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PURIFICATION OF A POLYENIC ANTIBIOTIC BY PREPARATIVE LIQUID CHROMATOGRAPHY

P. GAREIL, G. SALINIER, M. CAUDE* and R. ROSSET

Laboratoire de Chimie Analytique des Processus Industriels de l'Ecole Supérieure de Physique et Chimie Industrielles de la Ville de Paris, 10 Rue Vauquelin, 75231 Paris Cedex 05 (France)

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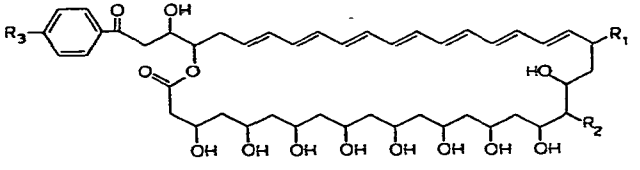
SUMMARY

High-resolution liquid chromatography was applied on the semi-preparative scale to separate some components of a polyenic antibiotic sample obtained by a fermentation procedure. A previous analytical separation by reversed-phase chromatography showed two main components and about seven impurities. The separation was adapted to the semi-preparative scale in order to establish precisely the structures of the components and their pharmacological properties. Through this example, the emphasis is placed upon the conversion procedure and the analysis of the optimization criteria and constraints.

INTRODUCTION

Polyenic antibiotics were introduced into medicine about 25 years and are now of great importance. Liquid chromatography is the best method for separating them because of their complicated chemical structures and their high molecular weights[1].

We have studied the chromatographic behaviour of the antibiotic A shown below, of molecular weight 1100, which has therapeutic properties against some infectious diseases. It is obtained by a fermentation procedure that leads to a complex mixture that has to be separated and analysed.



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The analytical separation of this antibiotic was recently performed in our laboratory. Our purpose now is to describe the conversion of the separation to the semi-preparative scale in order to determine the structures of the various components and their pharmacological properties.

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EXPERIMENTAL

Equipment

For preparative chromatography we used a home-made chromatograph including the following units: an Orlita MS 15/7 reciprocating pump (Orlita, Giessen, G.F.R.), a pulse damper made of a capillary tubing inserted between two pressure gauges, a Rheodyne six-way sampling valve (Rheodyne, Berkeley, CA, U.S.A.), an LDC Spectromonitor II spectrophotometer fitted with 3-mm pathlength preparative cells (LDC, Riviera Beach, FL, U.S.A.), a Siemens programmable fraction collector (Siemens, Karlsruhe, G.F.R.) and a Varian A 25 recorder (Varian, Palo Alto, CA, U.S.A.).

For analytical chromatography, a Varian 8510 chromatograph equipped with a stop-flow injector, a Varian Varichrom spectrophotometer (10-mm pathlength) and a Hewlett-Packard 3380 integrator (Hewlett-Packard, Palo Alto, CA, U.S.A.) were used.

Materials

The chromatographic stationary phases were 10-μm LiChrosorb RP-8 and 5–20-μm LiChroprep RP-8 (Merck, Darmstadt, G.F.R.). The mobile phases were prepared from distilled water, methanol (R.P. Normapur grade; Prolabo, Paris, France) and dried for analysis grade dimethyl sulphoxide (Merck). The antibiotic samples were kindly supplied by UCB Corp. (Brussels, Belgium).

Procedure

The optimal analytical and preparative operating conditions are given in Table I. The recovered effluent fractions were checked by analytical chromatography. For this purpose, the eluent was methanol–water (65:35), which gives a rapid analysis at the expense of poorer resolution. Subsequently, the fractions were evaporated to dryness with a vacuum rotary evaporator at 50°C under a pressure of 2–3 torr to remove the mobile phase. The solid materials obtained were kept in actinic glass-stoppered vessels to prevent exposure to light.

TABLE I
ANALYTICAL AND PREPARATIVE OPERATING CONDITIONS

Operating conditions	*Analytical*	*Preparative*
Column length	15 cm	47 cm
Column diameter	1/4 in.	1/2 in.
Stationary phase	LiChrosorb RP-8, 10 μm	LiChroprep RP-8, 5–20 μm
Mobile phase	Methanol–water (64:36)	Methanol–water–DMSO (65:30.5)
Flow-rate (ml h^{-1})	120	130
Pressure drop (bar)	40	110
Detection	UV 380 nm, l = 10 mm	UV 400 nm, l = 3 mm
Injection	1 μl of 4 mg·ml^{-1} solution in DMSO	4.14 ml of 4 mg·ml^{-1} solution in DMSO

RESULTS AND DISCUSSION

Analytical chromatography: preparative criteria and constraints

The analytical separation of the antibiotic was achieved by reversed-phase chromatography in our laboratory[2] (Fig. 1). This method seems to be the most suitable for the analysis of heptaenic compounds[3,4]. Nine components seem to be present in the mixture. The percentage composition given in Fig. 1 was calculated by assuming that the absorptivity coefficients are identical for all the components. Hence this composition is only approximate. A more accurate composition is indicated later.

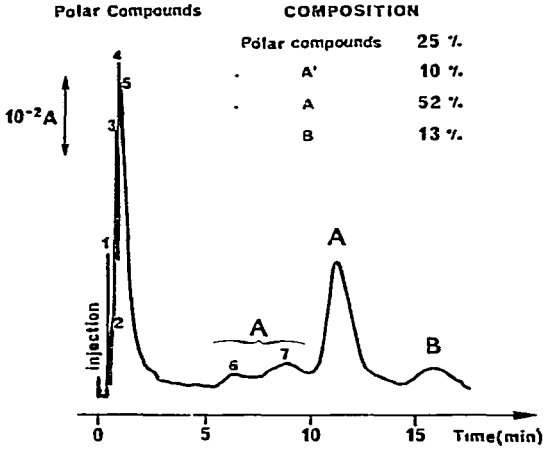

Fig 1 Analytical chromatogram of a polyenic antibiotic sample. Column, 15 cm × 1/4 in I D ; stationary phase, LiChrosorb RP-8, 10 μm; mobile phase, methanol–water (64:36); flow-rate, 120 ml h^{-1}; UV detection at 380 nm; injection, 1 μl of a 4 mg ml^{-1} solution in DMSO.

The conversion of the separation to the semi-preparative scale can be effected as follows.

Definition of the fractions: as the analytical chromatogram shows, it does not seem feasable to obtain the nine components of the mixture in high purity. Moreover, only components A and B seem to have interesting pharmaceutical properties. Thus the effluent fractions were defined as follows:

first fraction: the group of five polar components;

second fraction: the group called A′, eluted just before A, and consisting of two components;

third fraction: pure compound A;

fourth fraction: pure compound B (it was known that B is the product of a photochemical transformation of A).

Purity: for each fraction defined above, a purity consistent with pharmacological checks or mass spectrometry is required.

Amount required: a few hundred milligrams.

Production rate. optimization of this criterion is unnecessary as the procedure will not be inserted in a production line.

Recovery ratio: the fermentation product at our disposal is not limited in quantity, so we did not attempt to optimize this criterion at the expense of the others.

The constraints we met with in optimizing this separation are technological, chemical and physical in nature. The size of the column chosen was governed by the size of our available slurry packing reservoir. This column constraint often occurs in semi-preparative chromatography, when the casual need for a separation makes the purchase of a more suitable new column unprofitable.

With a preparative aim, the chromatographer also continuously meets a constraint of a chemical nature, *viz.*, the solubility of the mixture in the eluent. The antibiotic solubility is very poor in the methanol–water mixtures used as mobile phase in reversed-phase liquid chromatography. If we consider the molecular structure of A, we can expect low solubility in polar solvents owing to its polyenic chain and a low solubility in non-polar solvents also, owing to its polyol chain. These predictions are unfortunately verified by experiment: the best solvent is dimethyl sulphoxide (DMSO) but the solubility of A in it is only 8 $mg \cdot ml^{-1}$. In all other solvents tested, the solubility is lower than 1 $mg \cdot ml^{-1}$.

With stationary phases consisting of non-polar bonded silicas, the column lifetime often depends on the pressure drop. Such columns should not be operated at a pressure much higher than 100 bar. This physical constraint occurs in setting the flow-rate.

Finally, the detection method must be non-destructive. With UV spectrophotometric detection we have to check that the photochemical transformation of A into B does not take place at the wavelength chosen (400 nm).

Optimization of the mobile phase

In order to select the best mobile phase, the criteria involved are the resolution between A and B (related to the purity) and the separation time (related to the production rate). The constraints involved are the pressure drop and the solubility.

To enhance the sample solubility, ternary eluents such as methanol–water–DMSO were studied. Table II shows that an increase in the DMSO content gives a satisfactory improvement in solubility, but it also rapidly leads to too high an inlet pressure and too poor a resolution. Moreover, at a constant DMSO content, an increase in the water content causes the resolution to rise, but the pressure drop and the separation time remain too high. The best mobile phase seems to be methanol–water–DMSO (65:30:5).

Optimization of the injection

We have studied the influence of the volume, concentration and amount in-

TABLE II
MOBILE PHASE OPTIMIZATION

Composition of mobile phase. methanol–water–DMSO	*Pressure drop (bar)*	*Resolution*	*Separation time (min)*	*Solubility*
65:35 0	70	2	85	Very low
65:34·1	75	1	85	Low
65 30:5	95	0 9	80	Medium
65·27:8	120	0.6	60	Good
62:30·8	150	1 2	130	Good

jected on the quality of the separation. The sample was first dissolved in pure DMSO. Although the antibiotic solubility in this solvent is 8 $mg \cdot ml^{-1}$, it takes a long time to obtain solutions with concentrations greater than about 4 $mg \cdot ml^{-1}$, and such solutions need to be filtered. Alternatively, there is no way of injecting a large volume of a more dilute solution, because a large volume of DMSO leads to a sudden pressure surge at the time of injection that takes several minutes to disappear; the high viscosity of DMSO is responsible for this effect. The best concentration is about 4 $mg \cdot ml^{-1}$. Thus, above this value the solubility constraint and below this value the pressure drop constraint are involved.

If a linear behaviour of the chromatographic system is assumed[5], the maximum volume that can be injected in order to maintain a total separation of A and B is given by the relationship

$$V_{0\,lim} = V_R\,(B) - V_R\,(A) - 2\,[\sigma\,(A) + \sigma\,(B)]$$

where V_R and σ are the retention volumes and the standard deviations of the analytical peaks, respectively. With V_R (A) = 130 ml, σ (A) = 3.6 ml, V_R (B) = 174 ml and σ (B) = 4.3 ml a maximum volume of 28 ml can be injected. According to the standard deviation values, both close to 4 ml, we can also predict the following results: with injected volumes less than 4 ml the injection is seen by the column as a concentration impulse and the resolution remains constant, and with injected volumes varying from 4 to 28 ml the injection is seen as a pulse and the resolution decreases.

We injected solutions at a concentration of 4 $mg \cdot ml^{-1}$ and found that the recovery ratio of A decreases on increasing the volume above 4 ml. This decrease cannot be ascribed to the non-linearity of the solute distribution isotherms between the two phases, as the retention volumes of A and B remain constant. It can rather be explained by the fact that the analytical peaks are not gaussian in shape and by a profile broadening which results from the injection of this high-viscosity solvent.

The best amount injected was concluded to be about 16 mg, which represents 0.9 $mg \cdot g^{-1}$ of stationary phase.

Mass balance of the preparative injections

The resulting preparative chromatogram is shown in Fig. 2. In spite of the increased wavelength and the decreased pathlength, the response of the detector is non-linear. Consequently, the apparent resolution between A and B is low.

According to the definition of the fractions given at above, fraction 1 is the polar fraction, fraction 2 is fraction A′, fractions 5 and 8 are pooled in a single fraction named "pure A", fractions 6 and 7 in a fraction named "very pure A" and fractions 10 and 11 in a fraction named "B". Other fractions are discarded.

Table III gives the mass balance of the various preparative injections. We recovered two fractions of A of different purity, and an additional fraction by washing the column every tenth injection with methanol–DMSO (95:5). This last fraction contains the non-polar components of the sample and is called "non-polar" hereafter. These components cannot be detected by analytical chromatography because they are fixed at the top of the column. If a constant specific absorptivity coefficient is assumed for all the compounds, the mass of each fraction can be calculated by measuring its volume and its absorbance. Conversely, if the mass of each fraction is known, the

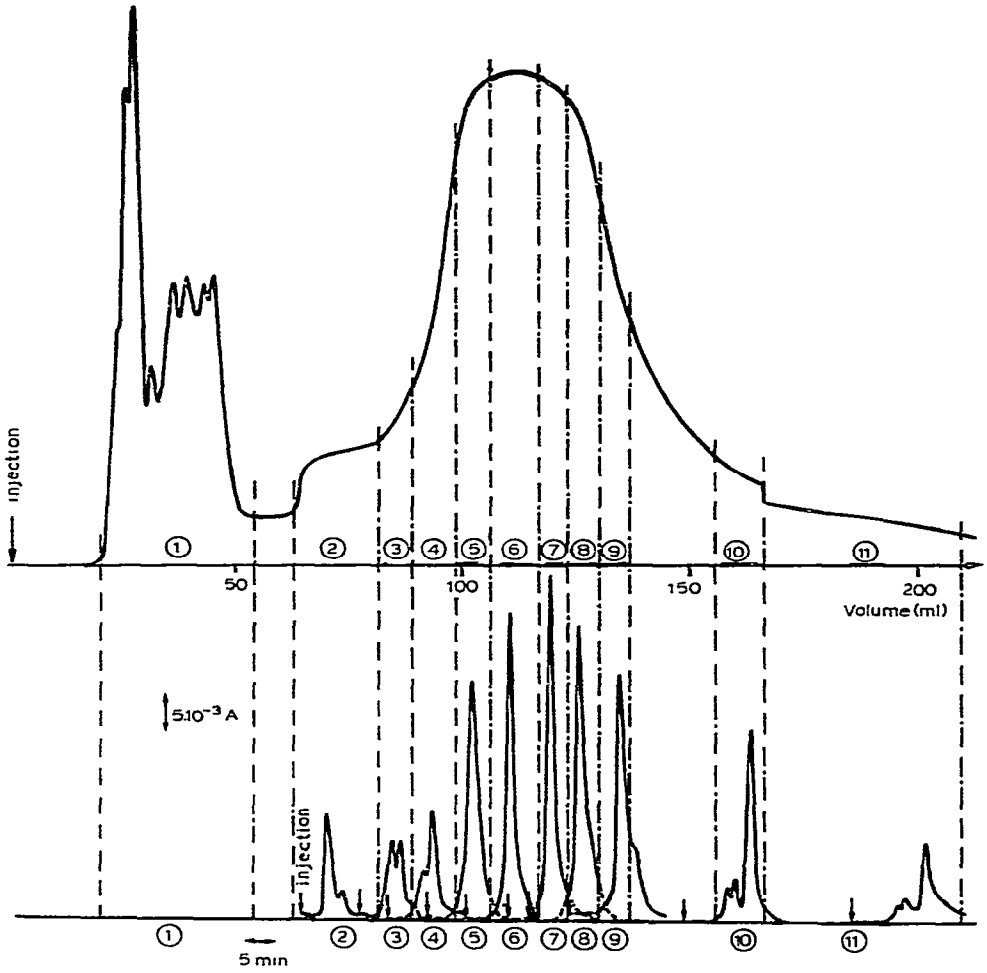

Fig 2. Preparative chromatogram and analytical checks. Operating conditions see Table II.

TABLE III
MASS BALANCE OF THE VARIOUS FRACTIONS ISOLATED BY PREPARATIVE LIQUID CHROMATOGRAPHY

Fraction	*Volume (ml)*	*Calculated amount* (mg)*	*Amount recovered (mg)*	*Specific absorptivity coefficient**, 388 nm ($cm^{-1} \cdot g^{-1} \cdot l$)*
Polar group	375	48	152	11
A′	282	15	17	31
Pure A	800	123	99	43
Very pure A	124	18	21	29
B	1010	27	159	6
Non-polar	150	7	70	3 7

* Assuming all the fractions have the same specific absorptivity coefficient as the initial mixture (ε = 35 $cm^{-1} \cdot g^{-1} \cdot l$ at 388 nm).

** Calculated from the absorbance and the mass of each fraction

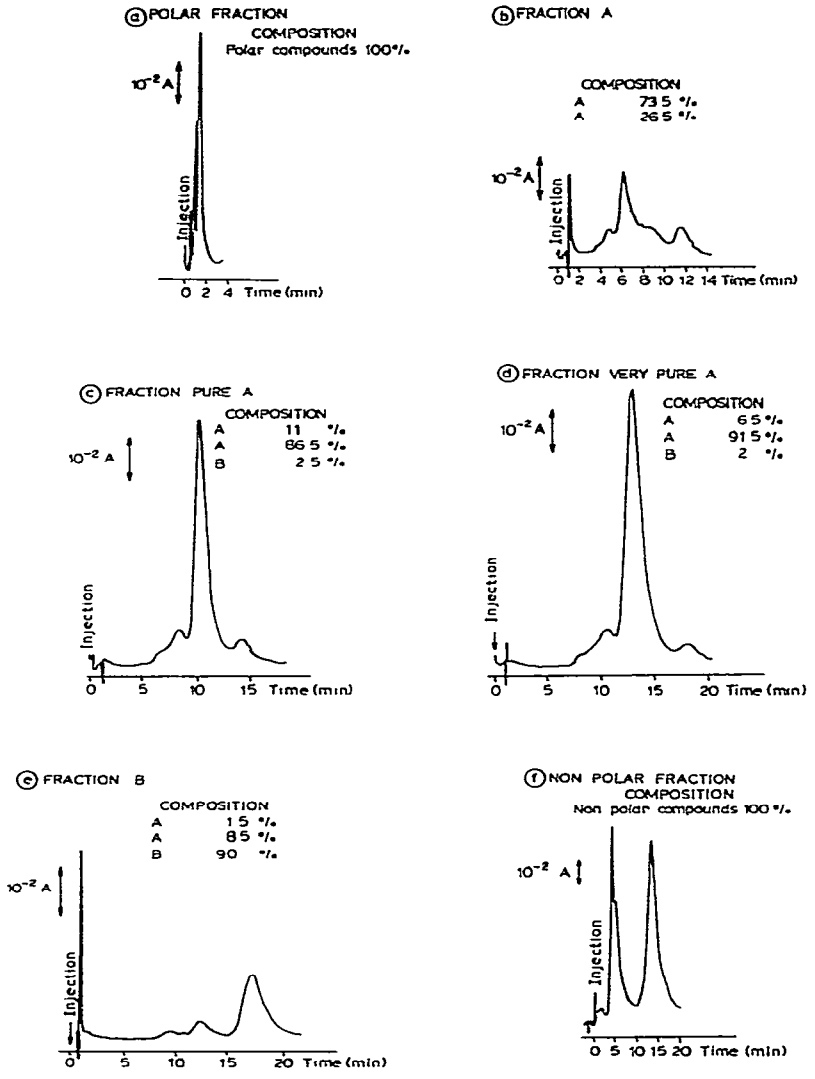

Fig 3. Analytical chromatograms of the recovered fractions Operating conditions as in Fig 1 except volume injected, (a) 30 μl, (b) 45 μl, (c) 20 μl, (d) 20 μl, (e) 80 μl, (f) 50 μl; mobile phase, (f) methanol–water (90.10).

specific absorptivity coefficient of each compound can be calculated. The results are given in Table III.

Then, for each fraction so recovered, a quantitative analysis can be carried out by liquid chromatography with the operating conditions given above (Fig. 3a–e). The non-polar fraction (Fig. 3f) requires methanol–water (90:10). Fig. 3f shows three compounds that have not been identified so far.

To conclude, the percentage composition of the initial sample can now be corrected with the data for the specific absorptivity coefficients:

Polar compounds	31%
A′	5%
A	21%
B	28%
Non-polar compounds	15%

The percentage of non-polar compounds was derived from the mass balance of the preparative injections. It is worth pointing out that the antibiotic seems to contain less of its active component A than expected. The amount of purified materials recovered was 518 mg and the recovery ratio was 94%.

All of this material was subjected to further investigations by mass spectrometry and other physico-chemical and pharmaceutical tests; the results will be reported elsewhere.

CONCLUSION

We have shown that semi-preparative liquid chromatography is suitable for the separation of a polyenic antibiotic from a complex mixture obtained by fermentation. Difficulties result from the very low solubility in most solvents and a general strategy has been devised for such cases.

More generally, through the present example we have tried to demonstrate one of the numerous ways of approaching a preparative separation problem, particularly the decisive role played by the criteria and the constraints which should be carefully analysed before any attempt at optimization is made.

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