Journal of Pharmaceutical and Biomedical Analysis 50 (2009) 426–431

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

A systematic stability evaluation of analytical RP-HPLC columns

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ARTICLE INFO

Article history:
Received 6 April 2009
Received in revised form 21 May 2009
Accepted 22 May 2009
Available online 30 May 2009

Keywords:
Column stability evaluation
Column stability
pH range
Silica
RP-HPLC method development

ABSTRACT

HPLC column stability is one of the critical factors that determine the success of a method while supporting the life cycle of a pharmaceutical product. A systematic approach for the evaluation of HPLC column stability has been developed with emphasis on the practicality of the application to pharmaceutical analysis. This paper describes the specifics of the experimental design, evaluation criteria used and result obtained for some of the most widely used analytical columns from highly reputable column manufacturers. A stability profile over the most commonly used pH range was established that may serve as a reference for column scouting during method development.

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1. Introduction

Quality by design (QbD) has become an area of increased focus in the pharmaceutical industry following the introduction of the risk-based quality initiative by the FDA [1] and publication of new guidance documents by the ICH [2–3]. The QbD principles are not merely confined to the development of pharmaceutical manufacturing processes but also apply to analytical method development to gain in-depth understanding of the link between method variables and the method performance. The performance characteristics to be studied cover those outlined in the ICH Q2 (R1) such as specificity, linearity, accuracy, precision, sensitivity, robustness and ruggedness [4]. Conventionally, HPLC method development was either trial and error based or relied on the experience and judgement of analysts [5]. By following QbD principles, the analyst obtains understanding of the method variables on method performance and of the risk associated with the method. This allows the risks to be managed by a control strategy. In addition, the requirements of a HPLC method are dependent on the development stage of the drug product. In early phase of drug development, requirements are less stringent. However, methods for late phase pharmaceutical development and commercialization need to be thoroughly understood, robust and rugged. A comprehensive example applying QbD principles to RP-HPLC method development has been described by Li et al. [6]. The authors provided a roadmap of a QbD method development emphasizing predefined objectives using sound development tool, and scientific understanding to achieve method control and to manage risk. HPLC column stability is one of the critical factors impacting the method performance and has been widely studied [7–10]. Column stability can be affected by temperature, type of aqueous buffer and their concentration, organic solvents, additives and mobile phase pH. This paper provides a systematic evaluation on column stability at different mobile phase pH. The results can be used as independent information in addition to that provided by the column manufacture. Column manufactures usually use various testing methods and criteria to evaluate column stability [9,10]. A robust HPLC method utilizing a durable column is highly desirable for ensuring timely turnaround of analytical results in support of successful clinical and commercial manufacturing.

This paper describes a systematic approach for evaluating the stability of RP-HPLC columns within the pH range claimed to be stable by the vendors. The evaluation was performed based on predefined objectives and criteria using an experimental design. The columns evaluated in this paper are Agilent Bonus RP C18 (pH 2–9) and Zorbax SB C8 (pH 1–8), Phenomenex Luna C18 (2) (pH 1.5–10), Waters XTerra MS C18 (pH 1–12), Waters XBridge C18 (pH 1–12), Waters Sunfire C18 (pH 1–7) and Waters XBridge Phenyl (pH 1–12). These columns were selected based on different column chemistry, such as functional group (C8, C18 and phenyl), selectivity, etc. The selected columns cover most applications of small molecule pharmaceutical analysis. In addition, column availability from secure suppliers was considered. We introduce a systematic approach for evaluating column stability that is practical and can be applied as an

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doi:10.1016/j.jpba.2009.05.028

Table 1
Test mixture components and their working concentrations.

Identifier	Test compounds	Diluent for stock solution (5 mg/mL)	Component concentration in the test mixture (mg/mL)
B1	Pyridine	Water	0.2
B2	Quinine	Acetonitrile	0.05
B3	Nortriptyline HCl	Water	0.05
A1	3-Methyl-4-nitrobenzoic acid	Acetonitrile	0.1
A2	4-Chlorocinnamic acid	Acetonitrile	0.05
A3	Phenol	Water	0.05
N1	Benzyl alcohol	Water	0.05
N2	Acetophenone	Acetonitrile	0.1
N3	Hexanophenone	Acetonitrile	0.15
N4	5-Methylsalicylaldehyde	Acetonitrile	0.1

Table 2
Experimental design for column stability studies.

	pH 2	pH 3	pH 6	pH 7	pH 7	pH 8	pH 9	pH 10	pH 11
Column	0.1% TFA	10mM phosphate	10mM phosphate	10 mM ammonium acetate	10mM phosphate	10mM bicarbonate	10mM Ammonia/Bicarbonate	0.1% ammonia	10 mM phosphate
Waters XBridge C18	x	–	–	–	x	x	x	x	x
Waters XTerra MS C18	x	–	x	x	x	x	x	–	–
Waters XBridge Phenyl	x	–	–	x	x	x	x	x	–
Phenomenex Luna C18	x	x	x	x	x	x	x	–	–
Agilent Zorbax Bonus RP C18	x	x	x	x	x	x	x	–	–
Waters Sunfire C18	x	x	x	x	x	–	–	–	–
Agilent Zorbax SB C8	x	–	–	–	x	x	–	–	–

Column dimension and particle size: 4.6 mm × 15 mm, 3.5 μm or 3.0 μm by vendors' availability.
"–" no experimentation was performed.
"x" stability evaluation.

integral part of method development. The resulting data can serve as a reference to aide column selection to achieve an overall optimal method performance.

2. Experimental

2.1. Chemicals and test compounds

Trifluoroacetic acid (TFA), ammonium hydroxide (ammonia, 28–30%) and ammonium bicarbonate (NH_4HCO_3, Baker analyzed reagent) were from J.T. Baker. Potassium phosphate monobasic (ACS reagent 99.0%), potassium phosphate dibasic trihydrate (ReagentPlus, ≥99.0%), potassium phosphate tribasic (reagent grade ≥98%), and phosphoric acid (≥85.0%) were from Sigma–Aldrich. The following chemicals, selected as the test compounds to be included in preparation of test mixture, were obtained from Sigma–Aldrich: 4-chlorocinnamic acid (99%, $pK_a < 4.0$), 3-methyl-4-nitrobenzoic acid (99%, $pK_a < 4.2$), pyridine anhydrous (99.0%, $pK_a = 5.19$), 5-methylsalicylaldehyde (98%, $pK_a = 8.47$), quinine (90%, $pK_a = 8.7$), nortriptyline hydrochride (98%, $pK_a = 9.7$), phenol (99+%, $pK_a = 9.86$), benzyl alcohol anhydrous (99.8%), acetophenone (reagent plus, 99%), and hexanophenone (99%).

The different test mixtures consisted of at least two acidic compounds and two basic compounds. At each chromatographic condition (Table 1), a scouting injection of 1 μL of individual stock solution of the above mentioned test compounds was made and the resulted chromatograms were examined. Only those test compounds that were baseline resolved ($R_s \geq 1.5$) were included in the preparation of test mixture. The test mixture solution was prepared in acetonitrile and water (1:1 v/v) by mixing the selected test compounds at the target working concentration as specified in Table 1.

2.2. Columns and mobile phases

Agilent 1100 HPLC system (Palo Alto, CA) equipped with quaternary pump, autosampler, column oven, degasser and diode array detector was used. Waters Empower software was used to acquire, store, and process the chromatographic data and to report results.

The HPLC columns used in this study included: Waters (Milford, MA, USA) XTerra MS C18 (4.6 mm × 150 mm, 3.5 μm), XBridge C18 (4.6 mm × 150 mm, 3.5 μm), XBridge phenyl (4.6 mm × 150 mm, 3.5 μm) and Sunfire C18 4.6 mm × 150 mm, 3.5 μm), Phenomenex (Torrance, CA, USA) Luna C18 (2) (4.6 mm × 150 mm, 3 μm), Agilent (Wilmington, DE, USA) Zorbax SB C8 (4.6 mm × 150 mm, 3.5 μm) and Zorbax Bonus RP (4.6 mm × 150 mm, 3.5 μm). Mobile phase A, which allowed studying of column stability in pH range from 2 to 11, was described in Table 2. Mobile phase B was acetonitrile, which was ramped from 5% to 95% in 10 min when organic buffers were used while the gradient for inorganic buffer was from 5% to 70%. The other chromatographic conditions were flow rate of 1.0 ml/min, column temperature of 40 °C, UV detection at 215 nm, and injection volume of 10 μL.

Table 3
Injection sequence.

	Events	Total number of injections
Stage 1	10 injections of test mixture solution at beginning 100 injections of blank (water/acetonitrile = 1/1, v/v) 10 injections of test mixture solution (end of Stage 1) Flow stopped 30 min	120
Stage 2	100 injections of blank 10 injections of test mixture solution Flow stopped 30 min	230
Stage 3	100 injections of blank 10 injections of test mixture solution Flow stopped 30 min	340
Stage 4	100 injections of blank 10 injections of test mixture solution Flow stopped 30 min	450
Stage 5	100 injections of blank 10 injections of test mixture solution Flow stopped 30 min	560

Table 4
Criteria for column stability evaluation.

Parameters	Acceptance criteria
Chromatograms	No peak splitting
Back column pressure, ΔP	No significantly change and ≤ 350 bar
Theoretical plate, N	RSD for all components ≤ 5.0%
Capacity factor, k'	RSD for all components ≤ 5.0%
Selectivity, α	RSD for all components ≤ 5.0%
USP tailing factor, T_f	$0.8 \le T_f \le 1.8$ and RSD for all components ≤ 5.0%
Resolution, R_s	$R_s \le 1.5$ and RSD for all components ≤5.0%
Peak area	RSD for all components ≤5.0%

2.3. Experimental design

Column stability was assessed within the pH range claimed by their respective vendors. The protocol is intended to evaluate the impact of mobile phase on column stability. The impact of sample solution is very minimal, as typically only a few μg of sample are loaded. The test mixture was chosen to assess column performance, not to assess the impact of the test probes themselves on column stability. Using injection number is a measure to indicate the residence time of mobile phase in the column. The stability of the column was first evaluated at pH 2 using 0.1% TFA, then at pH 7 using 10 mM phosphate buffer. If the column performance at pH 7 using 10 mM phosphate buffer did not sustain 200 injections, then stability of the corresponding column was evaluated using less aggressive buffers at pH 3 and 6, and lastly at pH 7 using 10 mM ammonium acetate. Only if a column was demonstrated to last over 200 injections at pH 7 using phosphate or ammonium acetate buffers did the stability testing continue at pH > 7 using organic buffers. The 5-stage injection sequence used is summarized in Table 3. On each stage, performance of column stability was evaluated against the predefined evaluated criteria in Table 4.

2.4. Evaluation criteria

Column stability was evaluated against the parameters listed in Table 4. In general, the performance parameters included theoretical plates (N), capacity factor (k), selectivity (α), tailing factor (T_f) and column back pressure (P) In this study, resolution and injection to injection reproducibility (evaluated in terms of variation of peak area) were also included in evaluation. The acceptance criteria were set to ensure a high degree of column performance during routine analysis. Table 4 lists those performance parameters and the corresponding acceptance criteria.

3. Results and discussion

For the purpose of an illustrative example, the details of the Agilent Zorbax Bonus RP C18 column at pH 7 using 10 mM phosphate buffer study are used to describe the evaluation process. The same approach was used to evaluate the other columns in Table 2. The test solution was a mixture of pyridine (B1), 3-methyl-4-nitrobenzoic acid (A1), 4-chlorocinnamic acid (A2), acetophenone (N2), nortriptyline (B3) and 5-methylsalicylaldehyde (N4) with their concentrations indicated in Table 1.

Typical overlaid chromatograms and column back pressures are displayed in Figs. 1 and 2. Fig. 1 shows the peaks of two acidic compounds, 3-methyl-4-nitrobenzoic acid (A1) and 4-chlorocinnamic acid (A2), tailed strongly at pH 7 (T_f is 2.8 and 2.7, respectively). At

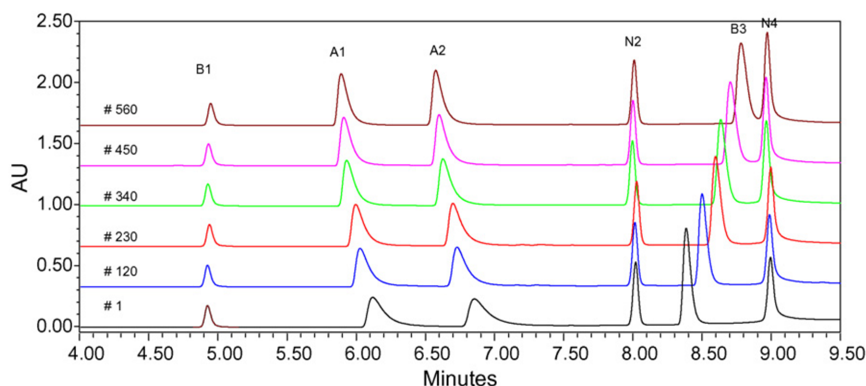

Fig. 1. Overlaid chromatograms of more than 500 injections at pH 7 (10 mM phosphate buffer, Zorbax Bonus RP C18). The injection number is shown within the figure.

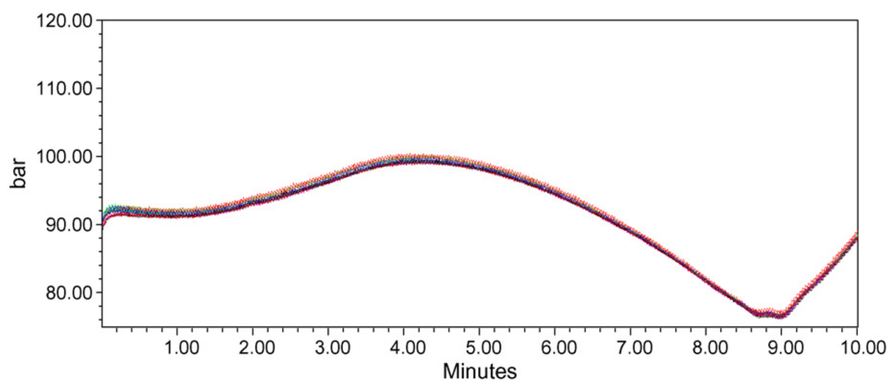

Fig. 2. Overlaid plot of column pressures of more than 500 injections at pH 7 (10 mM phosphate buffer, Zorbax Bonus RP C18). The injection numbers are #1, #120, #230, #340, #450 and #560.

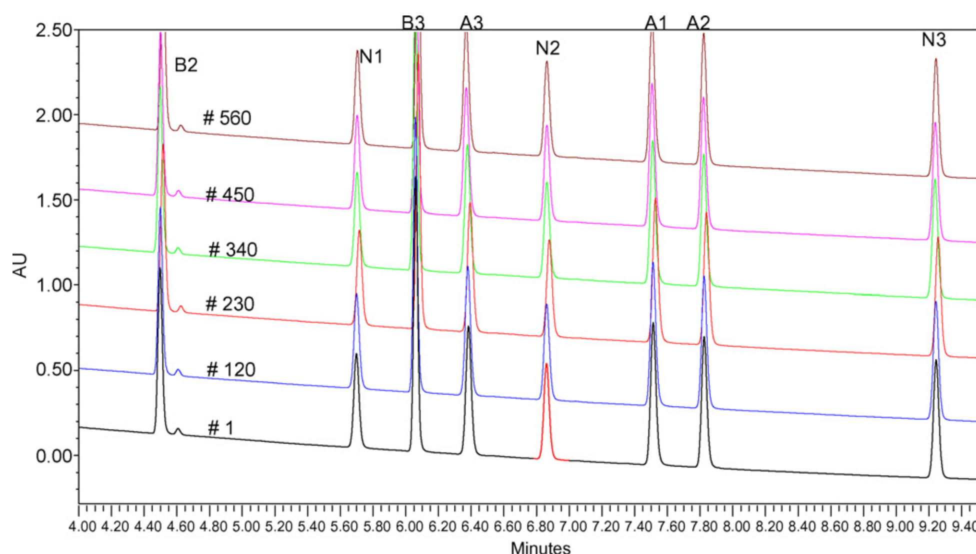

Fig. 3. Overlaid chromatograms of more than 500 injections at pH 2 (0.1% TFA, Zorbax Bonus RP C18). The injection number is shown within the figure.

Table 5
Average theoretical plate (N) at different stages using 10 mM phosphate bufferat pH 7 (Zorbax Bonus RP C18 column).

N	Beginning	End of Stage 1	Stage 2	Stage 3	Stage 4	Stage 5	%RSD of all stages
B1	58,417	59,210	59,292	59,905	59,721	60,926	1.5
A1	13,150	20,328	22,736	25,893	28,148	30,862	24.6
A2	12,402	22,043	25,609	29,399	33,804	37,805	31.1
N2	19,9456	199,606	199,365	197,569	199,591	199,670	0.54
B3	111,439	99,026	89,375	82,744	77,729	73,671	14.7
N4	201,331	205,747	204,790	206,867	209,769	215,173	2.46

Table 6
Average resolution (R_s) at different stages using 10 mM phosphate buffer at pH 7 (Zorbax Bonus RP C18 column).

R_s	Beginning	End of Stage 1	Stage 2	Stage 3	Stage 4	Stage 5	%RSD of all stages
A1, B1	8.19	8.85	8.82	8.83	8.82	8.81	2.8
A2, A1	3.21	4.03	4.30	4.58	4.86	5.08	14.2
N2, A2	7.27	10.12	11.10	12.14	13.04	13.90	19.0
B3, N2	4.16	5.38	6.06	6.64	7.15	7.67	19.0
N4,B3	6.88	5.22	4.15	3.34	2.57	1.87	42.2

this pH value, A1 and A2 were de-protonated leading to peak tailing due to strong interaction between the conjugated bases of A1 and A2 with silanol groups on the silica surface. The tailing gradually decreased with time (T_f is 2.2 and 2.3 after 560 injections), suggesting slightly decreased silanol activity. Of note is the significant increase of the theoretical plate number for both compounds. At the beginning of the evaluation, N values for A1 and A2 were approximately 13 K and 12 K, respectively, increasing to roughly 31 K and 38 K after 560 injections. Both peaks became narrower resulting in the increase in the N values, indicative of decreased silanol activity. Pyridine (B1, pK_a = 5.19) is a weaker base than nortriptyline (B3, pK_a = 9.7) and both tailed noticeably at pH 7. For pyridine, column efficiency, tailing factor and column capacity factor remained relatively unchanged throughout 560 injections. On the other hand, although column capacity factor and tailing factor for nortriptyline showed an insignificant change, the column gradually lost its efficiency. The data indicated more than 30% loss of column efficiency over the course of 560 injections. This loss of column efficiency was gradual due to broadening of the peak, rather than loss of retention (Fig. 1), indicating slight dissolution of silica packing [8]. This assumption is supported by back pressure data displayed in Fig. 2, which shows stable back pressure after more than 500 injections. If this dissolution continued to proceed beyond a critical point, the packed silica bed may possibly abruptly collapse causing voids and a concomitant steep loss of efficiency. Any void forming in the column would have been indicated by a drop in backpressure. The two neu-

Table 7
Average peak area different stages using 10 mM phosphate buffer at pH 7 (Zorbax Bonus RP C18 column).

Peak area	Beginning	End of Stage 1	Stage 2	Stage 3	Stage 4	Stage 5	%RSD of all stages
B1	3,973,898	2,945,456	2,975,709	2,994,498	2,977,754	3,055,593	1.5
A1	22,125,483	22,041,784	23,062,855	23,181,778	23,278,727	24,205,249	3.9
A2	43,915,048	45,339,381	47,265,543	48,419,179	47,959,385	49,943,253	4.5
N2	13,757,087	13,894,640	13,884,265	14,045,208	13,926,303	14,391,564	1.9
B3	60,603,764	60,776,218	61,509,793	61,834,122	61,175,902	61,406,408	1.7
N4	21,419,995	21,539,728	24,728,511	25,601,714	25,002,520	24,137,176	9.0

Table 8
Stability of columns at different mobile phase pH.

	pH 2	pH3	pH 6	pH 7	pH 7	pH 8	pH 9	pH 10	pH 11
Buffer / Column	0.1%TFA	phosphate	phosphate	ammonium acetate	phosphate	bicarbonate	ammonia / bicarbonate	ammonia	triphosphate
XBridge C18	Green	-	-	-	Green	Green	Green	Green	Yellow
Xterra MS C18	Green	-	Red	Green	Red	Green	Green	-	-
XBridge Phenyl	Green			Green	Yellow	Red	Red	Red	
Luna C18(2)	Green	-	Green	Yellow	Yellow	Yellow	Yellow	-	-
Bonus RP C18	Green	Green	Yellow	Yellow	Red	Red	Red	-	-
Sunfire C18	Green	Green	Red	Red	Red				
Zorbax SB C8	Green	-	-	-	Green	Yellow	-	-	-

Green—no major constraint on use, column demonstrated to hold up >500 injections.
Yellow—some constraint on use, column demonstrated to hold up >200 injections but <500 injections.
Red—avoid use (use only if no other choice), column holds up <200 injections.

tral compounds, acetophenone (N2) and 5-methylsalicylaldehyde (N4), showed little change in their chromatographic data along the course of increasing injections.

According to the predefined criteria in Table 4, the Agilent Zorbax Bonus RP C18 column was not considered stable at pH 7 using phosphate buffer (Tables 5–7). Its stability at pH 6 using 10 mM phosphate buffer and at pH 7 using 10 mM ammonium acetate was further evaluated. Experimental data demonstrated that the column could hold up for 200 injections (approximately 1400 column volumes) under both conditions. Under acidic conditions, at pH 3, using 10 mM phosphate buffer and pH 2 using 0.1% TFA, the column exhibited stable performance for over 560 injections. Fig. 3 showed super-imposable chromatograms to show sustained column stability over 560 injections. However, at pH 8 or pH 9 using ammonium bicarbonate as buffer, the column stability was found to fall short of 200 injections.

The stability results are summarized in Table 8. Columns stable for more than 500 injections are encoded in green. Column stable for more than 200 injections but less than 500 injections are encoded in yellow. Columns stable for less than 200 injections are encoded in red.

The results suggested that Waters XTerra MS C18 column was stable over a pH range from 2 to 9 when organic buffers were used at pH 8 and 9. However, its stability was greatly compromised in the presence of phosphate buffers. Its performance rapidly deteriorated at even pH 6 when potassium phosphate was the buffer. This observation came as no surprise because it has been well recognized that column stability can be significantly affected by the type and concentration of buffers [11,12–13]. Waters XBridge C18 column exhibited exceptional performance over a wide pH range from 2 to 10. It held up in its performance for over 500 injections at pH up to 9. Even when tested at pH 11, the XBridge C18 column showed an impressive stability of over 200 injections. The enhanced stability of XBridge C18 over XTerra MS C18 at high pH was realized by utilizing ethylene bridged hybrid (BEH) particle, a new second generation of HPT (hybrid particle technology). The BEH particle of XBridge HPLC column exhibits vastly improved efficiency, mechanical strength and pH range compared to the first generation methyl hybrid particle of XTerra columns [10,14–15]. Waters XBridge Phenyl column showed consistent performance for over 500 injections at pH 2 and pH 7 when using organic buffer. However, it could not hold up for 100 injections at pH 8 and above. While the Luna C18(2) column was stable under acidic conditions, without any problem for over 500 injections, its stability was compromised in the pH range from 7 to 9. Luna C18(2) was stable for over 200 injections at pH 7– 9. Contrary to the manufacturers' claims, the results showed that the Bonus RP column was stable only in the pH range of 2 and 3. Its stability failed to meet some of our evaluation criteria, as specified in Table 4, in the pH range from 7 to 9. Similarly, the Sunfire C18 column proved to be stable at pH of 2 and 3, falling far short of the claimed pH range up to 7. The Zorbax SB C8 column exhibited its claimed stability over pH 2–8.

4. Conclusion

HPLC column stability has been investigated since advent of liquid chromatography. However, there are many challenges in devising a standardized test protocol. For pharmaceutical anal-

ysis, column stability should ideally be evaluated against a test mixture that consists of compounds representative of the common organic functionalities in the active pharmaceutical ingredient and its expected related substances. This was the main consideration in the selection of ten test compounds that encompass a wide variety of functionalities. These include carboxylic acid, amine, phenol, aldehyde, ketone, etc. This paper proposes stringent evaluation criteria with focus on gaining column stability knowledge.

The column stability performance observed is quite different when compared with the information provided by the column manufacturers. For this very reason, we would recommend use of the stability data presented for column scouting purposes, prior to starting method development. Once a set of method conditions is selected and other chromatographic conditions have been finalized, an evaluation of column stability should be conducted against a representative sample matrix to gain knowledge of the stability performance of the column prior to validating the method.

A systematic, practical approach was used to evaluate seven commonly used RP-HPLC columns against predefined performance criteria. This approach is an integral component of a QbD method development. The data generated for commonly used columns provide an aide to practitioners faced with the challenge of developing robust and rugged methods for use in a QbD environment.

Acknowledgements

The authors would like to thank Dr. Mark Kleinman for comments and discussions and all the members of the Analytical Sciences Department in Chemical Development at GlaxoSmithKline for their support.

Appendix A.

$$N = 5.54\left(\frac{t_n}{w_n}\right)^2$$

where t_n = retention time of peak n (min), w_n = peak width at half height (min)

$$k' = \frac{(t_n - t_0)}{(t_0)}$$

where t_n = retention time of peak n (min), t_0 = dead time of column (min)

$$\alpha = \frac{k'_2}{k'_1}$$

where k'_2 = capacity factor of peak 2, k'_1 = capacity factor of peak 1

$$T_f = \frac{W_{0.05}}{2f}$$

where $W_{0.05}$ = peak width at 5% peak height, f = distance from the leading edge of peak to the midpoint (measured at 5% of peak height).

$$R_s = \frac{1.18\,(t_2 - t_1)}{(w_1 + w_2)}$$

where t = retention time of peak (min), w = peak width at half height (min)

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