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RESEARCH ARTICLE

Application of Multidimensional Screening and Analysis and Computer Simulation Software for Rapid HPLC Method Development

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Abstract A comprehensive automated strategy for the development of a reversed-phase high-performance liquid chromatography-UV chromatographic method for a basic drug candidate (pKa 7.7), its impurities, and degradants was demonstrated by using multidimensional screening and analysis (MeDuSA) and Drylab® computer optimization software. Phase 1 of the strategy employed an automated column selection system and solvent screening (MeDuSA). Samples were screened using ten columns of varying selectivity and high pH stability in combination with four mobile phases of differing pH (pH 2, 4, 7, and 10) containing acetonitrile and methanol as organic modifiers. The best chromatographic conditions (greater number of resolved impurities, tailing factor ~1.0, resolution >1.4) were obtained using the methyl hybrid organic–inorganic polymer phase Xbridge C18 and pH 7 and pH 10 ammonium acetate/methanol/acetonitrile mobile phase. Further screening in MeDuSA using mobile phases A: 0.01 M NH_4OAc in CH_3CN/water (5:95), and B: 0.01 M NH_4OAc in CH_3CN/water (95:5) at pH 7 demonstrated that CH_3CN is the more critical organic modifier for selectivity. In phase 2, DryLab® software was used to model the effect of temperature, pH, and ionic strength of the buffer on resolution and peak shape to determine the design space of the method and establish the optimal operating conditions. In silico optimization supported by DryLab® enabled the determination of the optimum conditions without carrying out trial-and-error simulations. Excellent agreement between Drylab® simulation and experimental results was obtained. As a result of this strategy, a method with the highest resolution of all components, optimum tailing factor (~1.05), and decreased run time (from 35 to 14 min compared to the original method) was developed faster and with higher efficiency in comparison to the traditional method development process.

Keywords Computer simulation · HPLC method development · Drylab · MultiDimensional screening and analysis

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Introduction

The several-fold increase in the number of projects in the development pipeline, shortened development timelines, the need to improve upon the understanding of processes being developed (Quality-by-Design), and increasing regulatory requirements have generated a number of challenges in the development of analytical methods. Since the goals of an analytical method vary according to the stage of a given product's development, challenges are quite different at each stage. At the drug discovery stage, where more than 10^4 drug candidates are produced [1], high throughput instrumentation for high-speed identification of analytes is required, and the concern is speed over accuracy. At the early development stage (where ten to 20 drug candidates are evaluated), the most important aspects of analytics involve recognition of reaction side products and mass balance issues, such as the impurity profile of isolated intermediates and drug products. At the late development stage, process chemists

conduct tens of experiments for final optimization and the determination of valid reaction parameter ranges. The goal of analytics is to optimize a method (usually based on high-performance liquid chromatography (HPLC) or a hyphenated technique such as liquid chromatography-mass spectrometry (LC/MS) or gas chromatography/MS) with high reproducibility and ruggedness [2]. It is recognized that traditional strategies for HPLC method development (relying on the abilities of the chromatographer) cannot solve all the challenges encountered today at early and late development stages. One variable at a time method development is labor intensive and time consuming and involves intensive exploitation of equipment and a substantial consumption of chemicals. Moreover, interactions between influencing parameters cannot be recognized. The determined optimum is in many cases not the "global optimum" [3]. Process understanding at the early and late development stages has been greatly enhanced by the use of fully automated method development using column selection systems and computer simulation software [4–15]. The objective of the automated method development is to provide experimental decision outcomes relevant to the optimization of complex separations as well as the control of the instrumentation, column selection, mobile phase choice, and other experimental parameters. The progress in computer software applications for method development was recently reviewed by Bączek and Tomasz [4]. Computer simulation programs based on experimental chromatographic measurements include Drylab® (Molnar-Institut für angewandte chromatographie, Berlin, Germany) and Chromsword (Dr. Galushko Software Entwicklung GmbH, Muehltal, Germany) [4–12,14,15]. Other software products such as Fusion AE use design of experiments to generate appropriate retention models to predict the most favorable chromatographic conditions [13]. In this paper, we demonstrate a comprehensive, two-phase strategy for the development of a reversed-phase HPLC-UV chromatographic method for a basic drug candidate (pKa 7.7), its impurities, and degradants, applicable in early and late development stages. Phase 1 consists of screening a sample using a defined set of columns of varying selectivity in combination with mobile phases of differing pH and solvent composition. Within Bristol-Myers Squibb, this screening protocol has been named MeDuSA (an acronym for multidimensional screening and analysis) [16]. At the end of phase 1, the fundamental chromatographic parameters are identified (stationary phase and pH range). Phase 2 addresses optimization of gradient, temperature, pH and ionic strength of the buffer to establish the ideal chromatographic parameters to ensure development of a robust method. Experiments in phase 2 were supported by DryLab® software to minimize the time-consuming empirical approach to method development.

Experimental

Materials and Methods

Acetonitrile (CH_3CN), methanol (MeOH), and trifluoroacetic acid (TFA) were HPLC grade from J. T. Baker (Phillipsburg, NJ, USA). Acetic acid glacial (HOAc), ammonium hydroxide (NH_4OH), and ammonium acetate (NH_4OAc) were American Chemical Society (ACS) grade reagents from EMD (Darmstadt, Germany). Potassium dihydrogen phosphate and potassium monohydrogen phosphate were ACS grade from Sigma Aldrich (St. Louis, MO, USA).

Ammonium acetate buffer (100 mM, pH 5.0) was prepared by dissolving 3.85 g of NH_4OAc in 500 mL of water and adjusting the pH to 5.0 with diluted acetic acid glacial. Potassium dihydrogen phosphate buffer (100 mM) was prepared by dissolving 6.86 g of KH_2PO_4 into 500 mL of water. Potassium monohydrogen phosphate buffer (100 mM) was prepared by dissolving 11.5 g of $K_2HPO_4{\cdot}3H_2O$ in 500 mL of water. Potassium phosphate buffer (10 mM) pH 7.0 was prepared by mixing 61.5 mL of 100 mM potassium dihydrogen phosphate and 38.5 mL of 100 mM potassium monohydrogen phosphate into a 1-L volumetric flask and filled to volume with water. The pH of the solution was adjusted with diluted phosphoric acid or KOH. Potassium phosphate buffer (10 mM) pH 7.5 was prepared by mixing 30.0 mL of 100 mM potassium dihydrogen phosphate and 60.0 mL of 100 mM potassium monohydrogen phosphate into a 1-L volumetric flask and filled to volume with water. The pH of the solution was adjusted with diluted phosphoric acid or KOH. Potassium phosphate buffer (10 mM) pH 8.0 was prepared by mixing 13.4 mL of 100 mM potassium dihydrogen phosphate and 86.6 mL of 100 mM potassium monohydrogen phosphate into a 1-L volumetric flask and filled to volume with water. The pH of the solution was adjusted with diluted phosphoric acid or KOH. Potassium phosphate buffer (10 mM) pH 8.5 was prepared by mixing 6.0 mL of 100 mM potassium dihydrogen phosphate and 94.0 mL of 100 mM potassium monohydrogen phosphate into a 1-L volumetric flask and filled to volume with water. The pH of the solution was adjusted with diluted phosphoric acid or KOH. Buffers were prepared and adjusted to the desired pH before the addition of organic solvent with a pH meter calibrated in aqueous buffers.

Eluent A1 for the MeDuSA instrument was prepared by mixing 200 mL of MeOH, 800 mL of H_2O, and 0.5 mL of TFA. The pH of the MeDuSA mobile phases were not adjusted. pH values are approximations and are listed for reference only. Eluent B1 was prepared as described above using CH_3CN instead of water. Eluent A2 was prepared by mixing 200 mL of MeOH, 800 mL of H_2O, and 1.0 mL of HOAc. Eluent B2 was prepared as described above using

CH_3CN instead of water. Eluent A3 was prepared by mixing 0.77 g NH_4OAc, 800 mL H_2O, and 200 mL MeOH. Eluent B3 was prepared by mixing 0.77 g NH_4OAc, 50 mL water, 200 mL MeOH, and 750 mL CH_3CN. Eluent A4 was prepared by mixing 200 mL of 0.05% NH_4OH (concentration, 28–30%) in MeOH and 800 mL of H_2O. Eluent B4 was prepared as described above using CH_3CN instead of water.

Tailing factors were calculated at 5% of the peak height, according to the USP General Chapter <621> [17]. Solute solutions were prepared in the appropriate mobile phase at concentrations ranging from 0.2 to 0.3 mg/mL and injected individually (20 μL).

All columns were sourced from the original manufacturer and stored as per the manufacturer's instructions. Hypersil GOLD was acquired from ThermoFisher Scientific (Pittsburgh, PA, USA). Synergi Fusion-RP and Luna phenyl hexyl were acquired from Phenomenex Inc (Torrance, CA, USA). Zorbax StableBond-C18 and Zorbax Extend C18 were acquired from Agilent Corporation (Santa Clara CA, USA). Xterra RP-18, Sunfire C18, XTerra Phenyl, XBridge C18 and Symmetry C18 were acquired from Waters Corporation (Milford MA, USA).

Instrumentation

A MeDuSA super-8 HPLC composed of a Shimadzu SIL-10A autosampler, a SPD-10AV VP UV-VIS detector, SCL-10A VP Shimadzu system controller, and two Shimadzu LC-10 AT VP binary solvent delivery systems with Shimadzu DGU-14A vacuum degasser was used. MeDuSA allows automated column switchability. The pumps deliver low and high organic solvent strength eluents of pH 2, 4, 7, and 10 that are paired using solvent lines A1B1, A2B2, A3B3, and A4B4, respectively. A detailed description of the MeDuSA instrument has been previously published [16] and will not be detailed here. The particular HPLC conditions for reversed-phase MeDuSA used in this study are given in Table 1. Waters Alliance HPLC system equipped with a PDA detector (model 2695) was used for the optimization experiments with Drylab®.

Computer Software

Process VP 7.3.1 instrument control software was developed by Bristol-Myers Squibb Company in collaboration with Shimadzu Scientific Instruments, Inc. [16]. Drylab®, the chromatographic optimization software, was purchased from the Molnar-Institut für angewandte chromatographie (Berlin, Germany).

Results and Discussion

Strategy for Rapid Method Development: MultiDimensional Screening and Analysis—Phase 1

The strategy for rapid method development used in this study is summarized in Fig. 1 and is illustrated with the

Table 1 HPLC conditions for reversed-phase MeDuSA

Eluents	pH 2	A1=0.05% TFA in MeOH/H_2O (20:80)
		B1=0.05% TFA in MeOH/CH_3CN (20:80)
	pH 4	A2=0.1 % HOAc in MeOH/H_2O (20:80)
		B2=0.1% HOAc in MeOH/CH_3CN (20:80)
	pH 7	A3=10 mM NH_4OAc in MeOH/H_2O (20:80)
		B3=10 mM NH_4OAc in MeOH/H_2O/CH_3CN (20:5:75)
	pH 10	A4=0.05% NH_4OH (Conc., 28–30%) in MeOH/H_2O (20:80)
		B4=0.05% NH_4OH (Conc., 28–30%) in MeOH/CH_3CN (20:80)
Columns		1. Hypersil GOLD 3 μm, 4.6×150 mm
		2. Synergi Fusion-RP 3 μm, 4.6×150 mm
		3. Zorbax StableBond-C18 3 μm 4.6×150 mm[a]
		4. Xterra RP-18 3.5 μm, 4.6×150 mm
		5. Luna phenyl hexyl 3 μm, 4.6×150 mm
		6. Sunfire C18 3.5 μm, 3.0×150 mm[a]
		7. XTerra Phenyl 3.5 μm, 4.6×150 mm
		8. Symmetry C18 3 μm 4.6×150 mm
		9. XBridge C18 3.5 μm, 4.6×150 mm
		10. Zorbax Extend C18 3.5 μm, 4.6×150 mm
Gradient		Starts 90%A, linear change to 90%B over 20 min, hold at 90%B for 5 min
Flow rate		1.0 mL/min
Detectors		UV at 225 and 256 nm

[a] Columns not used at pH 10 due to lower stability

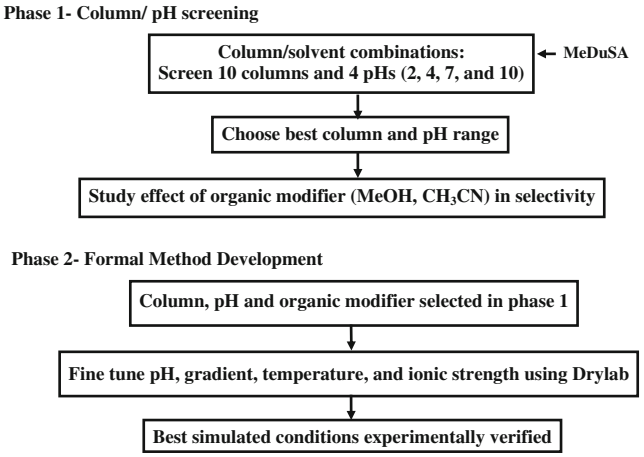


Fig. 1 Strategy for rapid method development

analysis of a basic drug candidate, its impurities, and degradants. The target drug candidate is an ionizable analyte containing a primary amine group and an amide group (pKa 7.7). Several impurities and degradation products with basic and acidic functionalities were identified upon analysis of batches from the proposed synthetic route and from stability samples under accelerated conditions.

Since the retention of ionizable compounds is highly dependent on their degree of ionization, it was expected that the pH would have a marked influence in the separation of the drug candidate and its impurities and degradation products. As seen with other basic compounds, this drug candidate produced poor chromatographic peak shape in typical RP "type A" silica columns (USP tailing >2.5). This tailing is attributed to the ionic interaction between the ionized basic solute and oppositely charged silanol groups, slow desorption kinetics for the retained solute, and ionic repulsion of sorbed cationic solutes [18–21]. For the retention of protonated bases on type-B alkyl-silica columns with low pH, non-ion pairing mobile phases, ionic repulsion seems to be the dominant factor causing peak tailing [21]. Pure "type B" silica (with fewer highly acidic silanols) and the use of acidic mobile phases (pH at least two units below the pKa) were selected in order to reduce peak tailing. As a result of traditional method development, a gradient method using a Luna phenyl hexyl 3 μm, 4.6 mm i.d. × 150 mm column, and mobile phases A: potassium phosphate buffer pH 5.0/CH_3CN (90:10) and B: potassium phosphate buffer pH 5.0/CH_3CN (20:80) was selected as the original method when this compound transitioned from drug discovery to early development. However, increased peak tailing (>2.8) at half the lifetime of the column and overloading effects with increasing mass sample were evident.

Multidimensional screening and analysis (MeDuSA) was explored to challenge the existing HPLC method. The objective was to quickly develop a method selective for all known impurities and degradants that maximized sensitivity without compromising peak shape. MeDuSA was originally introduced at Bristol-Myers Squibb (BMS) Process Research and Development (PR&D) to support process chemists [16]. MeDuSA, as designed by BMS, screens samples using a defined set of columns of varying selectivity in combination with mobile phases of differing pH and solvent composition. Columns used in MeDuSA were selected from a group of columns with different selectivity than the one used in the original method (Luna phenyl hexyl) on the basis of a large column-comparison

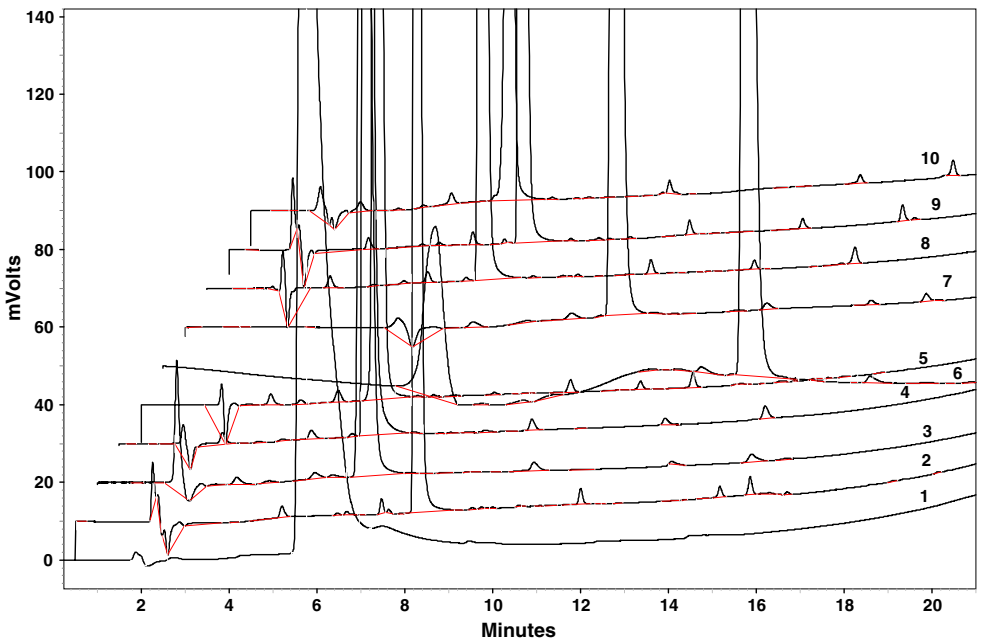


Fig. 2 Overlay chromatograms at pH 2 (Table 1). *1* Hypersil GOLD, *2* Luna phenyl hexyl, *3* Synergi Fusion-RP, *4* Zorbax Extend C18, *5* XTerra Phenyl, *6* Symmetry C18, *7* Xterra RP-18, *8* XBridge C18, *9* Zorbax StableBond-C18, *10* Sunfire C18

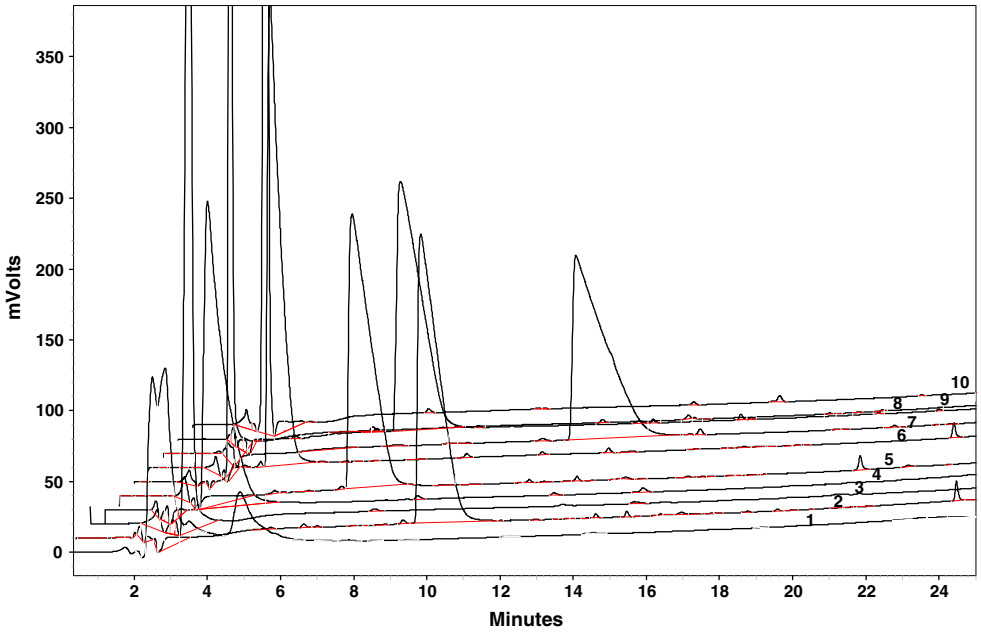


Fig. 3 Overlay chromatograms at pH 4 (Table 1). *1* Hypersil GOLD, *2* Luna phenyl hexyl, *3* Synergi Fusion-RP, *4* Zorbax Extend C18, *5* XTerra Phenyl, *6* Xterra RP-18, *7* XBridge C18, *8* Zorbax StableBond-C18, *9* Symmetry C18, *10* Sunfire C18

function (Fs) value, based on the hydrophobic-subtraction model of reversed-phase column selectivity. In this model, reversed-phase columns are characterized by five selectivity parameters (relative column hydrophobicity *H*, relative steric interaction *S**, column hydrogen-bond acidity *A*, column hydrogen-bond basicity *B*, and column ion-exchange capacity *C*). A column-comparison function (Fs) based on values of *H*, *S**, *A*, *B*, and *C* for columns 1 and 2 was described and summarized for more than 300 different columns [21,22]. Values of Fs>3 can be used to select columns of different selectivity. The vast differences in selectivity due to the column and mobile phase combinations lead to the appearance (or disappearance) of sample components which were not present (or were overlooked) during typical method development. Values of Fs of the columns selected ranged between 3 and 23 [21,22].

The MeDuSA software (known as Process-VP within BMS) and hardware is the result of a collaboration between BMS and Shimadzu [16]. Shimadzu marketed a variant of the original product under the name Discovery-VP® (now ProminenceMD®). Process-VP® controls Shimadzu's HPLC instrumentation and automatically collates and stacks the chromatograms into a stack-plot report. An active pharmaceutical ingredient (API) sample containing

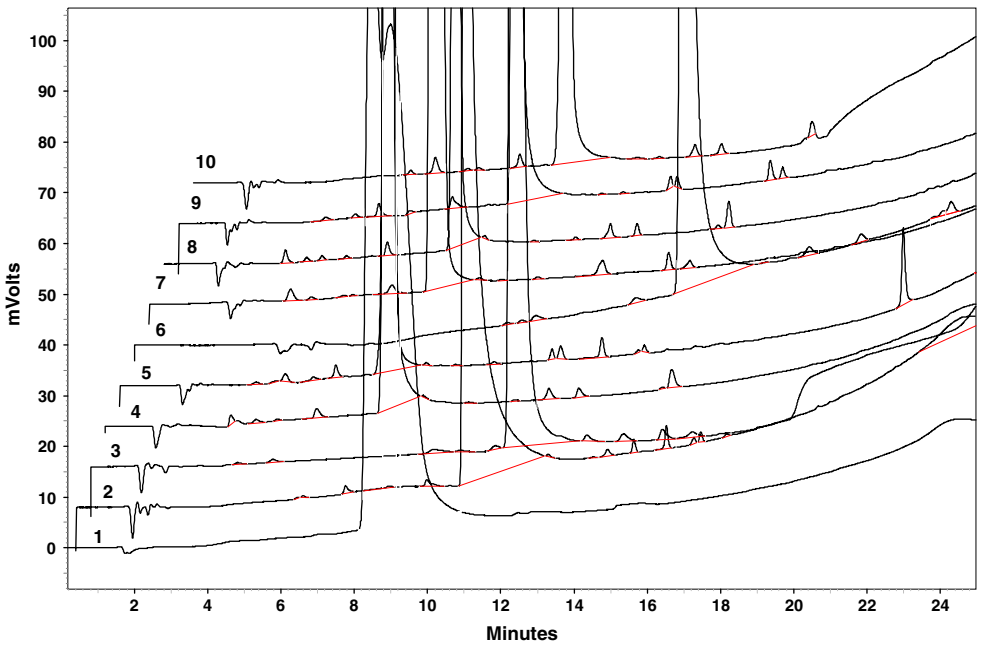


Fig. 4 Overlay chromatograms at pH 7 (Table 1). *1* Hypersil GOLD, *2* Luna phenyl hexyl, *3* Synergi Fusion-RP, *4* Zorbax Extend C18, *5* XTerra Phenyl, *6* Symmetry C18, *7* Xterra RP-18, *8* XBridge C18, *9* Zorbax StableBond-C18, *10* Sunfire C18

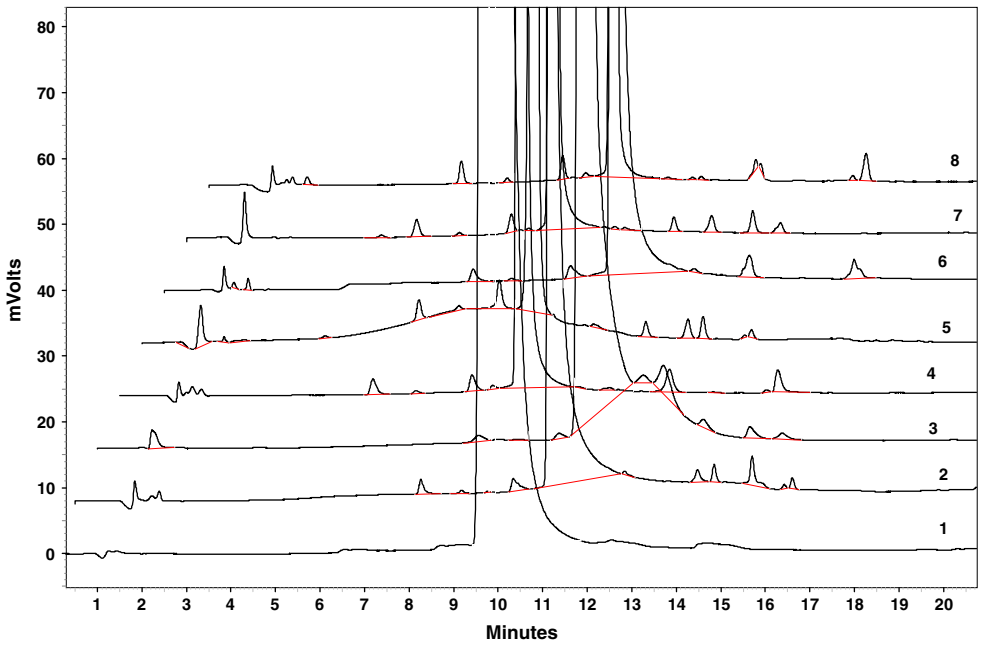


Fig. 5 Overlay chromatograms at pH 10. *1* Hypersil GOLD, *2* Luna phenyl hexyl, *3* Synergi Fusion-RP, *4* Zorbax Extend C18, *5* XTerra Phenyl, *6* Symmetry C18, *7* Xterra RP-18, *8* XBridge C18

all of the impurities and degradants known at this stage of development was screened, and the results were compared against a chromatogram recorded using the original method. The objectives of method development were to achieve minimum resolution (Rs) of 1.4 for the critical pair, selectivity for all relevant peaks, column plate number >5,000, tailing factor (USP tailing) less than 2.0, and analysis time less than 25 min. Figures 2, 3, 4, and 5 show overlay chromatograms obtained from all ten columns at pH 2, 4, 7, and 10, respectively.

Tables 2, 3, and 4 show resolution values between impurities and the tailing factor of the basic drug candidate (peak 6) at pH 2, 7, and 10. The results at pH 4 (Fig. 3) were not included in this presentation due to excessive peak tailing (ranging from 3.4 in XTerra RP 18 to 6.6 in XBridge C18), low retention of the basic drug candidate (such as in Synergi Fusion-RP, Zorbax Extend C18, Symmetry C18, XTerra RP18 and Sunfire C18), and poor resolution. Although the MeDuSA instrument is equipped with an MS detector, peaks in this study were not identified by mass, and the peak matching function was not used. Peak numbers in the table correspond to the number of peaks observed in the chromatogram, peak 1 corresponding to the peak with shortest retention time and peak 13 corresponding to the longest retention time.

At pH 2, the drug candidate showed excessive tailing on Hypersil GOLD, and the impurities were not resolved. Excessive peak tailing of some impurities was observed on Synergi Fusion-RP, Zorbax Extend C18, and XTerra RP-18. Excessive retention of the drug candidate was observed on

Table 2 Resolution values for main impurities and tailing factor of basic drug candidate (peak 6) at pH

Column	Resolution values for impurities of basic drug candidate by peak pair											Critical pair
Peak Pair	1–2	2–3	3–4	4–5	5–6	6–7	7–8	8–9	9–10	10–11	11–12	12–13	
Luna phenyl hexyl	7.5	1.4	5.4	1.3	3.8 (1.4)	7.5	1.7	7.1	10.3	20.6	4.5	1.7	4,5; 12,13
Synergi Fusion-RP	2.2	NR	3.0	1.2	2.0 (1.6)	3.6	2.9	7.3	NR	11.2	6.2	2.4	–[a]
Zorbax Extend C18	3.7	2.2	2.6	4.0	1.3 (1.5)	4.3	4.1	1.7	1.8	4.5	13.7	1.8	5,6; 12,13
XTerra Phenyl	3.2	NR	2.6	1.7	3.3 (1.5)	4.0	2.1	8.3	9.0	8.5	6.8	6.4	–[a]
Xterra RP 18	2.7	NR	2.3	2.4	1.12 (1.4)	2.7	5.5	NR	1.8	8.8	5.3	NR	–[a]
XBridge C18	5.1	3.4	2.7	4.6	1.60 (1.4)	5.6	3.6	1.8	9.9	13.5	11.5	1.7	5,6; 12,13
Zorbax SB-C18	5.0	1.6	4.4	5.0	1.3 (1.6)	5.8	3.6	3.9	8.4	15.9	14.0	1.7	5,6; 12,13
Sunfire C18	3.8	3.0	3.2	2.7	NR (0.9)	5.6	5.3	11.9	NR	25.2	12.0	NR	–[a]

For peak 6, value in parenthesis corresponds to tailing factor

NR not resolved

[a] Not all peaks were resolved. Critical pair not determined

Table 3 Resolution values for main impurities and tailing factor of basic drug candidate (peak 6) at pH 7

Column	Resolution values for impurities of basic drug candidate by peak pair												Critical pair
Peak Pair	1–2	2–3	3–4	4–5	5–6	6–7	7–8	8–9	9–10	10–11	11–12	12–13	
Luna phenyl hexyl	6.5	NR	5.4	4.4	4.01 (3.8)	3.8	6.5	1.7	3.9	5.8	4.7	1.2	–[a]
Synergi Fusion-RP	3.1	9.8	NR	3.0	1.19 (1.8)	NR	5.2	NR	2.9	3.1	2.2	NR	–[a]
Zorbax Extend C18	NR	3.1	2.4	4.2	6.75 (1.0)	3.8	5.8	5.6	4.0	3.2	10.4	NR	–[a]
XTerra Phenyl	2.1	NR	3.2	2.8	5.88 (0.8)	3.8	3.9	5.0	8.7	1.3	5.9	NR	–[a]
Symmetry C18	1.5	1.2	NR	7.0	3.19 (1.8)	NR	3.8	NR	4.4	6.2	0.6	NR	–[a]
Xterra RP 18	2.1	3.4	1.1	4.0	2.88 (1.4)	3.4	2.7	4.9	6.7	NR	6.9	2.6	–[a]
XBridge C18	3.0	2.1	3.5	5.9	9.34 (0.9)	4.1	8.2	6.6	5.1	3.9	12.8	1.6	12,13
Zorbax SB-C18	3.4	3.0	3.9	4.8	6.38 (1.0)	NR	9.4	3.3	7.7	NR	15.3	1.9	–[a]
Sunfire C18	3.1	3.8	1.4	5.6	5.97 (1.0)	6.7	2.8	2.5	4.6	3.3	13.8	NR	–[a]

For peak 6, value in parenthesis corresponds to tailing factor

NR not resolved

[a] Not all peaks were resolved. Critical pair not determined

Symmetry C18. Acceptable separation of impurities was observed on Zorbax SB-C18, Luna phenyl hexyl, and XBridge C18 columns (Fig. 2 and Table 2). However, pH 2 was not selected for further optimization since more than one critical pair of peaks was observed, which could complicate method optimization.

At pH 4, right-angled triangle-shape peaks were obtained in all the columns, with the exception of Synergi fusion-RP, Symmetry C18, and Sunfire C18, for which the retention of the basic candidate was drastically reduced (Fig. 3). At low pH, deterioration of peak shape has been observed even on some highly inert type B phases with few ionized silanols. This effect is proposed to be due to mutual repulsion of similarly charged ions on the hydrophobic surface of the phase ([18–20] and literature therein). While the exact mechanism by which overloading of ionized solutes occurs remains unclear, peak shape problems caused by this overloading should be reduced by increasing the mobile phase pH above the analyte pKa [18–20]. In fact, the best results were obtained at pH close to or above the pKa of the base (pH 7 and 10) in combination with methyl hybrid organic–inorganic polymer phases such as XTerra RP18 and Xbridge C18.

At pH 7, peak splitting was observed on Hypersil GOLD. Excessive peak tailing of the drug candidate and impurities was observed on Luna phenyl hexyl and Synergi Fusion-RP. Excessive retention of the drug candidate and impurities was observed on Symmetry C18, showing a similar behavior as observed at pH 2. The best separation and peak tailing was observed on XBridge C18 (Fig. 4 and Table 3).

At pH 10, excessive peak tailing of the drug candidate and impurities was observed on Hypersyl GOLD, Synergy

Table 4 Resolution values for main impurities and tailing factor of basic drug candidate (peak 6) at pH 10

Column	Resolution values for impurities of basic drug candidate by peak pair												Critical pair
Peak Pair	1–2	2–3	3–4	4–5	5–6	6–7	7–8	8–9	9–10	10–11	11–12	12–13	
Luna phenyl hexyl	5.2	3.9	2.9	NR	3.8 (1.9)	3.0	9.2	NR	2.1	5.3	4.6	1.3	–[a]
Synergi Fusion-RP	NR	22.7	2.2	2.7	1.3 (2.4)	NR	3.6	NR	NR	2.2	3.3	2.1	–[a]
Zorbax Extend C18	NR	4.0	5.3	2.0	2.6 (1.5)	5.4	2.6	NR	4.3	NR	5.6	NR	–[a]
XTerra Phenyl	19.2	11.0	3.6	3.5	3.9 (1.0)	3.5	6.3	5.1	4.9	1.7	6.1	NR	–[a]
Symmetry C18	2.8	NR	3.6	5.0	3.6 (1.7)	NR	NR	8.1	5.1	NR	8.8	NR	–[a]
Xterra RP 18	3.7	4.5	5.9	2.2	2.9 (1.1)	7.4	1.2	5.9	4.2	4.5	2.9	NR	–[a]
XBridge C18	21.1	6.0	7.6	2.9	3.5 (0.9)	7.1	3.3	1.2	9.0	0.9	14.7	1.6	10,11

For peak 6, value in parenthesis corresponds to tailing factor

NR not resolved

[a] Not all peaks were resolved. Critical pair not determined

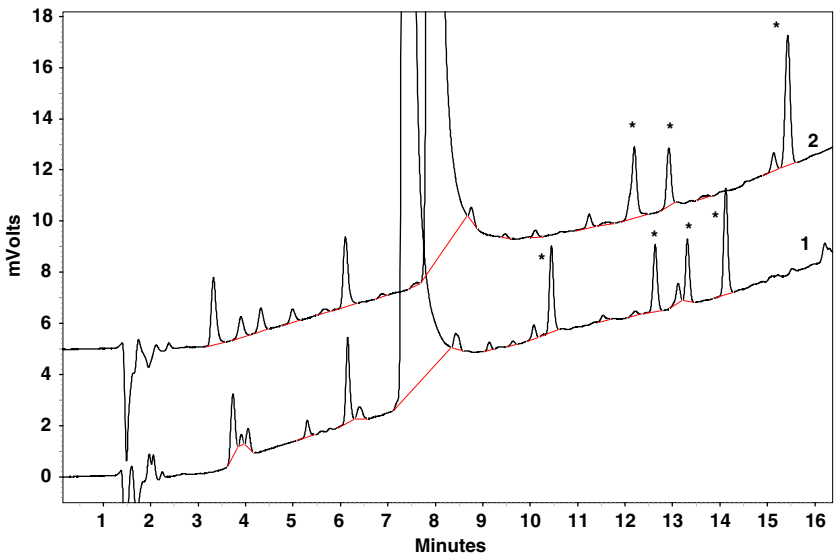


Fig. 6 Overlay chromatograms at pH 7 from MeDuSA scouting in XBridge C18. *1* Mobile phase A: 10 mM NH_4OAc in CH_3CN/water (5:95), mobile phase B: 10 mM NH_4OAc in CH_3CN/water (95:5); *2* Mobile phase A: 10 mM NH_4OAc in MeOH/H_2O (20:80), mobile phase B: 10 mM NH_4OAc in MeOH/H_2O/CH_3CN (20:5:75)

Fusion-RP, and Luna phenyl hexyl. The best separation and peak tailing was observed on XTerra RP-18 and XBridge C18 (Fig. 5 and Table 4).

XBridge C18 provided the best chromatography at pH 7 and acceptable resolution of all peaks at pH 10, suggesting robustness throughout this range. Chromatographic conditions between pH 7 and 9 were selected for further optimization and method development as (1) neutral pH conditions are expected to be more robust than pH 10, since higher sample stability and longer column lifetime are observed at neutral pH to slightly basic conditions, and (2) there was no difference between tailing factors of the basic drug candidate (0.9 at both pHs), demonstrating that peak tailing can be improved even if the concentration of the protonated base is above 50%.

Due to the increased solubility of the basic drug candidate in CH_3CN relative to MeOH, an additional analysis was performed using mobile phase A: 0.01 M NH_4OAc in CH_3CN/water (5:95), and mobile phase B: 0.01 M NH_4OAc in CH_3CN/water (95:5) at pH 7 in Xbridge C18, to determine the effect of MeOH in selectivity and the feasibility of using mobile phases containing only acetonitrile as the organic modifier. Positive attributes of acetonitrile include lower viscosity and lower back pressure of acetonitrile/water mixtures in comparison to methanol/water mixtures, higher elution strength, and lower solvent consumption since the same elution strength can be achieved using a lower percentage of acetonitrile compared to methanol. Additionally, lower solubility of air in acetonitrile leads to less effort for effective degassing [23]. As shown in Fig. 6, peak tailing of the basic compound is similar in both conditions (0.9). However, the chromatographic profiles significantly change for the peaks eluting after the basic candidate. An additional peak is observed when only acetonitrile is used in the mobile phase (Fig. 6 chromatogram 1) that is not observed when both methanol and acetonitrile are used (Fig. 6 chromatogram 2). This change in selectivity may be

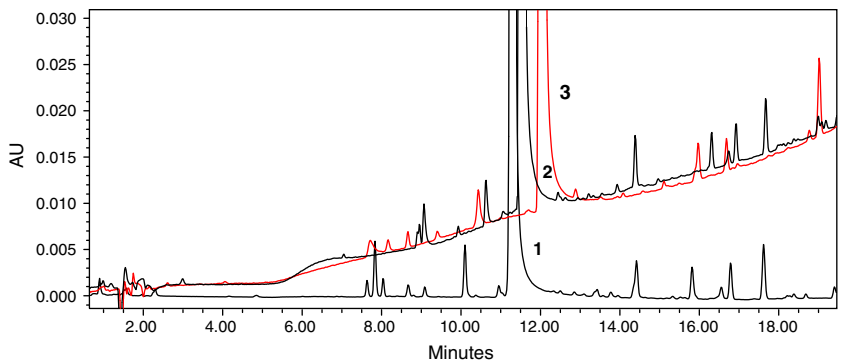
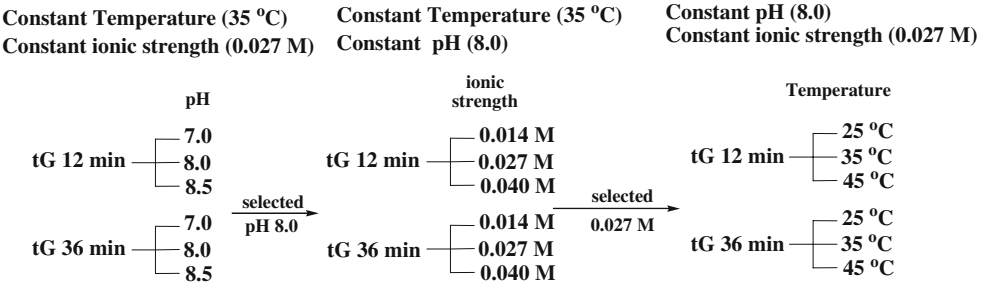


Fig. 7 Overlay chromatograms at pH 7 from Waters Alliance in XBridge C18. *1* Mobile phase A: 10 mM phosphate buffer pH 8.0 in H_2O/CH_3CN (90:10), Mobile phase B: 10 mM phosphate buffer pH 8.0 in H_2O/CH_3CN (80:20). *2* Mobile phase A: 10 mM NH_4OAc in CH_3CN/H_2O (5:95), mobile phase B: 10 mM NH_4OAc in CH_3CN/H_2O (95:5). *3* Mobile phase A: 10 mM NH_4OAc in MeOH/H_2O (20:80), mobile phase B: 10 mM NH_4OAc in MeOH/H_2O/CH_3CN (20:5:75). 3

Fig. 8 Drylab® experiments



attributed to a change in the pH of the aqueous portion of the mobile phase, as well as variation in the analyte's ionization state upon the addition of both or only one organic component. While resolution of the peaks that elute prior to the basic drug candidate is better when using methanol in the mobile phases, it is expected that Drylab can be used in phase 2 to optimize the chromatography and baseline resolve these peaks.

Phase 2: Drylab® Simulation

Drylab® experiments were used to model in silico the effect of varying chromatographic parameters such as gradient, pH, temperature, and ionic strength (buffer concentration) on the resulting chromatographic characteristics. Results from phase 1 suggest a Waters XBridge C18 column and ammonium acetate pH 7.0 and 10.0 buffers/CH_3CN mobile phase provide the best chromatographic conditions. In order to model the effect of pH and ionic strength, phosphate buffer was selected instead of ammonium acetate buffer. Phosphate buffer is preferred due to its lower UV absorption compared to ammonium acetate. As shown in Fig. 7, the use of phosphate buffer in combination with acetonitrile (Fig. 7 chromatogram 1) effectively reduces eluent absorbance at 225 nm and baseline drift, increases sensitivity, and demonstrates equivalent selectivity to that achieved during the MeDuSA screening (Fig. 7, chromatograms 2 and 3). Another advantage of the phosphate buffer is that high ionic strength can be reached at low concentrations, avoiding excessive salt concentration in the organic mobile phase and the risk of buffer precipitation. An additional advantage is that the pH of a phosphate buffer is not significantly affected by change in temperature [23].

Figure 8 illustrates the sequence of experiments run in phase 2, Drylab® studies to optimize chromatographic conditions and determine the most robust operating parameters. In experiment 1, temperature (35°C) and ionic strength (10 mM, I_c, 0.027 M in both mobile phases) were kept constant, while gradient time and pH were varied. To model pH changes in Drylab®, six runs were conducted at pH 7.0, 8.0, and 8.5 using two different gradient times (12 and 36 min).

Column data (name, length, diameter, particle size), eluent data (temperature, flow rate, and gradient) and dwell volume (1.7 mL) were input. All calibration runs were conducted over the same gradient range (initial and final %B), and flow rate (1.0 mL/min). In the Data Entry window, the pH mode was selected (LC-RP gradient/pH 6 runs). Retention time and area data were imported from the Empower software.

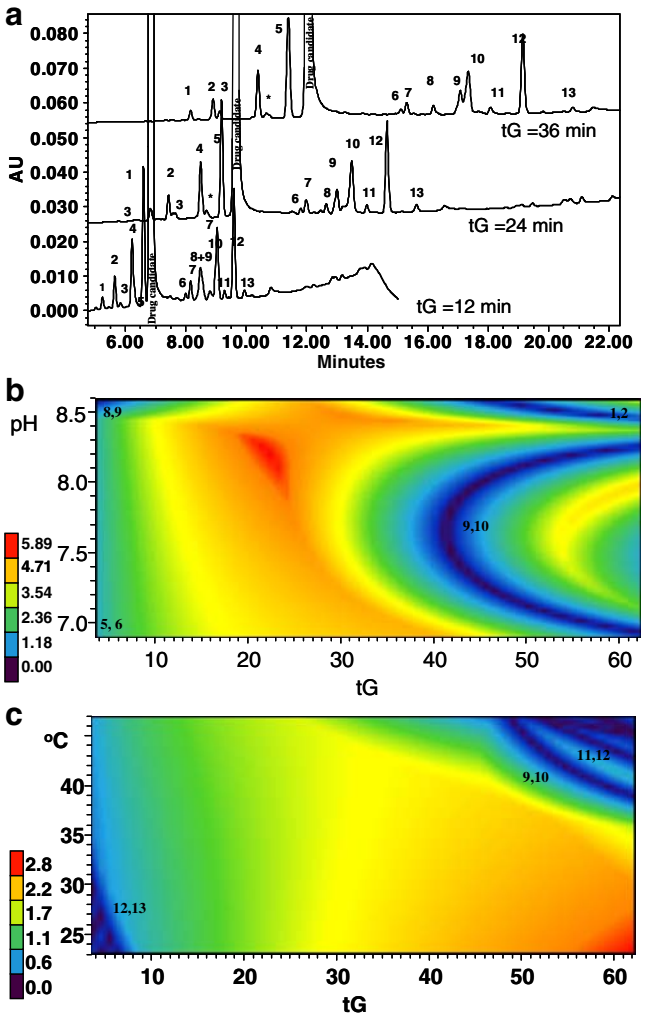


Fig. 9 **a** Chromatograms using phosphate buffer pH 8.0 (I_c, 0.027 M), gradient time 12, 24, and 36 min at 35°C. **b** Resolution map as a function of gradient time (tG) and pH. **c** Resolution map as a function of gradient time (tG) and temperature (°C)

Table 5 Experimentally determined (t_{exp}) and simulated (t_{sim}) retention times for each component in the mixture in Fig. 4a (tG24) and retention time comparisons ($r(tG24)=t_{exp}/t_{sim}$)

Peak	t_{sim}	t_{exp}	r(tG24)
Peak 1	6.79	6.84	1.007
Peak 2	7.40	7.43	1.004
Peak 3	8.65	8.69	1.005
Peak 4	8.46	8.50	1.005
Peak 5	9.15	9.19	1.004
Drug Candidate	9.61	9.65	1.004
Peak 6	11.85	11.95	1.008
Peak 7	12.31	12.65	1.028
Peak 8	12.52	12.61	1.007
Peak 9	12.94	12.99	1.004
Peak 10	13.45	13.49	1.003
Peak 11	13.95	14.06	1.008
Peak 12	14.63	14.66	1.002
Peak 13	15.70	15.81	1.007

Each peak in the sample was matched with the corresponding peak in each of the six input runs. Peak matching was done by visual comparison and by area deviation using the peak matching function in Drylab®. Results from a third run (tG= 24 min) were used to improve the accuracy of the peak matching process but was not included in the calibration. Figure 9 shows typical chromatograms at tG 12, 24, and 36 min at pH 8.0, and the resolution maps as a function of gradient time (tG) pH, and temperature.

The resolution map in Fig. 9b represents a plot of minimum sample resolution (for the most overlapped or "critical pair" of bands) as a function of gradient time and pH. Green, yellow, and orange regions (representing minimum peak resolution of 2.1, 4.2, and 5.6, respectively) indicate appropriate conditions of gradient and pH for full resolution of all the peaks in the chromatogram. Blue areas in the resolution map represent working conditions with minimum resolution (0.7 to 1.4) of the most overlapped or "critical pair" of bands. Different critical pairs are observed at different gradients and pHs (Fig. 9b). At pH 7 and short gradient times, the critical pair is peaks 5 and 6 (the drug candidate). The blue band observed at higher gradient times (tG~45 min) represent poor resolution for peaks 9 and 10. The simulation indicates robustness around pH 8.0. A resolution of 3.4 is achieved for the critical pair at pH 8.0 and gradient time of 10 min. Higher resolution can be achieved at pH 8.0 and gradient time of 25 min (>5.6). However, a 25-min run is not required to obtain baseline separation of all peaks.

Table 5 presents the actual (t_{exp}) and simulated (t_{sim}) retention times for each component in the mixture in Fig. 9a at a gradient time of 24 min (tG 24), together with their ratios ($r=t_{exp}/t_{sim}$). The ratio is a quantitative measure of the agreement between the experimentally determined separation and that predicted by DryLab® software. All ratios ranged from 1.002 to 1.028, indicating excellent agreement between the experimental and simulated results.

Based on results from experiment 1 of the plan described in Fig. 8, pH 8.0 was selected for further optimization. In experiment 2, temperature (35°C) and pH (8.0) were kept constant, while gradient time and ionic strength were varied. To model changes in ionic strength, six runs were conducted using phosphate buffer at 5 mM (I_c=0.014), 10 mM (I_c=0.027), and 15 mM (I_c=0.04) and two gradient times (12 and 36 min). The ionic strength of the phosphate buffer at 5 mM is approximately the same as the ionic strength of the NH_4OAc 10 mM solution used in the mobile phase at pH 7 in the MeDuSA screening. All calibration runs were conducted over the same gradient range (initial and final %B), and flow rate (1.0 mL/min). In the Data Entry window, the ionic strength mode was selected (LC-RP gradient-ionic strength six runs). Retention time and peak area results were imported from the Empower software. Each peak in the sample was matched with the corresponding peak in each of the six input runs, as described for experiment 1. A I_c, 0.027 M ionic strength (corresponding to 10 mM phosphate buffer) provided the best separation (data not shown).

Based on results from experiment 2, 10 mM phosphate buffer pH 8 was selected for further optimization. In

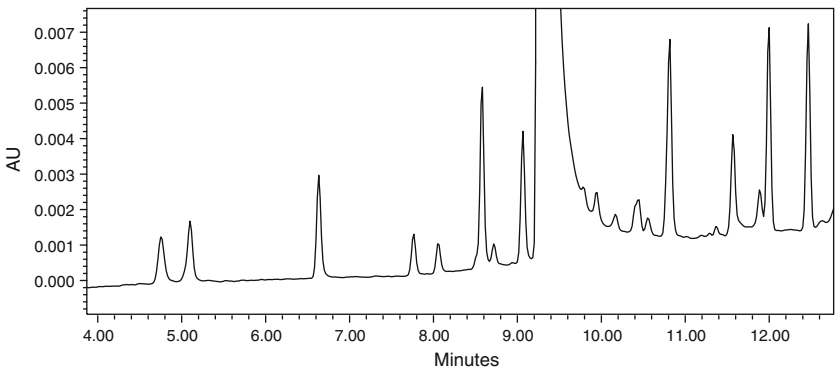


Fig. 10 Chromatogram showing resolution of all impurities and degradants in the sample using Drylab® optimized conditions

experiment 3, ionic strength (I_c, 0.027 M) and pH (8.0) were kept constant, while gradient time and temperature were varied. To model changes in temperature, four runs were conducted at 25°C and 45°C and two gradient times (12 and 36 min). All calibration runs were conducted using the chromatographic conditions previously described. In the Data Entry window, the temperature mode was selected (LC-RP gradient/temperature four runs). Retention time and area data were imported from the Empower software. Each peak in the sample was matched with the corresponding peak in each of the four input runs, as described for experiment 1. Figure 9c shows the resolution map as a function of gradient time (tG) and temperature (°C). In this resolution map, light green, yellow, and red regions represent appropriate resolution of all the peaks in the chromatogram, while blue represent working conditions with minimum resolution (0.6) of the most overlapped or "critical pair" of bands (as shown in Fig. 9c). As shown in Fig. 9b, c, the selected operating conditions (pH 8.0, 35°C, and 16 min gradient time) occur in an operating space that provides excellent method robustness.

Computer simulation in DryLab® allows the optimum gradient time or steepness to be determined without conducting trial-and-error simulations. Optimal HPLC separation of the drug candidate, impurities, and degradants was achieved on a Waters XBridge C18, 4.6×150 mm, 3.5 μm particle size column maintained at 35°C (Fig. 10). Mobile phases A and B consisted of 10 mM phosphate buffer pH 8.0/CH_3CN [90:10] and 10 mM phosphate buffer pH 8.0/CH_3CN [20:80], respectively. The analysis began with mobile phase A at 100%, then linear gradient from 100% A to 80% A in 6 min, then linear gradient from 80% A to 20% A in 5 min, then linear gradient from 20% A to 100% A in 1 min, and finally mobile phase A was held at 100% for 1 min, for a total run time of 14 min. The flow rate was 1.0 mL/min, and the UV detector wavelength was set at 225 nm.

These final conditions provide sufficient chromatographic resolution between detected impurities in a worst case sample with an efficient run time (RT). In addition, these conditions demonstrated the observation of a new peak (Fig. 10 RT 9.1 min), that was co-eluting with the API peak when using the originally developed HPLC method at pH 5.0. This peak was not detected by the DAD detector peak purity test, due to the similarity of the UV spectra of both substances and the low level of the impurity.

Conclusion

The advantages of using rapid method development approach that utilizes multidimensional screening and analysis and Drylab® computer optimization software were demonstrated. In silico optimization supported the development of a suitable HPLC method selective for all known impurities and degradants that optimized peak shape for a basic drug candidate. Rapid screening of chromatographic conditions was enabled through the use of ten columns of varying selectivity in combination with four mobile phases with pH ranging from pH 2 to pH 10. The best chromatographic conditions were obtained using methyl hybrid organic–inorganic polymer phases such as Xbridge C18 at a pH close to the pKa of the drug candidate. Utilizing this screening strategy, a robust method selective for all the known impurities was developed, ensuring an accurate impurity assessment. DryLab® allowed the optimum chromatographic conditions to be determined without conducting expensive and time-consuming trial-and-error experimentation.

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