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Research paper

Method transfer for fast liquid chromatography in pharmaceutical analysis: Application to short columns packed with small particle. Part II: Gradient experiments

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Abstract

Liquid chromatography (LC) is currently considered as the gold standard in pharmaceutical analysis. Today, there is an increasing need for fast and ultra-fast methods with good efficiency and resolution for achieving separations in a few minutes or even seconds. A previous article (i.e. method transfer for fast LC in pharmaceutical analysis. Part I: isocratic separation) described a simple methodology for performing a successful method transfer from conventional LC to fast and ultra-fast LC in isocratic mode. However, for performing complex separations, the gradient mode is often preferred. Thus, this article reports transfer rules for chromatographic separations in gradient mode. The methodology was applied for the impurity profiling of pharmaceutical compounds, following two strategies.

A first approach, using short columns (20–50 mm) packed with 3.5 μm particles and optimized HPLC instrumentation (with reduced extra-column and dwell volumes), was applied for the separation of a pharmaceutical drug and eight related impurities. Special attention was paid to the dwell (gradient delay) volume, which causes the most detrimental effect for transferring a gradient method. Therefore, the dwell volume was simultaneously decreased with the column dead volume. Under optimal conditions, it was possible to reduce the analysis time by a factor of 10, with an acceptable loss in resolution since the column length reduction is less critical in gradient than isocratic mode.

The second tested approach was Ultra Performance Liquid Chromatography (UPLC), where sub-2 μm particles were used simultaneously with very high pressures (up to 1000 bar). A complex pharmaceutical mixture containing 12 compounds was separated in only 1.5 min allowing a reduction of the analysis time by a factor of 15 in comparison to a conventional method, with similar peak capacity.

Keywords: High speed liquid chromatography; Method transfer; Gradient mode; Short columns; UPLC; Fast separation

1. Introduction

In a previous article [1], general rules were described for transferring, in isocratic mode, a conventional method to fast- or ultra-fast liquid chromatography (LC) in pharmaceutical analysis. It was shown that among the different tested approaches, the use of short columns (i.e. 5 cm length) packed with 1.7 μm particles was the best strategy to significantly reduce the analysis time while maintaining efficiency and, therefore, resolution constant. However, it was also demonstrated that dedicated instrumentation was necessary, with low extra-column volumes and high detection acquisition rates. Furthermore, very small particles induce a large back pressure (Darcy's law) and the equipment must be also compatible with the high generated pressure. As shown previously [1], fast- and ultra-fast separations were achieved by Ultra Performance Liquid Chromatography (UPLC) compatible with short columns packed with 1.7 μm particles and high pressure (1000 bar).

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List of abbreviations

Symbols

b	gradient steepness parameter
d_c	column internal diameter
D_m	diffusion coefficient of solute at very low concentration in solvent
d_p	particle size of the support
F	mobile phase flow rate
k_e	"average" or "effective" retention factor
k_0	isocratic retention factor for an organic modifier proportion corresponding to the beginning of the gradient
L	column length
N	plate number
n_{peaks}	peak capacity
R_s	resolution
S	characteristic constant of the solute in gradient mode (slope of the logarithmic plot: $d(\log k)/d\Phi$)
t_0	column dead time
t_d	system dwell time
t_{grad}	gradient time
t_{iso}	initial isocratic hold
t_r	analysis time
u	mobile phase linear velocity
V_d	system dwell volume
V_0	column dead volume
V_{inj}	injection volume

Greek symbols

α_0	chromatographic selectivity at a mobile phase composition corresponding to the beginning of the gradient
Φ	mobile phase composition in gradient mode
σ^2_{col}	column dispersion
σ^2_{ext}	extra-column band broadening
σ_t	gradient peak width at half height in time units
υ	reduced mobile phase linear velocity

Gradient elution is largely used in pharmaceutical analysis for performing complex separations (e.g. purity profiles, analyzing drugs and metabolites, etc.) where compounds possess a broad polarity range [2,3]. Generally, three variables must be optimized for achieving a separation in gradient mode: the initial and final proportion of the hydro-organic solvent as well as the gradient time. Advantages of gradient versus isocratic separation are well known [4–7] and include the sensitivity improvement (due to lower chromatographic dilution), the peak capacity increase (peak width being independent of retention), and the analysis time reduction. Gradient drawbacks are mainly related to the particular requirements of the LC instrumentation and to the laborious method development, even if optimization software can tackle this difficulty [8–11]. Furthermore, method transfer is often more complex since additional variables coming from the equipment can impact on selectivity such as the value of the dwell volume [5].

A large number of fast separations in gradient mode have been reported in the literature with short columns packed with small particles [12–15]. However, chromatographic resolution was often not sufficient and mass spectrometry (MS) was mandatory for quantitative purposes. Fast separations using short columns packed with sub-2 μm particles were also reported by Lurie [16]. A complex separation of 16 anabolic steroids was achieved in less than 3 min and a wide variety of drugs was separated in approximately 2 min, both with a column of 30 mm packed with 1.7 μm particles. Other authors demonstrated the potential of fast gradients in UPLC for pharmaceutical formulations and environmental compounds [17,18].

As previously reported for isocratic separations [1], this paper describes the basic equations for transferring a conventional method from a given system to a fast separation. Two strategies were applied in gradient mode to reduce the analysis time (short columns packed with 3.5 μm particles and UPLC) and, for achieving a successful transfer in gradient mode, a particular attention was paid to the instrumentation. In this paper, different examples were presented to illustrate the transfer gradient method from conventional HPLC to fast LC conditions.

2. Theory

2.1. Theory of gradient elution

The efficient use of gradient elution in reversed phase-liquid chromatography (RP-LC) requires an understanding of the relations between separation (retention factor, bandwidth, selectivity, resolution) and operating parameters such as gradient profile, column geometry, mobile phase flow rate, etc. For this purpose, several theories have been proposed to describe gradient elution by Jandera and Churacek [19], Schoenmakers and co-workers [6,7,20] and other groups [21,22]. These approaches, based on detailed mathematical expressions, allow precise calculations but offer little help for conventional LC users in pharmaceutical analysis.

Alternatively, Snyder et al. have developed an approximate theoretical treatment for understanding and controlling separation in RP-LC gradient elution [5,23,24]. The so-called Linear Solvent Strength (*LSS*) theory gives an explicit and simple solution to evaluate retention in gradient elution since it considers the gradient in a very similar way to isocratic elution. Therefore, the *LSS* theory presents a great advantage since it explains the similarities and the differences between isocratic and gradient separations. Among the set of equations proposed by Snyder and Dolan [5] to completely describe the gradient elution,

the main relationships for retention, bandwidth and resolution have been selected and summarized below.

2.1.1. Retention in gradient elution

As presented in Fig. 1, in the RP-LC gradient mode, the organic modifier proportion is generally modified from a low starting value ($\%B_{initial}$) to a higher final value ($\%B_{final}$). Since the eluent strength increases during the chromatographic process, the retention factor k for each sample component decreases with time. Therefore, k, as defined in isocratic mode, is an unsuitable parameter for retention in gradient elution. For this reason, the *LSS* theory introduces the concept of k_e, which represents the retention factor of the solute in the eluted mobile phase composition. The latter represents the "average" or "effective" value of k during gradient elution. It defines the peak width in gradient elution mode and could be estimated with:

$$k_e = \frac{1}{2.3 \cdot b}. \quad (1)$$

The b term is the gradient steepness, obtained by:

$$b = \frac{t_0 \cdot \Delta\Phi \cdot S}{t_{grad}}, \quad (2)$$

where t_{grad} is the gradient time, t_0 is the column dead time which depends on the column dead volume and mobile phase flow rate ($t_0 = V_0/F$), $\Delta\Phi = \%B_{final} - \%B_{initial}$ is the change in composition during the gradient, ranging from 0 to 100 (see Fig. 1), and S is a term almost constant which corresponds to the elution strength of the organic modifier (slope of the logarithmic plot: $d(\log k)/d\Phi$). S depends on the solute and organic modifier nature and could be considered equal to a constant comprised between 0.03 and 0.05 for a linear relationship of $\log k$ versus $\%B$, with associated k comprised between 1 and 15 [25]. S being constant, gradient steepness (b) should ideally remain constant for all compounds eluted during the chromatographic run. This assumption is valid for compounds of related structure but not in all cases (e.g. S depends on the chemical structure and MW of the compounds). With this assumption, and opposite to isocratic elution, the *LSS* gradient theory demonstrates approximately equal values of average retention factor for samples eluting at different times during separation.

Another difference in the case of gradient experiments is that the average retention factor (k_e) depends on the system geometry (gradient time, flow rate and column dimensions), according to Eqs. (1) and (2). For these reasons, the estimation of expected retention time is more complex and the observed retention time for a chromatographic peak in gradient RP-LC can be expressed by the following equation [5]:

$$t_R = \frac{t_0}{b} \cdot \log(2.3 k_0 b + 1) + t_0 + t_d \quad (3)$$

where t_R is the retention time in a linear gradient separation, k_0 is the retention factor at a mobile phase composition corresponding to the beginning of the gradient (i.e. for $\Phi = \%B_{initial}$) and t_d is the system dwell time for gradient elution (discussed in details in Section 4.1). All these parameters are presented in Fig. 1.

2.1.2. Peak width in gradient elution

Under isocratic conditions, the peak widths proportionally increase with retention (leading to broader peaks and lower sensitivity). Conversely, the bandwidths in gradient elution chromatography can be considered (at least approximately) constant for all compounds eluted during the gradient process and equivalent to the peak width of

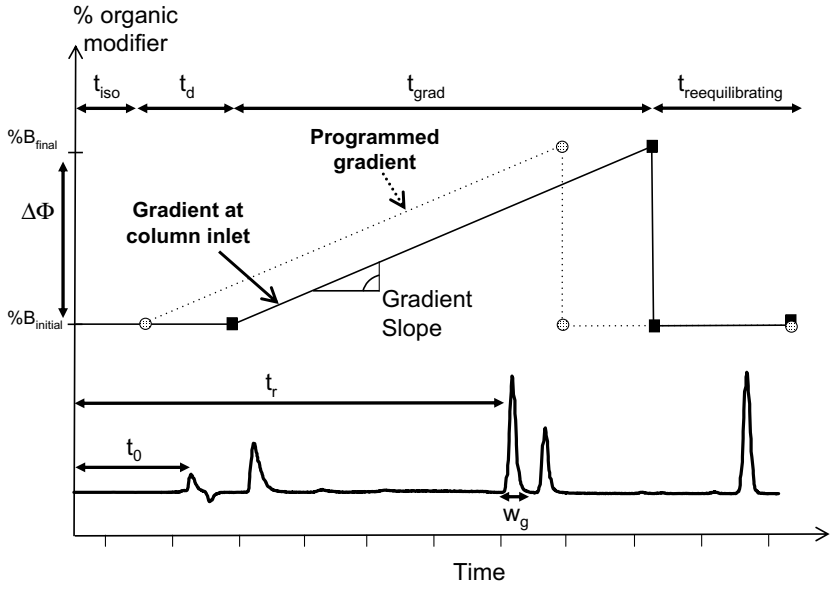

Fig. 1. Graphical representation of the main parameters used to describe gradient elution, in Section 2.

a compound eluted with $k = 1$–2, under isocratic conditions [26]. This phenomenon is attributed to the constant average retention factor (k_e) of all compounds, whatever their eluting time. Peak widths at half height in time units (σ_t) can be estimated with:

$$\sigma_t = \frac{t_0}{\sqrt{N}} \cdot (1 + k_e). \quad (4)$$

It must be noted that this estimation neglects the phenomenon of band compression seen in gradient elution [27]; namely, the trailing edge of the chromatographic peak migrates faster due to a more eluent mobile phase composition, compared to the leading edge of the peak eluted with a weaker mobile phase, during gradient process. The error caused by this approximation is expected to be in the range 10–20% [28], but can be partially corrected by the variation of viscosity and diffusion coefficients with mobile phase composition, stationary phase diffusion phenomena or extra-column peak broadening [27].

As previously described in the literature, the concept of efficiency is based on the number of theoretical plates ($N = 5.54 \cdot (t_r^2/\sigma^2)$) and is not adapted for gradient elution. Thus, the concept of peak capacity (n_{peaks}) must be used [29–31]. The latter, appropriate for isocratic and gradient elution, is defined as the number of peaks which can be resolved in a certain time interval, provided that all compounds are separated with an equal resolution, $R_s = 1$.

$$n_{peaks} = 1 + \frac{t_{grad}}{4 \cdot \sigma_t}. \quad (5)$$

According to Eqs. (4) and (5), peak capacity is related to the average number of plates, N, theoretically generated by the chromatographic column in isocratic experiments (i.e. $N = L/(h \cdot d_p)$, where h is the reduced plate height equivalent to a theoretical plate) and to the average retention factor k_e (see Eq. (1)). Because of the broader retention range and the narrower peaks, the peak capacity is considerably higher in gradient than in isocratic mode.

2.1.3. Resolution in gradient elution

The peak width in gradient mode, σ_t, can be assumed the same for each band and is given by Eq. (4). Therefore, the general equation for resolution (R_s) between two adjacent bands 1 and 2 in isocratic mode should be replaced in gradient elution by:

$$R_s = \frac{t_{r_2} - t_{r_1}}{4 \cdot \sigma_t}, \quad (6)$$

where t_{r_1} and t_{r_2} are the retention times of peaks 1 and 2, respectively. Another expression for R_s in gradient elution can be obtained thanks to Eqs. (3) and (4):

$$R_s = \frac{\sqrt{N}}{4} \cdot \frac{2.3 \cdot k_e}{1 + k_e} \cdot \log \alpha_0, \quad (7)$$

where α_0 represents the selectivity between peaks 1 and 2 at a mobile phase composition corresponding to the initial composition of the gradient (i.e. for $\Phi = \%B_{initial}$). As reported in Eq. (7), three different parameters influence resolution in gradient mode: the isocratic chromatographic efficiency, which depends on column geometry and flow rate (i.e. according to Knox curves); the selectivity, which is related to the gradient profile and column chemistry; and the capacity term, which is a function of the average retention factor, k_e

In conclusion, resolution and peak capacity are generally improved in gradient elution over the isocratic mode. The *LSS* gradient theory allows the demonstration of such features and represents a correct, simple and easy-to-use description of gradient elution in RP-LC.

2.2. Method transfer in gradient elution

Simple scaling equations are necessary in gradient mode to determine the injected volume and the flow rate to be used with a new column geometry.

The new injected volume is proportional to the square ratio of the column diameters (d_c), and the ratio of the column lengths (L), as indicated in [1]:

$$V_{inj_2} = V_{inj_1} \cdot \frac{d_{c_2}^2}{d_{c_1}^2} \cdot \frac{L_2}{L_1}, \quad (8)$$

where 1 and 2 are related to the original and transferred methods, respectively.

It can be noted that larger injection volumes than predicted can be used to maximize sensitivity. However, the sample should be dissolved in a solvent of weaker eluent strength than the initial mobile phase composition gradient. This approach described as sample focusing (peak compression) [32] allows the enrichment of the analytes on the top of the column.

To take into account changes in geometry (i.e. column length, L and particle size, d_p), the new flow-rate value, F_2, is estimated according to the following equation [1]:

$$F_2 = F_1 \cdot \frac{d_{c_2}^2}{d_{c_1}^2} \cdot \frac{d_{p_1}}{d_{p_2}}. \quad (9)$$

To maintain constant retention factors and resolution between an original and transferred method, other parameters must be optimized. In linear or multi-linear gradient elution, the gradient profile can be decomposed as the combination of two parts: isocratic and gradient segments. For both parts, the gradient volume should be scaled in proportion to the column volume, to yield identical elution patterns. The rules for efficient gradient transfer, introduced by Snyder and Dolan [33] and updated recently by Carr and Schellinger [34], should be strictly followed.

For the isocratic step (and also equilibrating time), the ratio between isocratic step time (t_{iso}) and column dead time (t_0) should be adapted with the help of Eq. (10):

$$t_{iso_2} = t_{iso_1} \cdot \frac{F_1}{F_2} \cdot \frac{V_{0_2}}{V_{0_1}}. \quad (10)$$

For slope segments, the initial and final gradient composition (%B) must be constant, and the new gradient time (t_{grad_2}) expressed as:

$$t_{grad_2} = \frac{(\%B_{final_1} - \%B_{initial_1})}{slope_2}. \quad (11)$$

The new gradient slope ($slope_2$) is estimated according to Eq. (12):

$$slope_1 \cdot t_{0_1} = slope_2 \cdot t_{0_2}. \quad (12)$$

Considering this equation, the new slope value is equivalent to:

$$slope_2 = slope_1 \cdot \frac{V_{0_1}}{V_{0_2}} \cdot \frac{F_2}{F_1}. \quad (13)$$

All these equations can be easily used for determining the new parameters of a transferred gradient method. A freely usable Excel program called ''HPLC calculator'', automatically establishing optimal conditions for method transfer in gradient mode, using Eqs. (8)–(13), has been distributed on an internal website [35]. In this calculator, system dwell volume can also be considered for calculation in gradient mode.

3. Experimental section

3.1. Materials

Compounds used throughout the study consist of an active substance and putative by-products with undisclosed structures.

Acetonitrile was of HPLC gradient grade from Panreac Quimica (Barcelona, Spain). Water was obtained from a Milli-Q water purification system from Millipore (Bedford, MA, USA). Formic acid was obtained from SDS (Peypin, France) and pH (in aqueous solution) was measured with a Metrohm pH meter (Herisau, Switzerland).

Columns used were all provided by Waters (Milford, MA, USA) and are reported in Table 1.

3.2. Samples and mobile phases

Two aqueous reconstituted solutions of the drug cocktail containing the active substance and its by-products were prepared by appropriate dilution of 1 mg/mL standard solution of each component in acetonitrile–water (50:50, v/v). The first solution contained the active substance at 100 ppm and eight by-products at 40 ppm. The second solution contained the active substance at 100 ppm and 11 by-products at 40 ppm.

The mobile phase consisted of a gradient mixture of 0.01% (v/v) formic acid in water and 0.01% (v/v) formic acid in acetonitrile. Experimental conditions are reported in Tables 2 and 3.

Table 1
Chromatographic columns used

Column type	Abbreviation	Particle size (μm)	Dimensions (mm)
XTerra RP_{18}	XT150	5	150 × 4.6
XTerra RP_{18}	XT50	3.5	50 × 4.6
XTerra RP_{18}	XT30	3.5	30 × 4.6
XTerra RP_{18}	XT20	3.5	20 × 4.6
XBridge C_{18}	XBD150	5	150 × 4.6
Acquity UPLC BEH C_{18}	ACQ50	1.7	50 × 2.1

3.3. Apparatus

3.3.1. HPLC

Separations were performed on a Merck LaChrom system (Merck, Darmstadt, Germany) constituted of L-7100 programmable pumps, a L-7200 autosampler with a 100 μL loop, a L-4250 UV–vis programmable detector and a L-7300 column oven. The UV–vis detector contained a 14 μL standard flow cell, the time constant was set at 0.1 s and data sampling rate at 20 points/s. Data acquisition, data handling and instrument control were performed by D-7000 HPLC System Manager Software. Extra-column band broadening of this instrument was estimated at approximately $\sigma^2_{ext} = 200\ \mu L^2$ at 1000 μL/min [1].

3.3.2. UPLC

Separations were performed on the Waters® Acquity UPLC system. This instrument included a binary solvent manager, an autosampler with a 2 μL loop, a UV–vis programmable detector and a column oven set at 30 °C. The UV–vis detector contained a 500 nL flow cell, the time constant was set at 25 ms and data sampling rate at 40 points/s. Data acquisition, data handling and instrument control were performed by Empower Software. Extra-column band broadening of this instrument was estimated at about $\sigma^2_{ext} = 5\ \mu L^2$ at 600 μL/min [1].

3.4. Methodology

Table 2 gives conditions used for carrying out HPLC and UPLC experiments. Reported values were calculated with the ''HPLC calculator'' using equations described in Section 2.2.

4. Results and discussion

In isocratic mode, the emphasis was made on extra-column band broadening contributions (σ^2_{ext}) which could significantly reduce chromatographic performance when short and thin columns (i.e. small σ^2_{col}) are considered. The negative influence of σ^2_{ext} on efficiency was more pronounced for less retained compounds (i.e. with low retention factors, k). For this reason, the problem was solved either by using optimized chromatographic instrumentation (e.g. low σ^2_{ext}) or by increasing retention factors k (e.g. to enhance σ^2_{col}) [1].

In gradient mode, extra-column effects also exist and influence chromatographic performance. However, because

Table 2
Experimental conditions for the gradient transfer of pharmaceutical formulation and eight related impurities from conventional HPLC to fast-LC

	Conventional HPLC	Fast-LC (short columns)
Columns	XT150	XT50
	XT20	XT30
		XT20
Flow rate	XT150: 1000 μL/min	XT50: 1430 μL/min
	XT20: 1430 μL/min	XT30: 1430 μL/min
		XT20: 1430 μL/min
Injection volume	XT150: 20 μL	XT50: 7 μL
	XT20: 3 μL	XT30: 4 μL
		XT20: 3 μL
Gradient profile	XT150: T_0 (100:0), T_{30} (40:60), T_{31} (100:0), T_{45} (100:0)	XT50: T_0 (100:0), $T_{7.0}$ (40:60), $T_{7.2}$ (100:0), $T_{10.5}$ (100:0)
$T_{(\text{time in min})}$ (A:B)	XT20: T_0 (100:0), $T_{2.8}$ (40:60), $T_{2.9}$ (100:0), $T_{4.2}$ (100:0)	XT30: T_0 (100:0), $T_{4.2}$ (40:60), $T_{4.3}$ (100:0), $T_{6.3}$ (100:0)
		XT20: T_0 (100:0), $T_{2.8}$ (40:60), $T_{2.9}$ (100:0), $T_{4.2}$ (100:0)
UV detection	Flow cell: 14 μL	Flow cell: 500 nL
	Constant time: 100 ms	Constant time: 25 ms
	Sampling rate: 20 points/s	Sampling rate: 40 points/s
Dwell volume	~1300 μL	~130 μL

A: 0.01% formic acid in H_2O.
B: 0.01% formic acid in ACN/H_2O (50:50% v/v).

apparent retention factors k_e are identical under gradient elution conditions Eq. (1), each peak in the chromatogram is similarly affected by extra-column contributions. Therefore, separations performed in gradient elution are less subjected to extra-column effects when k_e are shifted to high values to reach optimal performance (see Eq. (5)).

In this study, experiments in gradient mode were always performed with $k_e > 3$. For this reason, problems encountered during method transfer could only be partially attributed to extra-column volume effects, and mostly to the system dwell volume.

4.1. The system dwell volume

The system dwell volume (V_d) is also known as gradient delay volume. It refers to the volume of a HPLC system between the mixing point of solvents and the head of the analytical column. Low-pressure mixing systems possess generally larger dwell volumes than high-pressure mixing systems. After the starting of the gradient, it will take time until the selected proportion of solvent reaches the column. It means that the sample is subjected to an additional isocratic migration in the initial mobile phase (i.e. t_d in Fig. 1, which has to be added to a potential initial isocratic hold equal to t_{iso}). Since the gradient dwell volume may differ from one system to another, this extra isocratic step would be different and could result in retention time variations affecting resolution when transferring a method [25,33,34]. Some authors [33,34] have demonstrated that the basic law of gradient transfer, as described in Eq. (12), is only valid as long as the system dwell volume (V_d or dwell time, t_d) is negligible and/or when analytes possess high apparent retention factors. Significant variations in apparent retention time and resolution could occur for early eluting peaks which are strongly affected by the gradient delay volume. To overcome this problem, the t_d/t_0

Table 3
Experimental conditions for the gradient transfer of pharmaceutical formulation and 11 related impurities from conventional HPLC to UPLC

	HPLC assay	UPLC assay
Columns	XBD150	ACQ50
Flow rate	1000 μL/min	610 μL/min
		1000 μL/min
Injection volume	20 μL	1.4 μL
Gradient profile	T_0 (100:0), T_{30} (40:60), T_{31} (100:0), T_{45} (100:0)	610 μL/min: T_0 (100:0), $T_{3.4}$ (40:60), $T_{3.5}$ (100:0), $T_{5.1}$ (100:0)
$T_{(\text{time in min})}$ (A:B)		1000 μL/min[a]: T_0 (100:0), $T_{3.4}$ (40:60), $T_{3.5}$ (100:0), $T_{5.1}$ (100:0)
		1000 μL/min[b]: T_0 (100:0), $T_{2.1}$ (40:60), $T_{2.2}$ (100:0), $T_{3.1}$ (100:0)
UV detection	Flow cell: 14 μL	Flow cell: 500 nL
	Constant time: 100 ms	Constant time: 25 ms
	Sampling rate: 20 points/s	Sampling rate: 40points/s
Dwell volume	~1300 μL	~130 μL

A: 0.01% formic acid in H_2O.
B: 0.01% formic acid in ACN/H_2O (50:50% v/v).
[a] Incorrect conditions (gradient profile not adapted to the flow rate).
[b] Correct conditions (gradient profile adapted to the flow rate).

ratio must be held constant while changing column dimensions, particle size or mobile phase flow rate.

Separations of a pharmaceutical drug and eight impurities were carried out on conventional (150 × 4.6 mm, 5 μm) and short columns (20 × 4.6 mm, 3.5 μm) with the conventional HPLC system. Experimental conditions are reported in Table 2 and were adapted as a function of the column (e.g. injection volume, flow-rate and gradient profile, according to Eqs. (8), (9), (11) and (13)). Corresponding chromatograms are given in Fig. 2, showing differences between both patterns. It could be observed that column length reduction leads to lower resolution and conventional instrumentation was not adapted for transferring a method from long to short columns since selectivity modification occurred. The latter could be attributed to the negative impact of system dwell volume which induced a different gradient profile with the short column. In the configuration used in this study, V_d was experimentally determined (as described in [36]) at 1.3 mL. The dotted lines seen on both chromatograms illustrate the real gradient profile, taking into account the column dead time ($t_0 = V_0/F$) and the system dwell time ($t_d = V_d/F$). In the original method ($t_0 = 1.7$ min), only the first peak was eluted under isocratic conditions, while in the transferred method ($t_0 = 0.17$ min), the first six peaks were eluted in the isocratic portion.

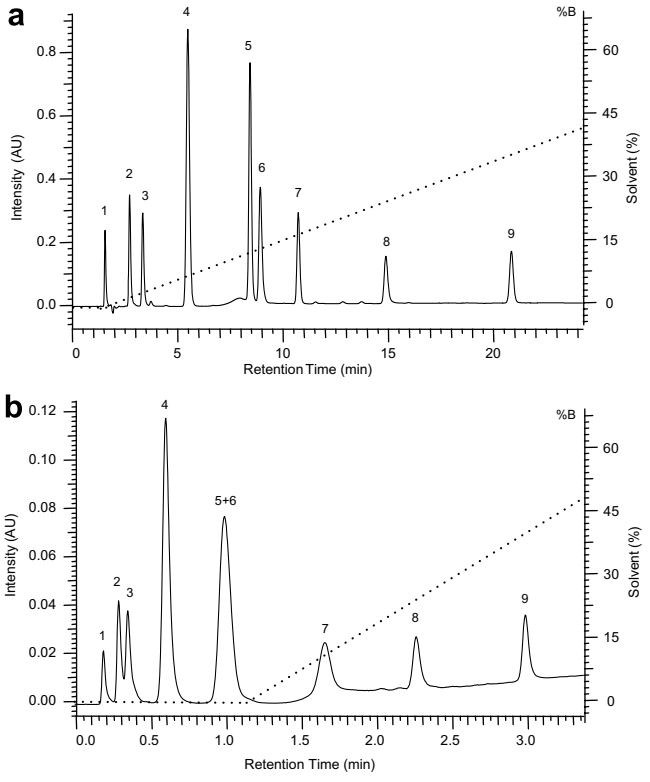

Fig. 2. Separation of a pharmaceutical formulation containing the main product (5) and eight impurities in gradient mode with conventional HPLC system: (a) column: XTerra RP_{18} 150 × 4.6 mm, 5 μm; flow rate: 1000 μL/min; injection volume: 20 μL; total gradient time: 45 min. (b) Column: XTerra RP_{18} 20 × 4.6 mm, 3.5 μm; flow rate: 1430 μL/min; injection volume: 3 μL; total gradient time: 4.2 min.

Since the column dead time was reduced by a factor of 10, the dwell time must be proportionally reduced to keep the t_d/t_0 ratio constant. For this purpose, it is mandatory to use an optimized system possessing a very low dwell volume. Other approaches could be applied to overcome this problem (i.e. isocratic hold at the beginning of the gradient, delay injection, etc. [5,25]).

4.2. Short columns packed with 3.5 μm particles on optimized system

Chromatograms obtained on columns of different lengths (comprised between 50 and 20 mm) with an optimized instrumentation possessing an appropriate reduced dwell volume demonstrated good separations (R_s always higher than 1.5) regardless of the column length, with a significant reduction of analysis time (i.e. Fig. 3). Experimental conditions are summarized in Table 2 and have been obtained according to Eqs. (8)–(13). These chromatograms were compared with the original separation reported in Fig. 2a. In order to evaluate the overall quality of the transferred method, analysis time must be considered simultaneously with sensitivity and resolution.

As expected from theory, the analysis time was proportionally reduced to the column length and the flow rate. The original separation was performed in 45 min (gradient time of 30 min and re-equilibrating time of 15 min, equivalent to 10 column volumes). With a smaller column (50 mm) packed with 3.5 μm particles, the optimal flow rate was increased and the total analysis time, including gradient and re-equilibrating times, was reduced to only 10 min. With the 30 and 20 mm columns packed with 3.5 μm particles, the total analysis time was strongly decreased to 6.3 and 4.2 min, respectively.

However, if short columns are beneficial for decreasing the analysis time, they are detrimental in terms of resolution and peak capacity (see Eqs. (5) and (7)) even if this loss is less important in gradient versus isocratic mode [30,31]. In gradient mode, with high average retention factors ($k_e > 3$), peak capacity remains acceptable. In reported examples, peak capacity and minimal resolution were, respectively, of 120 and 3.5 for the conventional column (Fig. 2a). Both values were reduced by about 20% with a 50 mm column packed with 3.5 μm particles. While, with 30 and 20 mm column lengths, performance was reduced by 40 and 60%. However, since the minimal resolution measured with the original method ($R_s = 3.5$) was high, separation remained acceptable in terms of peak capacity and resolution, and still allowed the separation of the nine compounds, whatever the column length. With the shortest column tested, peak capacity was reduced to 50 while minimal resolution was equivalent to 1.53.

Sensitivity of the methods was also investigated, and a linear decrease was observed as a function of the column length, leading to a reduction of sensitivity by a factor of 3–4. This behaviour was clearly due to the loss

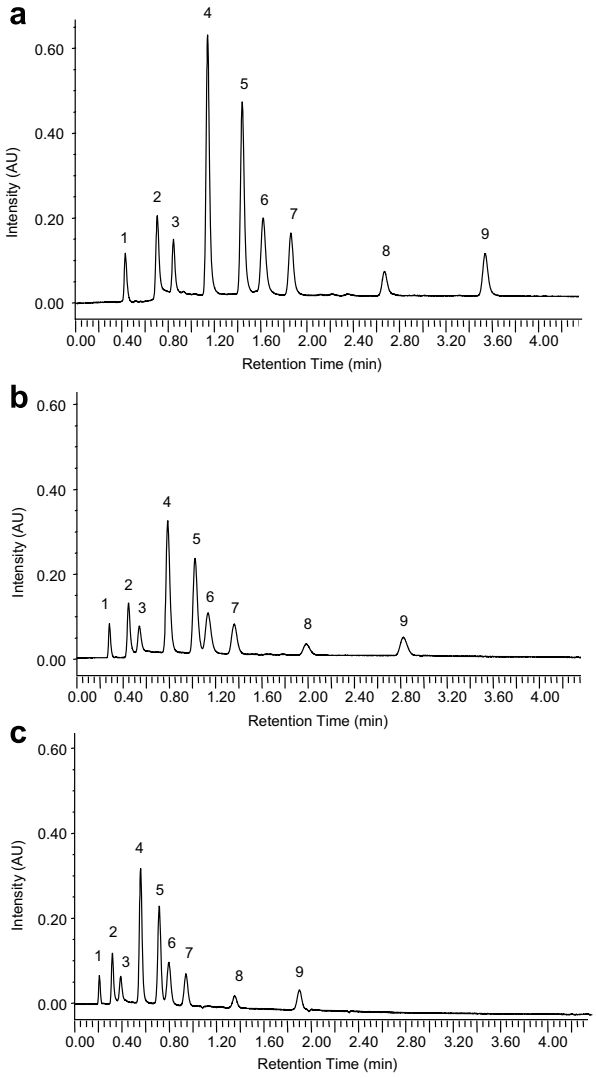

Fig. 3. Separation of a pharmaceutical formulation containing the main product (5) and eight impurities in gradient mode with optimized HPLC system: (a) column: XTerra RP_{18} 50 × 4.6 mm, 3.5 μm; flow rate: 1430 μL/min; total gradient in 10.5 min; injection volume: 7 μL. (b) Column: XTerra RP_{18} 30 × 4.6 mm, 3.5 μm; flow rate: 1430 μL/min; total gradient time: 6.3 min; injection volume: 4 μL. (c) Column: XTerra RP_{18} 20 × 4.6 mm, 3.5 μm; flow rate: 1430 μL/min; total gradient time: 4.2 min; injection volume: 3 μL.

of peak capacity and therefore, to the decrease of the peak height.

In order to summarize, the transfer of a method in gradient mode from a conventional to shorter columns requires the reduction of the dwell volume (i.e. Fig. 2b compared to Fig. 3c). Therefore, the use of short columns is compatible in the gradient mode for reducing the analysis time [1] with an optimized system (Eqs. (8)–(13)).

4.3. Short columns packed with small particles

To avoid the loss in peak capacity and resolution with short columns, it is necessary to simultaneously decrease the particle size. The theoretical peak capacity (measured in gradient mode with an optimized system) as a function of the column length and particle size was plotted (according to Eq. (5)) in Fig. 4. Each point of these curves was obtained by adapting the gradient time and the flow rate to the column geometry, according to Eqs. (9) and (11). Three curves are presented, corresponding to the selected particle sizes (5, 3.5 and 1.7 μm), showing that a short column packed with 5 μm particles should be used only for simple separations. When column length and particle size decreased simultaneously (see arrows in Fig. 4), peak capacity remains constant. A 70 mm column packed with 3.5 μm particles possesses the same peak capacity as the 50 mm column packed with 1.7 μm particles but the analysis time is strongly reduced with the latter.

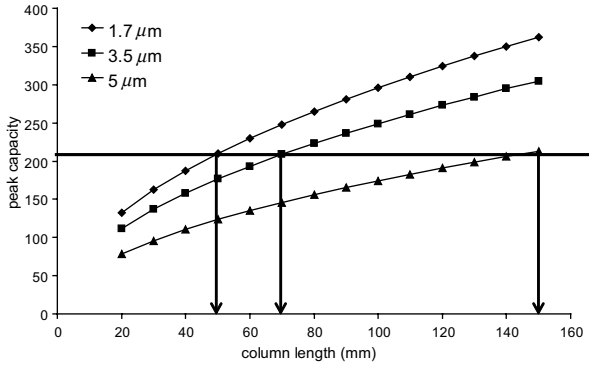

Fig. 4. Theoretical evolution of peak capacity as a function of column length and packing particle size – case of an initial gradient from 0% to 60% ACN in 30 min on a 150 × 4.6 mm, 5 μm column. Gradient duration was adapted to the column geometry, with equations of part, Section 2. ♦, particles of 1.7 μm with a flow rate of 3 mL/min; ■, particles of 3.5 μm with a flow rate of 1.4 mL/min; ▲, particles of 5 μm with a flow rate of 1 mL/min.

4.3.1. Method transfer from HPLC to UPLC

The selected sample used for transferring a method performed in a conventional column toward a short column packed with sub-2 μm particles consisted of a drug substance with 11 related impurities (impurity profiling).

The separation of the 12 compounds was originally achieved using a XBridge C_{18} column (150 × 4.6 mm, 5 μm) and further transferred to UPLC using an Acquity BEH C_{18} column (50 × 2.1 mm, 1.7 μm). In this case, the transfer was purely geometrical because XBridge and Acquity BEH supports are based on the same stationary phase chemistry and possess the same selectivity.

Fig. 5 presents the corresponding separations and Table 3 gives the conditions of the original HPLC and transferred gradient to UPLC. The original separation was performed in approximately 30 min and efficiently transferred to UPLC with less than 3 min of analysis time (reduction by a factor of 10). Sensitivity was not significantly affected between HPLC and UPLC conditions.

A 10% higher resolution was obtained with the XBridge support, compared to the Acquity BEH. Since resolution is

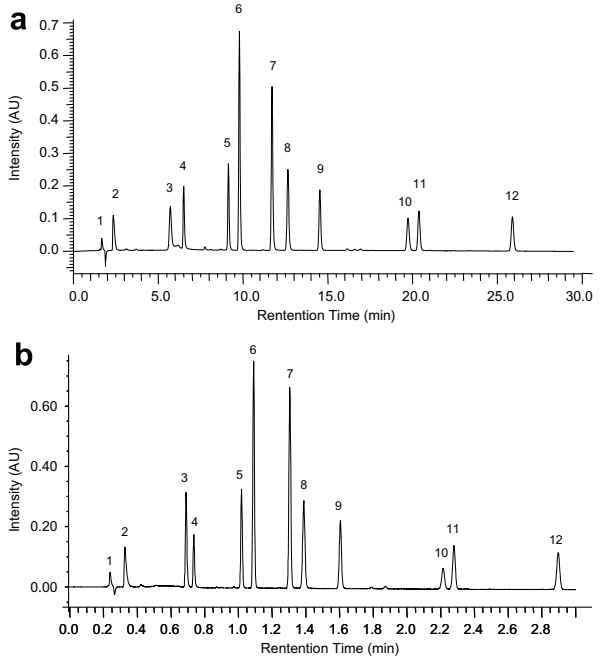

Fig. 5. Separation of a pharmaceutical formulation containing the main product (6) and 11 impurities in gradient mode with HPLC and UPLC system: (a) HPLC system; column: XBridge C_{18} 150 × 4.6 mm, 5 μm; flow rate: 1000 μL/min; injected volume: 20 μL; total gradient time: 45 min. (b) UPLC system; column: ACQUITY BEH C_{18} 50 × 2.1 mm, 1.7 μm; flow rate: 610 μL/min; injected volume: 1.4 μL; total gradient time: 5.1 min.

the sum of three main contributions (Eq. (7): efficiency, selectivity and average retention), these parameters were evaluated independently for each set of conditions. Efficiency was evaluated with the peak capacity in gradient elution. In the tested conditions, peak capacity was equal to 217 with XBridge, and to 205 with Acquity BEH. XBridge was slightly more efficient than Acquity BEH, probably due to the lower contribution of extra-column effects. Few changes in overall selectivity were observed because both columns are packed with the similar chromatographic support. Nevertheless, the first eluted compounds (resolutions between pairs 3–4 and 5–6) showed differences up to 10%. This variability, in the first part of the chromatogram, could be attributed to the dwell volume effect, as previously described. Finally, average retention factors (k_e) were estimated for the three separations according to Eq. (1). Almost no variability in retention was observed which could not explain decrease in resolution. The latter should mainly be attributed to decrease in peak capacity. However, for early eluted compounds, the dwell volume could affect the separation, even if this parameter was not critical with an optimized instrumentation maintaining a t_d/t_0 ratio constant.

Another relevant advantage of fast-LC is the re-equilibrating time reduction. In HPLC (150 × 4.6 mm column at 1 mL/min), re-equilibration took about 20 min, while using a short column packed with sub-2 μm particles (50 × 2.1 mm column at 613 μL/min), the re-equilibrating time decrease to only 2 min.

4.3.2. Optimization of the transferred method

With small particles, Knox curves are quite flat thanks to the improved mass transfer [37,38]. For this reason, it is possible to work beyond the optimum velocity without a significant loss in efficiency. Separation performed in UPLC at a flow rate of 610 μL/min (Fig. 5b) was tested at the maximal flow rate (1000 μL/min).

The chromatogram in Fig. 6a presents the separation in gradient mode not adapted to the mobile phase flow-rate (cf. Table 3) and therefore, a loss in resolution was observed. Separation between peaks 7 and 8 became critical ($R_s < 1.5$) while the separation between peaks 10 and 11 was improved. Furthermore, the analysis time decrease was limited (2.5 vs. 3 min).

For an adequate transfer, it is necessary to adapt the gradient profile and Table 3 gives conditions when flow rate increased to 1000 μL/min (Fig. 6b). Here, no significant chromatographic changes were observed and an important decrease in analysis time (about 50%) from about 3 to 1.7 min was achieved.

Finally, the separation of the 12 analytes was performed 15 times faster compared to the initial separation (27 vs. 1.7 min), without compromising the separation quality.

5. Conclusion

This paper describes some simple rules for transferring successfully a conventional chromatographic separation

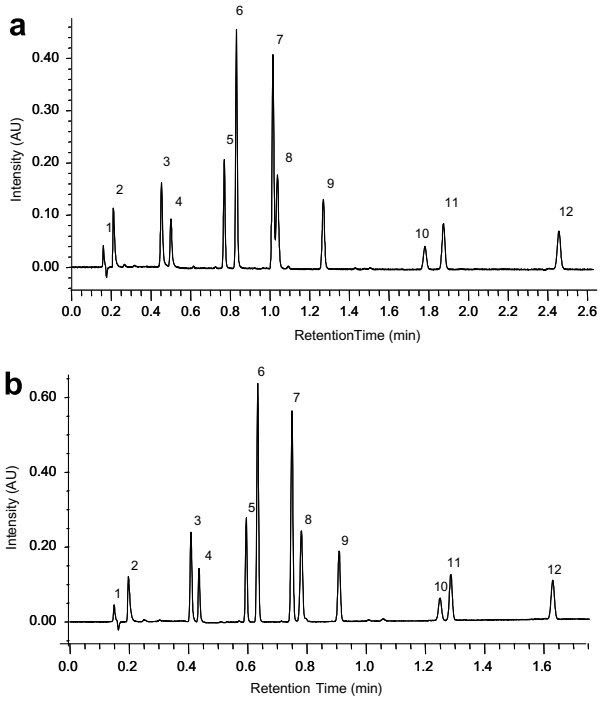

Fig. 6. Effect of a correct and incorrect gradient transfer when mobile phase flow rate was increased. (a) *Incorrect conditions* – column: Acquity BEH C_{18} 50 × 2.1 mm, 1.7 μm; flow rate: 1000 μL/min; injected volume: 1.4 μL; total gradient time: 5.1 min. (b) *Correct conditions* – column: Acquity BEH C_{18} 50 × 2.1 mm, 1.7 μm; flow rate: 1000 μL/min; injected volume: 1.4 μL; total gradient time: 3.1 min.

to a fast one with limited influence on resolution. In the case of gradient elution; injected volume, flow rate, isocratic step duration and gradient slope must be adapted. The presented methodology was applied to speed up impurity profiling in the pharmaceutical industry, following two strategies.

A first approach, dealing with the use of short columns (50–20 mm) packed with 3.5 μm particles and optimized HPLC instrumentation, was applied for the separation of a pharmaceutical drug and eight related impurities. It was possible to reduce the analysis time from 20 min in conventional mode to only 2 min with a short column, with an acceptable loss in resolution.

The second tested approach was Ultra Performance Liquid Chromatography (UPLC), where sub-2 μm particles were simultaneously used with very high pressures (up to 1000 bar) to maintain high efficiency. A complex pharmaceutical mixture was separated with conventional HPLC (column of 150 mm length, 5 μm particles) in 30 min and 9 times faster in UPLC (50 mm column, 1.7 μm particles) thanks to pure geometrical transfer. It was possible to further decrease analysis time (factor 15, i.e. run time equal to 1.7 min) at the maximal flow rate compatible with UPLC. However, chromatographic performance (peak capacity and resolution) was reduced by about 10%. It is important to note that gradient conditions must be adapted when mobile phase flow rate is increased beyond the optimal velocity.

Special attention should also be paid to the dwell volume, which represents the most detrimental parameter for transferring a gradient separation. It is important to simultaneously decrease the dwell volume with the column dead volume.

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