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Development of a Stability-Indicating Analytical Method for Determination of Venetoclax Using AQbD Principles

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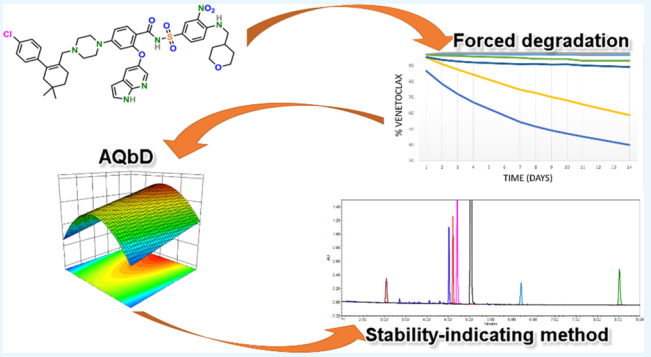
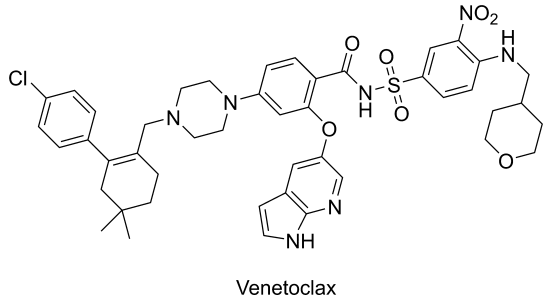
ABSTRACT: Venetoclax is an emerging drug for the treatment of various types of blood cancers. It was first approved in 2016 for the treatment of relapsed and refractory chronic lymphocytic leukemia. Later, the indications expanded, and multiple research as well as clinical studies are still conducted involving venetoclax. No analytical method for the determination of venetoclax can currently be found in the literature. We developed a mass spectrometry-compatible stability-indicating ultrahigh-performance liquid chromatography (LC) method for venetoclax. The LC method was developed using analytical quality by design principles. The developed method is able to separate venetoclax and its degradation products. The method was validated in the working point where a linearity range was established and accuracy, repeatability, and selectivity were assessed. Venetoclax is the only Bcl-2 protein inhibitor on the market. It is very effective in combinational therapy, so future drug development involving venetoclax may be expected. A stability-indicating method could aid in the development of new pharmaceutical products with venetoclax.



1. INTRODUCTION

Venetoclax (4-[4-[[2-(4-chlorophenyl)-4,4-dimethylcyclohex-en-1-yl]methyl]piperazin-1-yl]-*N*-[3-nitro-4-(oxan-4-ylmethylamino)phenyl]sulfonyl-2-(1*H*-pyrrolo[2,3-*b*]pyridin-5-yloxy)benzamide) (Figure 1) is an orally bioavailable, B-cell



Venetoclax

Figure 1. Molecular structure of venetoclax.

lymphoma-2 (Bcl-2) selective inhibitor.[1] The discovery of the antiapoptotic Bcl-2 protein began with an observation of the *t*(14; 18) chromosome translocation in follicular lymphoma and a suggestion of the involvement of gene *bcl-2* in B-cell malignancies with said translocation.[2] From there on, the Bcl-2 protein family began to grow and numerous research studies were conducted on the topic.[3,4]

Venetoclax was first approved by the U.S. Food and Drug Administration (FDA) and European Medicines Agency (EMA) in 2016 for the treatment of patients with relapsed and refractory chronic lymphocytic leukemia (CLL).[5] Currently, venetoclax is approved by the FDA for the treatment of adult patients with CLL or small lymphocytic lymphoma and for the treatment of newly diagnosed acute myeloid leukemia in adults who are 75 years old or older or who are not suitable for intensive induction chemotherapy, in combination with azacitidine or decitabine or low-dose cytarabine. EMA approved venetoclax for the treatment of patients with genetic changes that make them unsuitable for chemoimmunotherapy when B-cell-receptor-pathway inhibitors (such as ibrutinib and idelalisib) are not suitable or have failed and for the treatment of patients without these genetic changes after treatments with chemoimmunotherapy and a B-cell-receptor-pathway inhibitor have both failed. Additionally, EMA approved venetoclax in combination with rituximab in patients who have received at least one previous treatment. The use of venetoclax in numerous other indications is still being explored. Venetoclax has also shown to be effective in

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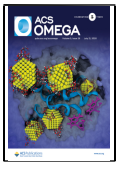

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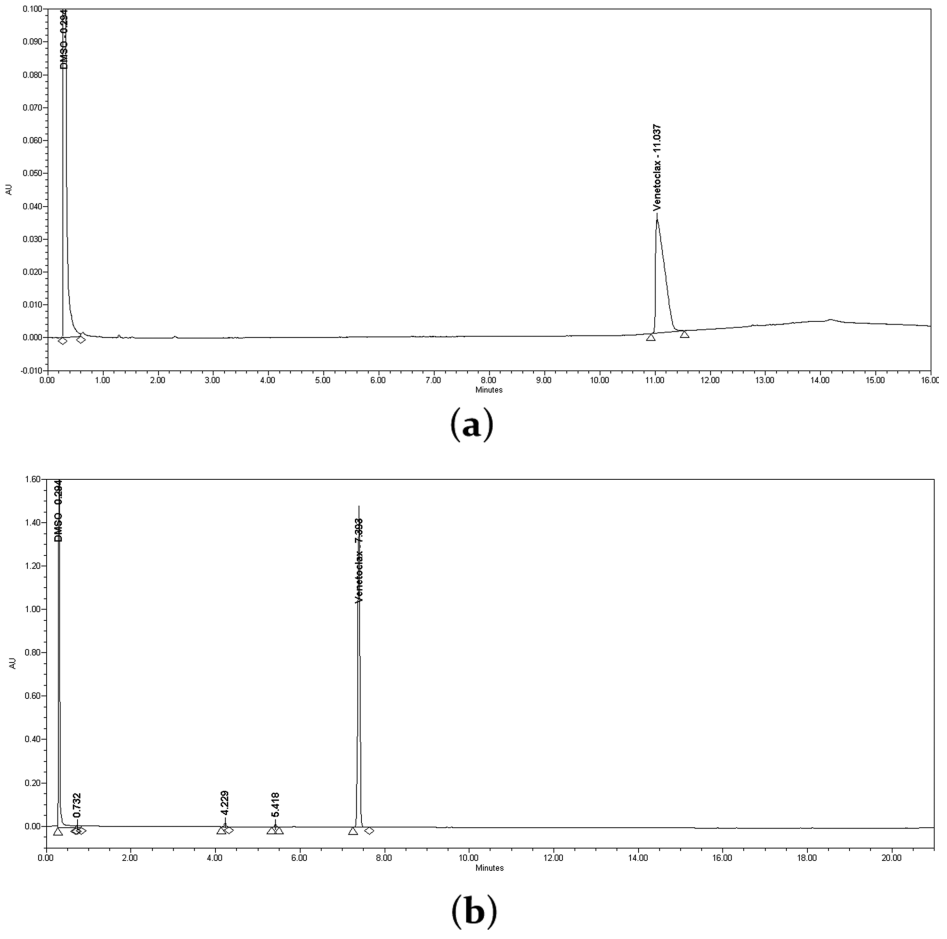

Figure 2. Chromatograms of the venetoclax drug substance analyzed with (a) initial chromatographic conditions and (b) using a mobile phase with a pH of 6.0. Peaks at t_R = 4.23 min and t_R = 5.42 min are two process-related impurities present in the venetoclax drug substance.

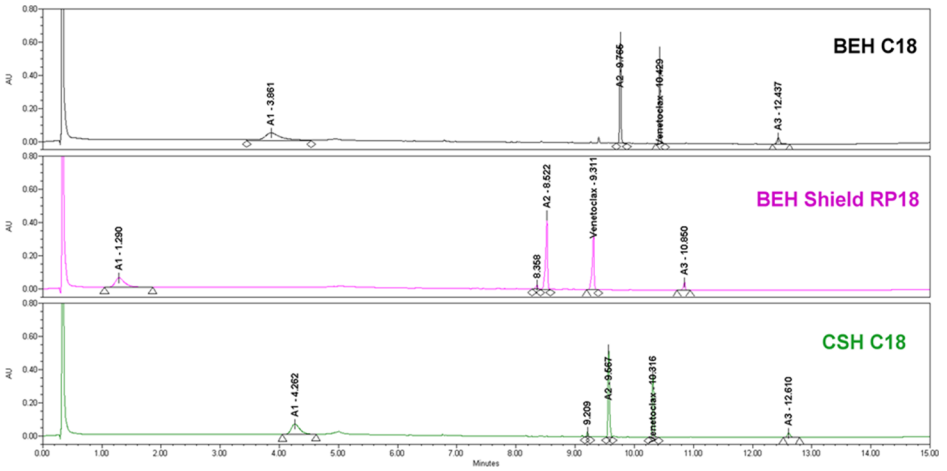

Figure 3. Chromatograms of the venetoclax sample degraded with 1 M HCl at 50 °C for 3 days using a UPLC BEH C18 (top), UPLC BEH Shield RP18 (middle), or UPLC CSH C18 (bottom) column. The peak eluting at approximately 0.3 min is a solvent peak of DMSO.

additional combinational therapies where a single agent may not be suitable.[6]

Venetoclax is insoluble or practically insoluble in aqueous solutions. The solubility is pH-dependent. It may have moderate permeability. As such, it is classified as a Biopharmaceutics Classification System (BCS) class IV compound. However, the absorption appears to be high (>85%) when administered with food.[7] The bioavailability of venetoclax tablets on the market is quite low: C_{max} = 0.387 μg/mL, AUC_t = 4.058 μg × h/mL, and AUC_∞ = 4.186 μg × h/mL.[8]

To the best of our knowledge, a suitable stability-indicating LC analytical method for the control of venetoclax degradation has not been reported yet. Therefore, the present study has been designed to develop a stability-indicating analytical method for determination of venetoclax. A stability-indicating

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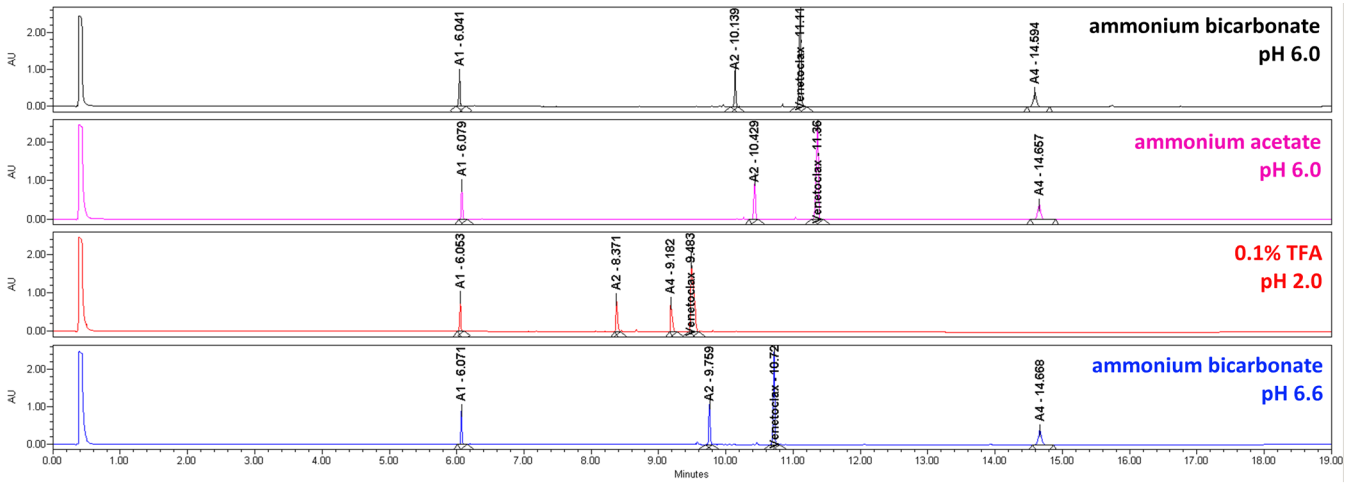
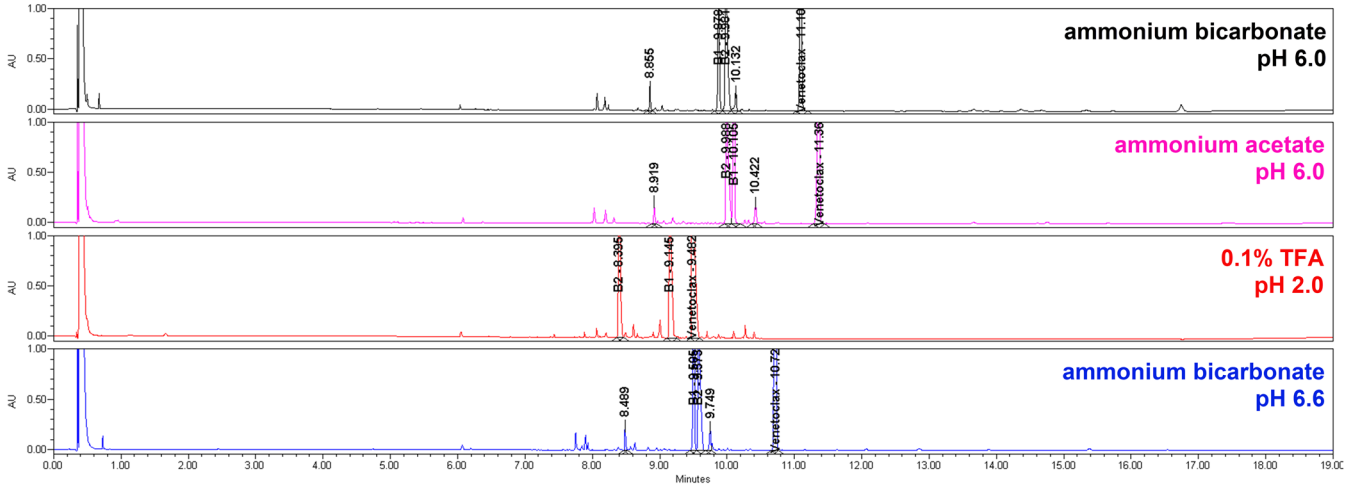


(a)



(b)

Figure 4. (a) Chromatograms of venetoclax degraded with 1 M HCl at 50 °C for 14 days and (b) chromatograms of venetoclax degraded with 1 M NaOH at 50 °C for 14 days, using different mobile phases A: *A* = NH_4HCO_3 (pH 6.0, 10 mM)-ACN (9:1, v/v) (top, black); CH_3COONH_4 (pH 6.0, 10 mM)-ACN (9:1, v/v) (middle, pink); 0.1% (v/v) TFA-ACN (9:1, v/v) (middle, red); NH_4HCO_3 (pH 6.6, 10 mM) (bottom, blue).

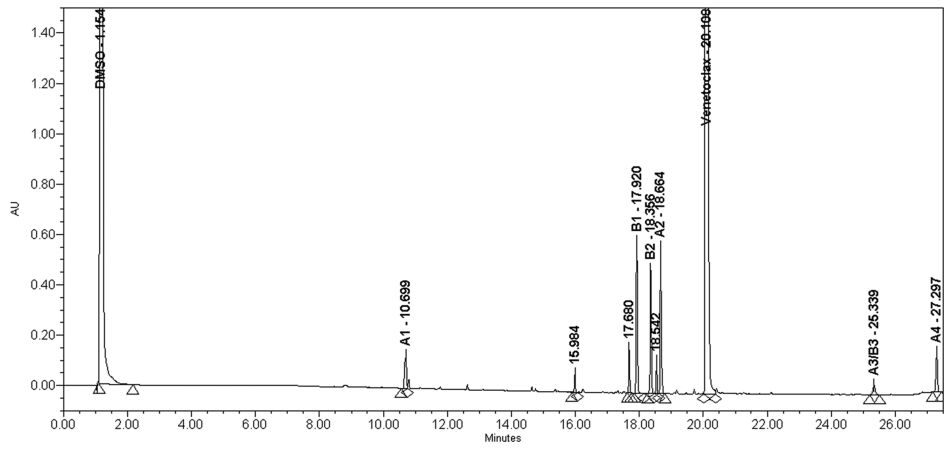


Figure 5. Chromatogram of the best result from method scouting DoE. Peaks eluting at 17.68 and 18.54 min are the process-related impurities originating from the active pharmaceutical ingredient.

test should be able to detect changes in quality attributes during storage.[9] Most importantly, a stability-indicating liquid chromatography (LC) method should be capable of discriminating between the active pharmaceutical ingredient and its degradation products.[10−14]

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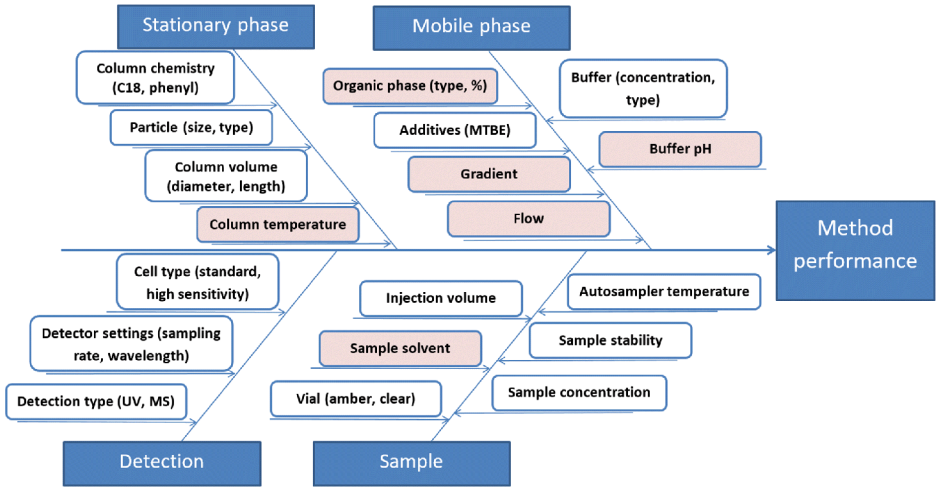

Figure 6. Ishikawa diagram for initial risk assessment. Factors considered as CMPs are marked with a light red color.

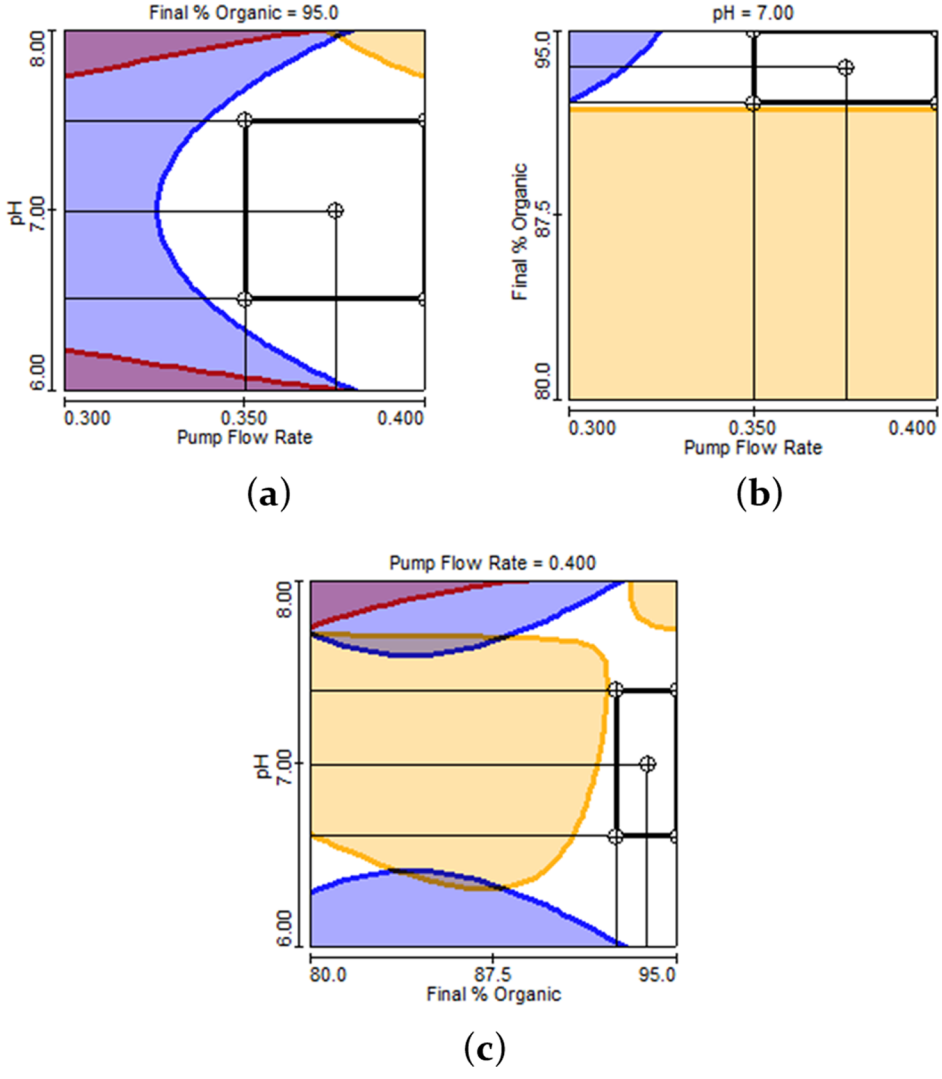

Figure 7. Graph representing an area where the defined criteria are met in white color. The colors are areas where the criteria are not met: red = number of peaks less than 10; blue = number of peaks with resolution ≥1.5 less than 9; orange = number of peaks with tailing ≤1.2 less than 7. Acceptable range of tested conditions is marked with a black rectangle. (a) Graph where x = pump flow rate; y = pH; at final percent of acetonitrile of 95%. (b) Graph where x = pump flow rate; y = final percent of acetonitrile; at pH 7.00. (c) Graph where x = final percent of acetonitrile; y = pH; at a pump flow rate 0.40 mL/min.

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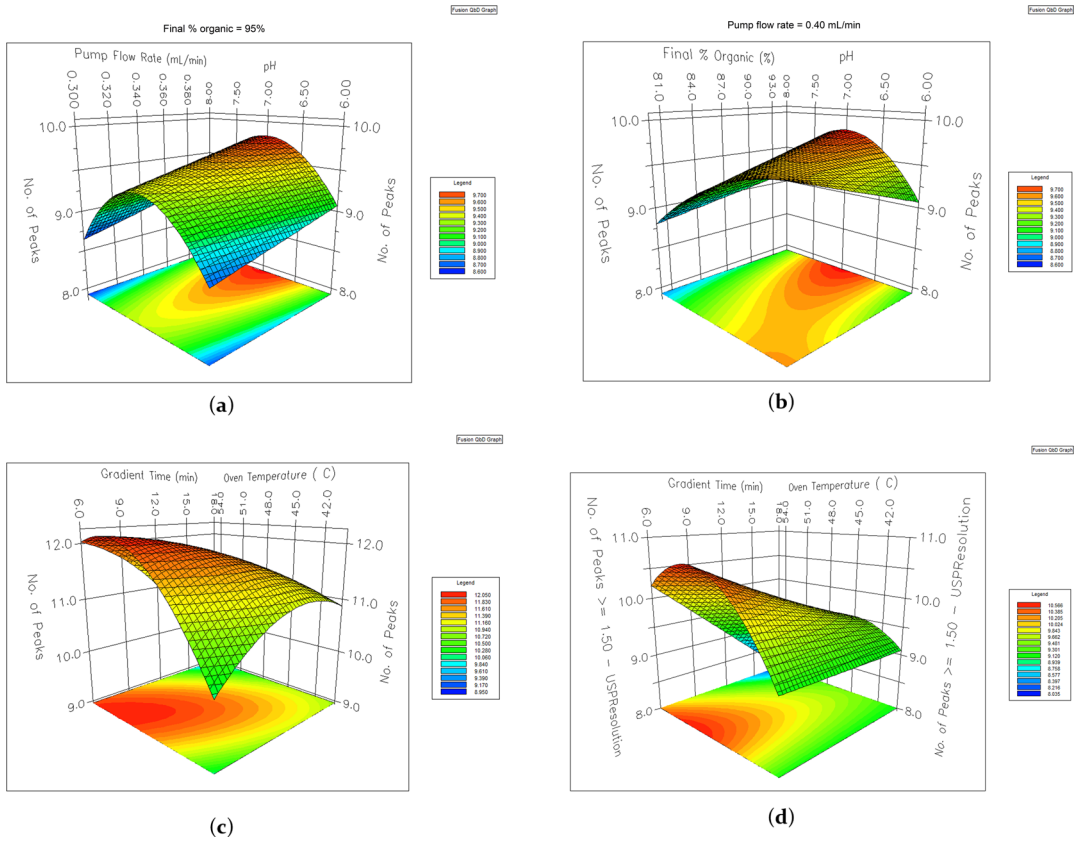

Figure 8. Surface plots from screening DoEs. (a,b) Surface plots from the first screening DoE, representing a number of peaks (a) in relation to pump flow rate (mL/min) and pH at a final percent of acetonitrile of 95% and (b) in relation to a final percent of acetonitrile and pH at a pump flow rate of 0.4 mL/min. (c,d) Surface plots from the second screening DoE, representing (c) a number of resolved peaks and (d) a number of peaks with resolution ≥1.5 in relation to gradient time (min) and column temperature (°C).

An analytical quality-by-design (AQbD) approach to method development was utilized. AQbD is an extension of quality-by-design (QbD). It is a systematic approach to the development of analytical procedures involving all the stages of the procedure's lifecycle.[15−22] The AQbD process includes the definition of the analytical target profile (ATP), selection of critical method attributes (CMAs), risk assessment, identification of critical method parameters (CMPs), screening and optimization using design of experiments (DoE), robustness testing, definition of method operable design region (MODR), and an establishment of the method control strategy.[19,22] As the term suggests, the analytical procedure lifecycle is a cyclic process resulting in a continuous improvement of the method.[20] The AQbD process has been implemented in the pharmaceutical industry as a new guideline by the International Conference on Harmonization (ICH)—Q14 is expected in 2021, which covers the topic of AQbD.[23,24] As there are no known venetoclax degradation products reported in the literature, forced degradation was conducted on the venetoclax drug substance to generate degradation products of venetoclax,[21] following directions in ICH guidelines.[25,26]

In addition to the developed analytical method being stability-indicating, the focus was to develop a mass spectrometry (MS)-compatible ultrahigh-performance liquid chromatography (UHPLC) method for resolving venetoclax and its main degradation products. As there are no known venetoclax degradation products reported in the literature, such a method could be useful in future degradation products' identification efforts.

2. RESULTS AND DISCUSSION

2.1. Sample Preparation. *2.1.1. Solvent Selection.* Sample preparation has shown to be a significant challenge in the method development process. Venetoclax is poorly soluble in many solvents usually used in the reversed-phase LC sample preparation, such as methanol (MeOH) and acetonitrile (ACN), and practically insoluble in aqueous solutions. The initial attempt to dissolve venetoclax in 80% ACN was thus not successful. We decided to add dimethyl sulfoxide (DMSO) to the solvent to improve the solubility. We wanted to maximize the amount of water in the solvent so as to minimize the solvent elution effect in the liquid chromatography, which can result in a poor peak shape. Venetoclax has successfully dissolved when we added 20% of the flask volume of DMSO and then topped the flask with 80% ACN, even in concentrations as high as 5 mg/mL. Later some solubility problems with the proposed solvent occurred. Crystals started to form after a week at room temperature and precipitation was observed when adding water solutions for degradation testing. Additional solvent testing was performed where higher amounts of DMSO, buffer (the same one as used in UHPLC) instead of water, and MeOH as a substitute for ACN were tested. Eight different solvent compositions were tested (Table S1). Initially, ACN-DMSO-buffer (7:2:1, v/v/v) was used as a solvent, but it was later changed for ACN-DMSO-buffer (6:3:1, v/v/v) after precipitation was observed in the vial after 2 months of storage at 5 °C.

2.1.2. Forced Degradation of Venetoclax. We conducted forced degradation studies on the venetoclax drug substance.

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Table 1. Method Model Equations Based on the First DoE from Screening

observed criteria	model[a]	ANOVA[b]
number of peaks	$y = 9.609 - 0.450(C)^2 + 0.213(A \times B) - 0.193(A \times C) - 0.274(B \times (C)^2) + 0.222(A \times B \times C)$	$R2 = 0.5686$ adj. $R^2 = 0.4659$ F-ratio = 5.5364 MS-LOF = 0.0978 (threshold 1.1481)
number of peaks with resolution ≥1.5	$y = 8.246 - 0.573(C)^2 + 0.242(A \times B) + 0.279(A \times (B)^2)$	$R2 = 0.4452$ adj. $R^2 = 0.3728$ F-ratio = 6.1518
number of peaks with tailing ≤1.2	$y = 147.886 + 81.595(B) + 69.728(B)^2 + 147.459(C)^2 + 61.599(B \times C) - 102.064((B)^2 \times C) - 162.169(B \times (C)^2) - 64.636(A \times B \times C)$	$R2 = 0.7118$ adj. $R^2 = 0.6056$ F-ratio = 6.7033 MS-LOF = 0.1163 (threshold 0.452)

[a] y = observed criteria, A = pump flow rate, B = final percent of mobile phase B, C = pH. [b] Regression ANOVA statistics, MS-LOF = mean square lack-of-fit, Adj. = Adjusted.

The primary stress conditions we chose were 0.1 M HCl, 1 M HCl, 0.1 M NaOH, 1 M NaOH, 0.3% H_2O_2, 3% H_2O_2, $FeCl_3$, and 22 h SUNTEST. All the stress testing, except for the SUNTEST, was conducted in a chamber at 50 °C for 1 day. This provided us with information about the stability characteristics of venetoclax. Method scouting was done using all the stress samples. For the AQbD process, we limited the stress conditions to those that achieved 10−20% of venetoclax degradation in a maximum of 7 days.[27] This resulted in venetoclax samples with added 1 M HCl and 1 M NaOH at 50 °C. We saw the rise of key degradation products that were marked as A1, A2, A3, and A4 in acidic conditions and B1, B2, and B3 in basic conditions. We later concluded that degradation products A3 and B3 are the same product based on retention times at different chromatographic conditions and the UV spectra. A mixture of an acidic and a basic stress solution of venetoclax was used for the AQbD process, which contained the key degradation products.

2.2. Analytical Target Profile. The analytical method should be able to quantify venetoclax in the presence of its degradation products over a range of 80−120% of the target concentration with an accuracy of 100 ± 2% and repeatability ≤2% RSD. A stability-indicating analytical method for the analysis of the venetoclax drug substance should be able to distinguish venetoclax and its degradation products with a resolution of more than 2.0.

Based on ATP, the UHPLC method with UV detection was chosen as the analytical technique. CMA chosen was the resolution between peaks.

2.3. Method Scouting. As there are no existing analytical methods for venetoclax in the literature, a couple of preliminary tests were done based on the knowledge gained through literature about the molecule itself.[7,28] Part of the method scouting consisted of multiple one-factor-at-a-time (OFAT) experiments. Reversed-phase LC was chosen based on the molecule structure and characteristics. The starting point for the development was an in-house method for the separation and analysis of a drug with similar physiochemical properties and its related substances and degradation products: mobile phase A: A = 0.1% H_3PO_4 (v/v); mobile phase B: B = ACN-methyl *tert*-butyl ether (MTBE) (850:80, v/v); Acquity UPLC BEH C18 (1.7 μm, 100 mm × 2.1 mm) column; column temperature 70 °C; flow rate 0.75 mL/min; autosampler temperature 5 °C; detection wavelength 220 nm; gradient: t = 0 min, 37% B; 1 min, 37% B; 9 min, 48% B; 11 min, 70% B; 13.5 min, 70% B; 14 min, 37% B; 2 min equilibration. It exhibited a poor peak shape with a significant tailing (Figure 2a).

Based on the predicted pH curves made by MarvinSketch (ChemAxon, Budapest, Hungary) (Figure S1), venetoclax exhibits many species throughout the pH spectrum. For LC, we want it to be in a single ionized form at the selected pH, to prevent tailing, which could occur if the molecule would shift from one ionized form to another at the selected mobile phase pH as the ionization influences the retention of the molecule on the stationary phase. There were three options: acidic pH of around 1, a pH of around 6, and a pH of around 11. As the acidic pH was tested, where venetoclax exhibited a significant tailing, and a basic pH of 11 is usually not compatible with most reversed-phase chromatographic columns, we decided to test an aqueous part of the mobile phase with a pH of around 6. We wanted to keep the method MS-compatible, so we decided to use ammonium bicarbonate as the buffer. The pH

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Table 2. Method Model Equations Based on the Second DoE from Screening

observed criteria	model[a]	ANOVA[b]
number of peaks	$y = 11.857 - 0.577(A) - 0.555(A)^2 - 0.816(B)^2 - 0.959(A \times B)$	$R^2 = 0.9668$ adj. $R^2 = 0.9536$ F-ratio = 72.9043
number of peaks with resolution ≥1.5	$y = 10.012 - 0.568(A) - 0.887(B)^2 - 0.522(A \times B)$	$R^2 = 0.7442$ adj. $R^2 = 0.6745$ F-ratio = 10.6702

[a] y = observed criteria, A = gradient time, B = column temperature. [b] Regression ANOVA statistics, Adj. = Adjusted.

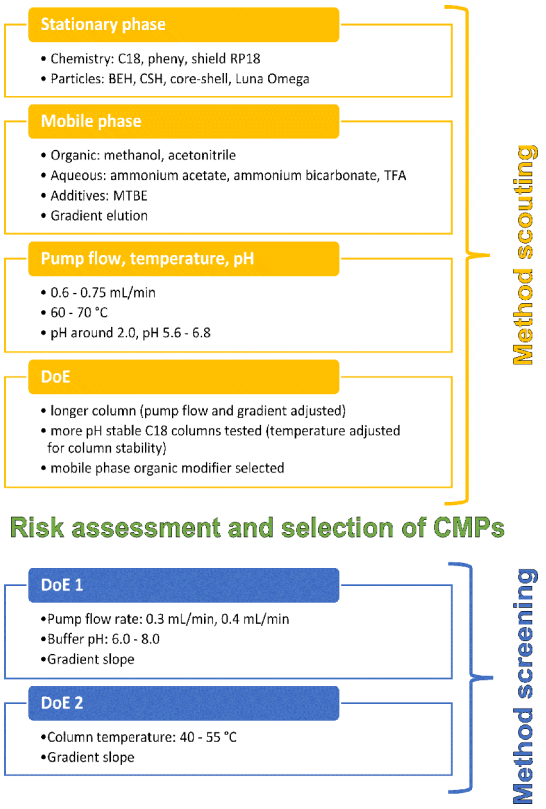

Figure 9. Summary of the work done and parameters studied up until method optimization.

was adjusted using acetic acid. The gradient was extended to better determine at what percentage of organic phase in the mobile phase venetoclax elutes. The peak shape of the venetoclax substance drastically improved. Furthermore, two process-related impurities were successfully separated from venetoclax (Figure 2b). At this stage, a phenyl stationary phase was tested. It was selected based on the venetoclax structure. It provided nice peak shapes but a smaller retention of venetoclax.

We tested the method further using degraded samples from degradation studies and the venetoclax substance solution, mainly to improve selectivity between venetoclax, its degradation products, and related substances. The degradation samples were first tested on two columns: BEH C18 and BEH Phenyl columns (1.7 μm, 100 mm × 2.1 mm) (Figure S2). The venetoclax peak had a better shape using a BEH C18 column and the separation of some degradation products seemed to be better with a BEH C18 column. Some peaks eluted very early, not showing much retention. The gradient was adjusted to start with a lower organic phase content to better retain the early-eluting peaks. The gradient was changed to: mobile phase A: A = NH_4HCO_3 (pH 6.0, 10 mM)-ACN (9:1, v/v); mobile phase B: B = ACN-MTBE (850:80, v/v); t = 0 min, 0% B; t = 3 min, 0% B; t = 6 min, 30% B; t = 10 min, 70% B; t = 13 min, 70% B; t = 15 min, 30% B.

Narrow pH changes of around pH 6 were tested to see if selectivity could be improved and to assess the influence of such changes. The narrow pH changes had an influence on the retention but not on peak shape or selectivity (Figure S3). The influence of MTBE in mobile phase B was evaluated (Figure S3). MTBE improved the peak shape of later-eluting compounds; however, early-eluting compounds showed better retention without MTBE (Figure S3). As MTBE did not prove to significantly improve the method performance, it was omitted from the mobile phase B.

Two additional stationary phases were tested. A more polar BEH Shield RP18 and a column with charged surface hybrid (CSH) technology—CSH C18 column, both of the same particle size and dimensions as previous columns. The C18 columns gave similar results, whereas a Shield column showed a slightly diminished retention. The early-eluting degradation

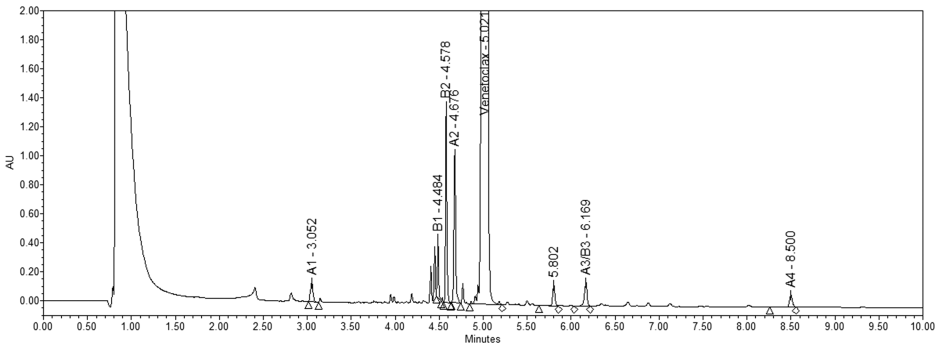

Figure 10. Chromatogram after the gradient split. The peak eluting at approximately 1 min is a solvent peak of DMSO. A couple of additional peaks are visible because of using the same sample throughout the AQbD process.

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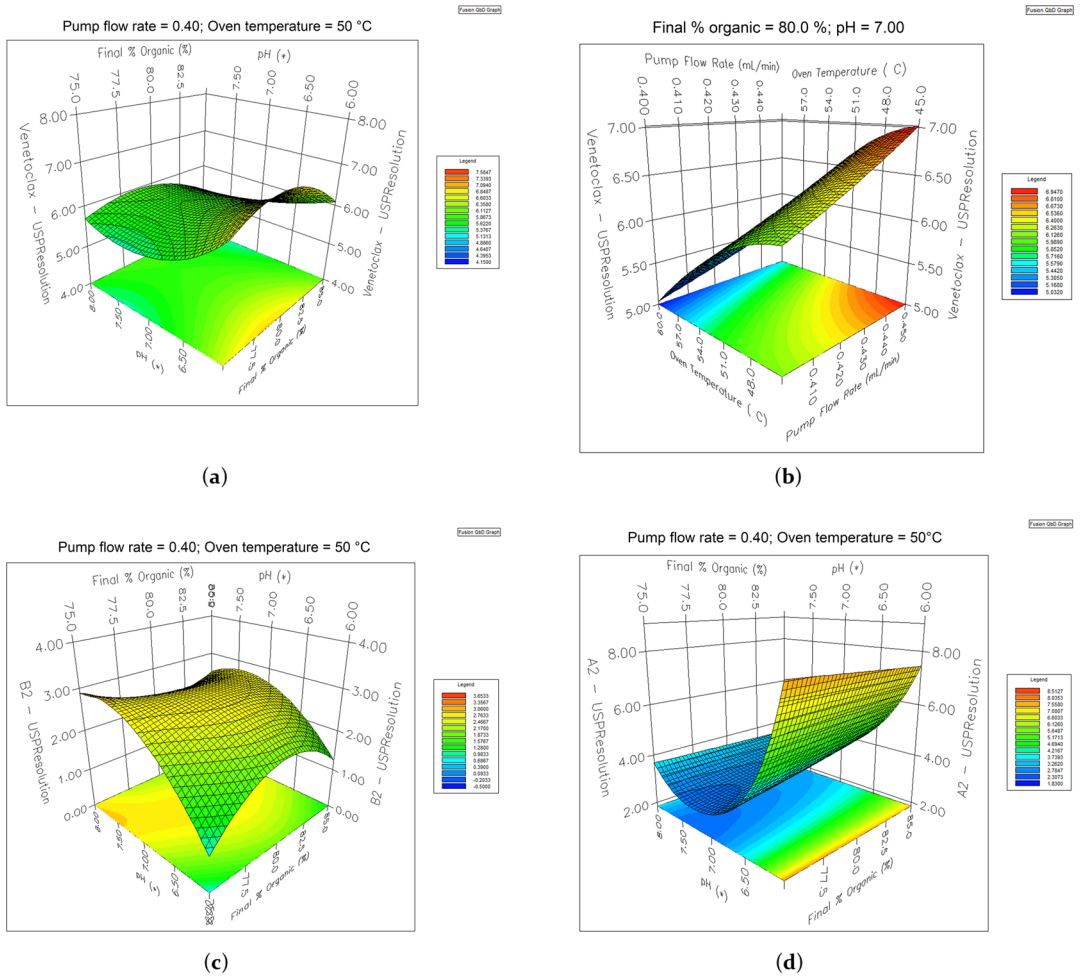

Figure 11. Models for CMAs represented with surface plots. (a,b) Surface plots for venetoclax resolution (R_v). (c) Surface plot for resolution of degradation product B2 (R_{c1}). (d) Surface plot for resolution of degradation product A2 (R_{c2}).

product (A1) showed better retention when using the CSH C18 column as well as a better peak shape—narrower and higher (Figure 3).

The starting pump flow and column temperature were quite high (0.75 mL/min and 70 °C). A high temperature can reduce the column lifetime especially with a higher pH of the mobile phase. Therefore, pump flow and temperature were lowered to extend the column lifetime (0.6 mL/min and 60 °C).

The effects of temperature change can be seen in the Supporting Information (Figure S4). The method time was extended to sufficiently elute all of the degradation products and the end of the gradient was modified—ending in a larger percentage of mobile phase B—to make the elution faster and the later eluting peaks narrower and higher. The modified gradient was t = 0 min, 0% B; t = 3 min, 0% B; t = 10 min, 70% B; t = 12 min, 70% B; t = 16 min, 80% B; t = 18 min, 80% B; t = 19 min, 0% B.

During the scouting phase, additional C18 columns were tested: Kinetex C18 (1.7 μm, 100 mm × 2.1 mm) with core shell technology and Luna Omega C18 (1.6 μm, 100 mm × 2.1 mm) (Figure S5). Luna Omega performed the best in terms of resolution between degradation products B1 and B2. The resolution between B1 and B2 was 1.88 for Luna Omega, 1.03 for Kinetex, and 1.65 for the previously used CSH column. The Luna Omega C18 column was thus chosen for further analysis.

The buffer capacity of ammonium bicarbonate is not maximal at the selected pH, so a 10 mM ammonium acetate buffer with a pH of 6.0 was tested on the Luna Omega C18 column as well as an acidic pH ∼ 0.1% TFA. Additionally, a 10 mM ammonium bicarbonate buffer with a pH of 6.6 was tested (Figure 4). Acidic pH provided alternative selectivity but the tailing of the venetoclax peak was not suitable (3.47), whereas the venetoclax peak shape was better at the pH of around 6. Peaks B1 and B2 switched when using ammonium acetate buffer, which worsened the resolution between them as peak B2 exhibited some tailing (Figure 4b).

Overall, C18 columns were found to be the most promising for the separation of venetoclax and its degradation products. A mobile phase with a pH in the range of 6−8 gave the best peak shape. Additionally, ammonium bicarbonate was the most appropriate buffer. An addition of MTBE did not contribute significantly to improve the peak shape.

At the end of the scouting phase, a DoE was utilized for the final selection of the chromatographic column and the type of mobile phase organic modifier. Because of a desire for a better separation of closely eluting peaks, a longer column (150 × 2.1 mm) was selected. The flow and gradient were calculated based on column volume and modified appropriately. A two-level full factorial design was employed for the DoE (Table

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Table 3. Method Models of CMAs Based on DoE from Optimization

CMAs	model coefficients[a]	*P*-value	*F*-ratio	regression ANOVA statistics[b]
R_v	+0.1725			$R2$ = 0.9083
	−0.0194(A)	<0.0001	143.7438	adjusted $R2$ = 0.8872
	+0.0184(C)	<0.0001	129.3516	*F*-ratio = 42.9426
	+0.0111(D)	<0.0001	44.6169	MS-LOF = 0.0231
	+0.0143(B)^2	0.0002	17.0421	
	+0.0079(C)^2	0.0185	6.0455	
	−0.0114(D)^2	0.0006	14.0420	
	−0.0060 ($A \times D$)	0.0035	9.6784	
	−0.0041 ($C \times D$)	0.0371	4.6598	
	+0.0066 ($B \times (D)^2$)	0.0010	12.5398	
R_{c1}	+2.4748			$R2$ = 0.8092
	−1.1496(C)	<0.0001	29.6262	adjusted R^2 = 0.7651
	−0.7667(D)^2	<0.0001	24.1305	*F*-ratio = 18.3752
	−0.2211 ($A \times C$)	0.0343	4.8113	MS-LOF = 0.0488
	−0.1993 ($B \times D$)	0.0477	4.1781	
	−0.2914 ($C \times D$)	0.0087	7.6448	
	+0.4805 ($A \times (B)^2$)	0.0033	9.8201	
	+0.6644 ($(B)^2 \times C$)	0.0069	8.1271	
	+0.7192 ($(B)^2 \times D$)	<0.0001	51.5833	
	−0.5337 ($A \times (D)^2$)	0.0021	10.8327	
R_{c2}	+1.2633			$R2$ = 0.9766
	+0.1436(B)	<0.0001	36.3502	adjusted $R2$ = 0.9697
	+0.3315(C)	<0.0001	105.6963	*F*-ratio = 140.4817
	−0.3513(D)	<0.0001	160.2558	MS-LOF = 0.0074
	−0.0946(C)^2	0.0007	13.8086	
	+0.4552(D)^2	<0.0001	347.0844	
	+0.0581 ($C \times D$)	0.0006	14.0238	
	−0.0671 ($(A)^2 \times C$)	0.0453	4.2925	
	−0.0784 ($A \times (C)^2$)	<0.0001	26.6275	
	−0.0845 ($(C)^2 \times D$)	0.0121	6.9661	
	−0.1703 ($B \times (D)^2$)	<0.0001	35.5127	
	−0.1759 ($C \times (D)^2$)	<0.0001	33.6721	

[a]A = pump flow rate, B = final percent of mobile phase B, C = column temperature, D = pH. [b]MS-LOF = mean square lack-of-fit.

S2). Because of the pH of the mobile phase used, there was a wish for a more pH stable chromatography column, as the Luna Omega C18 column pH range is 1.5−8.5. An addition of acetonitrile to the ammonium bicarbonate buffer raises the pH. When testing this effect, the pH of the initial buffer solution (pH 7.3) went as high as pH = 8.3. Thus, the pH on the column can be higher than the pH of the initial buffer solution. Screening parameters for DoE were chosen: column used [BEH C18 column (suitable pH range 1−12), CSH C18 column (suitable pH range 1−11)], type of organic modifier in the mobile phase (acetonitrile, methanol), and time of gradient (15−30 min). CSH C18 columns are more sensitive to higher temperatures at higher pH values of the mobile phase. We still wanted to test the column as it proved to provide suitable results in previous experiments. However, the column temperature was lowered to 50 °C to accommodate the suggested column temperatures provided by the column supplier. The criteria chosen were number of observed resolved peaks and number of peaks with a resolution greater than or equal to 1.5. The best overall answer search was executed with the response goal settings: maximize number of peaks with 8 peaks having desirability of 0 and 10 peaks having a desirability of 1 and maximize the number of peaks with a resolution ≥1.5 with 6 peaks having desirability of 0 and 10 peaks having a desirability of 1 (desirability is a function of Fusion QbD software, where results are graded on a desirability scale from 0 to 1). The best results were obtained using a BEH C18 column, acetonitrile, and a fast gradient (15 min), where the method was able to resolve all the key degradation products and venetoclax (Figure 5). The cumulative desirability result was 0.5229 (with target being 1.0000—which would be achieved if 10 peaks were resolved with a resolution ≥1.5). The overall predicted number of resolved peaks was 9.8, with 7.3 of them having a resolution ≥1.5 (excluding the DMSO peak eluting at approximately 1 min). When the experiment was run, 10 peaks were successfully resolved with 7 having a resolution ≥1.5.

Method conditions of the best run (Figure 5) were UPLC BEH C18 (1.7 μm, 150 mm × 2.1 mm) column; mobile phase A: A = NH_4HCO_3 (pH 6.0, 10 mM)-ACN = (9:1, v/v); mobile phase B: B = ACN; pump flow 0.3 mL/min; column temperature 50 °C; gradient: t = 0 min, 0% B; t = 8 min, 0% B; t = 23 min, 80% B, t = 28 min, 80% B; t = 28.5 min, 90% B; followed by a 2 min column wash at 90% B and 3 min re-equilibration.

2.4. Initial Method Risk Assessment. We performed method risk assessment using an "Ishikawa" diagram[16] (Figure 6). Method parameters were evaluated based on knowledge about the molecule itself gained through literature[7,28,29] and the scouting experiments. At this stage, we could better define CMAs based on method scouting: resolution of venetoclax (R_v) should be ≥1.5. Critical resolution (R_c) was the resolution between degradation products B1 and B2, which form in stress testing with added NaOH at 50 °C (R_{c1}), as well as the resolution between degradation products B2 and A2 (R_{c2}) as they elute rather closely and the resolution may prove important in a mixed sample (such as the one used in the AQbD process). Degradation product A2 can form in acidic conditions and in smaller amounts in basic conditions.

First, parameter categories/groups relating to LC, which can affect method performance, were established (stationary phase, mobile phase, detection, and sample). Then, possible parameters were placed on the diagram in their respective categories. Each change was evaluated based on the effect on our selected CMAs (R_v, R_{c1}, and R_{c2}) that we could notice in the initial experiments and the possibility for that change to occur. For example, the type of buffer has a significant effect on critical resolutions, as can be seen in Figure 4, but it can be easily controlled, so it was not considered as critical. On the other hand, MTBE is usually added to the mobile phase by hand and may result in more variation, but it showed little effect on the CMAs.

CMPs that effect CMAs were identified: sample solvent, column temperature, mobile phase pH, percent of organic modifier in the mobile phase, gradient slope, and mobile phase

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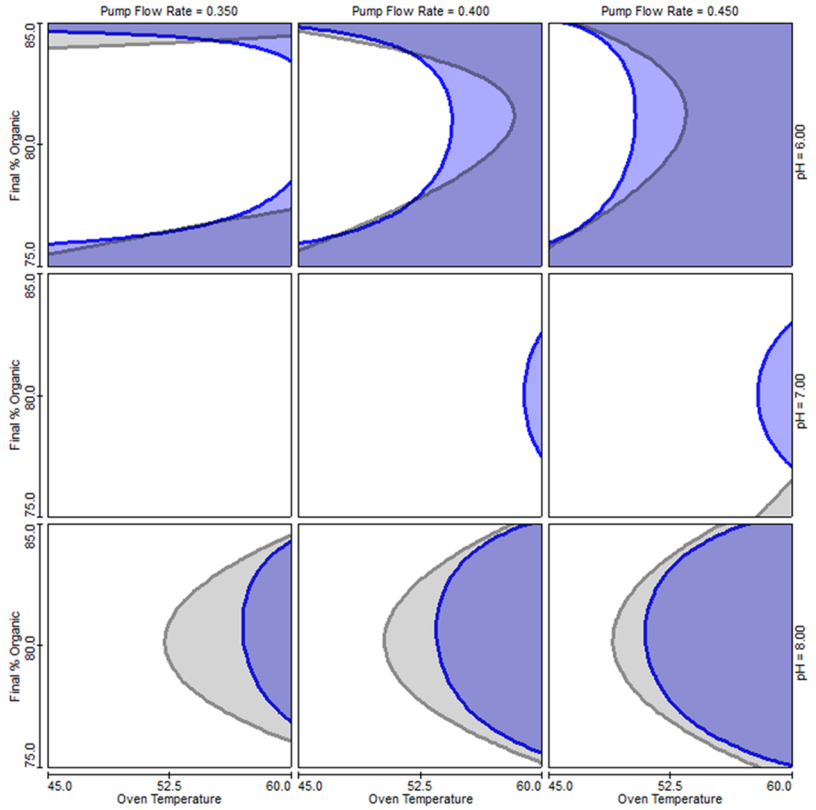

Figure 12. Trellis graphs from DoE to establish CMA models and robustness testing. Graph representing an area where the defined criteria are met in white color—design space. The colors are areas where the criteria are not met: blue = $R_{c1} \leq 1.5$, gray = $C_{pk} \leq 1.33$. x = column temperature (45−60 °C); y = final percent of mobile phase B in the first gradient (75−85%); at pH of 6.0 (top line), 7.0 (middle line) and 8.0 (bottom line); and pump flow of 0.35 (left column), 0.40 (middle column), and 0.45 mL/min (right column).

flow. The sample solvent is critical as venetoclax has low solubility in many solvents and a total solubility of the drug substance is needed for accuracy. Additionally, variability can occur as the sample solvent is usually mixed by hand. However, it was tested separate from the LC method development (see Section 2.1.1), which resulted in a suitable solvent even if variation in composition occurred. Thus, it was excluded from the LC AQbD process. The column temperature showed a minor, but not necessarily insignificant, effect on method performance (Figure S4), but it can exhibit significant variation. We felt more information about parameter interactions could prove useful, so the column temperature was marked as possibly critical and in need of further investigation. Pump flow can usually be well controlled and as such not considered critical. However, in combination with gradient change and percent of organic modifier in the mobile phase, it can show significant parameter interaction. As such, it was included in the selected CMPs. Lastly, percent of the organic modifier in the mobile phase, buffer pH, and gradient all had significant effects on method performance in terms of resolutions (CMAs), tailing, and length of the method. Furthermore, they are more difficult to control.

2.5. Method Screening. Method screening was performed by applying DoE to evaluate critical parameters and their interactions, using an Acquity BEH C18 column (1.7 μm; 150 × 2.1 mm) and acetonitrile as the organic modifier. Stationary and mobile phases have the most influence on retention and resolution. We chose the critical parameters based on the method risk assessment (Figure 6) and selected those related to the mobile phase for the first screening.

The parameters studied were pump flow rate (0.3 and 0.4 mL/min), pH of the buffer (6.0, 6.5, 7.0, 7.5, and 8.0), and the final percent of acetonitrile in the gradient (80−95%). The other method parameters were column temperature 50 °C; mobile phase A: A = NH_4HCO_3 (10 mM); mobile phase B: B = ACN; gradient: t = 0 min, 10% B; t = 1 min, 10% B; t = 16 min, 80−95% B; t = 26 min, 80−95% B; followed by a 2 min column wash with 99% B and a 6 min re-equilibration with 10% B.

The monitored criteria were the number of observed resolved peaks, the number of peaks with a resolution greater than or equal to 1.5, and the number of peaks with tailing less than 1.2. A wider set of criteria were chosen (such as number of resolved peaks), not merely CMAs, with a wish to achieve the best results and gain a wider knowledge about the method. DoE using an A- and G-optimal process design (Table S3)[30] was used with a cubic design model. An A-optimal design focuses on minimizing the average variance of predictions of the regression coefficients and a G-optimal design focuses on minimizing the maximum variance of the predicted values. According to the results (Figure 7), the best conditions were pH 7.00, final percent of acetonitrile 95%, and pump flow of either 0.38 mL/min or 0.40 mL/min.

The performed DoE experiment enabled us to establish the interactions of the parameters and their influences on the results through the models. The method models showed an interesting, nonlinear relation between the parameters (Figure 8a,b). The model equations are presented in Table 1. Model equations were statistically evaluated using analysis of variance

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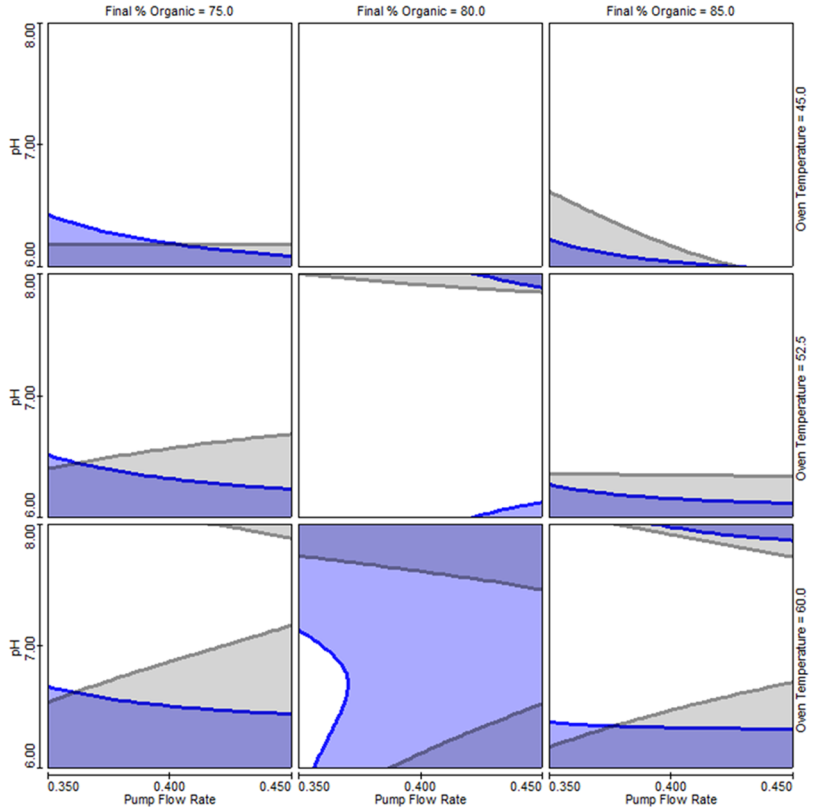

Figure 13. Trellis graphs from DoE to establish CMA models and robustness testing. Graph representing an area where the defined criteria are met in white color—design space. The colors are areas where the criteria are not met: blue = $R_{c1} \leq 1.5$, gray = $C_{pk} \leq 1.33$. x = pump flow rate (0.35−0.45 mL/min); y = buffer pH (6.0−8.0); at column temperature of 45 (top line), 52.5 (middle line) and 60 °C (bottom line); and final percent of mobile phase B in the first gradient of 75 (left column), 80 (middle column), and 85% (right column).

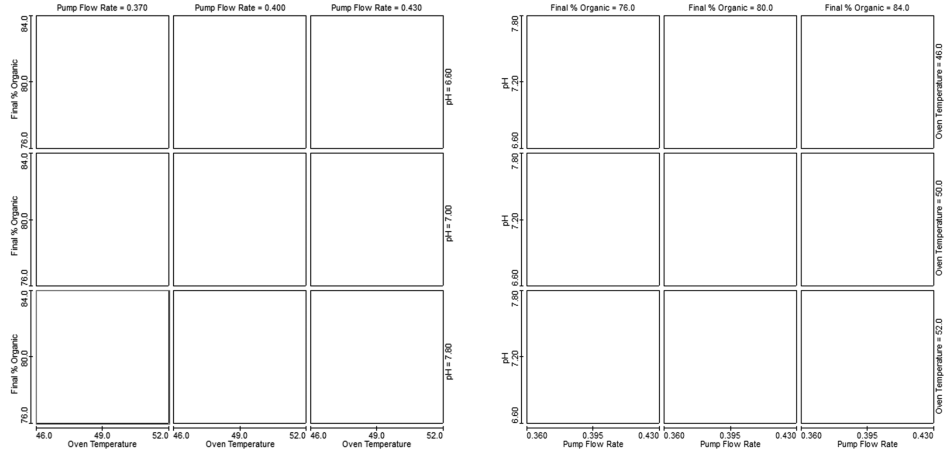

Figure 14. Trellis graphs of the MODR. Inside the MODR, all the criteria are met, which can be seen by the white color in the entire MODR region. (a) Axes parameters are the same as in Figure 12 and (b) axes parameters are the same as in Figure 13.

(ANOVA)[31] and showed good statistical significance with F-ratios > 4.00 and acceptable fit (R^2 and LOF analysis).

In the next step, mobile phase B was changed to 95% ACN because of the UHPLC pump check valve longevity. The method was somewhat shortened, beginning with a higher amount of ACN as no observed peaks eluted earlier. After that change, method parameters were UHPLC BEH C18 (1.7 μm, 150 mm × 2.1 mm) column; mobile phase A: A = NH_4HCO_3 (pH 7.0, 10 mM); mobile phase B: B = 95% ACN (v/v); column temperature 50 °C; flow rate 0.4 mL/min; gradient: t = 0 min, 20% B; t = 1 min, 20% B; t = 13 min, 100% B; t = 17 min, 100% B; t = 18 min, 20% B.

With those changes, we performed a second screening DoE (Table S4), where the column temperature (40−55 °C) was optimized in relation to gradient time (6−18 min). Column temperature was chosen based on the initial risk assessment (Figure 6) as it was not yet included in the first DoE because of reducing the parameters for a single experiment, and the

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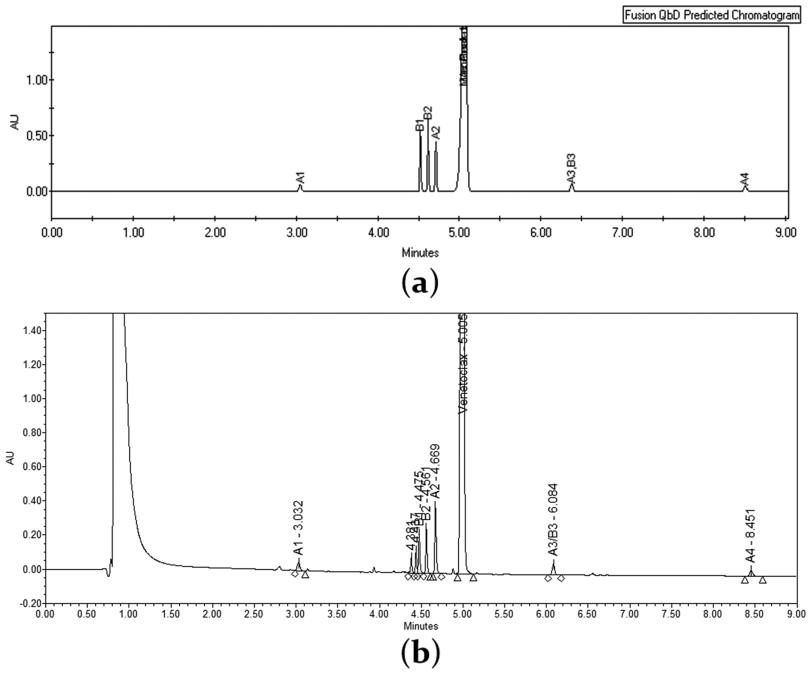

Figure 15. (a) Predicted chromatogram in the selected working point using Fusion QbD software and (b) actual chromatogram in the selected working point. The peak eluting at approximately 1 min is a solvent peak of DMSO. Peaks eluting at 4.38 and 4.48 min are the process-related impurities originating from the active pharmaceutical ingredient. Predicted chromatogram (a) contains only the key degradation products that were tracked during robustness testing.

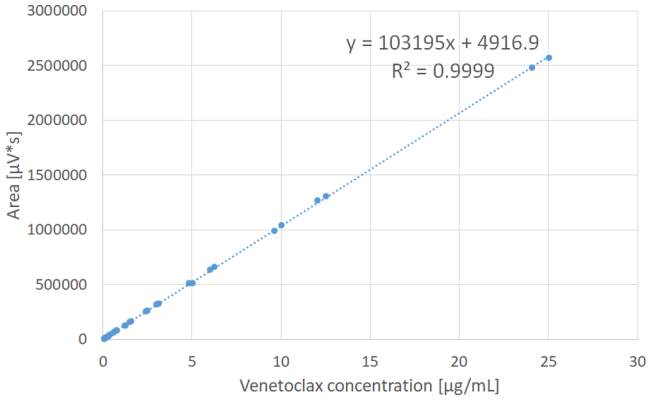

Figure 16. Graph representing the venetoclax linearity results including the linear equation.

gradient was included as the mobile phase B was changed to 95% ACN. Additionally, the first screening DoE showed that a steeper gradient was more efficient, so we wanted to further test if an additional change to the gradient could prove beneficial. An A- and G-optimal process design and a cubic design model were used in the DoE (Table 2, Figure 8c,d). A shorter gradient (6 min) and a higher temperature (49.7 °C) were indicated as the best answer.

The work so far is summarized in Figure 9. We studied various chromatographic parameters. Scouting was composed of multiple OFAT experiments to evaluate single parameter changes and evaluate their criticality as well as select the static parameters for the analytical method. A single DoE was utilized for the final selection of the chromatographic column and mobile phase organic modifier. The information gained assisted us with the method risk analysis and identification of critical parameters. The following was the method screening

Table 4. Accuracy and Precision Results from Method Validation

theoretical conc. of venetoclax (µg/mL)	actual conc. of venetoclax (µg/mL)	area (µV × s)	calculated conc. of venetoclax (µg/mL)	recovery (%)	repeatability (RSD %)
8	8.082	827,755	7.974	98.66	0.71
	8.229	843,195	8.123	98.71	
	8.480	879,142	8.472	99.91	
				average: 99.09	
10	10.6106	1,119,332	10.799	101.82	1.11
	9.624	994,240	9.587	99.62	
	10.016	1,043,173	10.061	100.45	
				average: 100.63	
12	12.373	1,275,218	12.310	99.49	1.09
	12.030	1,266,765	12.228	101.65	
	12.521	1,308,675	12.634	100.91	
				average: 100.68	

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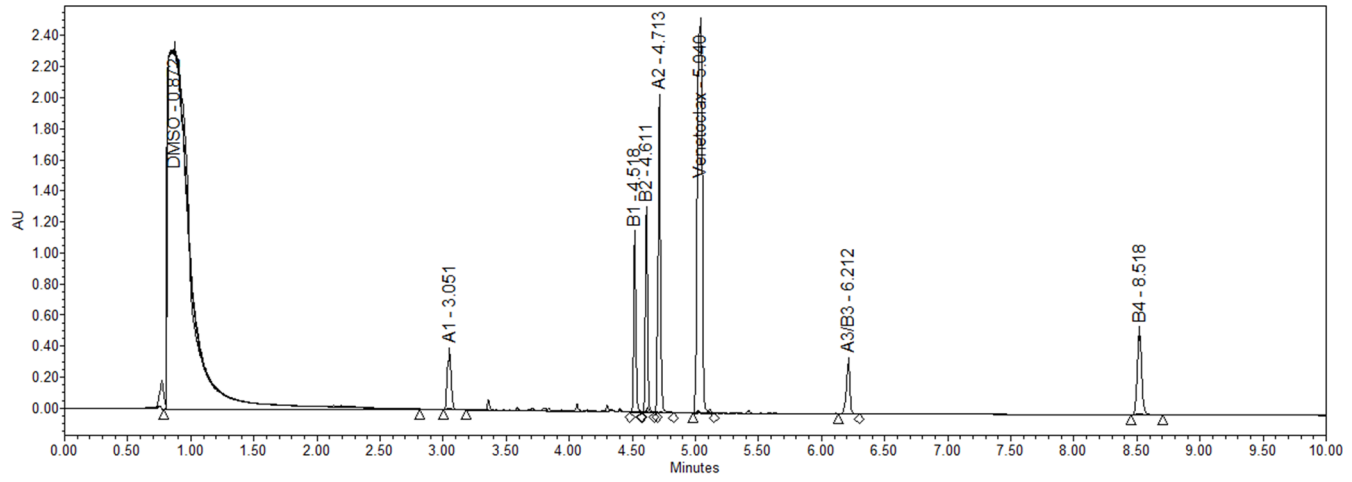
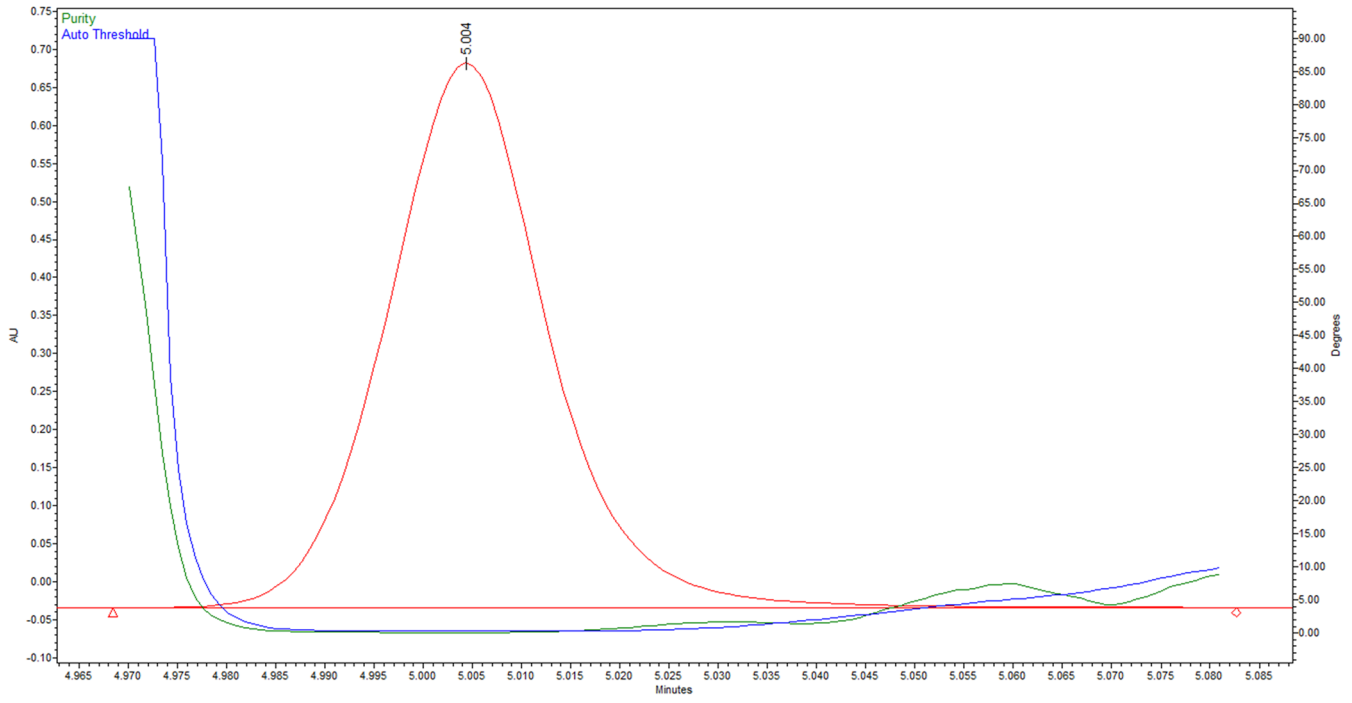


(a)



(b)

Figure 17. (a) Overlay chromatogram of degradation products A1, B1, B2, A2, A3/B3, A4, and venetoclax. (b) Purity plot of venetoclax.

process consisting of two experiments utilizing DoE. Combinations of parameters were studied and the method was further adjusted based on the performance.

2.6. Method Optimization. As a shorter gradient (6 min) and a higher temperature (49.7 °C) were indicated as the most promising in the screening process, the gradient time was further tested including shorter and longer times. Once again, the experiments showed the shortest gradient was the most suitable. The shorter gradient time has proven to be beneficial to the resolutions between degradation products B1 and B3 (R_{c1}) and between degradation products B2 and A2 (R_{c2}); however, the resolution of venetoclax (R_v) has worsened. Thus, the gradient was split into two gradient steps to optimize the separation of degradants in the first gradient but keep the resolution of venetoclax with the second gradient. The method parameters were UPLC BEH C18 (1.7 μm, 150 mm × 2.1 mm) column; mobile phase A: A = NH_4HCO_3 (pH 7.0, 10 mM); mobile phase B: B = 95% ACN (v/v); column temperature 50 °C; pump flow 0.4 mL/min; gradient: t = 0 min, 20% B; t = 1 min, 20% B; t = 4 min, 80% B; t = 9 min, 100% B; t = 10 min, 20% B (Figure 10). The resolutions between critical pairs were R_{c1} (between B1 and B2) = 3.17 and R_{c2} (between B2 and A2) = 3.18. Up to this point, the method was effectively shortened from 28 min (Figure 5) to 10 min (Figure 10). Shorter run times result in a smaller mobile phase consumption and an easier time management in the laboratory. They enable a higher analysis throughput.

Next, we employed a DoE including all of the CMPs of interest and we monitored their effect on the CMAs exclusively to calculate the appropriate method model equations. Parameters for the DoE study (Table S5) were chosen in regard to the initial method risk assessment (see Section 2.4)

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and further knowledge gained through the screening process: buffer pH (6.0−8.0), percent of acetonitrile, column temperature (45−60 °C), and flow rate (0.35−0.45 mL/min). The percent of acetonitrile was studied as a variation of mobile phase B in the first gradient as that is the most critical part of the method. The criteria chosen were the CMAs defined in the initial risk assessment (see Section 2.4): resolution of venetoclax (R_v) ≥ 1.5; critical resolutions R_{c1} (resolution between B1 and B2) and R_{c2} (resolution between B2 and A2) ≥ 1.5 (Figure 11, Table 3). An A- and G-optimal design was used for the DoE with a cubic design model.

Based on the DoE results, the method model equations were calculated and statistically evaluated using ANOVA (Table 3). Additionally, each model coefficient was evaluated. The included model coefficients show a statistical significance (*P*-values < 0.05, *F*-ratios > 4.00). Furthermore, the *F*-ratios show the level of significance of each individual coefficient in the model. High R^2 values and low lack-of-fit (LOF) values indicate a good fitting model and high *F*-ratios show that the model equations have statistical significance.

2.7. Robustness Study. The robustness simulator was used with enabled variation of all the critical parameters with a maximum expected variation set at ±3σ. Fusion QbD software uses process capability indices (C_p, C_{pk}) to quantify system robustness. For CMAs, C_{pk} were used as the process capability indices with a lower specification limit set (LSL) at 1.33, meaning 99.99% of measurements will fall inside the specification limits. The robustness simulator runs Monte Carlo simulations and presents C_{pk} in the graphs (Figures 12, 13).

The venetoclax resolution (R_v) and resolution of A2 (R_{c2}) were ≥1.5 in the whole experimental region. The resolution of B2 (R_{c1}) was <1.5 at higher temperatures with a higher pump flow and a pH of 6.0 (Figure 12) as well as at a column temperature of 60 °C at the percent of mobile phase B after the first gradient of 80% (Figure 13). A control space, where all three critical resolutions (R_v, R_{c1}, and R_{c2}) were suitable, was defined as MODR (Figure 14) (see Section 2.8).

2.8. Method Operable Design Region. The MODR, also known as control space, was established based on the CMA models and robustness simulations. The DoE region is presented in Figures 12 and 13. The MODR, where the method is robust is flow rate = 0.37−0.43 mL/min; column temperature = 46−52 °C; and pH = 6.6−7.8. The acceptable variation of percent of mobile phase B is ±4%. As the mobile phase B consists of 95% of acetonitrile (ACN), the acceptable variation of acetonitrile is ±3.8% (Figure 14).

A working optimal point was chosen inside MODR, which is flow rate = 0.4 mL/min; column temperature = 50 °C; and pH = 7 (Figure 15). The predicted CMAs at the working point were R_v = 5.98, R_{c1} = 2.86, and R_{c2} = 3.13. The actual CMAs at the working point were R_v = 6.03, R_{c1} = 2.49, and R_{c2} = 3.10.

2.9. Final Risk Assessment and Control Strategy. CMPs have proven to be mobile phase pH, percent of acetonitrile in the mobile phase, flow rate, and column temperature. The most critical parameter for venetoclax resolution is the flow rate, which is suggested to be kept at the optimal point. The least critical among the CMPs is the percent of acetonitrile. The resolution between degradation products B1 and B2 (R_{c1}) seems to be the most sensitive to the change of parameters out of the three CMAs, so this resolution might be considered as a good criterion for system suitability.

2.10. Method Validation. The developed stability-indicating method for venetoclax was validated in terms of linearity, accuracy, and repeatability at the selected working point. Method validation was performed according to the ICH Q2(R1) guidelines.[32] Venetoclax solutions for validation were prepared as described in Section 4.4.2.

The method proved to be linear in the venetoclax concentration range of LOD—25 μg/mL with a coefficient of determination (R^2) = 0.99987 (Figure 16). Limit of quantification (LOQ) and limit of detection (LOD) were determined by calculating the S/N ratios of the prepared venetoclax solutions. LOD was determined to be 0.075 μg/mL, where an S/N value was 3.5 and LOQ was determined to be 0.188 μg/mL with an S/N value of 10.48.

Accuracy was established based on the calculated recoveries at three concentration levels representing 80, 100, and 120% of the target value (10 μg/mL) (Table 4). The actual venetoclax concentration was calculated taking into account the weight, dilution, and purity of the drug substance. The accuracy was determined by dividing the concentration calculated from linearity with the actual concentration of venetoclax and is expressed as recovery in %. All of the recoveries are in the range of 100 ± 2%.

Repeatability was measured and calculated in three replicates at three concentrations (80, 100, and 120% of the target concentration) as the relative standard deviation (RSD). The method showed good repeatability with RSD <2.00%.

All of the key degradation products (A1, A2, A3/B3, A4, B1, and B2) were injected, and an overlay chromatogram was produced. Additionally, a degradation solution prepared for robustness testing was diluted to achieve an appropriate venetoclax concentration for purity testing (absorbance <1 AU), and peak purity was evaluated using Empower 3 software. Degradation products and venetoclax were well separated (Figure 17a). The calculated purity angle was 0.159, which was less than the purity threshold (0.292), indicating that the peak is spectrally pure (Figure 17b).

The method has proven to be accurate, repeatable, and specific in the range of LOD—25 μg/mL in the selected working point.

3. CONCLUSIONS

A stability-indicating reversed-phase UHPLC method for determination of venetoclax was developed using an AQbD approach. There were no previous stability-indicating analytical methods for venetoclax, no venetoclax degradation products nor venetoclax impurities available for the development process. This led to the approach where forced degradation samples were effectively used throughout the AQbD process.

A mathematical model was established for the CMAs in regards to the CMPs. A robust method region was proposed inside the design region—control space, also known as MODR: flow rate = 0.37−0.43 mL/min, column temperature = 46−52 °C, pH = 6.6−7.8, and variation of acetonitrile ±3.8%. The mathematical model enables us to get a better understanding of the effects of the method parameters on the results. The developed analytical method was validated in the selected working point in terms of accuracy, repeatability, sensitivity, and linearity. The developed method achieved the ATP set at the beginning of the AQbD process.

The developed LC method is able to separate six main venetoclax degradation products (A1, A2, A3/B3, A4, B1, and B2). Additionally, the method is MS-compatible, enabling an

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easy transition between different detection methods. This sets a good foundation for future investigation on the identification of degradation pathways of venetoclax, which could be established after structure elucidation of identified key degradation products. Work on the structural elucidation of venetoclax degradation products is underway in our laboratories, and results will be reported in due course.

4. MATERIALS AND METHODS

4.1. Chemicals and Reagents. Venetoclax was obtained from Selvita (Krakow, Poland) and MSN Laboratories (Hyderabad, India). Gradient grade acetonitrile (ACN) and methanol (MeOH) were purchased from J.T.Baker now part of Avantor (Radnor, PA, USA). Analytical grade glacial acetic acid, LiChrosolv MTBE, hydrochloric acid (HCl) Titrisol solution, sodium hydroxide (NaOH) Titrisol solution, analytical grade EMSURE 85% orthophosphoric acid, analytical grade 30% peroxide, iron (III) chloride hexahydrate, and analytical grade buffers were purchased from Merck KGaA (Darmstadt, Germany). Dimethyl sulfoxide (DMSO) was purchased from Honeywell (Charlotte, NC, USA). Purified water was obtained by filtrating through a Milli-Q system from Merck Millipore (Burlington, MA, USA).

4.2. Equipment and Software. LC method development and analyses were performed on Acquity UPLC systems (Waters, Millford, MA, USA) equipped with a binary solvent manager (BSM), sample manager (SM), temperature-controlled column compartment, and photodiode array detector (PDA); Acquity UPLC system (Waters, Millford, MA, USA) with BSM, SM, PDA using a high-sensitivity flowcell, column manager (CM), and an additional solvent switch; and Acquity UPLC H-Class systems (Waters, Millford, MA, USA) equipped with a quaternary solvent manager (QSM), sample manager with flow-through needle (SM-FTN), and either PDA or a tunable ultraviolet (TUV) optical detector.

The weighing was done on either XP4002S precision balance, XP205 DeltaRange analytical balance, AX205 DeltaRange analytical balance, or MX5 microbalance (Mettler Toledo, Columbus, OH, USA). Weighing of the venetoclax drug substance was done in a ventilated balance enclosure OK 15 (Iskra Pio, Šentjernej, Slovenia). The pH was measured using a SevenMulti pH meter (Mettler Toledo, Columbus, OH, USA). Pipettes used were Picus automatic pipettes (Sartorius, Göttingen, Germany) and Handystep electronic repetitive pipettes (Brand, Wertheim, Germany). Ultrasonic baths used were Branson 8510 (Emerson Electric, St. Louis, MO, USA), Sonic 10 and Sonic 20 (Iskra Pio, Šentjernej, Slovenia). Stress testing was done in a BF 720 standard incubator (Binder, Tuttlingen, Germany). Photostability was measured in a Suntest XLS+xenon test instrument (Atlas Material Testing Technology part of Ametek, Mount Prospect, IL, USA).

Chromatography columns used were Acquity UPLC BEH C18 (1.7 μm, 100 mm × 2.1 mm, and 150 mm × 2.1 mm), Acquity UPLC BEH Phenyl (1.7 μm, 100 mm × 2.1 mm), Acquity UPLC BEH Shield RP18 (1.7 μm, 100 mm × 2.1 mm), Acquity UPLC CSH C18 (1.7 μm, 100 mm × 2.1 mm, and 150 mm × 2.1 mm) (Waters, Millford, MA, USA); Kinetex C18 (1.7 μm, 100 mm × 2.1 mm), Luna Omega C18 (1.6 μm, 100 mm × 2.1 mm, and 150 mm × 2.1 mm), Luna Omega PS (1.6 μm, 100 mm × 2.1 mm) (Phenomenex, Torrance, CA, USA).

Waters LC systems were equipped with Empower 3 chromatography data software (Waters, Millford, MA, USA). AQbD was done with Fusion QbD software (S-Matrix, Eureka, CA, USA).

4.3. Final UHPLC Method Conditions. The final method conditions in the working point were Acquity UPLC BEH C18 (1.7 μm, 150 mm × 2.1 mm) column; mobile phase A: A = ammonium bicarbonate (pH 7.0; 10 mM) pH adjusted with acetic acid; mobile phase B: B = 95% ACN; strong needle wash = water−ACN−DMSO (5:4:1, v/v/v); pump flow 0.4 mL/min; injection volume 5 μL; column temperature 50 °C; autosampler temperature 5−10 °C; detection wavelength 220 nm; gradient: t = 0 min, 20% B; t = 1 min, 20% B; t = 4 min, 80% B; t = 9 min, 100% B; t = 10 min, 20% B; re-equilibration = 3 min.

4.4. Preparation of Sample Solutions. *4.4.1. Method Development and the AQbD Study Sample.* A stock solution of venetoclax in DMSO was prepared in a concentration of 5 mg/mL. The stock solution (1 mL) was transferred in a 5 mL flask, and 1 mL of 1 M HCl was added. The stock solution (1 mL) was transferred in a different 5 mL flask, and 1 mL of 1 M NaOH was added. The flasks were sealed and transferred to a standard incubator chamber set at 50 °C. After 3 days, the samples were removed from the chamber. The sample (1 mL) with added HCl and 1 mL of the sample with added NaOH were combined in a 10 mL flask. DMSO (2 mL) was added along with a few drops of 10 mM ammonium bicarbonate buffer (pH = 6). Then, the flask was topped off with ACN. The sample solution was then filtered through a 0.22 μm polyvinylidene fluoride (PVDF) filter into an amber vial.

4.4.2. UHPLC Method Validation Samples. All of the samples for validation were prepared as solutions in the UHPLC sample solvent (ACN-DMSO-buffer (6:3:1, v/v/v)). The venetoclax drug substance from MSN was used with a calculated purity of 98.20%. The calculation was done based on the certificate of analysis provided by the supplier. For linearity, a sample solution with a concentration of about 100 μg/mL was prepared in two replicates. They were diluted in a series of dilutions to achieve concentrations of about 50, 25, 12.5, 6.25, 3.125, 1.563, 0.781, 0.391, and 0.195 μg/mL. Initial solutions with a concentration of about 100 μg/mL were then diluted to a concentration of about 10 μg/mL. These were further diluted in a series of dilutions to achieve concentrations of about 5, 2.5, 1.25, 0.625, 0.313, 0.156, 0.078, 0.039, and 0.020 μg/mL. A linear range was established. A target concentration (10 μg/mL) inside the range was selected, and solutions for accuracy and repeatability were prepared (80, 100, and 120% of target concentration). Solutions from linearity were used for accuracy and repeatability (two replicates). Additional venetoclax solutions with a concentration of about 10 μg/m and 12 μg/mL were prepared to produce the third replicate needed for accuracy and repeatability. Additionally, a venetoclax solution with a concentration of about 8 μg/mL was prepared in triplicate to assess the accuracy and repeatability at 80% of the target concentration. For specificity, the key degradation products (A1, A2, A3/B3, A4, B1, and B2) were isolated. The key degradation products were injected, and an overlay chromatogram was produced. In addition, an AQbD study sample (see Section 4.4.1.) was diluted to achieve an absorbance of venetoclax <1 AU. This solution was used to calculate the peak purity.

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■ ASSOCIATED CONTENT

Supporting Information

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Solvent testing; predicted pH curves of venetoclax; chromatograms of the venetoclax sample degraded with 1 M HCl at 50 °C for 3 days using a BEH C18 column and BEH Phenyl column; chromatograms of the venetoclax sample degraded with 1 M HCl at 50 °C for 3 days—the influence of small pH changes as well as the omission of MTBE in the mobile phase B; effects of column temperature change; testing of different C18 chromatographic columns; DoE from the method scouting experiment; first DoE from method screening; second DoE from method screening; and DoE from method optimization (PDF)

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Author Contributions

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Notes

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