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Short communication

Some aspects of the purification of anthraquinone antibiotics by preparative reversed-phase liquid chromatography

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Abstract

Elloramycin was chosen as a representative anthraquinone antibiotic to determine the separation efficiency of preparative reversed-phase liquid chromatography with regard to dependency on sample solubility, sample solvent strength, sample concentration and particle size of the stationary phase. In the case of elloramycin, which shows moderate lipophilic properties, it was demonstrated that an optimal strength of the sample solvent avoided front elution and peak fronting, that a diluted sample led to a better separation, and that a small particle size like 10 μm resulted in a strong increase in the purity of the separated compound in contrast to low-cost 15–25-μm particles.

Keywords: Preparative chromatography; Pharmaceutical analysis; Solubility parameters; Anthraquinone; Antibiotics; Elloramycin

1. Introduction

The isolation of a pure antibiotic or another metabolite from a complex fermentation broth usually can be achieved only by the subsequent application of a combination of various separation steps such as polystyrene resin chromatography, organic solvent extraction, precipitation, adsorption and size-exclusion chromatography [1–3]. Pure compounds are needed both for structure elucidation and biological assays. To shorten this time-consuming isolation procedure on the one hand, and to obtain the compound in highest purity on the other hand, efficient preparative HPLC techniques are described [4,5].

Preparative HPLC separations of moderate lipophilic and lipophilic compounds such as anthraquinone antibiotics are mostly associated with problems in solubility of the sample in the mobile phase, and are caused by the necessity that the strength of the sample solvent should be nearly the same as the strength of the mobile phase to prevent front elution or unresolved peaks. Therefore, the solubility of the sample in the mobile phase is the most limiting factor for column loadability and product throughput.

As a representative compound, we have chosen the moderate lipophilic anthraquinone antibiotic elloramycin, whose structure is shown in Fig. 1, to determine the following parameters: first, the influence of organic solvents onto sample solubility, second, the influence of the sample solvent strength, third, the influence of the injected sample volume, and forth, the influence of the particle size of the stationary phase. Problems in preparative separations of other anthraquinone compounds are similar to those in the case of elloramycin.

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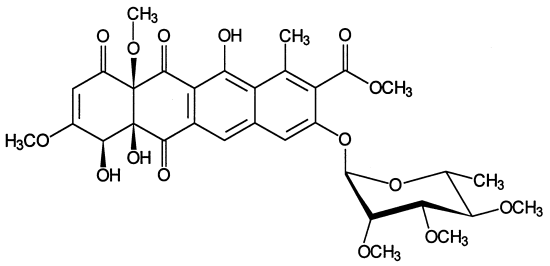

Fig. 1. Structure of elloramycin.

2. Experimental

2.1. Preparative HPLC equipment

The system consisted of two high-pressure pumps (Sepapress HPP-200/100; Kronwald), gradient unit (Sepacon GCU-311), and a Valco preparative injection valve (Model 6UW; VICI) with a 5-ml sample loop. The UV absorbance of the eluate was monitored at 288 nm by a Gilson spectrophotometer Model 116 equipped with a preparative flow cell (0.2 mm pathway, 0.7 μl volume). Fractions were collected manually according to the UV absorbance.

2.2. Preparative columns

A VarioPrep stainless steel column with dimensions of 250×21 mm I.D. was filled with Nucleosil-100 C_{18}, particle size 10 μm and 15–25 μm, respectively (Macherey-Nagel).

2.3. Mobile phases

The separations were done by isocratic elution with MeOH–water (70:30) at a flow-rate of 24 ml/min. Water was purified by means of a Milli-Q system (Millipore).

2.4. Elloramycin preparation

Elloramycin was isolated from the fermentation broth of *Streptomyces olivaceus* Tü 2353 by multiple sheet filtration, chromatography of the culture filtrate on Amberlite XAD-16, ethyl acetate extraction, silica gel chromatography and Sephadex LH-20 chromatography [6]. The elloramycin content of this prepurified batch was 96%, determined by HPLC in comparison with a pure reference sample as external standard.

A raw product with a defined elloramycin content of 20% was prepared from the culture filtrate by Amberlite XAD-16 chromatography and lyophilization.

3. Results and discussion

3.1. Influence of various organic solvents on the sample solubility

The solvent in which the sample is dissolved shows two limiting factors in preparative HPLC. First, the polarity of the sample solvent must have a similar polarity than the eluent to prevent front elution. Second, the solubility of the sample is a restrictive parameter to column loadability and therefore, limiting to sample overload in the preparative mode and sample throughput per time. Table 1 shows the dependence on the solubility of elloramycin from the nature of the organic solvent. It was an astonishing result, that solvents commonly used for anthraquinone antibiotics, such as alcohols showed the worst solubility for elloramycin. Compared to alcohols, acetonitrile, tetrahydrofuran and dimethyl sulfoxide showed a more than 10-fold higher solubility. The dipole and proton acceptor parameters of the solvent are the critical parameters which influence the solubility of elloramycin. A concentration in the range of 100 mg/ml which could be achieved using these solvents is an im-

Table 1
Maximal solubility of elloramycin in organic solvents

Solvent	P'	x_e	x_d	x_n	Elloramycin (mg per ml solvent)
Methanol	5.1	0.48	0.22	0.31	6.5
Ethanol	4.3	0.52	0.19	0.29	8.7
n-Propanol	4.0	0.54	0.19	0.27	9.7
Acetonitrile	5.8	0.31	0.27	0.42	118
Dioxan	4.8	0.36	0.24	0.40	79.5
Tetrahydrofuran	4.0	0.38	0.20	0.42	113.5
Dimethyl sulfoxide	7.2	0.39	0.23	0.39	117

Data from Meyer [7]: P'=solvent polarity parameter, x_e=solvent proton acceptor parameter, x_d=solvent proton donor parameter, x_n=solvent dipole parameter.

portant precondition for an efficient preparative separation.

3.2. Influence of the strength of sample solvent

The following experiment should give an answer as to whether or not the strength of the sample solvent influences the retention behaviour and peak symmetry of elloramycin, using a mobile phase of methanol–water (7:3). Ten mg of prepurified elloramycin (96%) were dissolved in 2 ml of the organic solvents listed in Table 1. This concentration guaranteed a complete dissolved sample. Methanol, acetonitrile and dimethyl sulfoxide showed a retarded, but a strong fronting elloramycin peak, whereas ethanol, propanol, dioxane and tetrahydrofuran resulted in a doubled elloramycin peak, one as a front-eluted and a second as a retarded peak. The best separation was obtained by a mixture of acetonitrile–water (1:1) as sample solvent, that led to a sharp retarded elloramycin peak. Fig. 2 shows the comparison of retention behaviour of the prepurified elloramycin sample using pure acetonitrile to acetonitrile–water (1:1). The addition of water to the diverse sample solvents up to a ratio of 75% prevented both, peak fronting and front elution. This result indicates the need to optimize the solubility parameters with reference to the strength of the sample solvent or mixtures to obtain a similar separation in the preparative as in the analytical mode.

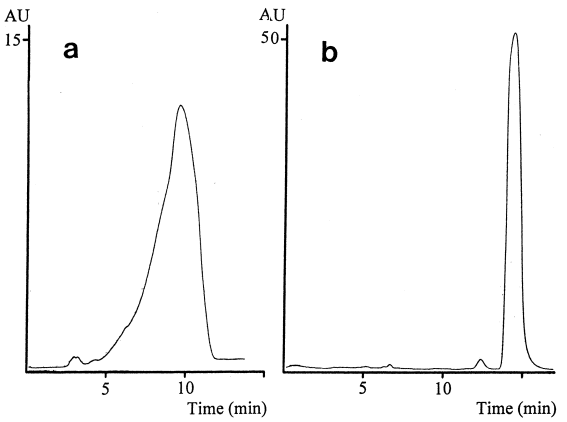

Fig. 2. Influence of the strength of the sample solvent on separation efficiency, (a) MeCN, (b) MeCN–water (1:1). Load: 10 mg prepurified elloramycin (96%) dissolved in 2 ml of sample solvent. Matrix: 10 μm Nucleosil C_{18}. Eluent: MeOH–water (7:3), flow-rate: 24 ml/min.

3.3. Influence of the injected sample volume

Another question was, if a small or a large sample volume will be more preferable for a preparative separation. A 100 mg-sample of prepurified elloramycin (96%) was dissolved in 2 ml and 4 ml, respectively, in the optimal solvent mixture acetonitrile–water (1:1). The result of this experiment indicated, that a larger sample volume and therefore a higher dilution of a sample showed a better resolution than the injection of a small volume with a concentrated sample (graphs not shown).

3.4. Influence of the particle size to separation efficiency and sample recovery

A further important question was, is there a need for expensive 10-μm particles in preparative separations or have low-cost 15–25-μm particles of the same efficiency in the case of column overload? In Fig. 3 is shown the comparison between a 10-μm and a 15–25-μm batch of the same reversed-phase material and identical chromatographic conditions regarding their efficiency in the separation of a raw product with an elloramycin content of 20%. The result indicates impressively that a small and homogenous particle size is superior to the larger and nonhomogenous so-called ‘preparative’ particles, even in the case of column overload. This result is in agreement with an earlier report [5], and contradicts ‘common wisdom’, that the use of small particles, e.g., 7 μm is only of advantage in the case of a low sample load, and that under heavily overloaded column conditions, larger particles such as 30 μm are preferable [8].

Not only the resolution, but also the purity and recovery of the separated compound resulted in a dramatic increase using small and homogenous particles, as is summarized in Table 2. A 100% purity of the elloramycin fraction was obtained exclusively by using 10-μm particles, both in the case of separation of the prepurified product having a content of 96%, and the raw product with a content of 20%. Using 15–25-μm particles, the purity of the

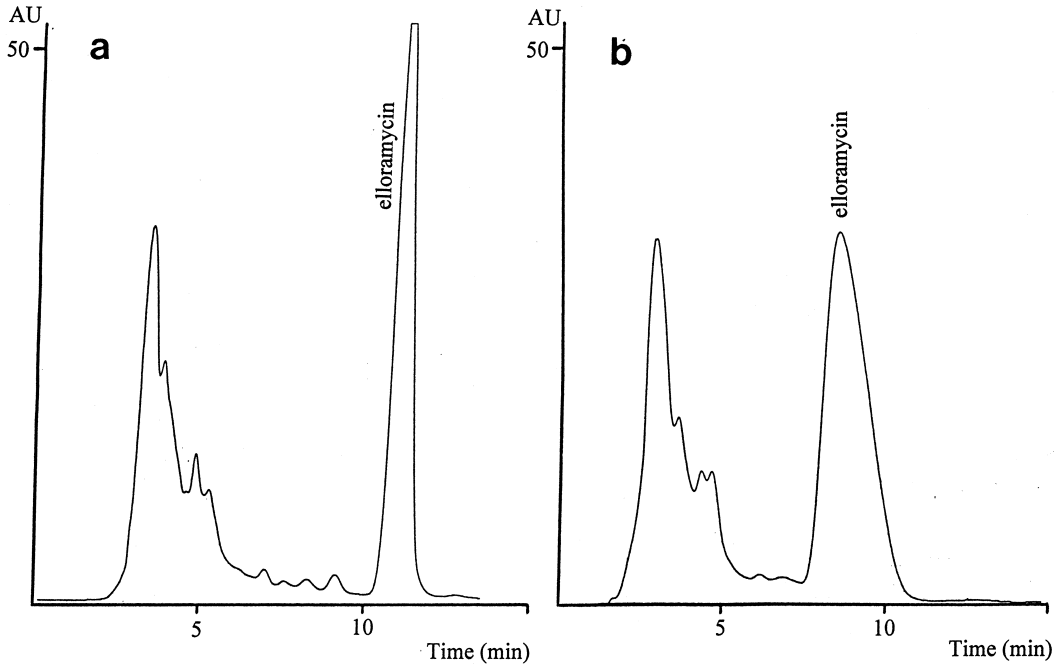

Fig. 3. Influence of the particle size on the separation efficiency, (a) 10-μm Nucleosil C_{18}, (b) 15–25-μm Nucleosil C_{18}. Load: 100 mg elloramycin raw product (20%) dissolved in 4 ml of MeCN–water (1:1). Eluent: MeOH–water (7:3), flow-rate: 24 ml/min.

96% product could be slightly increased to an elloramycin content of 97%, whereas injecting the raw product the elloramycin content increased from 20% only up to 80%.

The loss of elloramycin in the main fractions was substantial, as well as in the case of 10 μm as 15–25-μm particles, as shown in Table 2. However, the recovery can be increased to about 100% in a second step by rechromatography of the side fractions of the collected main peak.

The results indicate further, that the time-consuming procedure of a conventional isolation scheme, like subsequent organic solvent extraction, adsorption and size-exclusion chromatography can be shor-

Table 2
Purity and recovery with regard to dependence on the particle size. Comparison of elloramycin isolation from a prepurified and from a raw product

	Dry mass (mg)	Elloramycin content (mg)	Purity	Recovery (%)
10-μm particles				
Prepurified product	20.83	20.0	96%	
Elloramycin fraction	18.6	18.6	100%	93
Raw product	100	20.0	20%	
Elloramycin fraction	13.7	13.7	100%	69
15–25-μm particles				
Prepurified product	20.83	20.0	96%	
Elloramycin fraction	19.8	19.2	97%	92
Raw product	100	20.0	20%	
Elloramycin fraction	21	16.7	80%	83

tened very efficiently by replacing all these steps with preparative reversed-phase HPLC using 10-μm particles, which resulted in an elloramycin fraction of highest purity.

4. Conclusions

The efficiency of preparative HPLC separations can be increased enormously by choosing an optimal organic solvent or a mixture of solvents to dissolve a maximal amount of the sample, and by optimizing the strength of the sample solvent to prevent front elution and peak fronting.

The injection of a diluted sample onto the preparative column is more favourable and led to a better resolution of the separated compounds in comparison with injection of a concentrated sample.

The particle size of the stationary phase has a strong influence on the separation efficiency, especially on the purity of the separated compounds. The use of small and homogenous particles has an impressive advantage towards low-cost, so-called 'preparative' particles, even under heavily overloaded column conditions.

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