Development of an UHPLC Method for Azithromycin Tablets Using ChromSword Auto Software

Application Note

Pharmaceutical QA/QC

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Abstract

In this Application Note, we used the Agilent 1200 Infinity Series LC Method Development System with Agilent Method Scouting Wizard Software and ChromSword Auto Software to automate the method development for impurities in Azithromycin tablets. The Agilent Method Scouting Wizard was used to screen columns, organic solvents, and buffers. ChromSword Software was used to determine gradient profiles automatically. Using the Design of Experiment (DoE) and chromatographic resolution as a critical method attribute (CMA), a design space was generated. The developed HPLC method is based on Agilent Poroshell HPH C8 columns, and shows excellent area and retention time precision for the API during 250 injections. With this method, the runtime from the USP method on conventional column types was reduced from 93 minutes to 6 minutes.

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Introduction

HPLC method development is frequently a time-consuming process. Typical method optimization includes optimizing parameters such as columns, mobile phases, column temperatures, and others. This can result in a considerable number of trial-and-error experiments[1]. In addition, optimizing a gradient separation is also based on an analyst's intuitive knowledge of the chromatographic process. Thus, achieving a good gradient method that separates samples with many peaks is generally not possible by a trial-and-error approach.

Automated method development systems provide an alternative solution. ChromSword Auto Software in combination with Agilent 1200 Infinity Series LC Method Development System and Agilent OpenLab CDS Software, creates a powerful, specialized method development system capable of developing new HPLC methods, or improving existing methods, fully automatically[2]. In addition, Agilent Method Scouting Wizard helps in scouting a large number of conditions, significantly improving the method development process.

Azithromycin is a macrolide antibiotic used against bacterial infections[3]. The United States Pharmacopeia (USP) method of azithromycin drug product uses a C18 column, a mobile phase at pH 8.9, and an LC run time of 93 minutes[4]. Time, cost of analysis, and reproducibility are serious concerns. In this Application Note, an Agilent 1200 Infinity Series LC Method Development System was integrated with ChromSword Auto to develop a short, robust UHPLC method for azithromycin tablet and three spiked impurities: Imp M, Imp A, and Imp C (listed in the USP).

Experimental

Instrumentation

Agilent 1200 Infinity Series LC Method Development Solution. The following individual modules and components were used:

- Agilent 1290 Infinity Binary Pump (G4220A)
- Agilent 1290 Infinity Valve Drive (G1170A) and Agilent Quick-Change 12-position/13-port solvent selection valve (G4235A)
- Agilent 1290 Infinity Autosampler (G4226A) maintained at 4 °C using thermostat (G1330B)
- Agilent 1290 Infinity Thermostatted Column Compartment (G1316C) cluster with two Agilent Quick-Change 8-position/9-port valves (G4230B)
- Solvent Selection Tubing Kit for four solvent (p/n 5067-4601)
- Agilent 1290 Infinity Dioide Array Detector (G4212A)

Software

- ChromSword Auto 4.0 Automated Method Development Software (Version 4.30.10.13) was used for the automated experimentation.
- ChromSword AutoRobust (Version 3.6 build 30-06-13) was used to do robustness experiments.
- ChromSword Report Viewer (Version 4.1-22-03-13) was used to analyze robustness results.
- Agilent Method Scouting Wizard (Version A.02.02). Add on to OpenLab CDS system
- Agilent OpenLab CDS System (Version C.01.05 [38])

Reagents and materials

Azithromycin tablets (Azithral) were purchased from a local store. Azithromycin impurities M, E, and A were purchased from Anant Pharmaceuticals Pvt Ltd, India. All solvents used for analysis were of LC/MS grade, and were purchased from Fluka. Additives and reagents were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Purified water was obtained from a Milli-Q water purification system (Billerica, MA, USA).

Sample preparation (azithromycin tablets USP protocol)[4]

Two tablets of azithromycin were crushed. An equivalent of 133.5 mg of azithromycin was accurately weighed and transferred to a 10-mL volumetric flask. A 7.5 mL amount of acetonitrile was added to the flask, sonicated for 5 minutes, and shaken for 15 minutes. The solution was allowed to equilibrate to room temperature, and then diluted with acetonitrile to volume. An aliquot of sample was centrifuged at 9,500 rpm for 15 minutes, and the supernatant was transferred to a 10-mL volumetric flask. 300 µL of the supernatant was mixed with 700 µL of solvent containing a 1:1 ratio of methanol and Solution B (1.7 mg/mL of monobasic ammonium phosphate adjusted with ammonium hydroxide to a pH of 10.00 ± 0.05) to obtain a solution having a nominal concentration of about 4 mg of azithromycin per mL. Three impurities (Imp M, E, A), prepared in 100 % acetonitrile, except for Imp A in which 50 % water was used, was spiked to obtain 2.5 % height of the API.

Workflow

The workflow contains four steps:

- Screening
- Rapid optimization
- Robustness tests
- Verification (Figure 1).

Screening was performed using Method Scouting Wizard, in which all possible combinations of different column chemistries, organic solvents, and pH ranges (aqueous solvents) were tested, excluding incompatible combinations of column chemistry and pH, and column chemistry and temperature. Based on the separation results, the two best conditions were selected for the rapid optimization phase by the ChromSword algorithm feature of ChromSword Auto 4.0. The optimized method was selected for robustness tests using the ChromSword AutoRobust tool, in which multiple method parameters were changed using the full factorial design. The results from the robustness tests were analyzed using ChromSword Report Viewer. A 2D contour plot of the experimental results was used to determine the design space (multidimensional combination and interaction of input variables and process parameters that have been demonstrated to provide assurance of quality (ICH Q8 R2)) and the robust region. The robust region was verified in a verification step. The reproducibility of resolution of the critical impurity pair (Imp M and E), relative retention time of all other impurities, and precision of area and retention time of the API peak were verified within the robust region.

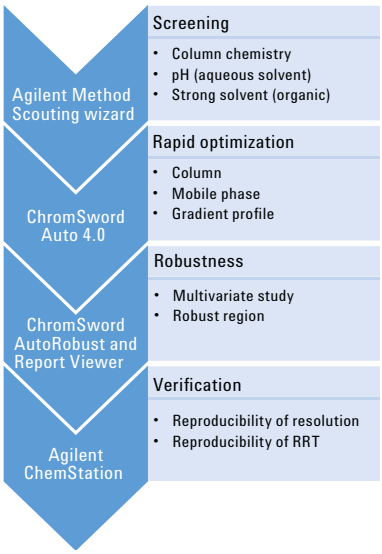

Figure 1. Overall workflow used for the study which consists of four main steps: screening, rapid optimization, robustness tests, and verification. Software packages used are shown on left side of the flow chart while detailed optimizing steps are shown on the right side.

Results and Discussion

Step 1: Screening

The column chemistry, the choice of the organic solvents, and the buffers has the largest impact on selectivity. The Method Scouting Wizard can test all possible combinations of method parameters, similar to a full factorial design. The software will intuitively avoid incompatible combinations of column and pH, as well as column and temperature. It also scales up the flow and run time according to the column diameter and length. In addition, the Method Scouting Wizard has the flexibility to flush and equilibrate the column after each change of the eluent composition.

Eight different Poroshell columns, eight different pH ranges (aqueous solvents), and two different organic solvents were screened (Table 1). The starting and ending point in % B, flow rate, and temperature setting for the screening experiments were obtained from literature, and kept as constant.

Table 1. Different column chemistries, mobile phases, buffers, and method parameters used in the screening process.

Columns (3.0 × 50 mm, 2.7 µm)	
Pos 1: Agilent Poroshell HPH-C18	
Pos 2: Agilent Poroshell 120 Sb Aq	
Pos 3: Agilent Poroshell 120 Phenyl Hexyl	
Pos 4: Agilent Poroshell 120 Bonus RP	
Pos 5: Agilent Poroshell 120 EC-C8	
Pos 6: Agilent Poroshell 120 EC-C18	
Pos 7: Agilent Poroshell 120 EC-CN	
Pos 8: Agilent Poroshell HPH-C8	
Mobile phases	
A1:01 -pH 2.1, 10 mM TFA	
A1:02 -pH 2.7, 20 mM formic acid in water	
A1:03 -pH 4.0, 5 mM formic acid and 10 mM ammonium formate in water	
A1:04 -pH 5.0, 5 mM acetic acid and 10 mM ammonium acetate in water	
A1:05 -pH 6.8, 10 mM ammonium acetate	
A1:06 -pH 8.0, 10 mM ammonium hydrogencarbonate in water	
A1:07 -pH 9.0, 10 mM ammonium acetate and 5 mM ammonia	
A1:08 -pH 10.8, 10 mM Ammonia	
B1: Acetonitrile	
B2: ACN/Methanol (1:1)	
Other parameters	
Pump flow	0.8 mL/min
Injection volume	2 µL
Column temperature	45 °C
Wavelength	210 nm ± 4 nm (ref off)
Gradient	Time % B 1.00 45 6.00 95 8.00 95 8.01 45
Gradient time	8 minutes

The results from the screening experiments delivered the two most suitable column chemistries: Agilent Poroshell HPH-C8 and Agilent Poroshell 120 EC-C18, which showed good separation and peak shape at pH 10.8 using acetonitrile as organic solvent (Figures 2A and 2B). Other combinations of column chemistries and pH ranges were neglected due to poor separation results and poor peak shapes. The results show that a combination of high pH and reverse phase C18 or C8 columns leads to an acceptable separation of azithromycin and its three impurities. The high pH range used in the USP method was not tested here in the first screening experiments, but was tested later in the second, the rapid optimization phase.

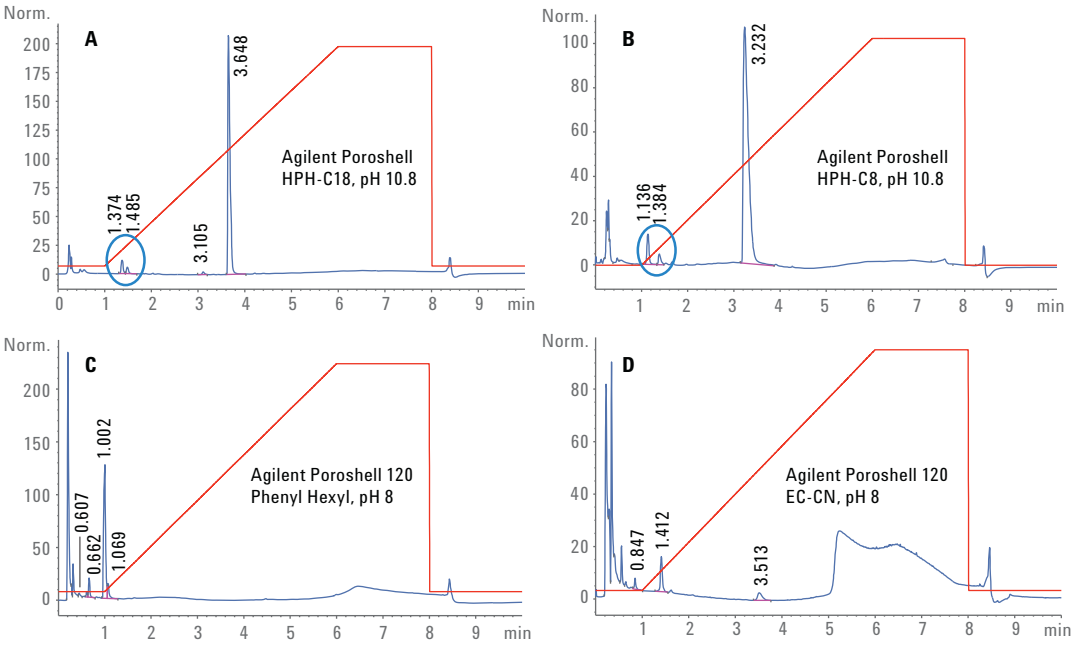

Figure 2. Results from the screening experiments. A) Agilent Poroshell HPH-C18 with pH 10.8, B) Agilent Poroshell HPH-C8 with pH 10.8, C) Agilent Poroshell 120 Phenyl Hexyl with pH 8, and D) Agilent Poroshell 120 EC-CN with pH 8. Good separation results have been obtained in A and B, where the impurities were baseline separated in A.

Step 2: Rapid optimization

Using the results from the screening step, the ChromSword Algorithm feature of the ChromSword Auto Software was applied for the rapid optimization phase. The ChromSword Algorithm automatically performs gradient runs while tracking the peaks of interest. The second gradient had been further optimized, and was based on the results from the first run. Within three or four runs, the software suggested an optimal gradient to obtain a good separation within a short run time. Here, we checked whether the nonvolatile buffers and experimental conditions based on the USP method gave a better separation compared to the volatile buffers (which could be used in combination with MS detection) used in screening experiments. Hence, the USP mobile phase (dibasic sodium phosphate (pH 8.9)) and (acetonitrile:methanol (3:1)) was incorporated as a study parameter. The criteria for filtering out the best method was the USP tailing factor < 2, and the resolution > 2 of a critical pair (Imp M and E). The Agilent Poroshell HPH-C8 column with the USP mobile phase showed the best results because the resolution of the critical pair was found to be 2.00 and USP tailing factor was found to be 1.8 (Figure 3). However, in the case of volatile buffers, the peak tailing was > 2, therefore, they were not chosen for further validation experiments. No further separation improvements were required as the rapid optimization process suggested a suitable gradient profile to separate azithromycin and its three impurities. However, in cases where a larger number of peaks need to be separated, the *fine optimization* option of ChromSword Software could be additionally chosen.

Table 2. Columns, mobile phases, buffers, and method parameters used in the rapid optimization phase.

Columns (3.0 × 50 mm, 2.7 um)	
Pos 1: Agilent Poroshell HPH-C18 Pos 2: Agilent Poroshell HPH-C8	
Mobile phases	
A1:01 -pH 10.8, 10 mM Ammonia (Volatile buffer) A1:02 -pH 8.9, (USP Buffer) B1: Acetonitrile B2: USP solvent (Acetonitrile/Methanol in 3:1 ratio)	
Other parameters	
Pump flow	0.8 mL/min
Injection volume	4 µL
Column temperature	50 °C
Wavelength	210 nm ± 4 nm (ref off)
Concentration limit	35 %–75 %
Equilibration time	5.00 minutes

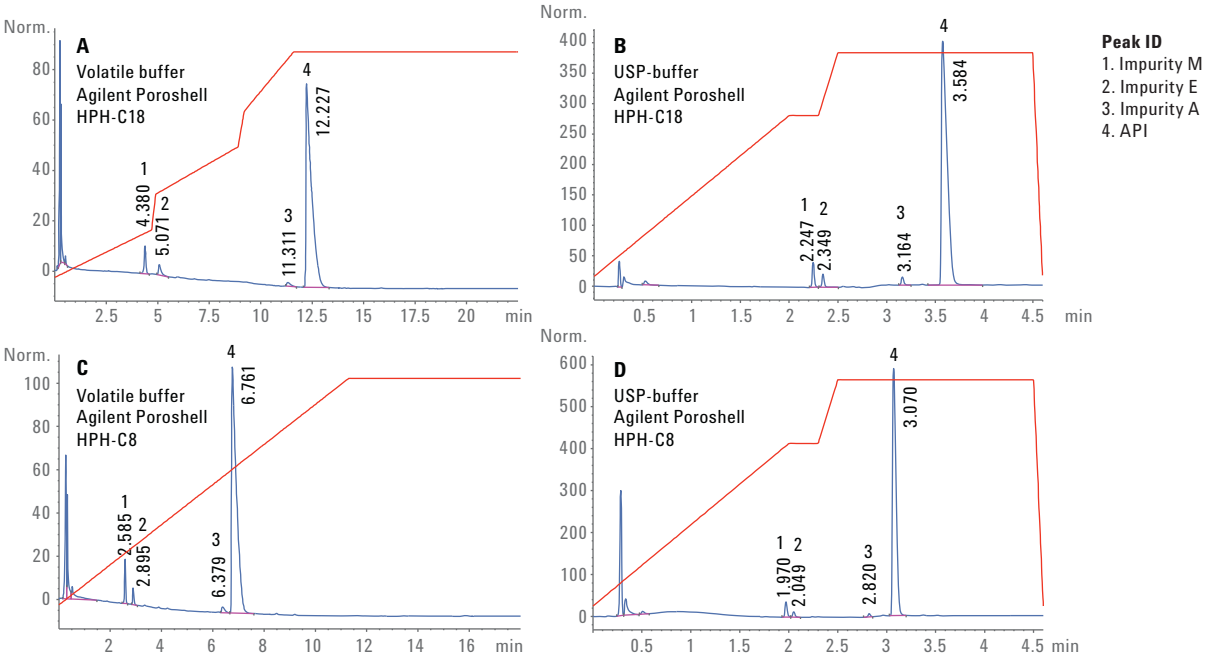

Figure 3. Results of the rapid optimization phase (A-D). The Agilent Poroshell HPH-C8 column with the USP mobile phase delivered the best results (D). The gradient was further optimized with ChromSword Algorithm. The USP tailing factor of the API and the resolution of the critical pair (Impurities M and E) was 1.8 and 2.0, as shown in chromatogram D.

Step 3: Robustness test

The ChromSword AutoRobust Software was used to perform robustness experiments as well as to create a design space. The method conditions obtained from the rapid screening experiments were imported into ChromSword AutoRobust Software. This software permits deliberate changes of parameters using different DoE based statistical design experiments. The parameters and range tested for robustness are listed in Table 3A. The design covered three different pH values (8.7, 8.9, and 9.1). In this work, a three-level full factorial design was used, in which three different parameters were changed simultaneously in all possible combinations (Figure 4). Each of these combinations was repeated for the three pH values mentioned above.

Table 3A. Method parameters and pH ranges varied in the robustness study. The break point refers to the gradient time settings where the time points of the different steps in the gradient were changed (± 0.2 minutes).

Agilent Poroshell HPH- C8 USP buffer with pH 8.7, 8.9, and 9.1	
Parameters	Range varied
Temperature	± 5 °C
Flow rate	± 0.2 mL/min
Break point*	± 0.2 minutes

Table 3B. Gradient with and without break point changes are shown, time points (bold) were changed ± 0.2 minutes.

Gradient without break point change		Gradient with break point change (± 0.2 min)		
Time	% B	Time (+0.2)	Time (–0.2)	% B
0	35	0	0	35
2.0	63	**2.2**	**1.8**	63
2.3	63	**2.5**	**2.1**	63
2.5	74	**2.7**	**2.3**	74
4.5	74	**4.7**	**4.3**	74

Full Design

T(°C)	Flow	Break Point, change time. min	Pattern
45	0.600	-0.200	---
45	0.600	0.000	--0
45	0.600	0.200	--+
45	0.800	-0.200	-0-
45	0.800	0.000	-00
45	0.800	0.200	-0+
45	1.000	-0.200	-+-
45	1.000	0.000	-+0
45	1.000	0.200	-++
50	0.600	-0.200	0--
50	0.600	0.000	0-0
50	0.600	0.200	0-+
50	0.800	-0.200	00-
50	0.800	0.000	000
50	0.800	0.200	00+
50	1.000	-0.200	0+-
50	1.000	0.000	0+0
50	1.000	0.200	0++
55	0.600	-0.200	+--
55	0.600	0.000	+-0
55	0.600	0.200	+-+
55	0.800	-0.200	+0-
55	0.800	0.000	+00
55	0.800	0.200	+0+
55	1.000	-0.200	++-
55	1.000	0.000	++0
55	1.000	0.200	+++

Design End

Figure 4. A screenshot from ChromSword Autorobust shows the three-level full factorial design created for finding the best method robustness. This design consists of individual experiments. Each experiment was repeated at three different pH (8.7, 8.9, and 9.1) values.

The data from robustness experiments were analyzed by ChromSword Report Viewer. The ChromSword Report Viewer analyzes resolution, retention time, area, and area % of all integrated peaks. It draws 2D contour plots that show resolution maps based on the interaction between different robustness parameters. For example, the results of the robustness test for pH 8.9 are shown in Figure 5. The resolution > 2 is indicated by a blue (dark blue or light blue) region. The yellow box is a range where the resolution at pH (from 8.7 to 9.1) values is consistently > 2. The final robust method used a flow rate of 1 mL/minute, and a TCC temperature of 53 °C. The gradient profiles before and after the robustness test are listed in Table 4. The gradient profile was modified by the software to guarantee a robust method.

Table 4. Gradient before and after robustness study. As a result of the robustness tests, the time scale was slightly changed to guarantee a robust method.

Gradient before robustness		Gradient after robustness	
Time	%B	Time	%B
0	35	0	35
2.0	63	2.1	63
2.3	63	2.4	63
2.5	74	2.6	74
4.5	74	4.6	74
4.6	35	4.7	35
6.0	35	6.0	35

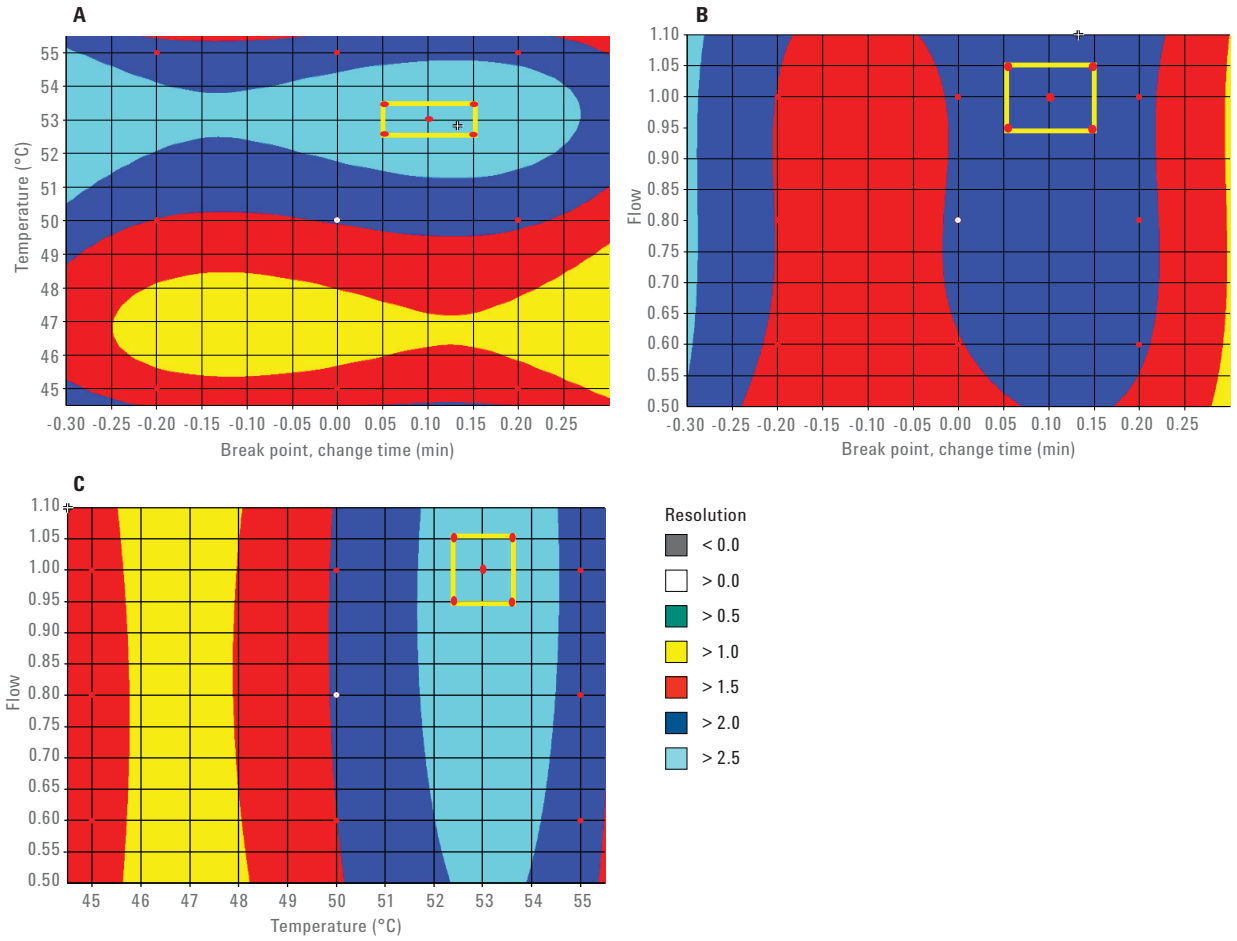

Figure 5. A 2D plot depicts the design space and robust regions (yellow boxes drawn manually). Each color reflects a region having resolution as shown in the caption. A) Impact of temperature and break point, B) flow rate and break point, and C) flow rate and temperature.

Step 4: Verification

The robust region was tested for reproducibility of the critical pair resolution and the relative retention times of the spiked impurities. The conditions at the center and edges of the box were tested. The results show that the resolution of the critical pair was maintained at > 2 under all conditions. The RRTs of all impurities were also consistent. In addition, the API peak purity was determined to be 99.9 % pure (Figure 6) by UV spectral data.

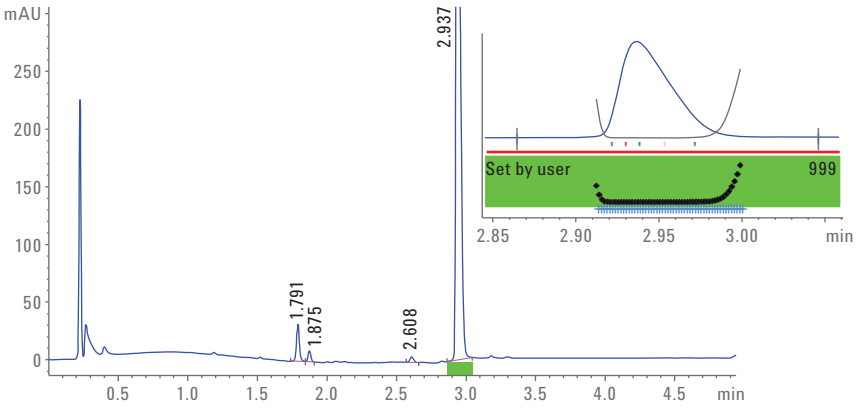

Figure 6. Peak purity plot: The peak purity of the API was determined to be 99.9 %, based on UV spectral data.

To prove the consistency of the method for a large number of runs, 250 injections applying the final method were done, and showed an excellent reproducibility for the target compound retention times (RSD < 0.05 %) and area precisions (RSD < 0.85 %) (Figure 7). This shows that Agilent Poroshell HPH-C8 columns are designed for high pH applications (Figure 7B). The backpressure observed when using Poroshell columns was approximately 150 bar. Poroshell columns fit perfectly with all HPLC and UHPLC systems.

Conclusions

Automated method development systems save time and costs. The Agilent 1200 Infinity Series Method Development System combined with the Agilent Method Scouting Wizard was used to screen different columns chemistries, mobile phases, flow rates, and temperature conditions. The ChromSword Auto 4.0 Software was used in the next step to develop an optimized analytical separation method for azithromycin from three spiked impurities (Imp M, E, and A) by automatic peak tracking. The runtime of the USP azithromycin method, based on a conventional C18 column, was decreased from 93 minutes to 6 minutes using an Agilent Poroshell HPH-C8, 2.7 µm column. The impact of the interaction of multiple factors on the robustness was evaluated, and a design space was generated in which the resolution was the CMA. The deliberate changes in the method conditions within the robust region were verified. The RRTs and resolution of impurities were reproducible in the newly developed method. A Poroshell HPH-C8 column was shown to match the chromatographic requirements. The reproducibility of the method was tested over 250 injections. A combination of intelligent software and flexible HPLC instrumentation allows rapid development of both robust HPLC and UHPLC methods.

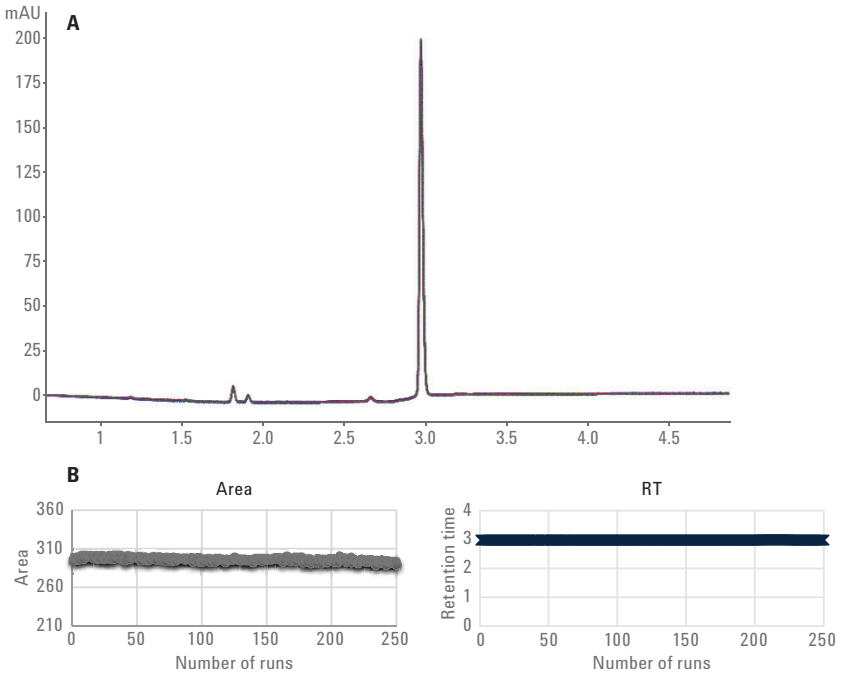

Figure 7. The reproducibility of an Agilent Poroshell HPH-C8 Column and method is shown. A) Overlay of 250 injections of the final method. B) RSD of API area and RT of 250 injections were 0.81 % and 0.04 %. Peak tailing and backpressure observed was 1.3 and 150 bar.

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