ELSEVIER

Journal of Chromatography A, 831 (1999) 203–216

JOURNAL OF CHROMATOGRAPHY A

Preparative purification of antibiotics for comparing hydrostatic and hydrodynamic mode counter-current chromatography and preparative high-performance liquid chromatography

M.-C. Menet[a,*], D. Thiebaut[b]

[a]*Laboratoire de Chimie Analytique UFR des Sciences Pharmaceutiques et Biologiques, Av. de l'Observatoire, 75006 Paris, France*
[b]*Laboratoire de Chimie Analytique et des Processus Industriels, ESPCI, 10 rue Vauquelin, 75005 Paris, France*

Received 6 April 1998; received in revised form 2 October 1998; accepted 2 October 1998

Abstract

Two modes allow the retention of the stationary phase in counter-current chromatography (CCC): one is called ''hydrodynamic'' and the other ''hydrostatic''. The analytical comparison between the two modes of CCC shows that it is necessary to carefully choose the mobile phase and the composition of the solvent system depending on the mode of retention of the stationary phase. On a semi-preparative scale, the two modes are similar towards obtained purities and yields (hourly and volumetric). CCC consequently consumes three times less solvents than semi-preparative HPLC and enables the recovery of milligram amounts of pure compounds with purities higher than 95%. No preliminary extraction is required as opposed to semi-preparative HPLC.

Keywords: Counter-current chromatography; Preparative chromatography; Antibiotics

1. Introduction

Counter-current chromatography (CCC) is a chromatographic separation method based on the partition of solutes between two immiscible liquid phases. One of the two phases is retained in the column by a centrifugal force field and is called the stationary phase. The second phase, which is called the mobile phase, percolates through the stationary one. Two modes allow the retention of the stationary phase. In the hydrodynamic mode [1], the stationary phase is retained in the column by a centrifugal force field varying in intensity and direction. On the contrary, in the hydrostatic mode, the centrifugal force field is constant [2].

This technique has been revealed as a very interesting preparative chromatographic method [3]. Indeed, the volume of the stationary phase retained in the column is at least 50% of its total volume, while reversed-phase high-performance liquid chromatography (HPLC) is commonly based on 10% of stationary phase inside the whole column. Moreover, commercial devices for the hydrostatic mode are available with columns of 5.4 l or more. Another striking advantage is the use of a liquid stationary phase, which prevents the denaturation of fragile solutes and irreversible adsorption and therefore allows the use of raw samples.

In this paper, we will first introduce the two

*Corresponding author.

0021-9673/99/$ – see front matter
PII: S0021-9673(98)00795-X

modes for the retention of the stationary phase and the corresponding devices we used in our laboratory. Secondly, we will describe the design of a selective solvent system for the separation of two antibiotics X and Y from their fermentation broth and we will compare its use in the two modes. Then we will explain the optimization of the running conditions with regard to the injection solvent, injection volume and injection mode to obtain the highest resolution in the two modes (keeping in mind the preparative purpose of the separation). Finally, we will compare the preparative chromatographic parameters (yield per hour and volumetric yield) obtained in the two modes of CCC and in preparative HPLC.

2. Experimental

2.1. Chromatographic systems

2.1.1. CCC

The chromatographic system consisted of a Shimadzu Model LC 5 A HPLC pump (Touzart et Matignon, Vitry sur Seine, France) and a Constametric II G pump (Milton-Roy, Villepinte, France) for pumping the aqueous and the organic phases, respectively. The pumps were connected to the CCC column.

In the hydrostatic mode, the CCC apparatus was a Model LLN centrifugal partition chromatograph (CPC) (Fig. 1) manufactured by Sanki Engineering, Yamazaki, Japan. It was used with six or twelve Type 250 W cartridges put in a rotor and providing an internal volume of 125 and 250 ml, respectively. Two rotary seals were set in the head and end of the column and allowed the junctions with the pumps and the detector. A strong centrifugal force was applied to the liquid phases inside the column by rotation of the rotor at various speeds, from 200 to 2000 rpm [2,4]. In the hydrostatic mode, the constant centrifugal force field did not allow a chromatographic efficiency of 500 theoretical plates to be obtained. Indeed, it was shown that the mobile phase only percolates through the stationary one without a real mixing of the two phases [5].

In the hydrodynamic mode (Fig. 2), two devices were used: a Model CPHV 2000 system (derived from Pharmatech 2000) and a Model CCC 800 (Pharma-Tech. Research, Baltimore, MD, USA). The first one was equipped with three identical and independent columns symmetrically arranged around the central axis of the centrifuge. Each column was prepared from 93.5 m×0.80 mm I.D. PTFE tubing wound onto a holder in a multilayer coil to give a capacity of 47 ml. The maximum speed was 2000 rpm. The second one was equipped with two identical and independent columns symmetrically arranged around the central axis of the centrifuge. Each column was prepared from 2.6 mm I.D. PTFE tubing to obtain a capacity around 500 ml. The maximum

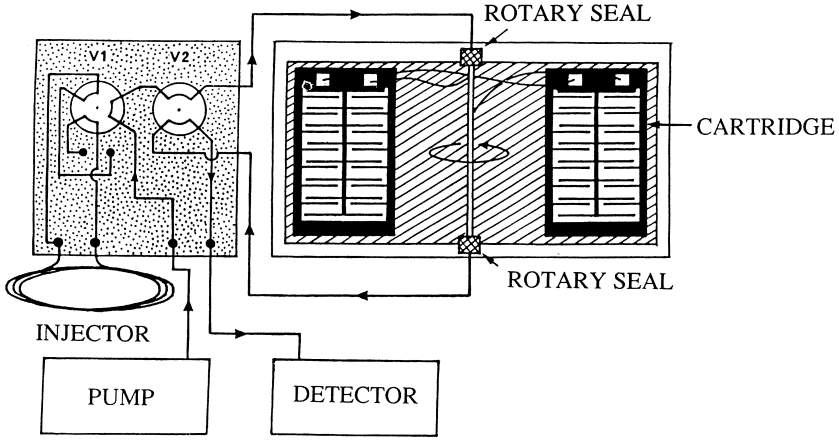

Fig. 1. CCC apparatus in the hydrostatic mode (“Sanki”) 1=rotary seal, 2=connecting tube, 3=separation column, 4=rotor, g=centrifugal force.

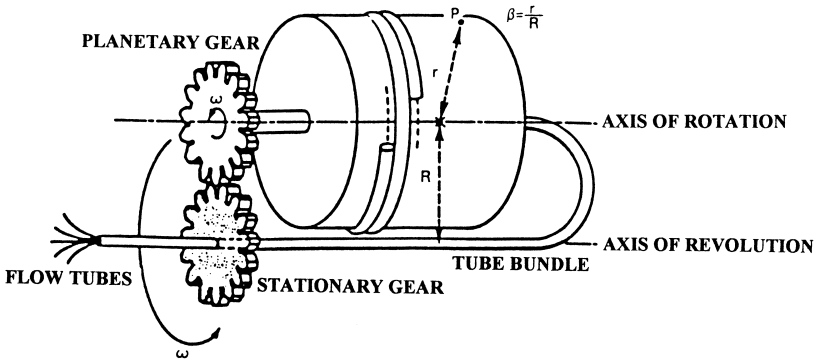

Fig. 2. CCC in the hydrodynamic mode.

attainable speed was 1000 rpm. It was shown that variable centrifugal forces create two phases settling and mixing zones allowing a higher chromatographic efficiency than in hydrostatic mode (1000 theoretical plates) to be obtained.

A third pump allowed the injection of the sample to the head of the column. The backflush valve allowed the elution mode of the mobile phase to be chosen. In the hydrostatic mode, two elution modes are available: the ascending mode referring to the flow of the mobile lighter phase through the channels in an inward direction, opposite to the direction of the centrifugal force and the descending mode referring to the flow of the mobile heavier phase through the channels in the same direction as the centrifugal force. In the hydrodynamic mode, the ends of the column are labelled ''head'' or ''tail'', depending on both the type of helix (right-handed or left-handed) and the direction of rotation [1]. It was found experimentally that in order to retain the stationary phase in the column, the lighter phase had to be pumped either from the tail to the head or from the head to the tail, depending on the nature of the two-phase solvent system and on the β ratio described Fig. 2 [1].

The detection of the solutes was based on evaporative light scattering detection (ELSD) [6] on-line with the CCC apparatus. The unit was a Sedex 45 ELSD system manufactured by Sédéré, Vitry sur Seine, France, designed for HPLC and used without modification. The nebulizer of the ELSD system was supplied with nitrogen by L'Air Liquide, Paris, France. The outlet of the column could also be connected to a fraction collector Model Helirac 2212 (LKB, Les Ulis, France).

2.1.2. Analytical HPLC

HPLC analyses of sample solutions were performed at 35°C on a Pecosphere 5CR C_8 column (5 μm, 150×4.6 mm) (Perkin-Elmer, Saint Quentin en Yvelines, France) with 0.1 *M* phosphate buffer (pH= 2.9)–acetonitrile (67.5: 32.5 v/v) as the mobile phase at a flow-rate of 1 ml/min.

2.1.3. Preparative HPLC

For comparison between CCC and preparative HPLC, Y was purified on octyl bonded silica LiChrospher 100 RP8 column (100 μm, 34×6 cm) (Merck, Nogent sur Maine, France) with a Prochrom axial compression system, Model LC6O.VE900 (Champigneulles, France). A step gradient was performed: after injection, 2 l of water–acetonitrile (84:16, v/v) were pumped, then the mobile phase made of water–acetonitrile (70:30, v/v) were pumped at a flow-rate of 80–90 ml/min. The pressure drop was 40 bar. Solutes to be injected were dissolved in 500 ml of water–acetonitrile (80:20, v/v) and detected by UV absorption at 250 nm (Spectromonitor 3100, Milton-Roy, Riviera Beach, Florida).

2.2. Reagents

All organic solvents were of HPLC grade. Methanol and acetonitrile were purchased from Prolabo, Paris, France; chloroform and ethyl acetate from

Rathburn (Chromoptic, Montpellier, France). Water was bidistilled. The solvents were filtered before use.

2.3. Solvent systems and sample solution

Solvent mixtures were equilibrated at room temperature and the phases were separated shortly before use. The solutions of pure antibiotics or crude extracts were prepared by dissolving the samples in the mobile phase or the stationary one.

Two raw materials were used: a crude ethyl acetate extract of fermentation medium containing 7% (w/w) in Y or a purified extract containing 25% (w/w) in Y. The one-day purification of the extract at 7% gave the extract at 25% in many steps: it included a dissolution followed by a filtration, then an extraction by methylene chloride, a treatment by alumina and a final drying [5].

2.4. Injection procedures

Two different injection procedures were followed for the CCC devices.

The standard method consisted in first filling entirely the CCC column with the stationary phase. Then the apparatus was rotated at the desired speed. After that, the mobile phase was pumped into the column at the desired flow-rate. Finally, after the equilibrium between the two phases was reached, a sample solution was injected and the mobile phase pumped into the column.

The second procedure was called the ''sandwich'' injection. The column was first entirely filled with the stationary phase and the apparatus rotated at the desired speed. Then the sample was injected into the column head and the mobile phase was pumped at the desired flow-rate. The separation occurred during the phase equilibrium.

The sample was injected via a Rheodyne Model 7125 injection valve in the hydrodynamic mode and via a Sanki injection valve (Model FCU II valve connection) or a pump in the hydrostatic mode. Injection with a valve indicates that the sample is introduced into the rotating column through a loop attached to the injection valve whereas the injection with the pump indicates that the sample is first introduced into the stationary column using a pump and then the column is rotated to start separation.

Injected volumes were limited to about 2 ml in analytical chromatography and to 10% of the volume of the column in preparative chromatography [7].

3. Theoretical background

In this part, we remind readers of the formulas used to calculate the efficiency and the resolution. The classical parameters which are used in Section 4 are gathered in the glossary.

3.1. Efficiency

The number of theoretical plates N for a symmetrical peak (as observed during our analytical studies) is computed using the Gaussian peak dispersion equation.

The number of theoretical plates N for an asymmetrical peak (as observed during our semi-preparative studies) is computed using the Foley and Dorsey formula [8]

$$N = 41.7 \cdot \frac{\left(\frac{t_r}{w_{0.1}}\right)^2}{\frac{A}{B} + 1.25} \quad (1)$$

where $w_{0.1}$ is the peak width at 10% of the peak height and A/B the asymmetry factor with $A+B=w_{0.1}$.

3.2. Resolution

In the analytical studies, the resolution between two peaks, R_s, can be calculated using:

$$R_s = \frac{\sqrt{N}}{4} \cdot \frac{\alpha - 1}{\alpha} \cdot \frac{k'_2}{k'_2 + 1} \quad (2)$$

if we assume the widths of the two peaks are equal, where N is the efficiency calculated with the second peak, k'_2 the retention factor of the second peak and α the separation factor. R_s can be calculated by the conventional formula:

$$R_s = \frac{2(t_{r_2} - t_{r_1})}{\omega_1 + \omega_2} \quad (3)$$

where t_{r_1} and t_{r_2} are the retention time of the first

and the second solutes, respectively, and w_1 and w_2 the widths of the two peaks.

4. Results and discussion

Firstly, the separation was optimized in the hydrodynamic mode prior its adaptation to the hydrostatic mode.

4.1. Design of a selective solvent system

Taking into account the relative hydrophobicity of the solutes X and Y we had to separate and according to Ito's classification [1], we chose an intermediate polarity solvent system, made of chloroform–ethyl acetate–methanol–water. Such a solvent composition has already been described for antibiotics separation [1].

4.1.1. Hydrodynamic mode

The volumetric composition of the solvent system was varied to obtain the best resolution for the separation. For each composition, an analytical separation of the two antibiotics was carried out and the partition coefficients computed according to (10) Section 6), the retention factors according to (8), the separation factor α and the resolution R_{s_1} according to classical formulae (3). The partition coefficients K were defined as the ratio of the concentration in the aqueous phase on the concentration in the organic mobile phase.

Table 1 shows the partition coefficients K_X and K_Y were smaller than one (i.e., <0.35) which indicated that the antibiotics are more soluble in the organic phase. The separation factors α were constant (1.7), even if the partition coefficients increased with the proportion of ethyl acetate. Therefore, the nature of the interactions which allows the solubility of the solutes in the organic phase is not modified by addition of ethyl acetate. Whatever the composition, the resolution was satisfactory. Thus, a satisfactory separation of the two antibiotics in less than 30 min was obtained with a resolution close to 2 (Fig. 3a).

4.1.2. Hydrostatic mode

We used the partition coefficients obtained in hydrodynamic mode to select the best solvent system in the hydrostatic mode.

Table 1
Selection of solvent system

	Solvent system: $CHCl_3$–$C_2H_5OCOCH_3$–CH_3OH–water		
	3:1:3:2 (v/v)	2.4:1.6:3:2 (v/v)	2:2:3:2 (v/v)
K_X	0.07	0.13	0.2
K_Y	0.12	0.21	0.35
α	1.7	1.6	1.7
R_{s_1}	1.8	2.1	2.6
R_{s_2}	0.56	0.75	1.1
K'_X	14.3	7.7	5
K'_Y	8.3	4.8	2.9
k'_X	33.2	15.4	10
R_{s_3}	2.4	2.1	2.2

Firstly, we calculated the Y and X retention factors k' according to (8), by using an average value for the S_F in hydrostatic mode (commonly 70% versus 90% for hydrodynamic one) and by using the organic mobile phase. Then, we estimated the resolution R_{s_2} of the antibiotics separation by using relation (2) with a 500 value for the efficiency (versus 1000 in the hydrodynamic mode) (Table 1). Indeed, the measured efficiency in the hydrodynamic mode is higher than that in the hydrostatic mode because the improved mixing in the hydrodynamic mode increases the liquid–liquid interface and facilitates the exchange of the X and Y solutes through the interface [5].

Because the retention of the stationary phase and the efficiency are higher in the hydrodynamic mode compared to the hydrostatic mode, a very good resolution R_{s_1} was obtained for the hydrodynamic mode with an aqueous stationary phase. Indeed, the estimated resolution R_{s_2} for the hydrostatic mode was not satisfactory (<1.1). Hence, to compensate for the lower values of S_F and efficiency, it was necessary to increase the partition coefficient. By exchanging the roles of the stationary phase and the mobile one, the partition coefficients K' were then defined as the ratio of the concentration in the organic phase to the concentration in the aqueous phase and is the inverse of previous ones while α remains constant. Estimated resolutions R_{s_3} are given in Table 1 by using relation (2).

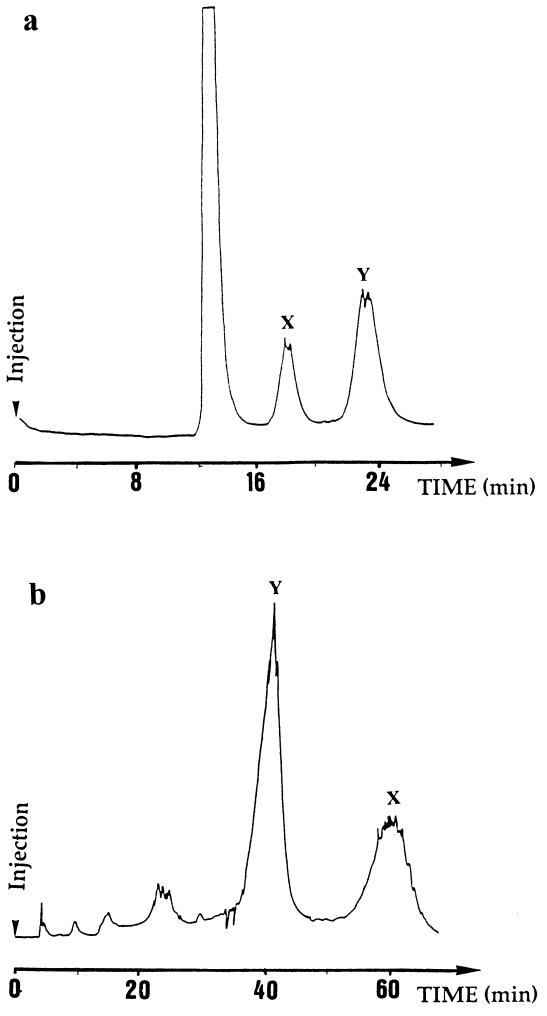

Fig. 3. (a) Chromatogram of the separation of X and Y on CPHV 2000. Internal volume: 110 ml. Solvent system: chloroform–ethyl acetate–methanol–water (2.4:1.6:3:2, v/v). Organic mobile phase is pumped into the head. Flow-rate: 2 ml/min. Injection volume 2 ml. Injected quantities: 4 mg of a pure antibiotics mixture. Rotational speed: 900 rpm. S_F: 52%. ELSD: 40°C, nitrogen pressure: 2 bar. (b) Chromatogram of the separation of Y and X on Sanki LLN. Internal volume: 125 ml. Solvent system: chloroform–ethyl acetate–methanol–water (2:2:3:2, v/v). Ascending mode. Aqueous mobile phase flow-rate: 4 ml/min. Injection volume: 2 ml. Injected quantities: 4 mg of a pure antibiotics mixture. Rotational speed: 900 rpm. S_F: 52%. ELSD: 40°C, nitrogen pressure: 2 bar.

An increase of the resolution was observed by using an organic stationary phase instead of an aqueous one. We chose the $CHCl_3$–$C_2H_5OCOCH_3$–CH_3OH–water (2:2:3:2, v/v) composition for the separation, which gave the smallest retention factors [calculated according to (10)] and therefore the shortest separation time (Fig. 3b).

These results show that the optimized solvent composition for the hydrodynamic mode cannot be directly applied to the hydrostatic mode because the latter lacks efficiency to obtain a satisfactory resolution ($R_s > 1.5$) [7]. It was demonstrated therefore to be necessary to choose the mobile phase and the composition of the solvent system carefully.

Remark: The organic stationary phase could also be used in the hydrodynamic system with an expected higher resolution than with the aqueous stationary phase.

These preliminary studies allowed the optimization of the composition of the solvent system for analytical scale purposes and a more than satisfactory resolution to ensure the further quality of the preparative scale separation to be obtained. Various running conditions remained to be optimized. The following studies were intended to determine if the resolution depended only on the selection of the solvent system or if it also involved the choice of other parameters such as the injection solvent, the injection volume and the injection mode.

4.2. Running conditions

Keeping in mind the preparative purpose of CCC, the analytical separation should show a higher than 1.5 resolution.

For these studies with an organic stationary phase, the Sanki LLN with six cartridges was used.

4.2.1. Injection solvent

A mixture of the two pure antibiotics was dissolved either in the aqueous mobile phase (first case: a) or in the organic stationary phase (second case: b) (Fig. 4). A 1.5 ml volume of the samples at 2 g/l was injected by the sandwich method.

We have calculated the stationary phase retention S_F according to formula (6), the efficiency and the resolution according to Eq. (3) for each case. The injection in the aqueous phase disturbed the two-phase equilibrium inside the column, leading to leaks

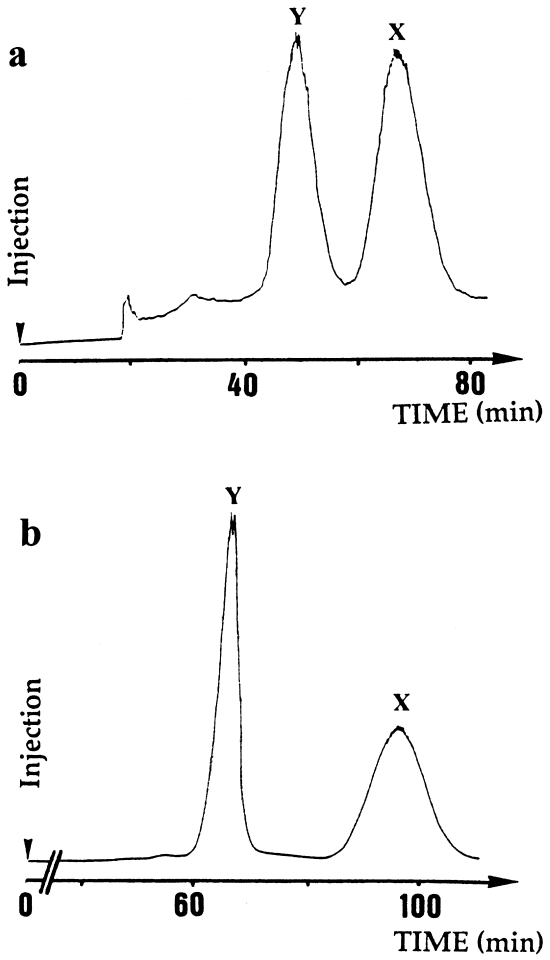

Fig. 4. Chromatogram of the separation of X and Y on Sanki LLN versus injection solvent. Internal volume: 125 ml. Solvent system: chloroform–ethyl acetate–methanol–water (2:2:3:2, v/v). Ascending mode. Aqueous mobile phase. Flow-rate: 3 ml/min. Injection volume 1.5 ml. Injected quantities: 3 mg of pure antibiotics mixture. Rotational speed: 800 rpm. ELSD: 40°C, nitrogen pressure: 2 bar. (a) Injection solvent: aqueous mobile phase. S_F: 44%. (b) Injection solvent: organic stationary phase. S_F: 52%.

of the organic stationary phase ($S_F = 44\%$ versus 52% for injection in the stationary organic phase). The values of the efficiency were lower for sample injected in the aqueous phase (280 versus 1410 plates for Y) because the solutes had to distribute in the stationary phase before their separation and their elution by the mobile phase.

The best values of the resolution (2.4 versus 1.4) and the efficiency (1410 versus 300) were obtained with the sample dissolved in the organic phase which is the stationary one. This result was interesting because the antibiotics are highly soluble in this organic phase.

4.2.2. Injection volume

According to the first study, the organic stationary phase was chosen as the injection solvent. The second condition that needed to be optimized was the injection volume. We studied the variation of the efficiency and of the resolution calculated according to (1) and (3), respectively, versus the injected volume (with a constant injected quantity) for three injection modes: after the phase equilibrium with an injection loop (a), sandwich mode with an injection loop (b) and sandwich method injecting to the head of the column with a pump (c) (Fig. 5).

These were classical decreases as encountered for preparative HPLC [8]. When using injection loops, efficiencies (maxima of 300 and 450 theoretical plates for Y and X, respectively) were smaller than in the case of the injection with a pump. Considering a 1.5 resolution was necessary for the separation of X and Y, the maximum injected volume should be no more than 10% of the total volume of the column. The same results were obtained in the hydrodynamic mode [9].

4.2.3. Injection mode

Also, a sample injections with a higher volume (15 ml) injected by a pump or via a Sanki injection valve were studied for both injection procedures (after and before the two-phase equilibrium) (Table 2). The calculated resolution [according to (3)] was higher when the sample was injected after the two-phase equilibrium, owing to an artificial increase of the S_F by the sample injection in the stationary phase and, consequently, of k'. Just after injection, the true S_F were higher than the values measured after the two-phase equilibrium (42.5% and 41.6% respectively for injection with the loop and a pump).

Afterwards, for preparative scale separation the samples were injected using a pump after the phase equilibrium was reached.

4.3. Preparative-scale separation

After the optimization of the running conditions,

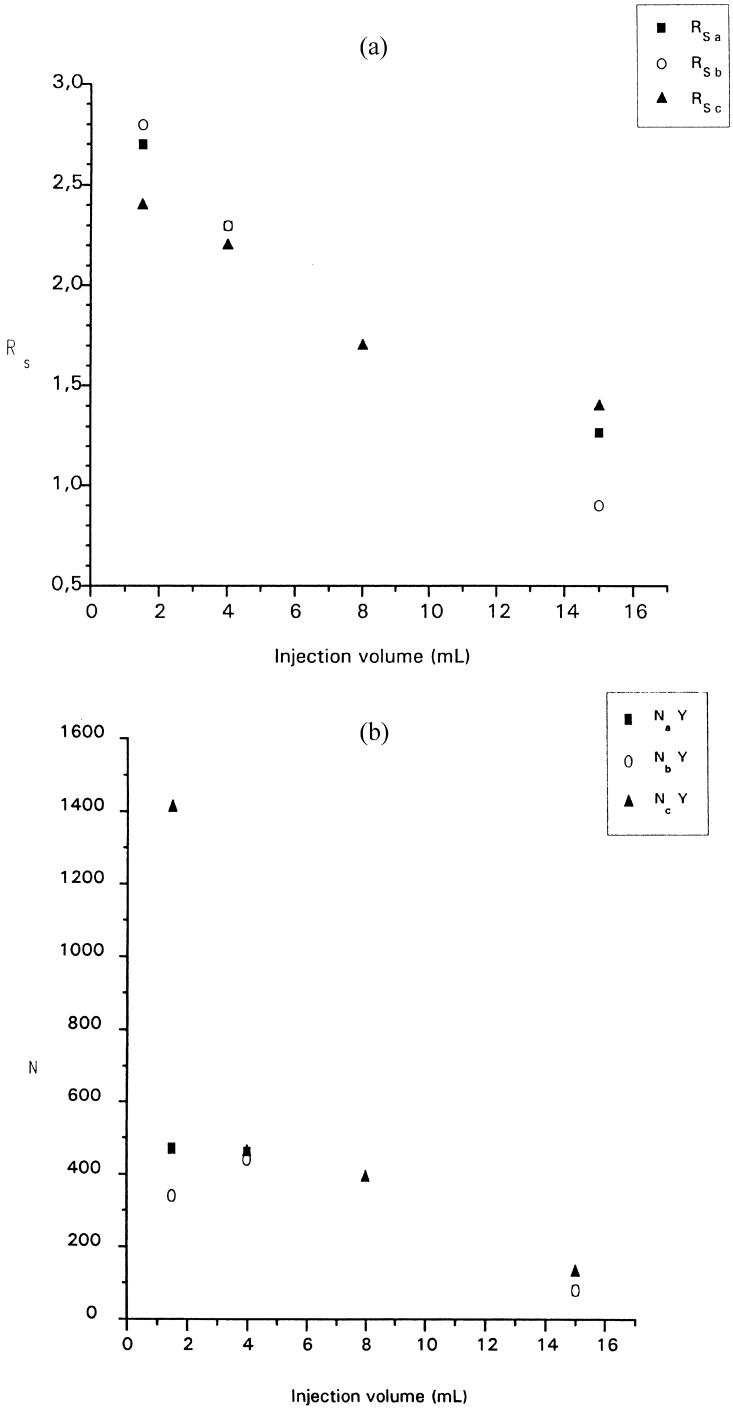

Fig. 5. Variation of the efficiencies and resolution versus the injected volume and three injection modes: after the phase equilibrium with an injection loop (a), sandwich method with an injection loop (b) and injection of the sample to the head of the column with a pump (c). Internal volume: 125 ml. Solvent system: chloroform–ethyl acetate–methanol–water (2:2:3:2, v/v). Aqueous mobile phase flow-rate: 6 ml/min. Injected quantities: 1 mg of a pure antibiotics mixture. Rotational speed: 800 rpm. ELSD: 40°C, nitrogen pressure: 2 bar.

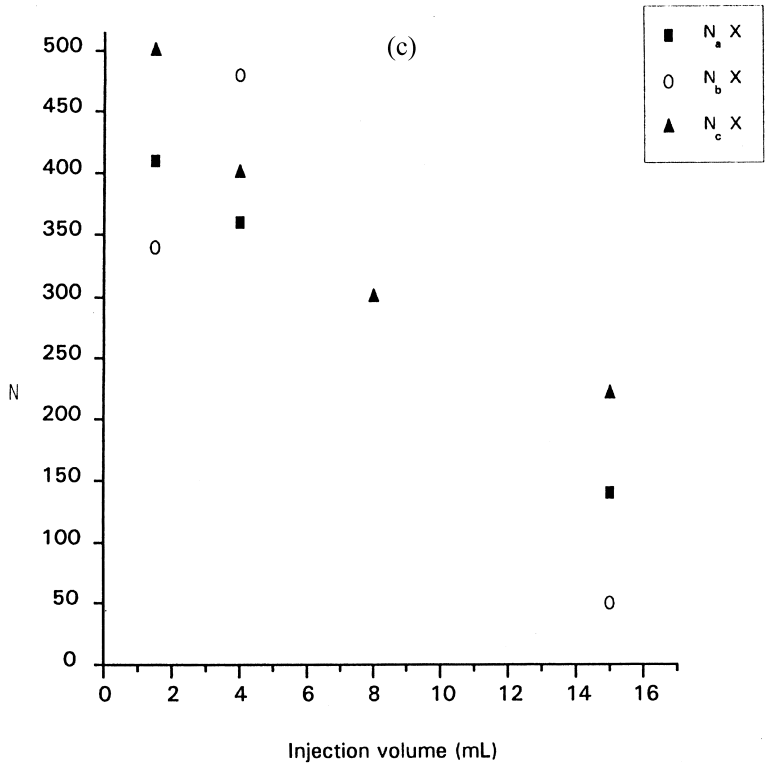

Fig. 5. (*continued*)

preparative scale separations were carried out with the two CCC modes and preparative HPLC. To compare the separations, classical parameters of preparative scale chromatography were calculated: the experimental duration including the sample preparation and the separation time, the solvent consumption including the volume of the mobile phase, the stationary phase and the injection solvent, the purity of the purest fraction in Y. The parameter ''purity in Y'' was chosen because Y is the solute which is the most difficult to purify because of its physico-chemical properties (particularly hydrophobicity) which are close to those of the main impurities. The hourly yield (g/h) is defined as the ratio of the recovered quantity to the experimental duration. The volumetric yield (g/l) is defined as the ratio of the recovered quantity to the solvent consumption [7].

4.3.1. CCC

According to the previous studies, the samples to

Table 2
Efficiency, resolution and S_F versus the injection mode

15 ml injection	Loop		Pump	
	After equilibrium	Before equilibrium	After equilibrium	Before equilibrium
N_1	80	80	140	130
N_2	140	50	80	220
R_s	1.3	0.9	1.5	1.4
S_F (%)	42.5[a]	37.6[b]	41.6[a]	40[b]

[a] measured after the two- phase equilibrium, [b] measured after the column emptying.

be injected were prepared in the organic phase (which is the stationary phase in the two modes). Injected volume was up to 10% of the total volume of the column, i.e., 10 ml, 20 ml and 100 ml (for 125 ml and 250 ml internal column volume in hydrostatic mode and 1000 ml in the hydrodynamic mode, respectively) injected with a pump and after the phase equilibrium was established. The collection of the fractions allowed the effluent to be analyzed and potential stationary phase leaks to be monitored.

4.3.1.1. Hydrostatic mode

Two purifications of raw material at 7% in Y were achieved with two different internal volumes, i.e., 125 ml (six cartridges) (Fig. 6a) and 250 ml (twelve cartridges) (Fig. 6b). Both products were completely separated with a column with an internal volume of 250 ml. During the separations leaks of stationary phase were observed: a mixture of both phases was expelled from the column in the collector tubes, particularly at the end of the separations. The fraction collected at 290 mm (Fig. 6b) was very rich in X, which is very soluble in the stationary phase but scarcely soluble in the mobile one. Such an enrichment was related to an important leak of stationary phase which was observed in the collection tube. The corresponding fractions contained 50% of stationary phase. The leak could be explained by the surfactant properties of X which disturbs the two-phase equilibrium.

The parameters of the preparative scale chromatography are shown Table 3. The highest purity in Y was obtained for the second separation, i.e., for a 250-ml internal volume versus a 125-ml one. It can be explained by a higher retention of the stationary phase during the separation of Y in the case of a 250-ml internal volume. The fractions collected before the elution of X contained a small amount of stationary phase as opposed to a 125-ml internal volume. An injection up to 10% of the total volume of the column disturbs the two-phase equilibrium for the column with the smallest internal volume. The ''capacity'' of the column was exceeded in terms of solvent stability.

The experimental duration, the solvent consumption and the yields were similar and showed that, as expected, an increase of the column length (12 cartridges as opposed to 6) increased the resolution [4]. Finally, the preparative scale separation was directly scaled up from a small internal volume to a higher one as in the hydrodynamic mode [9]. This scale up is very interesting in the hydrostatic mode because a column with a volume up to 5 l is commercially available.

CCC was revealed as a very suitable method for the purification of selected compounds from complex mixtures because fractions at 96% in Y were obtained from crude extract with a 7% content in Y [8].

4.3.1.2. Hydrodynamic mode

One experiment was carried out by using the organic stationary phase to increase the resolution. 10.8 g of raw material were solubilized in organic stationary phase and injected in CCC 800 (Fig. 7). The results are gathered in Table 3 to compare the preparative chromatographic parameters obtained in the two modes and in preparative HPLC.

4.3.2. Preparative HPLC

The chromatogram shown in Fig. 8 corresponds to the injection of 1.5 g of Y (in about 6.3 g of raw material). Although peaks of Y and solute which eluted before appeared partially resolved, fractions 3 and 4 contained 87% injected Y. These optimal conditions led to a recovery of 1.3 g of Y at a 95.7% purity per run.

4.4. Comparison and discussion of CCC and HPLC results obtained for raw samples

Table 3 summarizes the results of separations of Y by CCC and preparative HPLC. The solvent volume consumption is the volume of the stationary and mobile phases in CCC or the volume of the mobile phase used in HPLC and the samples.

The injected sample in the hydrostatic mode was not prepurified to concentrate it in Y from 7% to 25%. So the injected quantity in Y in the Sanki apparatus is lower (0.28 g in hydrostatic mode as against 2.70 g in hydrodynamic mode and 1.59 g in preparative HPLC).

For the same enrichment in Y, i.e. from 25% (purity of the injected solution) to 95% (purity of the purest fraction) hydrodynamic CCC leads to a 0.4 g/l volumetric yield which is about three times higher than the 0.15 g/l yield of preparative HPLC.

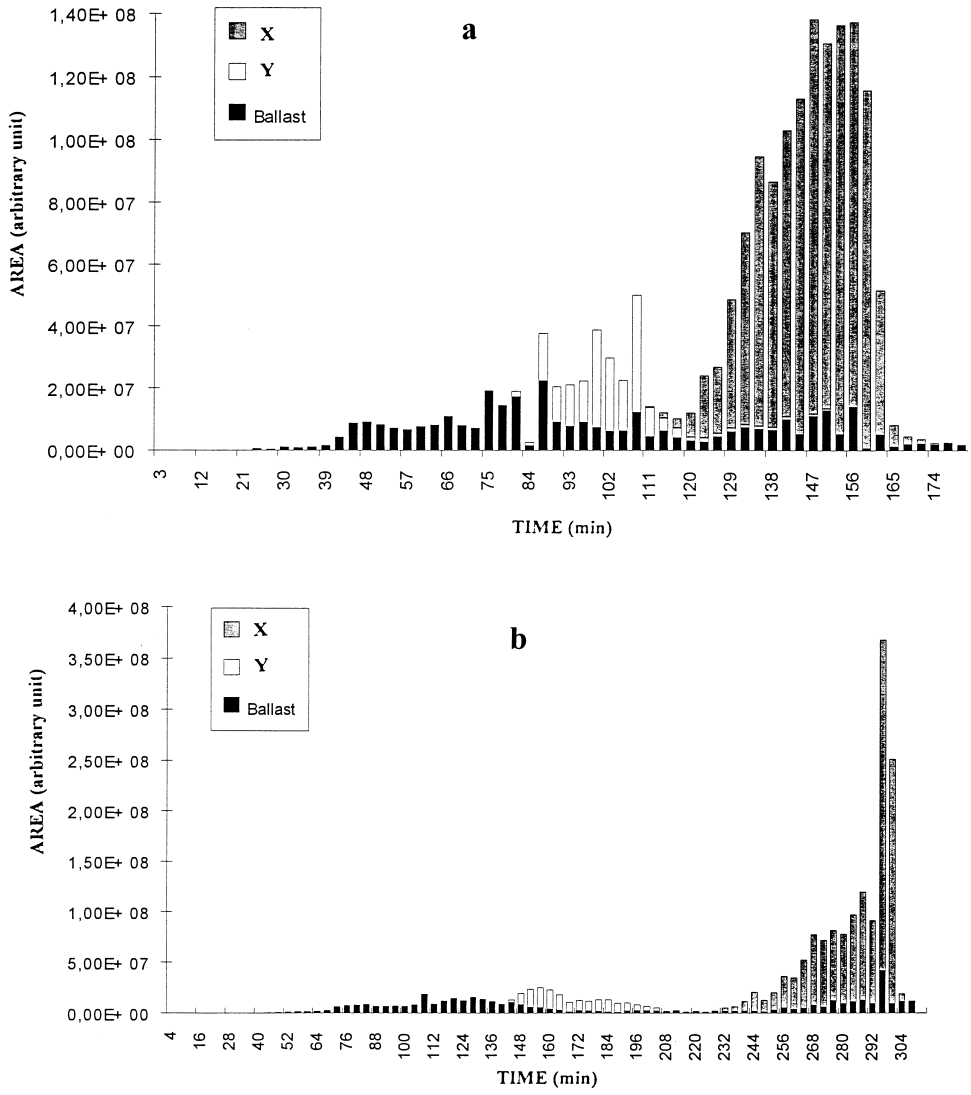

Fig. 6. Reconstituted chromatograms for preparative separations of Y and X in hydrostatic mode. Solvent system: chloroform–ethyl acetate–methanol–water (2:2:3:2, v/v). (a) Internal volume: 125 ml. Aqueous mobile phase flow-rate: 2.4 ml/min. Rotational speed: 800 rpm. Initial S_F: 55%. Injection volume: 10 ml with a pump. Injection solvent: organic stationary phase. Injected crude extract concentration 200 g/l (2 g of raw material). Y injected quantity: 0.14 g. X injected quantity: 0.8 g. Volume of the collected fractions: 7.5 ml. (b) Internal volume: 250 ml. Aqueous mobile phase flow-rate: 3 ml/min. Rotational speed: 700 rpm. Initial S_F: 50%. Injection volume: 20 ml with a pump. Injection solvent: organic stationary phase. Injected crude extract concentration: 200 g/l (4 g of raw material). Y injected quantity: 0.28 g. X injected quantity: 1.6 g. Volume of the collected fractions: 12 ml.

Hydrodynamic CCC consequently consumes three times less solvent than preparative HPLC.

For similar volumetric yields, i.e. 0.20 g/l in hydrostatic CCC and 0.15 g/l in preparative HPLC, the enrichment in Y is higher with hydrostatic CCC than with preparative HPLC. Indeed starting from a crude extract at 7% in Y with hydrostatic CCC or from a 25% in Y extract with preparative HPLC leads to the same 95% highest purity. These results demonstrate the potential of hydrostatic CCC in directly purifying crude extracts. No preliminary purification of the extract is required, as opposed to

Table 3
Comparison hydrostatic and hydrodynamic CCC and HPLC

	Hydrostatic mode (125 ml)	Hydrostatic mode (250 ml)	Hydrodynamic mode	HPLC
Crude extract purity in Y (%)	7	7	25	25
Injected quantity of Y (g)	0.14	0.28	2.70	1.59
Experimental duration (h)	3	6.2	9.5	2.2[a]
Solvent volume consumption (L)	0.7	1.4	6	10.8
Purity of the purest fraction in Y	73	>95%	>95%	>95%
Hourly yield (g/h)	0.05	0.035	0.28	0.72
Volumetric yield (g/l)	0.2	0.20	0.45	0.15

[a] One hour for the column equilibration at the 90 ml/min flow-rate + 1 h for the separation.

preparative HPLC which requires a 1 day enrichment of the crude extract from 7% to 25% in Y.

Definitely, CCC is not the right tool for analytical purposes. However, CCC enables very dirty material to be handled directly, as shown in this paper, with centrifugal partition chromatography or, in a coming paper, with the coil planet centrifuge [10]. In contrast to CCC preparative HPLC required a sample treatment before separation, which enables very efficient purification of high value materials, in one step. Moreover, taking into account the time required for the purification steps before preparative HPLC, the productivity of CCC is higher than that of HPLC.

5. Conclusion

The analytical comparison between the two modes of CCC has shown that it is necessary to choose the mobile phase carefully and to fit the composition of

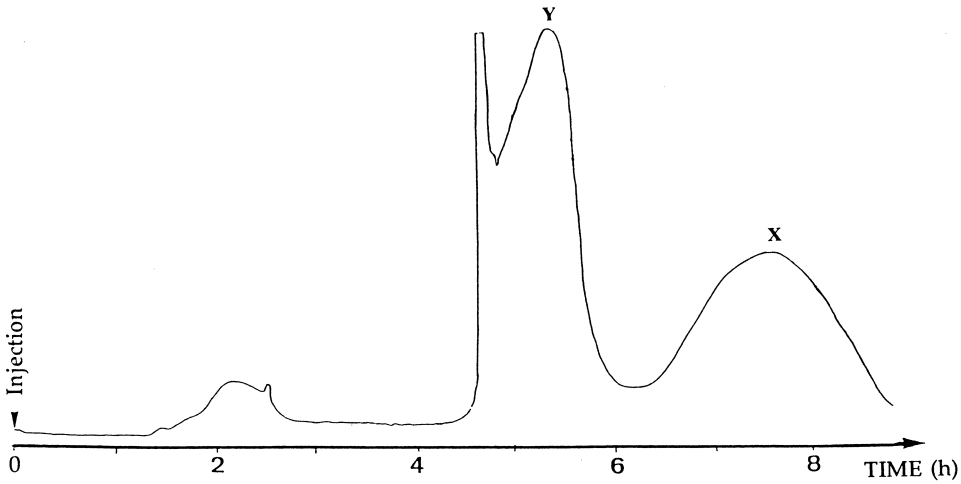

Fig. 7. Chromatogram for preparative separations of Y and X in hydrodynamic mode. Solvent system: chloroform–ethyl acetate–methanol–water (2.5:1.5:3:2, v/v). Internal volume: 1000 ml. Aqueous mobile phase flow-rate: 9 ml/min. Rotational speed: 700 rpm. Injection volume: 100 ml. Injection solvent: organic stationary phase. Injected crude extract 10.8 g. ELSD: 40°C, nitrogen pressure: 2 bar.

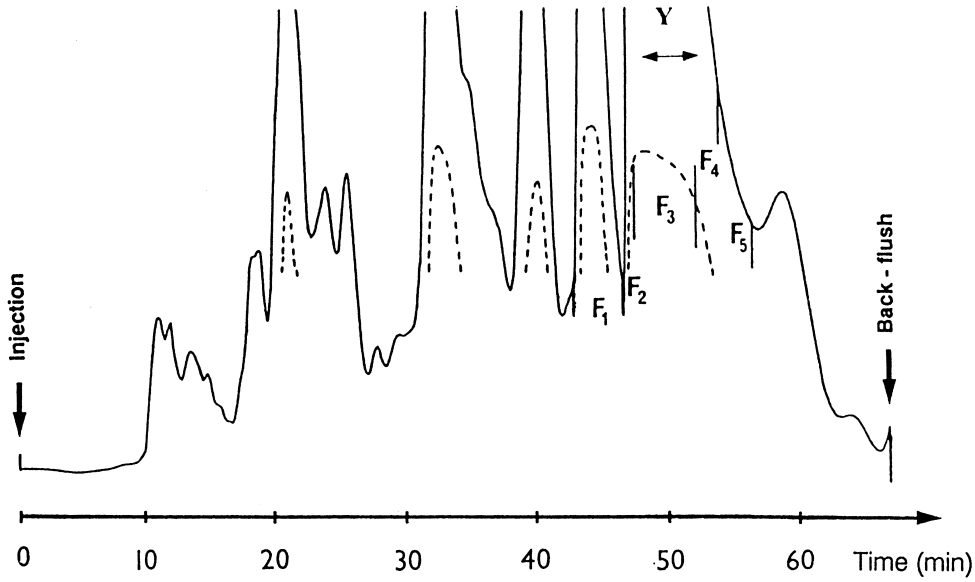

Fig. 8. Preparative HPLC chromatogram of Y. Column 34×6 cm I.D. with axial compression, stationary phase: octyl bonded silica, LiChrospher 100 RP8, 100 μm. Mobile phase: water–acetonitrile (70:30, v/v), flow-rate: 80–90 ml/min. Pressure drop: 40 bar. Injection: 6.3 g of raw material containing 1.55 g of Y. Detection: UV at 250 nm.

the solvent system, because efficiency and retention of the stationary phase in the hydrostatic mode are lower than in the hydrodynamic mode. On a semi-preparative scale, the two modes of CCC are similar in terms of purities and of yields obtained (hourly and volumetric).

Using small scale devices as described in this paper, CCC has enabled the recovery of an amount (about 1 g) of pure compound with a higher purity than 95%, without previous extraction as opposed to semi-preparative HPLC. It is a unique technique for the fractionation and the purification of compounds from raw samples.

6. Symbols

V_t internal volume of the column.

V_e volume of the stationary phase expelled from the column by the mobile phase during the equilibrium period.

V_s Volume of the stationary phase: $V_s = V_t - V_e$ (4).

V_m mobile phase volume in the column: $V_m = V_t - V_s$ (5) can be measured after the column emptying.

S_F retention of the stationary phase: $S_F = (V_s/V_m)$ (6)

t_0 hold-up time: $t_0 = (V_m/F$ (7), where F (ml/min) is the flow-rate of the mobile phase

k' retention factor: $t_r = t_0\ (1 + k')$ (8), where t_r is the retention time of the solute.

K partition coefficient: $K = (C_s/C_m)$ (9) and $K = k'(V_m/V_s) = k'[(1/S_F) - 1]$ (10), where C_s is the concentration of the solute in the stationary phase and C_m its concentration in the mobile phase.

α separation factor, which is related to the solvent selectivity, is defined as the ratio of the peak retention factor to the first peak retention factor.

Acknowledgements

The authors wish to thank Rhône-Poulenc Rorer who furnished crude and purified extracts of X and Y.

References

[1] B. N. Mandava, Y. Ito, Countercurrent Chromatography. Theory and Practice, Chromatographic Science Series, vol. 44, Marcel Dekker, New York, 1988.
[2] Manual for Centrifugal Partition Chromatography, Model LLN, Sharon Hill, PA, 1987.
[3] K. Hostettman, A. Marston, Anal. Chim. Acta 236 (1990) 63.
[4] W. Marayama, T. Kobayashi, Y. Kosuge, J. Chromatogr. 239 (1982) 643.
[5] J.-M. Menet, M.-C. Rolet, D. Thiébaut, R. Rosset, Y. Ito, J. Liq. Chromatogr. 15 (1992) 2883.
[6] S. Drogue, M.-C. Rolet, D. Thiébaut, R. Rosset, J. Chromatogr. 538 (1991) 91.
[7] R. Rosset, M. Caude, A. Jardy, Chromatographies en Phases Liquide et Supercritique, Masson, Paris, 1991.
[8] J.P. Foley, J.G. Dorsey, Anal. Chem. 55 (1983) 730.
[9] S. Drogue, Thèse de Doctorat, Université de Paris VI, 1992.
[10] M. Agnely, D. Thiébaut, J. Chromatogr. A 790 (1997) 17.