Creating an optimized preparative method set based on a pre-preparative analytical run

Application Note

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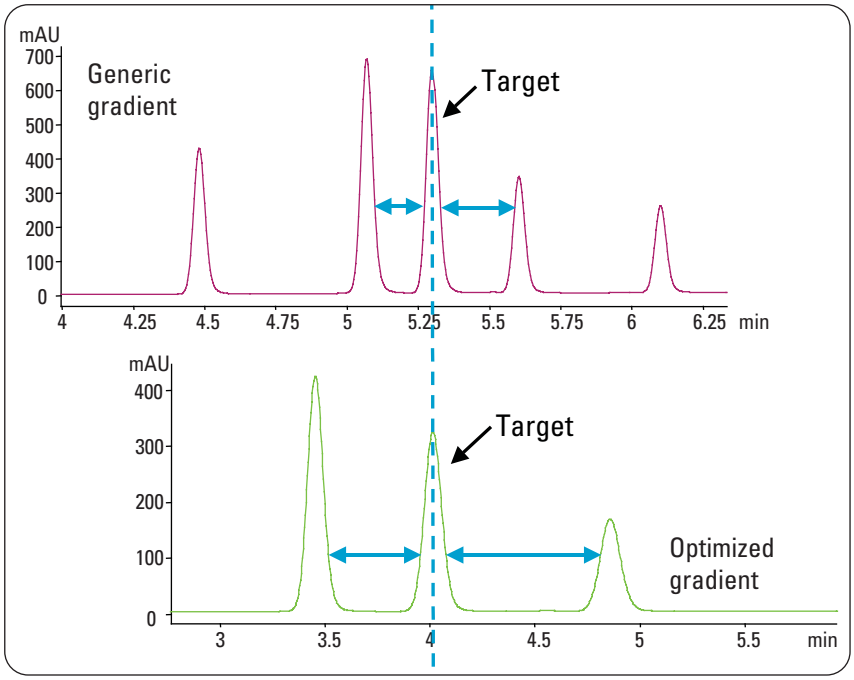

Abstract

In analytical HPLC good resolution is required over the entire gradient; in preparative HPLC it is only important to achieve good resolution around the target compound, which should be collected in highest purity. With a small set of optimized gradient methods a much better resolution can be achieved than with a single generic method. The generation of a small method set based on retention time windows in the pre-preparative analytical run is described in this Application Note. Using Agilent standards with known elution properties makes this an easy four-step process, which can be accomplished in less than one hour. An application example demonstrating how the resolution of the target compound can be improved is also shown.

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Introduction

Baseline separation of peaks is required for accurate quantification of compounds in analytical HPLC. Therefore, a resolution of about 1.5 is needed if the peak areas are not too dissimilar. A good resolution is only required for the target compound in preparative HPLC[1], and that compound is required with high purity. The separation of the remainder of compounds in the chromatogram is not of interest. Generic gradients of 5 – 95 % organic mobile phase are frequently used for purification runs, which do not always lead to sufficient resolution for the target compound. To increase the resolution an optimized method with a shallower gradient around the elution composition of the target compound could be used. Since the preparative method cannot be optimized for each compound this is achieved in practice by using a small set of methods. The preparative method is selected according to a retention time window, in which the compound elutes in the pre-preparative analytical run. The process of generating an optimized preparative method set for retention time windows in the pre-preparative analytical run is described in an *article by Blom et al.*[2] In this Application Note the process described in the article is simplified by using Agilent standards but it can also be performed with any other standard compounds.

Experimental

Equipment

The experiments were done on an Agilent 1200 Series purification system containing the following modules:

- Agilent 1200 Series quaternary pump with degasser
- Agilent 1200 Series well-plate autosampler
- Agilent 1200 Series thermostated column compartment
- Agilent 1200 Series diode array detector (flow cell: 10-mm path length)
- Agilent 1200 Series fraction collector AS

The system was controlled using the Agilent ChemStation (rev. B.02.01).

Results and discussion

To produce a method set for retention time windows, the retention times of several standard compounds were measured using an analytical method. A diagram was generated showing the relation of retention time and true elution composition for these standards. The elution composition of any compound can be extracted or calculated from this diagram by knowing its retention time in the analytical run. The same approach can be taken for the initial and final gradient composition for retention time windows. As the elution composition for eight Agilent standards (Isocratic Test Sample 01080-68704, Electrospray LC Demo Sample 59987-20033) is provided in this Application Note, the generation of a preparative method set involves of four easy steps:

1. Run the Isocratic Test Sample and the Electrospray LC Demo Sample with the analytical method.
2. Generate a diagram with the measured retention times and the known gradient elution compositions.
3. Calculate or extract the initial and final gradient composition for the optimized preparative methods from the graph.
4. Set up the dedicated gradient for each preparative method.

Conditions:

Column:	ZORBAX SB-C18, 4.6 x 50 mm, 3.5 µm
Mobile phases:	water + 0.1 % HCOOH = A acetonitrile + 0.1 % HCOOH = B
Gradient:	at 0 min 5 % B at 7 min 95 % B at 9 min 95 % B
Stop time:	9 min
Post time:	5 min
Flow:	1 mL/min
Injection:	2 µL
Column temp.:	25 °C
UV detector:	DAD 245 nm/10 (ref. 360 nm/100) flow cell 10-mm pathlength

Step 1: Run Agilent standards with the analytical method

An analytical method was set up on a 4.6-mm id column running a gradient from 5 – 95 % organic mobile phase in 7 minutes at a flow rate of 1 mL/min. The chromatograms for the Isocratic Test Sample and the Electrospray LC Demo Sample are shown in figure 1.

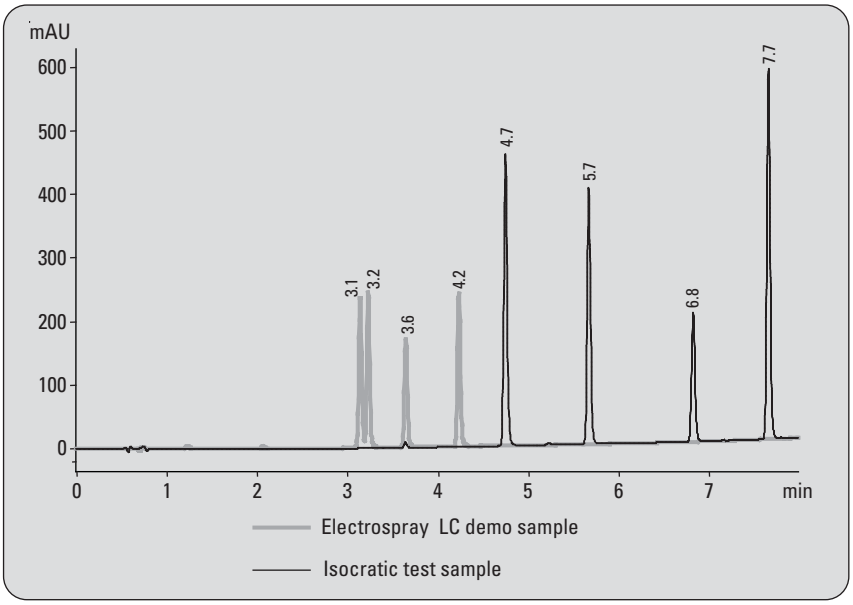

Figure 1
Analytical run.

Step 2: Generate diagram of retention times and elution composition

Using the known elution composition (measured as described in *Procedure with non-Agilent standards* on page 6) and the retention times of the Agilent standards (table 1) the diagram in figure 2 can be created.

Compound	RT [min]	% B
ES 1	3.1	15
ES 2	3.2	16
ES 3	3.6	19
ES 4	4.2	27
Iso 1	4.7	30
Iso 2	5.7	41
Iso 3	6.8	54
Iso 4	7.7	65

Table 1
Retention times and elution composition.

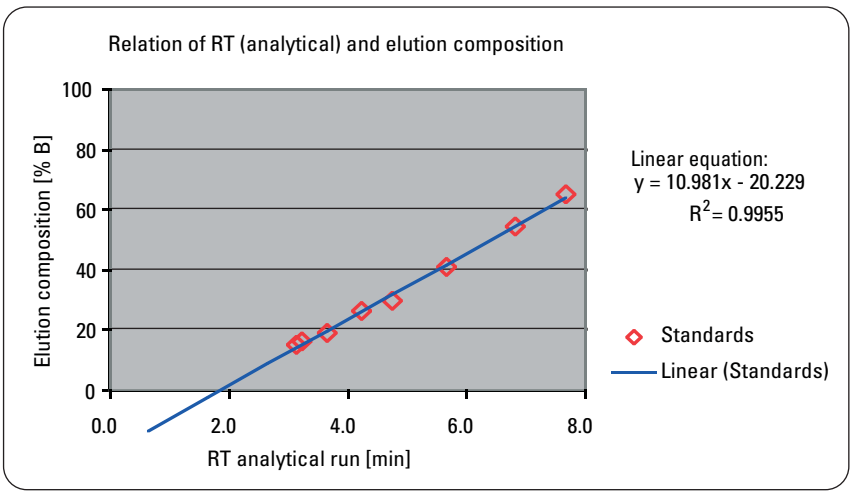

Figure 2
Relation of retention times and elution composition.

Step 3: Get initial and final gradient compositions for preparative methods

To generate a preparative method set, the initial and final gradient compositions are calculated by entering the start and end times of retention time windows in the linear equation. If the result is lower than the initial gradient composition in the analytical run the initial composition is used. For the last method in the method set the same final composition as in the analytical run is used. If the analytical method used above is divided into time windows of one minute, for example, the results of the initial and final gradient composition for the corresponding optimized preparative methods are shown in table 2. The results were calculated using the linear equation from figure 2.

RT window [min]	% B
0 – 2	5
2 – 3	5 – 13
3 – 4	13 – 24
4 – 5	24 – 35
5 – 6	35 – 46
6 – 7	46 – 57
7 – 8	57 – 95

Table 2
Calculated initial and final gradient compositions.

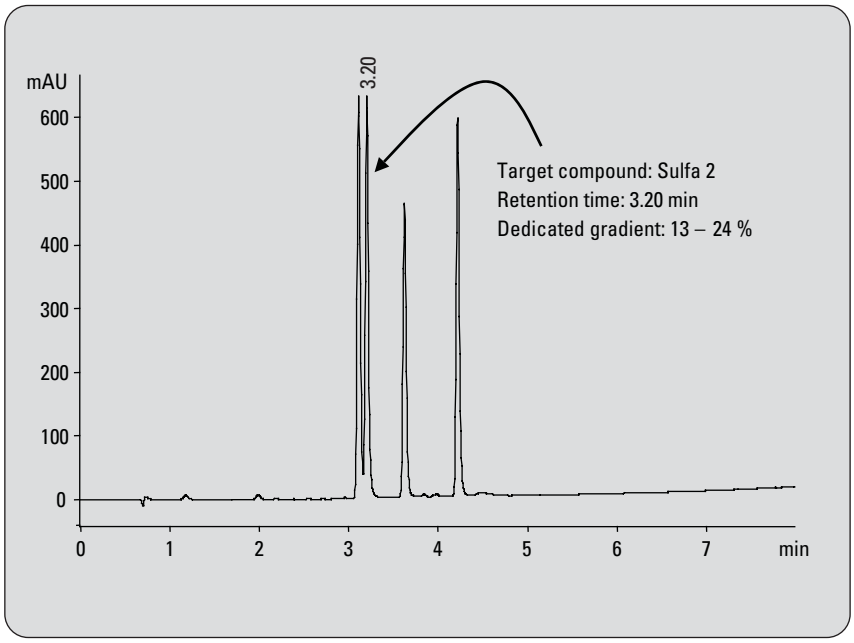

Figure 3
Analytical run of sulfa drugs.

Step 4: Set up the optimized preparative methods
The optimized preparative gradient methods were set up as follows:
Gradient:
at 0 min 5 % B
at 0.1 min initial % B (table 2)
at 1 min initial % B
at 7 min final % B (table 2)
at 7.1 min 95 % B
at 8 min 95 % B

- All methods start at 5 % B to make column equilibration easier. B was increased to the initial conditions at 0.1 min.
- The initial conditions were held for 1 minute to allow the early eluting compounds to come off the column. The length of this hold time depends on column length and flow rate.
- The gradient from the initial to the final conditions was run over 6 minutes. This time depends again on the column length and flow rate. It can be extended but should not be shortened.
- The final composition of 95 % B was also held for 1 minute to allow the late eluting compounds to come off the column. Depending on column length and flow rate this time should be extended.

Purification example
As an example four sulfa drugs were analyzed using the method from figure 1. The result is shown in figure 3.

To purify the target compound sulfa 2, the sample was applied to a preparative column using a generic gradient and an optimized gradient as described in *Step 4: Set up the optimized preparative methods*. The results are shown in figure 4 (A,B).

The target compound partly co-elutes with the previous peak using the generic gradient, however, baseline separation could be achieved with the optimized method. As shown in figures 5 the resolution between the previous peak and sulfa 2 is more than doubled, the resolution between sulfa 2 and the following peak increases by about 50 %.

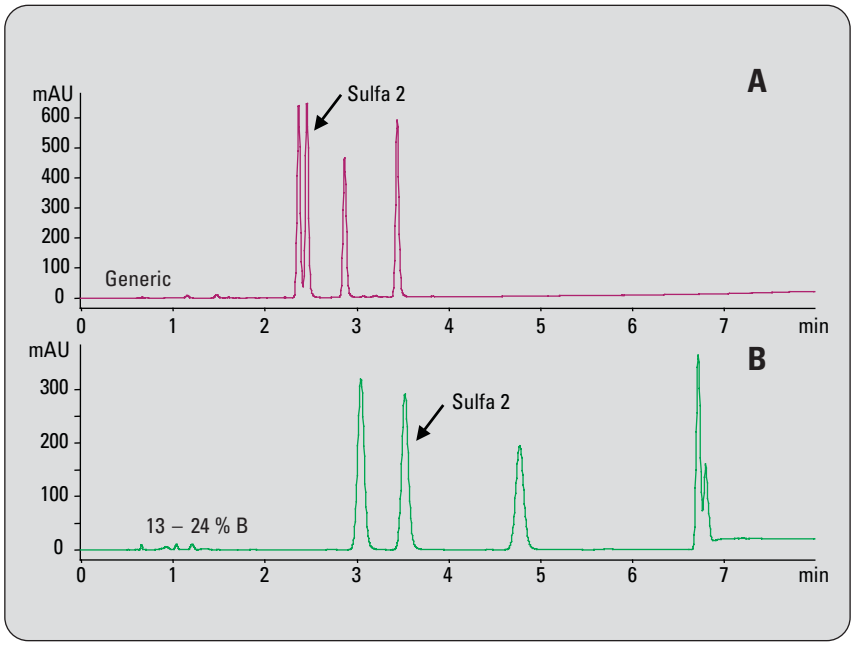

Figure 4
Preparative run with generic (A) and optimized gradient (B).

Conditions:			
Column:	ZORBAX SB- C18, 9.4 x 50 mm, 5 µm		
Mobile phases:	water + 0.1 % HCOOH = A		
	acetonitrile + 0.1 % HCOOH = B		
Gradient 4A:	at 0 min 5 % B	Gradient 4B:	at 0 min 5 % B
	at 7 min 95 % B		at 0.1 min 13 % B
	at 9 min 95 % B		at 1 min 13 % B
			at 7 min 24 % B
			at 7.1 min 95 % B
			at 9 min 95 % B
Stop time:	9 min		
Post time:	5 min		
Flow:	4.2 mL/min		
Injection:	20 µL		
Column temp.:	25 °C		
UV detector:	DAD 245 nm/10 (ref. 360 nm/100) flow cell 10-mm pathlength		

Procedure with non-Agilent standards

If non-Agilent standards must be used to generate the analytical retention time/elution composition diagram, the elution compositions of these standards have to be measured first. It is important for this procedure to run a shallow gradient to make sure the measured elution composition is accurate. Figure 6 shows the measured elution composition for the Agilent standards at different gradient slopes. If the gradient is too steep, the measured elution composition is too high. As a result, the target compounds would not elute in the shallow gradient area but right after the solvent front in the preparative run. The resolution of the target compound would probably be worse than with a generic gradient. In general the slope of the gradient in measuring the elution composition should not be higher than 2 – 4 %/min.

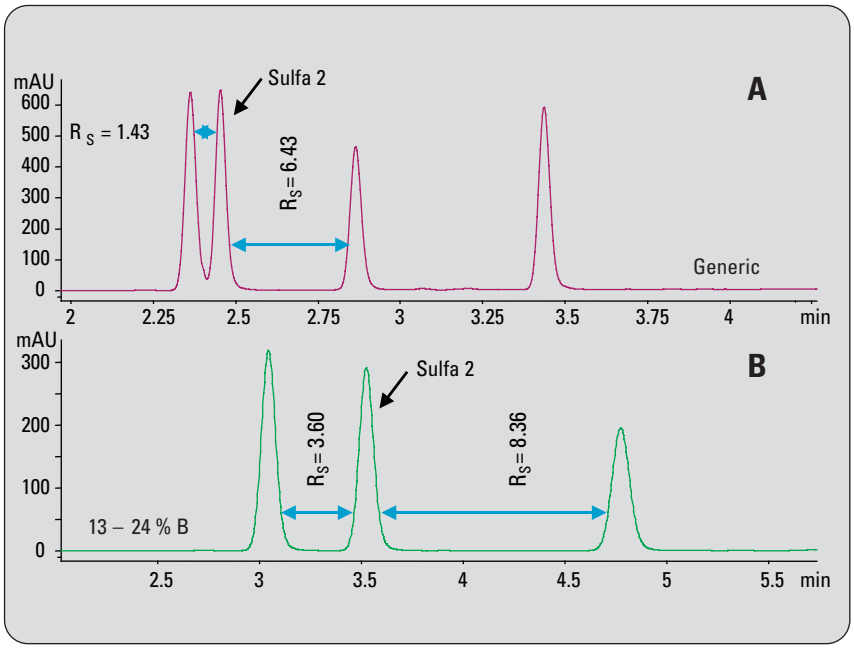

Figure 5
Increasing resolution with optimized gradient.

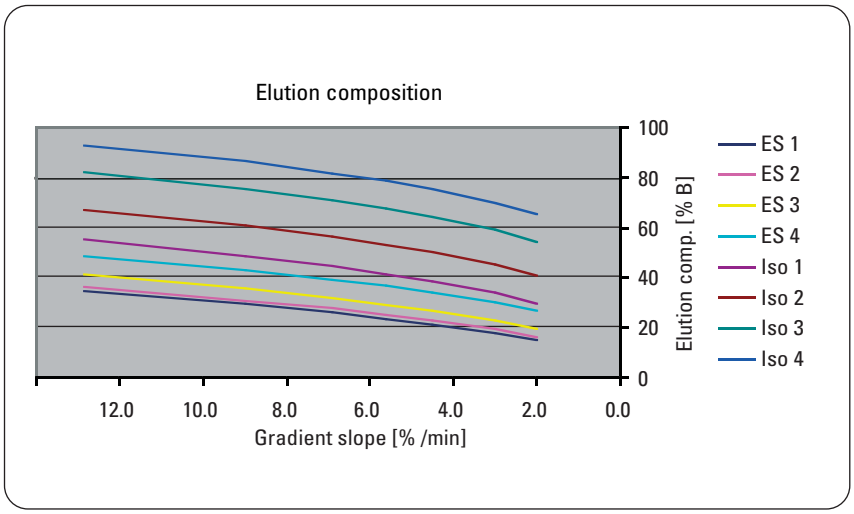

Figure 6
Gradient elution composition.

Conclusion

In this Application Note the generation of a small set of optimized preparative methods based on retention time windows in the pre-preparative run was explained. By using the Isocratic Test Sample and the Electrospray LC Demo Sample the complete process was done in four easy steps taking less than one hour to accomplish. The improved resolution of or near the target compound using optimized gradients was presented in an application example.

References

1.
"Principles in preparative HPLC", *Agilent Technologies primer*, *publication number 5989-0652EN*, **2004.**

2.
Karl F. Blom, Brian Glass, Richard Sparks, and Andrew P. Combs, J. *Comb. Chem.*, *6*, *874-883*, **2004.**

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