Computer-assisted rapid development of gradient high-performance liquid chromatographic methods for the analysis of antibiotics

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Abstract

Computer simulation by DryLab G/plus has proved to be an invaluable tool in the rapid development of analytical HPLC methods for antibiotics. A glycopeptide antibiotic under study at this Research Center was taken into consideration as a case study. Retention data from two preliminary experiments have allowed us to perform several simulations which greatly shortened the time normally required for the identification of the optimum gradient conditions. The "key steps" in the simulation process have been experimentally verified and a more than satisfactory agreement between calculated and experimental retention times was consistently found.

This article presents the preliminary results obtained and proposes an HPLC method for the analysis of a glycopeptide antibiotic.

Because of its general approach, the DryLab concept can be extended to any class of chemical compounds not only shortening impressively the HPLC method development time, but also making it possible to adjust/develop a method which takes into account the typical features of the instrument to be used. This last aspect means, among other things, that: (1) HPLC methods already optimized can be transferred from one laboratory to another, differently equipped, without any "local" further adjustment or development work and (2) HPLC methods can be optimized "at a distance" for other laboratories once a few instrumental parameters have been determined and two exploratory runs have been carried out.

1. Introduction

Glycopeptide antibiotics are a family of antibiotics which have recently acquired relevant clinical importance as a result of a specific and high pharmacological activity against many Gram-positive pathogens and highly gentamicin-resistant Enterococci. Among the members of this family, teicoplanin represents the last member introduced into clinical practice [1-10].

Glycopeptide antibiotics are usually (i) char-acterized by a highly modified sugar-containing heptapeptidic structure and are
(ii) naturally produced by fermentation where they occur as mixtures, called complexes, of closely chemically related substances or factors.

Because of the intrinsic structural complexity and the large number of components normally constituting these mixtures, the development of a reliable and robust analytical method is normally a challenging and time-consuming task.

In spite of protracted development efforts, the

final chromatogram often provides inadequate overall resolution. An example which supports this statement is the HPLC chromatogram (Fig. 1) of a glycopeptide antibiotic complex currently under study at this Research Center, which has shown pharmacological activity against aerobic and anaerobic Gram-positive microorganisms.

The chromatogram shown in Fig. 1, shows bands which are poorly resolved in spite of a gradient time (t_g) of 45 min. Resolution, in particular, is very low for the bands which elute close to the main peak, or factor B of the complex. Chromatograms shown later in this article will in fact demonstrate that close to the main peak several other factors of the complex are eluted (e.g., factor B1, demannosyl factor B, etc.) for which separation and quantification are essential for a correct definition of the quantitative HPLC profile.

The achievement of high chromatographic resolution is a prerequisite that cannot be disregarded particularly when dealing with complex

mixtures of natural substances such as the glycopeptide antibiotics. In fact the chromatograms, usually quite confused, are often further complicated by the onset of new peaks related to new chemical substances synthesized by the strains as a result of even slight changes in the fermentation conditions.

Recent reports in the literature have shown the successful applicability of the predictive software DryLab G/plus [11] to the rapid development of reliable and robust HPLC methods concerning complex mixtures of natural substances [12,13].

On the basis of the previous considerations, and (i) the task of our laboratory being the development of analytical methods to be used at both the development and production levels, and (ii) the HPLC method available for the antibiotic under evaluation, still used at the time this study was started, being unsatisfactory, we have chosen DryLab G/plus to rapidly develop and optimize an HPLC method for the glycopeptide antibiotic under study.
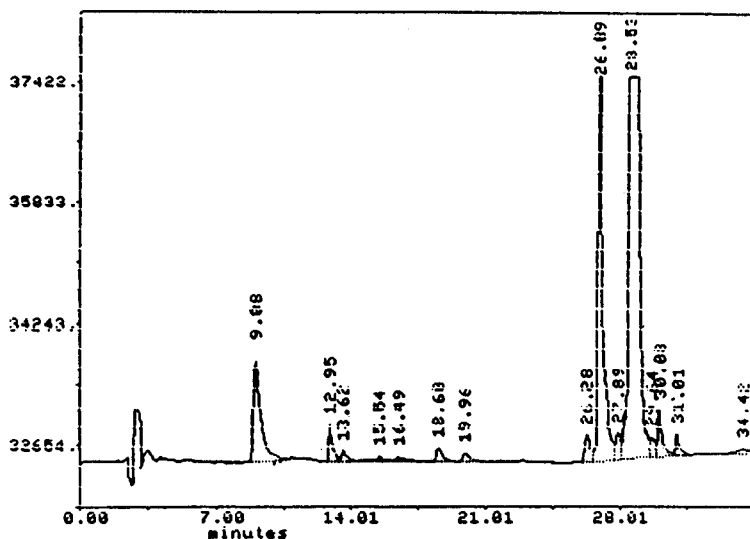

Fig. 1. HPLC chromatogram of the gycopeptide antibiotic under evaluation (batch 1) available at the beginning of this study. Instrument, Hewlett-Packard Model 1084; column, Bakerbond C_8 (250 × 4.6 mm); mobile phase A, NaH_2PO_4 20 mM-CH_3CN (95:5, v/v); mobile phase B, NaH_2PO_4 20 mM-CH_3CN (25:75, v/v); gradient profile: %B (t, min): 10 (0), 30 (10), 30 (20), 65 (30), 90 (40), 90 (45), 10 (48); flow-rate, 1 ml/min.

2. Experimental

Conditions: instrument, liquid chromatograph Model 1090A (Hewlett-Packard); column, Hypersil ODS 5 μm (Shandon) 250 mm $\times$ 4.6 mm I.D.; mobile phase A, phosphate buffer 12.5 mM pH 7.8–CH_3CN (90:10, v/v); mobile phase B, phosphate buffer 12.5 mM pH 7.8–CH_3CN (50:50, v/v). Gradient profile: at 0 min, 16% B; at 5 min, 37% B; at 15 min, 45% B; at 19.3 min, 45% B; at 21.3 min, 60% B; at 26.3 min, 70% B. Flow-rate, 1 ml/min; detector, UV diode array; plotted wavelength, 254 nm; injected volume, 10 μl; temperature, 22°C. *Sample preparation:* a solution, the concentration of which was about 1 mg/ml, was prepared by dissolving the sample in mobile phase A. No further pH adjustments were required, while 15 min sonication were, at least, necessary. *Phosphate buffer (12.5 mM, pH 7.8) solution preparation:* 2 l of 12.5 mM pH 7.8 phosphate buffer were prepared by diluting with water 870 ml of a 25 mM solution of Na_2HPO_4 to which were added 130 ml of a 25 mM solution of NaH_2PO_4. The two mother solutions were prepared as follows: 25 mM Na_2HPO_4: 17.92 g of $Na_2HPO_4 \cdot 12$ H_2O to 2 l with water; 25 mM NaH_2PO_4: 6.9 g of $NaH_2PO_4 \cdot H_2O$ to 2 l with water. Acetonitrile used was of HPLC grade, water was from Milli-Q and phosphate salts were of analytical-reagent grade.

2.1. Instrumentation

The HPLC system used for this study was a Hewlett-Packard (Palo Alto, CA, USA) Model 1090A liquid chromatograph equipped with a UV photodiode array detector. Although chromatograms were monitored at 254, 280 and 350 nm with a sampling interval of 320 ms, the plotted wavelength is always 254 nm. Data were collected and integrated on a Hewlett-Packard Model 79994A analytical workstation and plotted on a Hewlett-Packard Model 7470A plotter.

DryLab G/plus software (LC Resources, Lafayette, CA, USA; in Europe: Molnar Institute for Applied Chromatography, Berlin, Germany) was operated on an IBM PS/2 Model 57 SX personal computer equipped with a Hewlett-Packard (Avondale, CA, USA) Laser Jet Model IIIp PCL5 printer.

2.2. Reagents and materials

The batches numbered 1 and 2 of the antibiotic under study were obtained from our Chemical Development Department (Lepetit Research Center). All the solvents used during this study were of HPLC grade (Carlo Erba, Milan, Italy) while the salts were reagents of analytical grade (Carlo Erba). Water was obtained from a Milli-Q purification system (Millipore, USA).

All the chromatograms were obtained by using a 250 $\times$ 4.6 mm I.D. Hypersil ODS 5 μm (Shandon Scientific, Runcorn, UK).

2.3. Chromatographic parameters of the analytical system

Beside some more basic chromatographic parameters (e.g., column length, column internal diameter, flow-rate, etc.) the DryLab G/plus software also requires as input data: dwell (or gradient delay) volume, extra-column band broadening and efficiency of the column.

The dwell (or gradient delay) volume of the equipment [14] was measured by running a 10-min gradient from CH_3CN–(CH_3CN + 0.2%, v/v, acetone) (90:10, v/v) to CH_3CN–(CH_3CN + 0.2%, v/v, acetone) (10:90, v/v) without the column and was shown to be $V_D = 0.6$ ml (vs. a 0.3–0.5 ml value for V_D as measured by the Manufacturer —courtesy of Hewlett-Packard).

Extra-column band broadening, σ_{ex}, was measured by injecting, without column and with MeOH–water (60:40, v/v) as mobile phase, 5 μl of a solution containing 10 μl of toluene in 10 ml of methanol at different flow-rates (i.e., 0.9, 1,

1.1, 1.2 ml/min). The σ_{ex} value used for DryLab G/plus simulations was that determined by extrapolation of previous experimental data at zero flow-rate and was shown to be: $\sigma_{ex} = 59$ μl.

Despite the very well known limitations of the approach used for σ_{ex} determination, the good agreement between simulated and experimental data, consistently found in the course of this study, has implicitly confirmed the validity of the value of 59 μl obtained.

The efficiency of the chromatographic column was determined by measuring the number of theoretical plates corresponding to the toluene peak. The theoretical plate number was calculated by using the classical expression $N = 5.54(t_R/w_{1/2})^2$ where t_R and $w_{1/2}$ indicate, respectively, the retention time and the width at half height of the toluene peak. In practice, efficiency was measured by injecting 10 μl of a solution containing 10 μl of toluene in 10 ml of methanol and using MeOH–water (60:40, v/v) as mobile phase. The flow-rate was 0.5 ml/min. Under these conditions an average value of 5000 theoretical plates was obtained. As was the case for extra-column band-broadening determination, the approach for measuring the column efficiency could also be a matter of opinion. However, as previously noted, the good agreement consistently found during this study between simulated and experimental data has implicitly confirmed the correctness of the efficiency value used. For the purposes of the simulation, an estimate of a suitable value for N can also be made by simply comparing the shape of the two initial exploratory chromatograms and how they look when represented by DryLab G/plus. The N value can then be changed with respect to the default value ($N = 10\ 000$) so that experimental and DryLab chromatograms will look nearly the same.

For X = ratio of the volume of mobile phase outside the pores to the total volume of mobile phase, Y = ratio of solute diffusion coefficients inside and outside the pores, A = Knox parameter (sometimes called the "multipath term") which describes the effects of eddies and voids within the column packing and which value depends primarily on how well a particular column was packed, values are required for

computer band-width predictions; the following data were respectively used: $X = 0.75$, $Y = 0.40$ and $A = 0.80$.

2.4. Chromatographic conditions and sample preparation

On the basis of previous experience, suitable HPLC solvents were prepared to run the preliminary separations. Mobile phase A was phosphate buffer 12.5 mM pH 7.84–CH$_3$CN (90:10, v/v) while mobile phase B was phosphate buffer 12.5 mM pH 7.84–CH$_3$CN (50:50, v/v). The Na$_2$HPO$_4$–NaH$_2$PO$_4$ buffer solution used for mobile phases preparation was that according to Gomori [14] and Sørensen [15,16], i.e., known amounts of a 25 mM Na$_2$HPO$_4$ solution and of a 25 mM NaH$_2$PO$_4$ solution were mixed and diluted to reach the desired pH of 7.8.

Other preliminary trials performed by using mobile phase B containing progressively increasing amounts of CH$_3$CN have not eluted further peaks from the column.

The glycopeptide antibiotic samples were dissolved in mobile phase A. All the experiments were carried out by injecting 10 μl of a sample solution with an average concentration of ca. 1 mg/ml (equivalent to ca. 6 nmol directly injected).

3. Results and discussion

As the first step in the method development process, two linear gradients characterized by different steepnesses (i.e., $t_g = 20$ min and $t_g = 60$ min) were run varying the percentage of component B of the mobile phase in the range 0–100% and using batch 1 as a sample.

The two chromatograms shown in Figs. 2a and b displayed the same peak sequence and this made an easy identification of twelve bands possible which were used as a starting set for carrying out the optimization work. The retention data for the twelve bands selected in the two initial runs and the area values (those which were measured in the run with $t_g = 60$ min) are summarized in Table 1.
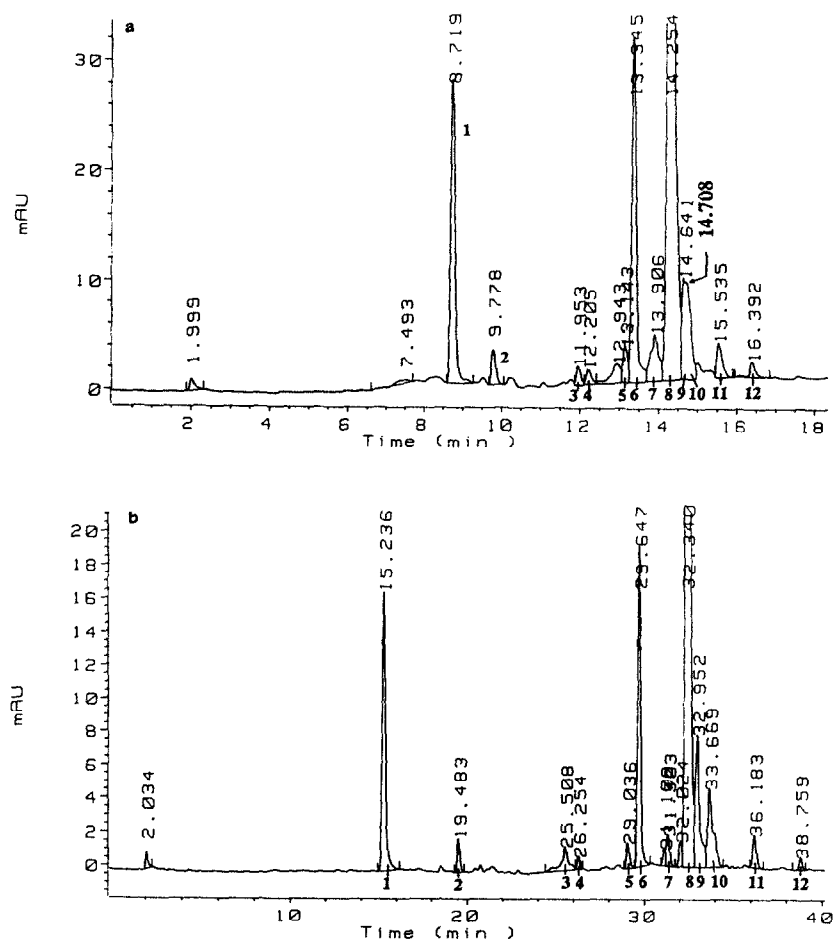
To begin the optimization process the data of

Fig. 2. (a) First exploratory HPLC chromatogram, $t_g = 20$ min, of batch 1. Column, Hypersil ODS 5 μm (250 × 4.6 mm); mobile phase A, phosphate buffer 12.5 mM pH 7.8–CH$_3$CN (90:10, v/v); mobile phase B, phosphate buffer 12.5 mM pH 7.8–CH$_3$CN (50:50, v/v); gradient profile: %B (t, min): 0 (0), 100 (20), 0 (25), 0 (35); flow-rate, 1 ml/min. (b) Second exploratory HPLC chromatogram, $t_g = 60$ min, of batch 1. Column, mobile phases and flow-rate as in (a); gradient profile: %B (t, min): 0 (0), 100 (60), 0 (65), 0 (75).

Table 1 and the chromatographic parameters of the analytical system (see the corresponding paragraph) were entered. On the basis of these input data the relative resolution map (RRM), calculated by DryLab G/plus, is depicted in Fig. 3. It clearly shows that (i) on the whole, a fairly acceptable resolution ($R_s \approx 1.4$), at least at the beginning, can be achieved at a gradient time of ca. 50 min, (ii) the resolution for bands 9 and 10 increases as the gradient time increases and (iii) the separation of the critical pair 8 and 9 can be improved by short and steep gradients.

As a direct consequence of the previous considerations a segmented gradient should be used to achieve a final successful separation.

As a starting point for the development of a segmented gradient the minimum gradient time for which the critical pair 5 and 6 (Fig. 3) shows $R_s \approx 1.4$ was chosen. This minimum gradient time is 23.8 min and it was automatically determined by DryLab G/plus starting from the gradient time corresponding to the maximum of the RRM shown in Fig. 3 and trimming off any waste time.

Table 1
Input retention data for DryLab G/plus simulations

Band	Run 1 (t_R, min)	Run 2 (t_R, min)	Area (counts)
1 (mannosyl aglycone)	8.72	15.24	224.63
2	9.78	19.48	22.36
3	11.95	22.51	34.29
4	12.21	26.25	12.48
5 (factor A1)	13.14	29.04	19.00
6 (factor A)	13.35	29.65	225.81
7	13.91	31.30	25.14
8 (factor B)	14.25	32.34	2133.94
9 (factor B1)	14.64	32.95	139.78
10	14.71	33.67	102.34
11	15.54	36.18	29.10
12	16.39	38.76	16.69

Run 1: $t_g = 20$ min; run 2: $t_g = 60$ min.

Table 2
Comparison between calculated (DryLab G/plus) and experimentally determined retention times when running the linear gradient from 15.6 to 63.2% mobile phase B in 23.8 min

Band	DryLab G (t_R, min)	Experimental (t_R, min)
1 (mannosyl aglycone)	7.30	6.84
2	9.77	9.50
3	14.67	14.56
4	15.28	15.17
5 (factor A1)	17.57	17.47
6 (factor A)	18.07	17.97
7	19.44	19.36
8 (factor B)	20.30	20.18
9 (factor B1)	20.90	20.69

$t_g = 23.8$ min; 15.6–63.2% B linear gradient.

The retention times of the twelve bands of interest, estimated by DryLab G/plus and those experimentally determined for the minimum gradient time, are compared in Table 2. The agreement between the two data sets is more than satisfactory. The examination of the experimental chromatogram, depicted in Fig. 4, still shows, however, an unresolved cluster of bands eluted after the main peak ($t_R = 20.18$ min) and characterized by an overall $\Delta t_R \approx 1.4$

min measured between the shoulder of band 10 and the main peak (or band 8). In order to resolve this cluster of bands better, several simulations using linear segmented gradients were attempted. The experimental chromatogram corresponding to the final simulation is shown in Fig. 5 and displays a $\Delta t_R \approx 2.5$ min between the peak eluted at 19.87 min (which corresponds to the shoulder of band 10 in Fig. 4) and the main peak ($t_R = 17.38$ min, Fig. 5). The
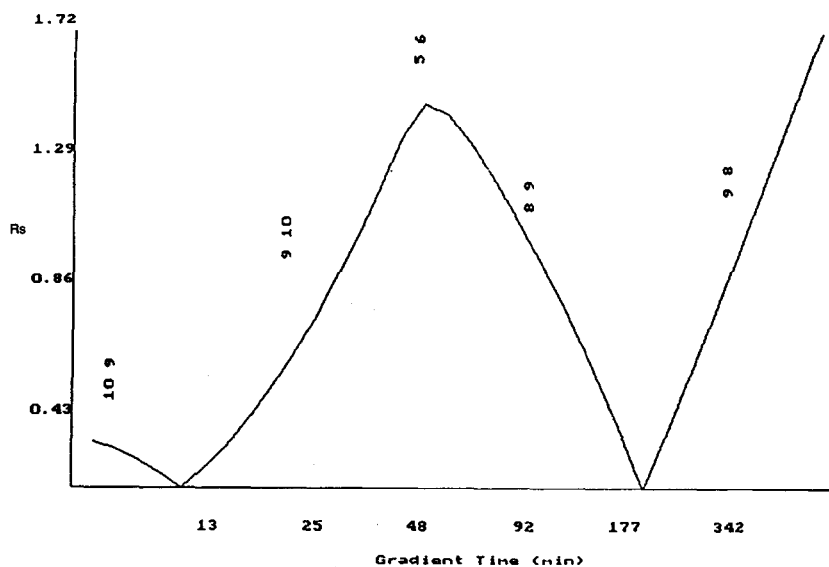

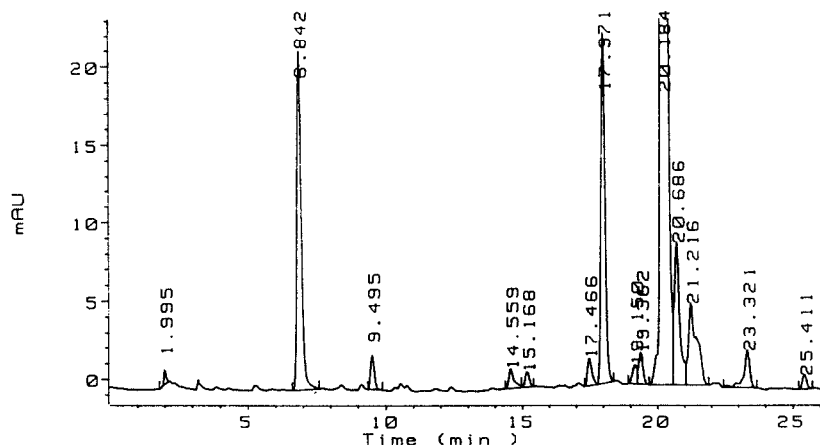Fig. 3. Relative resolution map (0 to 100% B gradient) calculated by DryLab G/plus with $N = 5000$.

Fig. 4. HPLC chromatogram corresponding to the minimum linear gradient time $t_g = 23.8$ min, automatically calculated by DryLab G/plus in correspondence with the maximum of the relative resolution map shown in Fig. 3. Sample, batch 1; column, Hypersil ODS 5 μm (250 × 4.6 mm); mobile phase A, phosphate buffer 12.5 mM pH 7.8–CH$_3$CN (90:10, v/v); mobile phase B, phosphate buffer 12.5 mM pH 7.8–CH$_3$CN (50:50, v/v); gradient profile: %B (t, min): 15.6 (0), 63.2 (23.8), 63.2 (23.8), 15.6 (30), 15.6 (35); flow-rate, 1 ml/min.

peak separated at $t_R = 19.87$ min in Fig. 5 is particularly important because it corresponds to the demannosyl derivative of factor B.

The calculated and experimentally determined retention times for the most relevant bands are shown in Table 3. Again good agreement between the two data sets was obtained.

The elution conditions, under which the chromatogram depicted in Fig. 5 was obtained, were

then slightly changed in order to partially smooth a variation on the baseline observed in the range $t_R \approx 20$–22.5 min and caused by the steepness of the gradient in that time interval.

This adjustment, which has increased the overall gradient time by 4 min [$t_g = 26.3$ min (Fig. 6) vs. $t_g = 22.3$ min (Fig. 5)], has however improved at 3.3 min the separation, Δt_R, between the peaks corresponding to factor B (main
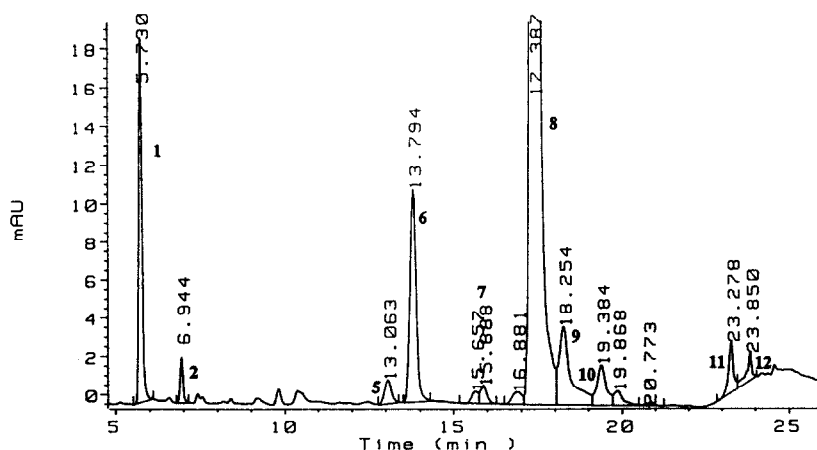

Fig. 5. HPLC chromatogram corresponding to the optimized linear segmented gradient: %B (t, min): 16 (0), 37 (5), 48 (16.6), 48 (19.3), 98 (22.3); column: Hypersil ODS 5 μm (250 × 4.6 mm); mobile phase A, phosphate buffer 12.5 mM pH 7.8–CH$_3$CN (90:10, v/v); mobile phase B, phosphate buffer 12.5 mM pH 7.8–CH$_3$CN (50:50, v/v); flow-rate, 1 ml/min.

Table 3
Comparison between calculated (DryLab G/plus) and experimentally determined retention times when running the optimized linear gradient: %B (t, min): 16 (0), 37 (5), 48 (16.6), 48 (19.3), 98 (22.3).

Band	DryLab G (t_R, min)	Experimental (t_R, min)
5 (factor A1)	13.89	13.06
6 (factor A)	14.61	13.79
7	16.69	15.89
8 (factor B)	18.11	17.38
9 (factor B1)	18.97	18.25
10	20.05	19.38
11	23.06	23.28
12	23.62	23.85

$t_g = 22.3$ min. Only the data regarding the most relevant bands are reported.

peak or band 8) and its demannosyl derivative ($t_R = 21.41$ min, Fig. 6). The chromatogram displayed by the batch 1 under the new conditions is plotted in Fig. 6. The gradients employed in Figs. 5 and 6 represent the last steps in this optimization process. The same gradient profile which was adopted in Fig. 6 was then used for the analysis of the batch 2, Fig. 7, allowing a clear identification of the peak due to the demannosyl B derivative and confirming the presence of a further peak which elutes at $t_R \approx$

20.7 min. The elution conditions under which the chromatograms reported in Figs. 6 and 7 were obtained, are summarized in the Appendix and they represent a proposed method for the HPLC analysis of glycopeptide antibiotic subject of this study.

4. Conclusions

DryLab G/plus software has proved to be a very practical and useful tool for shortening the time required to develop analytical HPLC methods including analysis of complicated mixtures of natural substances such as the glycopeptide antibiotics.

A method has been rapidly developed which allows a satisfactory chromatographic resolution of the most important factors of the complex glycopeptide antibiotic.

It must also be pointed out that the usefulness of the DryLab approach is even more evident if it is taken into account that (i) it does not refer to any definite class of chemical substances, (ii) once a few equipment parameters required for computer simulations are determined (i.e., dwell volume, extra-column band broadening, plate number of the chromatographic column) and if parts, when changed, are replaced with their
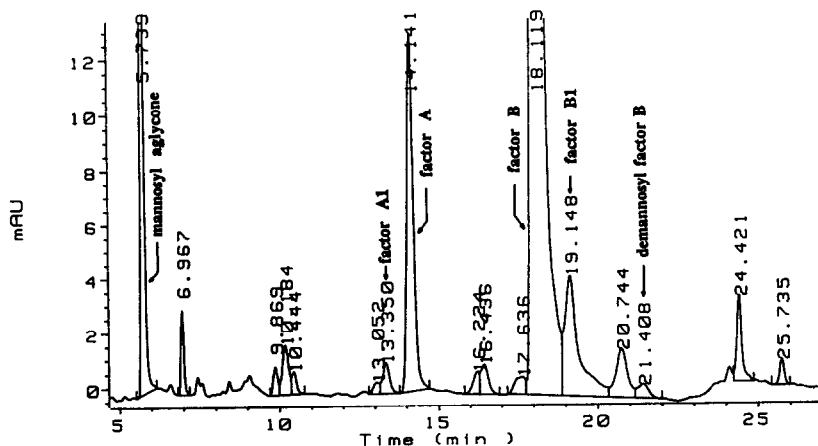

Fig. 6. HPLC chromatogram corresponding to the optimized linear segmented gradient: %B (t, min): 16 (0), 37 (5), 45 (15), 45 (19.3), 60 (21.3), 70 (26.3); sample, batch 1; column: Hypersil ODS 5 μm (250 × 4.6 mm); mobile phase A, phosphate buffer 12.5 mM pH 7.8–CH$_3$CN (90:10, v/v); mobile phase B, phosphate buffer 12.5 mM pH 7.8–CH$_3$CN (50:50, v/v); flow-rate, 1 ml/min.
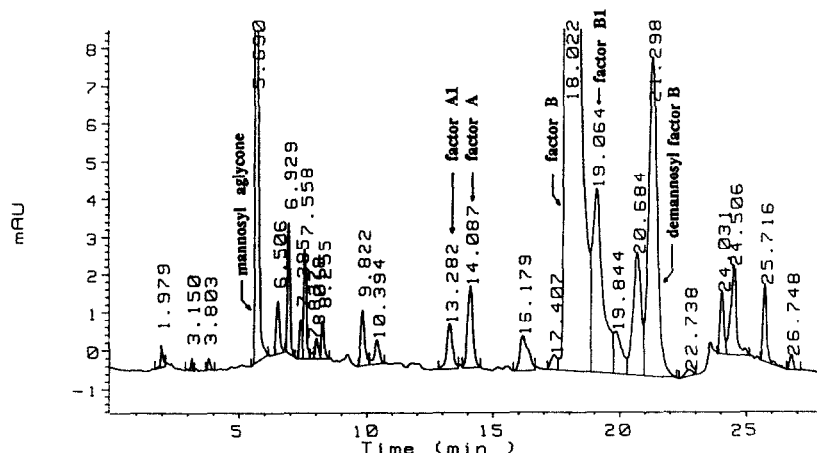
Fig. 7. HPLC chromatogram corresponding to the optimized linear segmented gradient: %B (t, min): 16 (0), 37 (5), 45 (15), 45 (19.3), 60 (21.3), 70 (26.3); sample, batch 2; column: Hypersil ODS 5 μm (250 × 4.6 mm); mobile phase A, phosphate buffer 12.5 mM pH 7.8–CH₃CN (90:10, v/v); mobile phase B, phosphate buffer 12.5 mM pH 7.8–CH₃CN (50:50, v/v); flow-rate, 1 ml/min.

equivalent, any further method developed on that instrument only requires two initial exploratory gradient runs (e.g., see those shown in Fig. 2a and b) [17], (iii) the RRM that can be easily calculated from the initial retention data shows not only the poorest-resolved band-pair, but also indicates the success probabilities that the elution conditions chosen will have, and (iv) any HPLC gradient method developed on a certain instrument can easily be adjusted to any other equipment, wherever located, once its characteristics (basically: dwell volume and extra-column band broadening) are known [17].

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