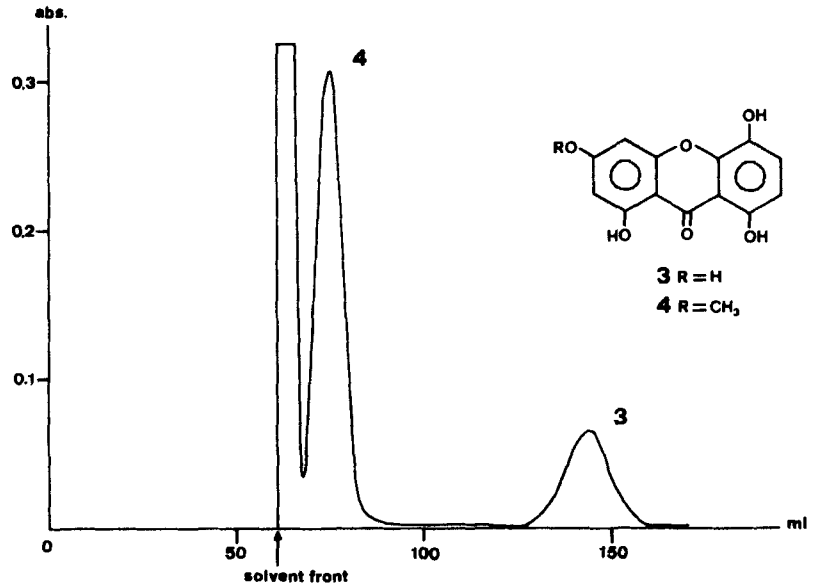

Fig. 2. *DCCC. of a crude $CHCl_3$ extract of* Gentiana strictiflora *with $CHCl_3$/MeOH/H_2O 65:35:20.* Descending mode: mobile phase, lower layer.

Direct separation of xanthone aglycones from a crude chloroform extract of Gentiana strictiflora. Xanthones are characteristic constituents of plants such as *Guttiferae* and *Gentianaceae* which are used in folk medicine [12]. There is at present great interest in these compounds since *Suzuki et al.* [13] demonstrated that xanthones isolated from *Gentiana* inhibit rat brain mitochondrial monoamine oxydase (MAO) *in vitro*. As no American *Gentiana* species have been studied until now, we examined several Rocky Mountains gentians in order to identify their xanthones. The crude $CHCl_3$ extract (200 mg) of *Gentiana strictiflora* (Rydb.) A. NELS was submitted to DCCC. with $CHCl_3/MeOH/H_2O$ 65:35:20, mobile phase: lower layer. Three fractions were obtained *(Fig. 2)*. The less polar constituents (colour pigments, fatty acids, triterpenes) were not separated under the chosen conditions and were eluted with the solvent front, whereas fraction 1 (29 mg) and 2 (10 mg) showed single spots on TLC. Spectroscopic studies of the pure compounds led to the following structures: **3**, desmethylbellidifolin (1,3,5,8-tetrahydroxyxanthone) and **4**, bellidifolin (1,5-dihydroxy-3-methoxyxanthone) [11]. These xanthone aglycones were separated far more readily than by column chromatography and with a smaller solvent consumption (the total amount of mobile phase used being 160 ml).

Isolation of the bitter principles of Gentiana algida. Besides xanthones, all *Gentiana* species investigated up to now contain bitter principles of the secoiridoid type [14]. The isolation of these glycosides has been achieved by silica gel or aluminium oxide column chromatography, with time-consuming solvent gradients. Recently, the procedure has been improved by *Meier* [15] who used preparative high-performance liquid chromatography (HPLC.) on chemically-bonded silica with $MeOH/H_2O$ mixtures. Preparative HPLC. is very fast, but requires tedious purifications of the sample prior to injection in order to avoid column contamination. In the present study, DCCC. has been applied to the isolation of the bitter principles of *Gentiana algida* PALL. (arctic gentian). A crude fraction (1.2 g) obtained

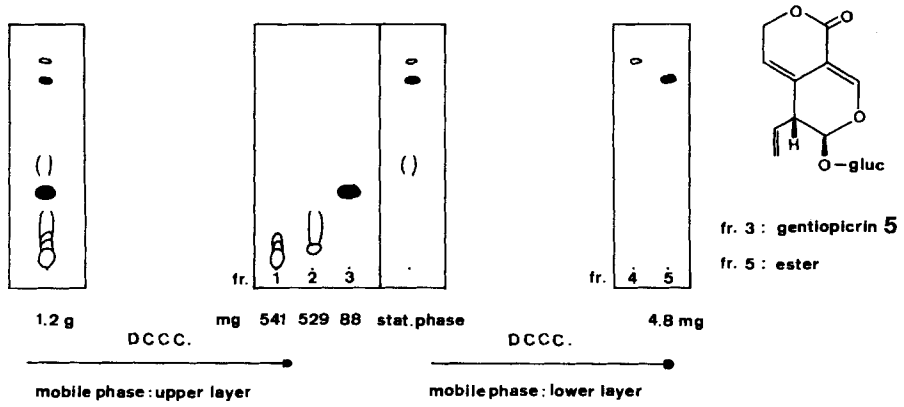

Fig. 3. *TLC. analysis of fractions collected from DCCC. separation of the bitter principles of* Gentiana algida *on silica with $CHCl_3/MeOH/PrOH/H_2O$ 45:60:5:40 (lower layer).*

after column chromatography on *Sephadex* LH-20 was submitted to DCCC. with the solvent system $CHCl_3$/MeOH/PrOH/H_2O 45:60:5:40. As the mixture consisted of components with a wide range of polarity, the separation had to be achieved in two steps. A first run with the more polar upper layer as the mobile phase afforded fractions 1–3 *(Fig. 3)*. Fractions 1 and 2 contained the most polar constituents (polysaccharides, free sugars) whereas fraction 3 (88 mg) showed a single component (TLC., HPLC.) identified as gentiopicrin **5** [16]. After elution of this fraction, the remaining stationary phase, formed of the less polar constituents, was recovered. A second run with the less polar lower layer as the mobile phase resulted in the separation of fraction 4 (traces) and 5 (4,8 mg). Spectroscopic studies indicate that fraction 5 consists of a phenolic secoiridoid glycoside with the ester attached to the sugar moiety. The UV. spectrum (λ_{max} (MeOH): 210, 245, 316 nm) is similar to that of amaropanin [17], but HPLC. showed the isolated glycoside to be different from all the known *Gentiana* bitter principles. The total volume of mobile phase used in the first run was 254 ml, whereas 125 ml were necessary for the elution of fraction 5. The solvent consumption for separation by preparative HPLC. on the same scale was much higher.

Isolation of iridoid glycosides from Ajuga pyramidalis. 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 $CHCl_3$ and MeOH. After concentration the methanolic extract was partitioned between butanol and water. The butanol extract (1.4 g) was submitted to DCCC. using the upper layer (more polar) of the system $CHCl_3$/MeOH/H_2O 43:37:20 as mobile phase and the fractions were monitored by TLC. This gave three major fractions *(Fig. 4)*. The fraction eluted with the solvent front (680 mg) was a mixture of the

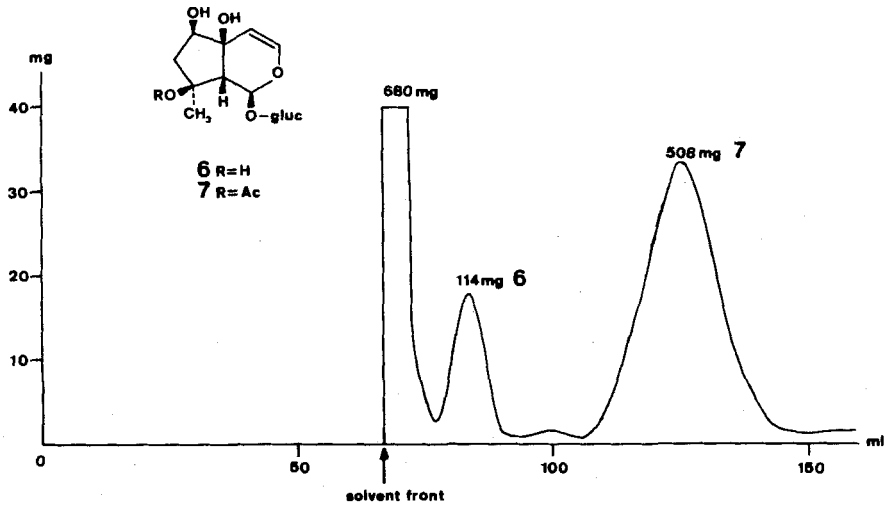

Fig. 4. *DCCC. of a crude extract of* Ajuga pyramidalis *(1.4 g) with $CHCl_3$/MeOH/H_2O 43:37:20* (Ascending mode: mobile phase, upper layer).

most polar constituents, whereas fractions 1 (114 mg) and 2 (508 mg) showed single spots on TLC. The pure compounds were identified by means of spectroscopic studies (UV., ^{13}C-NMR.) and by comparison with authentic samples as harpagide **6** and 8-*O*-acetylharpagide **7** [10], 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. Thus, DCCC. is complementary to the various methods for isolation of iridoids which have recently been reviewed [18].

Discussion. – Droplet counter-current chromatography has proved to be a very efficient and reproducible method for the isolation of various natural products. Xanthone glycosides and the related aglycones, as well as secoiridoid and iridoid glycosides could be separated far more readily than by conventional chromatography. Major plant constituents can be obtained directly in a pure form from crude extracts. Furthermore, the solvent consumption is smaller than with other chromatographic methods (open column chromatography, preparative HPLC.). There is no doubt that in the near future, DCCC. will become increasingly popular and be applied in many other fields of natural products, as well as in biochemistry. The method is particularly indicated for polar compounds since there is no solid packing material where irreversible adsorption can occur. However, there are some restrictions owing to the fact that all the solvent systems employed so far contain water. We are currently attempting to extend the application of DCCC. to water-sensitive substances.

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Experimental Part

Gentiana strictiflora (Rydb.) A. NELS (35 g dry material) was obtained near Albany, Wyoming, USA. *Gentiana algida* PALL. was collected at Mount Evans, Colorado, USA at an altitude of 13,500 ft. (14 g dried leaves) and *Ajuga pyramidalis* L. was collected near Haute-Nendaz, Valais, Switzerland (50 g dry material). The aerial parts of the plants were extracted successively with cold petroleum ether, $CHCl_3$ and MeOH. *Sephadex* LH-20 (Pharmacia) was used for column chromatography with MeOH. All the DCCC. separations were carried out on a Model A 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 Teflon tubes (I.D. 0.5 mm). 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 aluminium sheets *(Merck)*. The solvent systems were (lower layer) $CHCl_3$/MeOH/PrOH/H_2O 45:60:5:40, $CHCl_3$/MeOH/H_2O 65:35:20, or $CHCl_3$/MeOH/H_2O 43:37:20. The compounds were detected with cerium(IV) sulfate in sulfuric acid or with *Godin* reagent [19]. In the separation of xanthones, the fractions were monitored by UV. spectroscopy at 300 nm.

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