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Rapid high performance liquid chromatography method development with high prediction accuracy, using 5 cm long narrow bore columns packed with sub-2 μm particles and Design Space computer modeling

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ABSTRACT

Many different strategies of reversed phase high performance liquid chromatographic (RP-HPLC) method development are used today. This paper describes a strategy for the systematic development of ultrahigh-pressure liquid chromatographic (UHPLC or UPLC) methods using 5 cm × 2.1 mm columns packed with sub-2 μm particles and computer simulation (DryLab® package). Data for the accuracy of computer modeling in the Design Space under ultrahigh-pressure conditions are reported. An acceptable accuracy for these predictions of the computer models is presented. This work illustrates a method development strategy, focusing on time reduction up to a factor 3–5, compared to the conventional HPLC method development and exhibits parts of the Design Space elaboration as requested by the FDA and ICH Q8R1. Furthermore this paper demonstrates the accuracy of retention time prediction at elevated pressure (enhanced flow-rate) and shows that the computer-assisted simulation can be applied with sufficient precision for UHPLC applications ($p > 400$ bar). Examples of fast and effective method development in pharmaceutical analysis, both for gradient and isocratic separations are presented.

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1. Introduction

The expression "high performance liquid chromatography" was created by Horváth et al. in 1967 [1]. As experimenting in his lab at Yale with superficially porous "pellicular" materials, the pressure went up the first time above 1000 psi, Horváth said, "this is not LC anymore, this is high-pressure LC (HPLC)". The consequent and visionary work of another Hungarian, Halász with small particles 35 years ago laid down the fundaments for columns packed with fine particles and he made separations of 15 compounds possible in 60 s as early as in 1974 [2]. The understanding of the fundamentals of reversed phase chromatography (RPC) performed by Csaba Horváth and his team at Yale, was strongly influencing the future developments in HPLC such as the concepts of the Design Space and DryLab® already in 1976 [3]. The work towards smaller and smaller particles was continued and in the year of 2004 Waters introduced the new technology called UPLC offering new possibilities to reduce analysis time by a factor of 3–4 using higher pressures up to 1000 bar.

Optimization of the selectivity in HPLC practice today means to find the most excellent conditions for a given separation. The FDA is requesting the scientific support of methods as described in the ICH Q8R1 [4]. Practically the first step is to select an appropriate stationary phase, which provides a reasonable separation factor ($k > 1$). Other column performance criteria are a minimum plate number of ca. 10,000, which is necessary for the separation with a suitable peak symmetry. Currently more than 400 commercially available reversed phase columns have been characterized in terms of their relative selectivity, based on five solute-column interactions by the hydrophobic-subtraction model [5]. After selecting the column, the mobile phase composition, the gradient time and temperature and other factors have to be optimized to get a satisfactory separation and establish the Design Space. According to ICH Q8 Design Space means the multidimensional combination and interaction of input variables (e.g., material attributes) and process parameters that have been demonstrated to provide assurance of quality. In most cases the separation can be achieved with an appropriate column and different variants of the mobile phase parameters.

There are different strategies of HPLC method development used today. All isocratic approaches are time consuming and laborious and require considerable solvent consumption. Computer modeling is a helpful tool to optimize the separation [6]. Without reviewing and giving an exhausting report about this segment of

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method development and optimization a short summary is given here. In an effort to improve the efficiency of method development and maximize information about method specificity, several computer modeling programs have been developed in the last 20 years. The most successful and widespread strategy optimizes the Design Space mainly by measuring and visualizing the effects of the mobile phase composition: gradient time and shape, pH, ionic strength, ternary eluent, additive concentrations and temperature. Similar programs are also available, such as ACD's LC-Simulator (Advanced Chemistry Development) and ChromSword (Merck Darmstadt). The programs use a small well-defined number of experiments on a particular stationary phase, predict the separation in parts of the Design Space based on changes in mobile phase composition, mode of elution (either isocratic or gradient), temperature, pH or column parameters such as column length, internal diameter, particle size and flow-rate [7].

Snyder, Dolan and co-workers recommended basic runs for multifactorial experimental designs already in 1996 [8]. A typical approach is to simultaneously model the selectivity of temperature and gradient steepness on a selected RP column for initial basic runs [9,10]. In this case basic runs are those experiments, on which the computer-models are "based" (calculated) to be able to model ca. 5000 experiments with a precision > 97% accuracy. For conventional 25 and 15 cm long columns, 1–2 mL/min flow-rate and a 5–100% acetonitrile–water gradient in time t_{g1} = 20–30 min and t_{g2} = 60–90 min were suggested to provide accurate prediction for further method development. With the help of resolution maps – which show the critical resolution of the peaks to be separated [11] – the gradient program and column temperature can be rapidly and efficiently optimized.

Other software packages use a customized database or vendor database of chromatographic methods, where method conditions can be predicted from compound structures [12,13]. Expert systems (e.g. EluEx) predict pK_a and log P (octanol–water partition coefficients) of the solutes and suggest a mobile phase composition for the separation [14]. These approaches work only, when all of the structures in a sample to be separated are known. Due to the decrease of isocratic method optimization routines and the informative value of linear gradients from 5 to 100% acetonitrile or methanol, most user prefer to run a gradient and find the substance peak experimentally rather quickly. However in the area of drug design the pK_a and log P values play an important role to find drugs with high biological activity.

An automated strategy for HPLC method development (ChromSword) was reported by Galushko et al. [15], who combined different strategies. Krisko et al. presented a strategy, which employs an automated column selection system and a series of HPLC columns, varying in hydrophobicity and silanol activity, in combination with modeling software to develop chromatographic methods [7].

Theoretically these approaches are independent from column type and geometry. After a few number of initial basic runs the optimal chromatographic conditions can be found shortly via computer simulation and prediction. The use of short columns (20–50 mm) and small (sub-2 μm) particles offer the possibility to reduce the analysis time without loosing the resolution [16–18]. Because of its speed and sensitivity, small particles are gaining considerable attention in recent years for pharmaceutical and biomedical analysis [19–23].

The aim of our work was to show, that computer-assisted method development tools can be applied in fast liquid chromatography with high authenticity and efficiency to explore parts of the Design Space. Methods were developed for hybrid type stationary phase (BEH C18), which is a popular column in UHPLC practice, and for other silica-based sub-2 μm packed materials using the DryLab® package. Data are presented for the accuracy of computer prediction, when sub-2 μm packed columns were applied for systematic method development. Examples from the pharmaceutical industry (impurity/degradation profiling, cleaning control analysis) are reported for the separation of neutral and also for basic compounds. At ultrahigh-pressure the retention time is theoretically a complex function of the pressure. This phenomenon was reported several times [24], therefore it was motivating to study how the computer simulation program is functioning at elevated pressure.

2. Experimental

2.1. Chemicals, columns

Acetonitrile and methanol (gradient grade) were purchased from Merck (Darmstadt, Germany). For measurements water was prepared freshly using Milli-Q equipment (Milli-Q gradient A10 by Millipore).

The reference materials and samples, such as ethinylestradiol and its impurities and degradation products, estradiol, dienogest, finasteride, gestodene, norethisterone acetate, levonorgestrel and bicalutamid and its impurities were produced by Gedeon Richter Plc (Budapest, Hungary). Duloxetine and its impurities and degradation products were purchased from Nosch Labs (Hyderabad, India).

Waters UPLC™ BEH C18 column with a particle size of 1.7 μm (50 mm × 2.1 mm) was purchased from Waters Ltd., Budapest. Restek Pinnacle DB C18 column, 50 mm × 2.1 mm, 1.9 μm and Restek Pinnacle DB Biphenyl column, 50 mm × 2.1 mm, 1.9 μm were purchased from Lab-Comp Ltd., Budapest. Zorbax SB C18 column (Agilent) 50 mm × 2.1 mm, 1.8 μm was purchased from Kromat Ltd., Budapest.

2.2. Equipment, software

UPLC was performed using a Waters Acquity system equipped with binary solvent delivery pump, an auto sampler, a photo diode array detector and Empower software. The UPLC system was purchased from Waters Ltd. Budapest, Hungary. The UPLC system had a 5 μL injection loop and a 500 nL flow cell (path length = 10 mm). The dwell volume of the system was measured to be 0.12 mL.

Method development was performed using DryLab® 2010 chromatographic optimization software (Molnar-Institute, Berlin, Germany). The log P (octanol–water partition coefficients) values were predicted by ChemDesk (Medicinal Chemistry at your Desk), which is granted by Computer-aided design and drafting (CADD) and was available at Gedeon Richter Plc.

2.3. Samples applied for method development

In the first example (Section 3.1) spiked tablet samples were chromatographed. The known impurities and degradants of ethinylestradiol as 6-alpha-hydroxy-ethinylestradiol, 6-beta-hydroxy-ethinylestradiol, 6-keto-ethinylestradiol, estradiol and 9,11-didehydro-ethinylestradiol stock solutions were solved in acetonitrile, then spiked to tablet sample solution and diluted with acetonitrile–water (50:50) (V:V).

For the example described in Section 3.2, representative samples for cleaning validation sampling were made. The surface of the equipment line in our plant consists of mostly (>95%) stainless steel but there are other surfaces, which are made of plexi-glass, polytetrafluorethylene (PTFE), silicone and textile. Reference solutions of the compounds of our interest (dienogest, estradiol, ethinylestradiol, finasterid, gestodene, levonorgestrel and norethisterone acetate), blank and spiked solutions sampled from the above mentioned surfaces and placebo solutions were injected during the initial basic runs. The steroids were dissolved in

acetonitrile then diluted with acetonitrile–water 40–60 V/V%. The swabs applied for sampling were soaked in methanol.

In the third example (Section 3.3) spiked and stressed (heat, light and pH) capsule samples were chromatographed. The known impurities and degradants of duloxetine as 1-naphtol, duloxetine-3-isomer impurity, dimethyl-duloxetine impurity, and duloxetine impurity "A" stock solutions were set up in methanol, then spiked to capsule sample solution and diluted with methanol. Then the spiked sample was diluted with water.

In the fourth example (Section 3.4) representative samples for impurity profiling of tablets containing bicalutamid were chromatographed. The known impurities of bicalutamid as bicalutamid-beta-anilin and bicalutamid-beta-sulphenyl stock solutions were solved in methanol, then spiked to tablet sample solution and diluted with acetonitrile-water 30–70 V/V%.

3. Results and discussion

3.1. Example for gradient method development for neutral compounds (simultaneous optimization of gradient program and column temperature for UHPLC separation)

This present example describes a fast and efficient development of a method applied for the determination of impurities and degradation products of a steroid active pharmaceutical ingredient (ethinylestradiol) from tablet, utilizing the separation power of a sub-2 μm packed column. The active ingredient and its impurities and degradants (6-alpha-hydroxy-ethinylestradiol, 6-beta-hydroxy-ethinylestradiol, 6-keto-ethinylestradiol, 9,11-didehydro-ethinylestradiol and estradiol) are polar neutral compounds, so it is not necessary to add buffer to the mobile phase.

Acetonitrile was chosen as organic modifier because its low viscosity and favorable UV cutoff. Simultaneous optimization of gradient program and column temperature was performed using a Restek Pinnacle C18 column with a particle size of 1.9 μm (50 mm × 2.1 mm) with aqueous acetonitrile as mobile phase. The flow-rate was set at 0.5 mL/min. The mobile phase "A" consisted of 5% acetonitrile and 95% water, the mobile phase "B" was acetonitrile. Two basic gradients with different slopes (7 and 21 min gradient time) were carried out at two different column temperatures (35 and 65 °C). The injection volume was 1 μL. Tablet samples spiked with the known impurities and degradation products were chromatographed. Our purpose was to achieve baseline resolution within the analysis time as short as possible.

The result is shown as a resolution map in Fig. 1, where the smallest value of resolution (R_s) of any two critical peaks in the chromatogram is plotted as a function of two simultaneously varied experimental parameters. In this case the parameters are gradient time and column temperature. It can be seen that a fast gradient with very steep slope (gradient time = 2.3 min) can provide the highest resolution if the column temperature is kept at 50 °C. The predicted optimum condition was set and experimental chromatograms were recorded. Fig. 2 shows the predicted and experimental chromatograms.

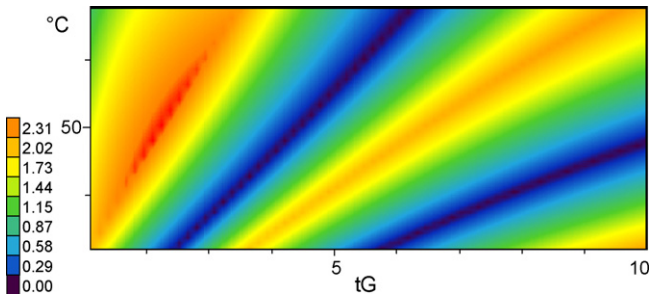


Fig. 1. Two-dimensional resolution map of the column temperature (°C) against gradient time (t_G, min) for the separation of steroid API and its related impurities and degradation products.

To establish the accuracy of our new fast approach (7 and 21 min basic gradient runs) applied for 5 cm × 2.1 mm packed (sub-2 μm) column, the predicted and experimentally derived chromatograms (retention times and resolution) were compared (Table 1). The predicted retention times were in good agreement with the experimental ones; the average of retention time errors was 1.6% (see Table 1), which can be considered as a highly accurate prediction with such rapid gradient profiles. The mean of predicted resolution (R_s) errors was 6.6%. The error of resolution values contains the retention time error and also the uncertainty of peak width prediction. Thus this prediction can be considered as an accurate support from the software package DryLab® and the suggested fast gradient basic runs (7 and 21 min) can be applied in daily routine work resulting in significant time saving. The time spent for method development in this example was approximately 5 h (2 gradient time × 2 temperature × 5 samples), and then the predicted method was verified in experiments and proved to be a suitable separation. Since then this method was validated and applied in our laboratory for routine analysis. Previously a 25 min long conventional separation was applied for this task, so the analysis time was shortened with a factor of about 10.

3.2. Example for isocratic method development for neutral compounds (simultaneous optimization of isocratic % B and column temperature for UHPLC separation)

Our second example describes the development process of a generic method, which is applied for simultaneous determination

Table 1
Experimental retention times and resolutions vs. predicted from the two-dimensional gradient time–temperature model.

Peaks	Retention time (min)				Resolution			
	Experimental	Predicted	Difference[a]	% error[b]	Experimental	Predicted	Difference[a]	% error[b]
1	0.65	0.64	0.01	1.56	–	–	–	–
2	0.72	0.73	−0.01	−1.37	2.61	3.09	−0.48	−15.53
3	0.94	0.97	−0.03	−3.09	8.41	8.62	−0.21	−2.44
4	1.14	1.13	0.01	0.88	5.56	5.38	0.18	3.35
5	1.49	1.48	0.01	0.68	9.9	9.68	0.22	2.27
6	1.61	1.59	0.02	1.26	2.75	2.80	−0.05	−1.79
7	1.73	1.69	0.04	2.37	2.98	2.51	0.47	18.73
8	1.86	1.81	0.05	2.76	2.05	1.94	0.11	5.67
9	2.30	2.29	0.01	0.44	11.52	11.83	−0.31	−2.62
Average			0.02	1.60			0.25	6.55

[a] Difference = experimental − predicted.
[b] % error = [(experimental − predicted)/predicted] × 100.

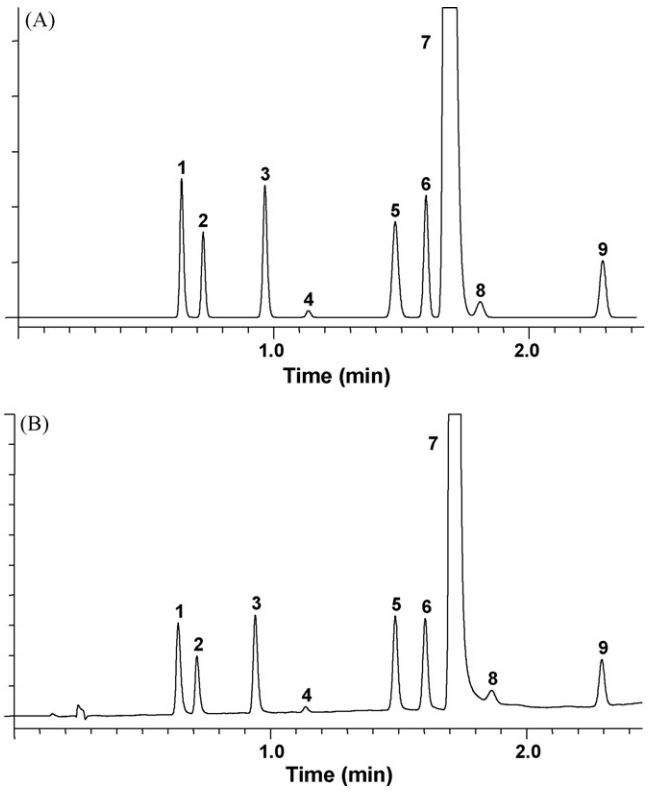


Fig. 2. Predicted (A) and experimental (B) chromatograms were optimized by 7 and 21 min gradient basic runs at two different column temperature (35 and 65 °C). Column: Restek Pinnacle C18 1.9 μm (50 mm × 2.1 mm), mobile phase "A": acetonitrile–water 5–95 V/V%, mobile phase "B": acetonitrile, gradient elution (35–70% B, in 2.3 min), flow: 0.5 mL/min (p = 299 bar), column temperature: 50 °C, injection volume: 1 μL, detection: 220 nm, analytes: a neutral polar API (steroid) and its related impurities and degradation products: (1) 6-alpha-hydroxy-ethinylestradiol, (2) 6-beta-hydroxy-ethinylestradiol, (3) 6-keto-ethinylestradiol, (4) unknown degradant, (5) estradiol, (6) 9,11-didehydro-ethinylestradiol, (7) ethinylestradiol, (8) unknown degradant and (9) unknown impurity.

of seven steroid API residues (dienogest, estradiol, ethinylestradiol, finasterid, gestodene, levonorgestrel and norethisterone acetate) in support of cleaning control analysis in formulation area. In the case of steroids (neutral compounds) the solubility (log P octanol–water partition coefficient) is one of the most important properties regarding selectivity. Under suitable chromatographic conditions isocratic and extrapolated retention factors correlate well with octanol–water partition (log P) or distribution coefficients [25]. In this case the log P values of the compounds to be separated were previously predicted (with ChemDesk) and because of the small differences in log P values ($\log P_{\text{dienogest}}$: 3.013, $\log P_{\text{estradiol}}$: 3.784, $\log P_{\text{ethinylestradiol}}$: 3.860, $\log P_{\text{finasterid}}$: 3.813, $\log P_{\text{gestodene}}$: 3.215, $\log P_{\text{levonorgestrel}}$: 3.490 and $\log P_{\text{norethisterone acetate}}$: 4.466) isocratic basic runs were considered.

Simultaneous optimization of isocratic % B and column temperature was performed using a Waters UPLC™ BEH C18 column with a particle size of 1.7 μm (50 mm × 2.1 mm) with aqueous acetonitrile as mobile phase. The flow-rate was set at 0.5 mL/min. The mobile phase "A" consisted of 5% acetonitrile and 95% water, the mobile phase "B" was acetonitrile. Two basic isocratic runs (35 and 55% B) at two different column temperature (45 and 60 °C) were achieved. The injection volume was 1 μL. The obtained resolution map is shown in Fig. 3. In this case the critical resolution in the chromatogram is plotted as a function of mobile phase % "B" and temperature. The resolution map shows that a separation achieved with 40% "B" eluent at 50 °C would give a sufficient resolution. The flow-rate was elevated to 0.65 mL/min to perform a really fast

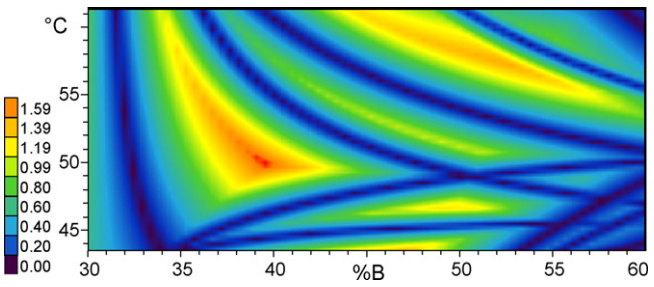


Fig. 3. Two-dimensional resolution map of the column temperature (°C) against mobile phase % "B" for the separation of steroid API residues.

separation. The predicted optimum condition was set and experimental chromatograms were recorded. Fig. 4 shows the predicted and experimental chromatograms.

The precision of prediction in the case of isocratic % "B"—temperature model under UHPLC conditions was evaluated with the comparison of predicted and experimentally obtained retention times and resolutions (Table 2).

The predicted and experimentally obtained chromatograms (retention times and resolution) were compared. The predicted retention times were in excellent agreement with the experimental ones, the average of retention time errors was 0.74% (see Table 2). The resolutions also were predicted with high accuracy (average of errors was 3.76%). So we can state that computer-assisted simulation can be applied with high precision for UHPLC conditions in the case of simultaneous optimization of isocratic % B and column tem-

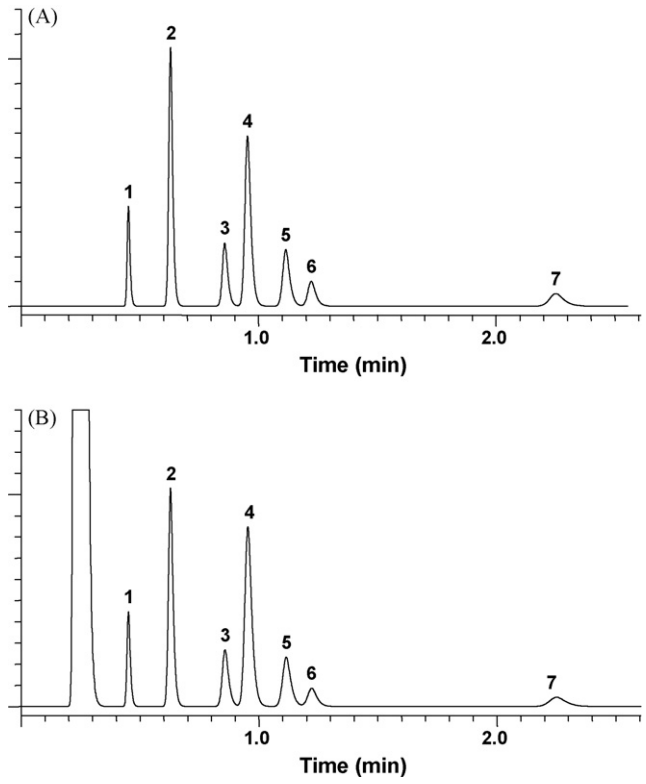


Fig. 4. Predicted (A) and experimental (B) chromatograms were optimized by 35 and 55% B isocratic basic runs at two different column temperature (45 and 60 °C). Column: Waters UPLC BEH C18 1.7 μm (50 mm × 2.1 mm), mobile phase "A": acetonitrile–water 5–95 V/V%, mobile phase "B": acetonitrile, isocratic elution with 40% B, flow: 0.65 mL/min (p = 435 bar), column temperature: 50 °C, injection volume: 1 μL, detection: 220 nm, analytes: neutral polar APIs (steroids): (1) dienogest, (2) estradiol, (3) ethinylestradiol, (4) finasterid, (5) gestodene, (6) levonorgestrel and (7) norethisterone acetate.

Table 2
Experimental retention times and resolutions vs. predicted from the two-dimensional isocratic % B—temperature model.

Peaks	Retention time (min)				Resolution			
	Experimental	Predicted	Difference[a]	% error[b]	Experimental	Predicted	Difference[a]	% error[b]
1	0.45	0.45	0.00	0.00	–	–	–	–
2	0.62	0.63	−0.01	−1.59	6.02	6.11	−0.09	−1.47
3	0.87	0.86	0.01	1.16	5.84	5.95	−0.11	−1.85
4	0.96	0.95	0.01	1.05	1.80	1.83	−0.03	−1.64
5	1.12	1.11	0.01	0.90	2.44	2.70	−0.26	−9.63
6	1.22	1.22	0.00	0.00	1.50	1.58	−0.08	−5.06
7	2.23	2.24	−0.01	−0.45	10.40	10.71	−0.31	−2.89
Average			0.01	0.74			0.15	3.76

[a] Difference = experimental − predicted.
[b] % error = [(experimental − predicted)/predicted] × 100.

perature. In this example the time spent on method development was less than 4 h.

3.3. *Example for gradient method development for basic compounds (simultaneous optimization of gradient program and mobile phase pH for UHPLC separation)*

Simultaneous optimization of gradient program and mobile phase pH was performed using a Zorbax SB C18 column with a particle size of 1.8 μm (50 mm × 2.1 mm) with methanol and buffer as mobile phase. The flow-rate was set at 0.5 mL/min. The mobile phase "A" consisted of 5% methanol and 95% buffer (10 mM phosphate + 0.1% triethylamine), the mobile phase "B" was 80% methanol and 20% buffer. Two basic gradients with different slopes (7 and 21 min gradient time) were run at three different mobile phase pH values (pH_1 6.2, pH_2 6.6 and pH_3 7.0). The injection volume was 3 μL. Capsule samples spiked with the known impurities and degradation products were chromatographed. Our purpose was to achieve baseline resolution focusing on duloxetine and duloxetine-3-isomer peaks. Earlier several attempts were made to separate this peak-pair on conventional systems in our laboratory, but these attempts were unsuccessful. Now the effect of pH on selectivity was systematically evaluated and simulated. With the help of resolution map as a function of pH, and the efficiency (peak capacity) of sub-2 μm particles, baseline separation can be achieved between the mentioned critical peak-pair.

The obtained resolution map is shown in Fig. 5. In this case the critical resolution in the chromatogram is plotted as a function of gradient time (min) and mobile phase pH. The resolution map shows that a separation achieved at pH 6.7 would give a sufficient resolution within 10 min. The predicted optimum condition was set and experimental chromatograms were recorded. Fig. 6 shows the predicted and experimental chromatograms.

The precision of prediction in the case of gradient program—mobile phase pH model under UHPLC conditions was evaluated with the comparison of predicted and experimentally obtained retention times and resolutions (Table 3).

The predicted retention times were also in excellent agreement with the experimental ones. When mobile phase pH was optimized the average of retention time errors was under 2% (see Table 3). The mean of predicted resolution (R_s) errors was 6.5%. The time spent for method development in this example required approximately 7 h (2 gradient time × 3 pH), then the predicted method was verified in experiments and proved to be an appropriate separation. Previously the peaks of duloxetine and duloxetine-3-isomer failed to separate with conventional methods but with this UHPLC method a separation of this critical peak-pair with a resolution of R_s = 1.89 was possible to achieve within 10 min.

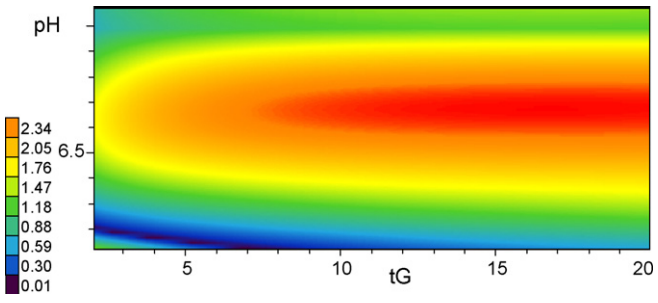


Fig. 5. Two-dimensional resolution map of the gradient time (min) against mobile phase pH for the separation of basic API and its related impurities and degradation products.

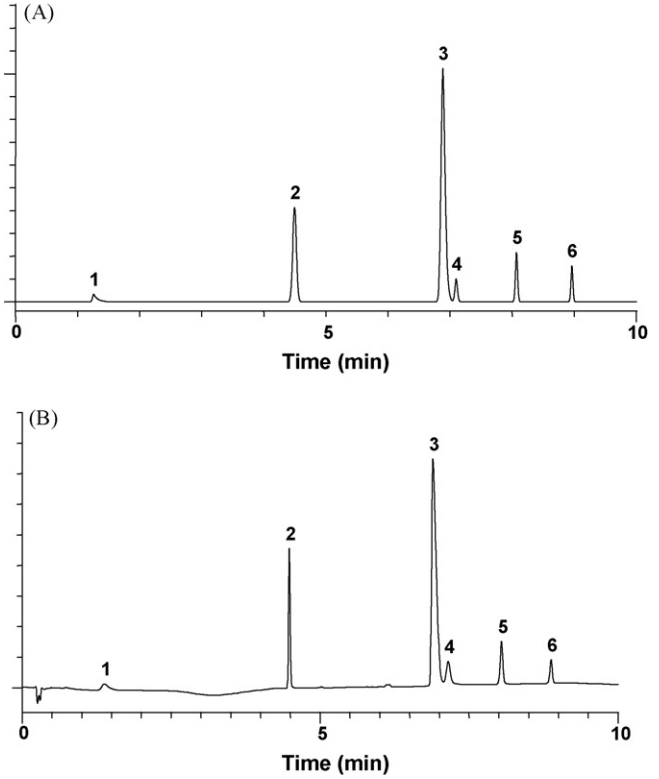


Fig. 6. Predicted (A) and experimental (B) chromatograms. Column: Zorbax SB C18 50 mm × 2.1 mm, 1.8 μm, mobile phase "A": methanol–buffer 5–95 V/V% (buffer: 10 mM phosphate + 0.1% triethylamine, pH 6.7), mobile phase "B": methanol–buffer 80–20 V/V% (buffer: 10 mM phosphate + 0.1% triethylamine, pH 6.7), gradient elution (initial 0% B, at 0.7 min 0% B, at 3.1 min 65% B and 100% B at 10 min), flow: 0.5 mL/min (p = 531 bar), column temperature: 30 °C, injection volume: 3 μL, detection: 230 nm, analytes: basic drug API and its related impurities and degradation products: (1) peak of light stress origin (unknown) (2) 1-naphtol (3) duloxetine (4) duloxetine-3-isomer impurity (5) dimethyl-duloxetine impurity and (6) duloxetine impurity "A".

Table 3
Experimental retention times and resolutions vs. predicted from the two-dimensional gradient time–mobile phase pH model.

Peaks	Retention time (min)				Resolution			
	Experimental	Predicted	Difference[a]	% error[b]	Experimental	Predicted	Difference[a]	% error[b]
1	1.38	1.27	0.1	8.66	–	–	–	–
2	4.52	4.5	0.02	0.44	25.13	23.94	1.19	4.97
3	6.9	6.95	−0.05	−0.72	25.31	26.80	−1.49	−5.56
4	7.17	7.12	0.05	0.70	1.92	2.13	−0.21	−9.86
5	8.08	8.04	0.04	0.50	8.66	9.34	−0.68	−7.28
6	8.94	8.98	−0.04	−0.45	10.78	11.33	−0.55	−4.85
Average			0.05	1.91			0.82	6.50

[a] Difference = experimental − predicted.
[b] % error = [(experimental − predicted)/predicted] × 100.

3.4. Prediction accuracy at elevated pressure by varying the flow-rate (simultaneous optimization of gradient program and column temperature for UHPLC separation)

This example illustrates the accuracy of retention time prediction when the flow-rate was changed compared to, as it was set during the basic experimental runs. A fast and efficient development of a method applied for the determination of impurities of an active pharmaceutical ingredient (bicalutamid) from tablet, utilizing the separation power of a sub-2 μm packed column was performed. The active ingredient and its impurities (bicalutamid, bicalutamid-beta-anilin and bicalutamid-beta-sulphenyl) are polar neutral compounds.

Simultaneous optimization of gradient program and column temperature was performed using a Restek Pinnacle DB Biphenyl column with a particle size of 1.9 μm (50 mm × 2.1 mm) with aqueous acetonitrile as the mobile phase. The flow-rate was set at 0.4 mL/min. The mobile phase "A" consisted of 5% acetonitrile and 95% water, the mobile phase "B" was acetonitrile. Two basic gradients with different slopes (7 and 21 min gradient time) were carried out at two different column temperatures (35 and 65 °C).

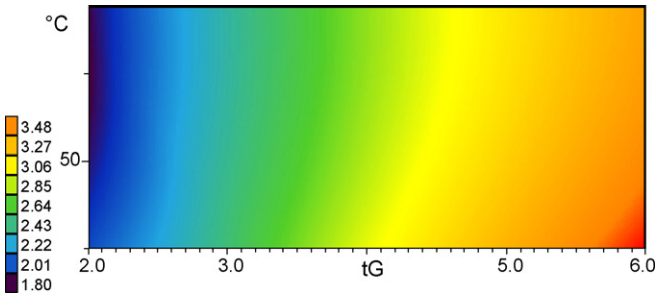


Fig. 7. Two-dimensional resolution map of the column temperature (°C) against gradient time (t_G, min) for the separation of bicalutamid and its related impurities.

The injection volume was 2 μL. Tablet samples spiked with the known impurities were chromatographed. Our purpose was to achieve a fast separation and then to predict the retention times for different (elevated) flow-rates based on the initial runs performed at 0.4 mL/min. A fast gradient separation (30–68% B, in 6 min) could provide suitable resolution if the column tempera-

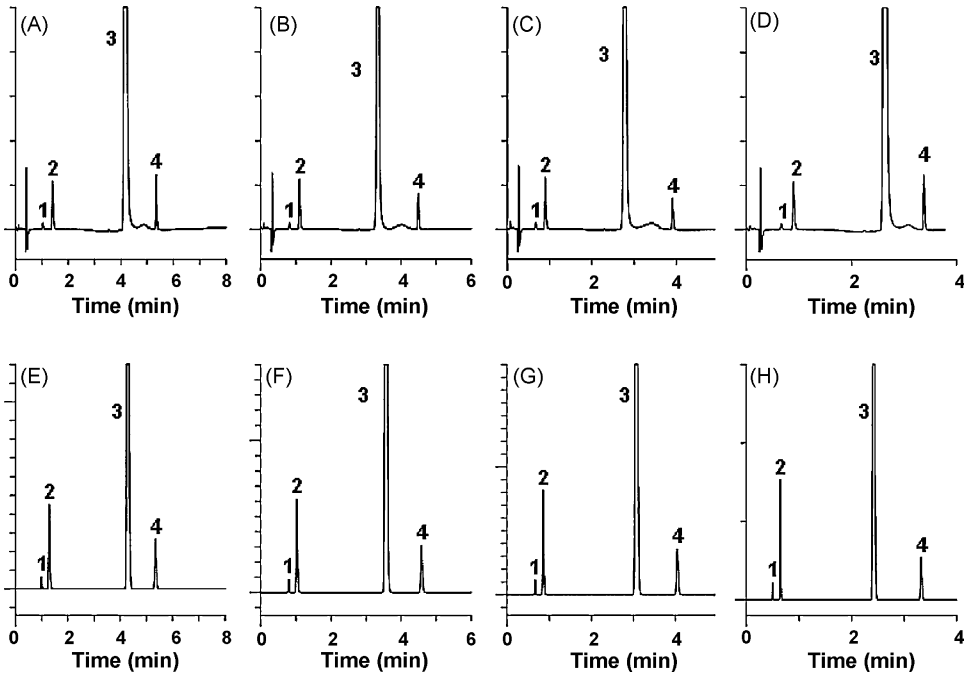


Fig. 8. Experimental (A–D) and predicted (E–H) chromatograms. Column: Restek Pinnacle DB Biphenyl 1.9 μm (50 mm × 2.1 mm), mobile phase "A": acetonitrile–water 5–95 V/V%, mobile phase "B": acetonitrile, gradient elution (30–68% B, in 6 min), flow: 0.4 mL/min (A and E), 0.5 mL/min (B and F), 0.6 mL/min (C and G) and 0.8 mL/min (D and H), column temperature: 45 °C, injection volume: 2 μL, detection: 270 nm, analytes: a neutral polar API and its related impurities: (1) unknown impurity, (2) bicalutamid-beta-anilin, (3) bicalutamid, and (4) bicalutamid-beta-sulphenyl. The prediction is based on basic runs performed with the flow-rate of 0.4 mL/min.

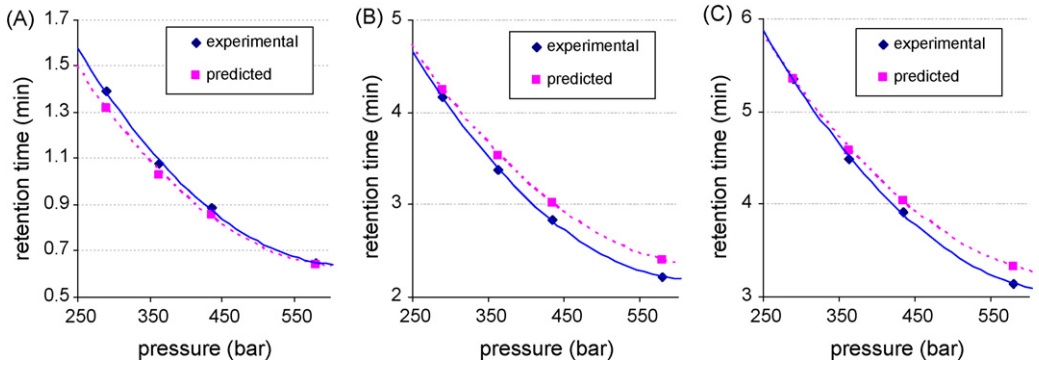


Fig. 9. Experimental and predicted retention times plotted against pressure. Analytes: (A) bicalutamid-beta-anilin, (B) bicalutamid, and (C) bicalutamid-beta-sulphenyl.

ture is kept at 45 °C (Fig. 7). The predicted optimum condition was set and experimental chromatograms were recorded with different flow-rates (0.4, 0.5, 0.6 and 0.8 mL/min). Fig. 8 shows the predicted and experimental chromatograms.

At first sight the predicted retention times are in good agreement with the experimental ones. But, when the predicted and experimental values are plotted against the pressure (Fig. 9), significant differences can be seen between the slopes of the fitted curves. The curves fitted on experimental retention times have steeper slope than the curves fitted on predicted values in each case. So the accuracy of computer prediction is really depends on the applied pressure (flow-rate), but when the flow-rate is enhanced with a factor of 1.2–2.0 compared to the value applied for basic runs—the computer-assisted simulation can be applied with sufficient precision for UHPLC applications. The average of retention time errors did not exceed 5% when the flow-rate (pressure) was duplicated (Fig. 10). When the flow-rate was enhanced with a factor of 1.25 and 1.50 – compared to the flow applied for basic runs – the prediction error was approximately 3 and 4% (respectively). Further experiments are going to be achieved to study this phenomenon.

Further work is planned towards three-dimensional optimization of the above examples to be able to show the influence of more then the basic factors (gradient slope and column temperature) by the systematic study of the ternary composition and the pH at the same time. In this way we want to explore the Design Space more detailed according to the requests of the regulatory authorities to prove scientifically, that the methods are robust and reliable.

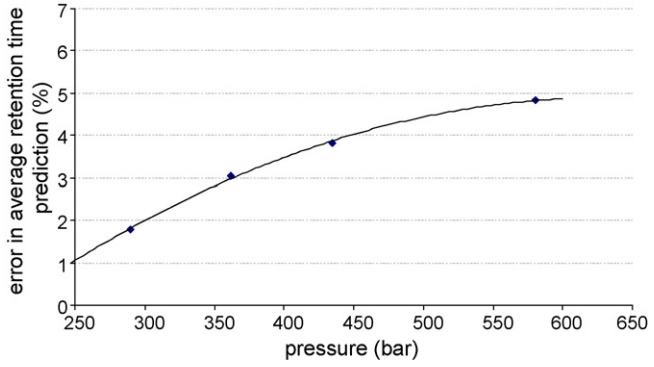


Fig. 10. Retention time prediction error (average) against pressure. Column: Restek Pinnacle DB Biphenyl 1.9 μm (50 mm × 2.1 mm), mobile phase "A": acetonitrile–water 5–95 V/V%, mobile phase "B": acetonitrile, gradient elution (30–68% B, in 6 min), flow: 0.4 mL/min (p = 290 bar), 0.5 mL/min (p = 362 bar), 0.6 mL/min (p = 435 bar) and 0.8 mL/min (p = 581 bar), column temperature: 45 °C, analytes: a neutral polar API and its related impurities.

4. Conclusion

The separation power of short columns packed with sub-2 μm particles are reported many times. But in this study it is also proved that by using computer modeling, the time required for method development can be considerably reduced.

It is possible to develop methods for pharmaceutical analysis (assay, impurity profiling, cleaning validation) within a day or even in a few hours. If a 50 mm × 2.1 mm sub-2 μm column is applied during the systematic method development, basic gradient runs with 7 and 21 min (at a flow-rate of 0.4–0.5 mL/min) can provide reliable accuracy for the computer model simulation under ultrahigh-pressure conditions if gradient separation is necessary. The average of predicted retention time errors was lower than 2%, which can be considered as a highly accurate prediction, so the suggested fast gradient initial basic runs can be applied in daily routine work resulting in significant time saving. Based on our experiments we can state that DryLab® separation modeling can be applied for elevated pressure (not only in HPLC practice) with high accuracy. The average of retention time errors did not exceed 5% when the flow-rate (pressure) was duplicated (p ~600 bar). When the flow-rate was enhanced with a factor of 1.25 and 1.50 – compared to the flow applied for basic runs – the prediction error was approximately 3 and 4% (respectively).

The second point is that the column technology used for sub-2 μm particles is well developed to reduce the silanol activity of the stationary phase and thus the computer simulation can be applicable also for the separation of basic solutes with reliable precision.

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