Application note | 000754

Chromatography

Automated UHPLC method development for mebendazole and related impurities, from method scouting to robustness testing

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Keywords

ChromSword Chromeleon Connect, Vanquish Flex, mebendazole, impurity analysis, UHPLC, automated method development, robustness test

Application benefits

- The Thermo Scientific™ Vanquish™ UHPLC Method Development system in combination with ChromSword Chromeleon Connect enables automated and unattended method development.
- The proposed method development workflow for the analysis of mebendazole and related impurities considerably reduces method development time and cost.
- The complete workflow includes method scouting, method optimization, and method robustness testing.
- The AutoRobust module of ChromSword Chromeleon Connect provides the robust region to afford assurance of quality of the final method via the design space between method parameters.

Goal

To develop a fast, robust UHPLC method for mebendazole and related impurities using an automated method development workflow.

Introduction

Mebendazole (methyl(5-benzoyl-1H-benzimidazol-2-yl) carbamate) belongs to a class of anthelmintic drugs and is widely used in the treatment of nematode infestations such as hookworm, roundworm, whipworm, pinworm, and threadworm. It functions by blocking tubulin formation within parasitic intestinal cells, which disrupts glucose uptake, digestion, and reproduction and eventually leads to parasite death.[1]

To ensure safety and efficacy of drugs like mebendazole, it is essential to monitor product- and process-related impurities throughout the drug lifecycle, from initial screening to quality control and quality assurance. According to the International Council for Harmonisation (ICH) guidelines, in quantitative tests for impurity content, the active pharmaceutical ingredient (API) and related impurities must be well resolved for accurate quantification.[2,3]

Developing LC methods that meet ICH requirements and which provide sufficient sample throughput demands significant effort even from experienced chromatographers.

HPLC method development typically consists of two steps: method scouting and method optimization. Specifically, key chromatographic parameters such as column, aqueous mobile phase composition and pH, and organic solvent type are first screened during method scouting. Once a viable set of starting conditions is identified, method optimization occurs in which chromatographic parameters (e.g., gradient profile, flow rate, and column temperature) are iteratively adjusted with the ultimate goal of providing a fit-for-purpose method. To ensure long-term method stability and facilitate future method transfer between instruments, robustness testing explores the effects of method parameter variation on the method variability. Robustness is generally evaluated during late-stage method development or early-stage method validation.[4]

Due to the labor- and resource-intensive nature of HPLC method development, process automation and acceleration are areas of constant interest. ChromSword Chromeleon Connect software combined with a Thermo Scientific™ Vanquish™ Flex UHPLC system enables automated method scouting, optimization, and robustness testing as well as enhanced data visualization and reporting. A flow scheme for the Vanquish system is shown in Figure 1.

In this application note, we present an automated method development workflow utilizing a Vanquish Flex UHPLC system combined with ChromSword Chromeleon Connect. A fast, robust UHPLC method was developed to quantify mebendazole and related impurities, which highlights the benefit of Vanquish Method Development systems for streamlining method development and minimizing manual user-instrument interaction.

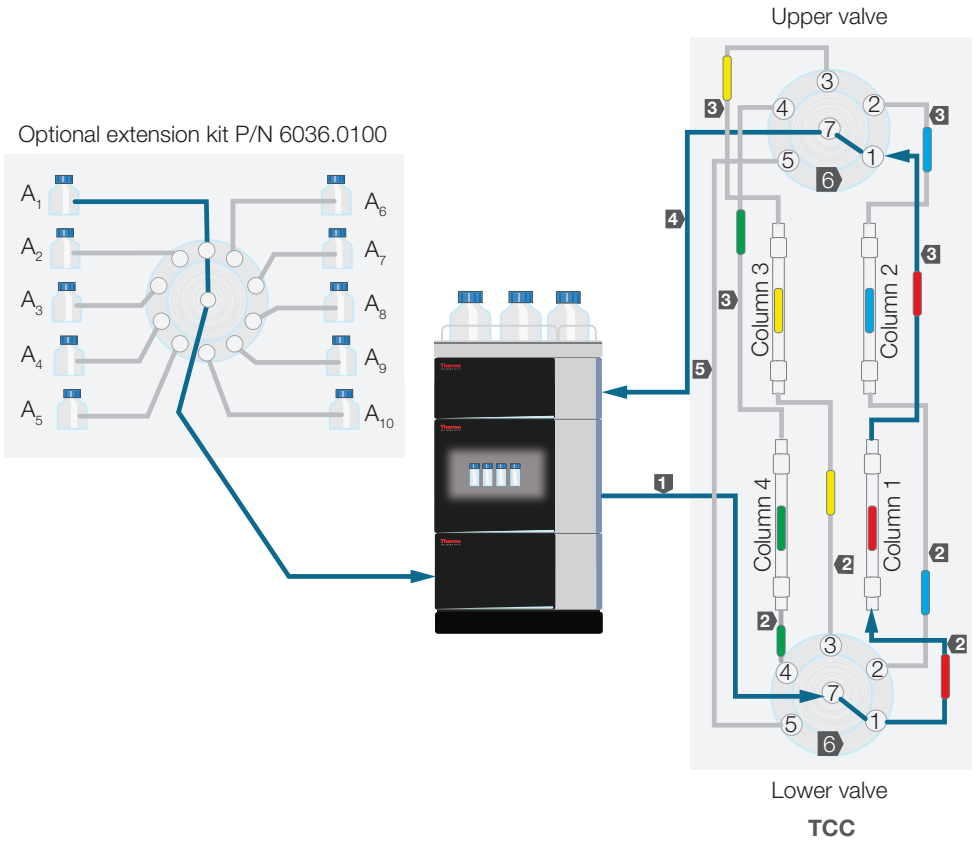

Figure 1. Flow scheme for the Vanquish Flex Quaternary UHPLC System with Automated Viper Method Scouting kit for Vanquish Systems

Experimental

Chemicals

Name	Part number
Deionized water, 18.2 MΩ·cm at 25 °C, Thermo Scientific™ Barnstead™ GenPure™ xCAD Plus Ultrapure Water Purification	50136149
Acetonitrile, Optima™ LC/MS grade, Fisher Chemical™	**A955**
Methanol, Optima™ LC/MS grade, Fisher Chemical™	**A456-212**
Formic acid, Optima™ LC/MS grade, Fisher Chemical™	**A117**
Ammonium acetate, Optima™ LC/MS grade, Fisher Chemical™	**A114-50**
Acetic acid, Optima™ LC/MS grade, Fisher Chemical™	**A113-50**
Ammonium bicarbonate, Fisher Bioreagents, Fisher Chemical™	10532775
N, N-Dimethylformamide, Fisher Chemical™, Acros Organics™, ACS reagent	10567942
EP reference standard: Mebendazole for system suitability CRS batch 1, catalogue code Y0000144[5]	EDQM
Ammonium hydroxide (NH4OH), >25%, purchased from a reputable vendor	

Sample handling

Name	Part number
Thermo Scientific™ Orion 3 Star™ pH Benchtop Meter	**13-644-928**
Fisher Scientific™ Fisherbrand™ Mini Vortex Mixer	**14-955-152**
Thermo Scientific™ Finpipette™ F1 Variable Volume Single-Channel Pipettes: 100-1000 µL, 10–100 µL, 1–10 µL	**4641100N** **4641070N** **4641030N**
Vials (amber, 2 mL), Fisher Scientific™	**15508760**
Snap Cap with Septum (Silicone/PTFE), Fisher Scientific™	**10547445**

Instrumentation

Module	Part number
Thermo Scientific™ Vanquish™ Quaternary Flex system consisting of:	
System Base Vanquish Horizon/Flex	VF-S01-A
Quaternary Pump F	VF-P20-A
Split Sampler FT	VF-A10-A
Column Compartment H	VH-C10-A-02
Diode Array Detector FG with standard flow cell, 13 µL (P/N 6083.0510)	VF-D11-A
Thermo Scientific™ Automated Viper™ Method Scouting kit for Vanquish Systems	6036.2807
Vanquish Switching Valve with 6-position/7-port	6036.2530 (2x)
Extension Kit for Automated Method Scouting, Vanquish Systems	6036.0100

Standard preparation

1 mg of the reference standard, consisting of the API mebendazole and related impurities A, B, C, D, E, F, and G, was dissolved in 1 mL dimethylformamide (DMF).

Aqueous mobile phase preparation

For the purpose of method scouting, four aqueous solutions were used as mobile phase A:

- 0.1% (v/v) formic acid in water
- 20 mM aqueous ammonium acetate buffer at pH 4.7
- 20 mM ammonium bicarbonate in water at pH 6.7
- 20 mM ammonium bicarbonate in water pH 7.7

The pH was adjusted with formic acid or ammonium hydroxide.

Table 1. Columns, aqueous mobile phases, organic solvents, and other method parameters used for method scouting

Column	• Thermo Scientific™ Acclaim™ Polar Advantage II (PA2) (100 × 2.1 mm, 2.2 µm) **P/N 068990** • Thermo Scientific™ Accucore™ Phenyl-Hexyl (100 × 2.1 mm, 2.6 µm) **P/N 17926-102130** • Thermo Scientific™ Accucore™ Phenyl-X (100 × 2.1 mm, 2.6 µm) **P/N 27926-102130** • Thermo Scientific™ Hypersil GOLD™ (100 × 2.1 mm, 1.9 µm) **P/N 25002-102130**
Aqueous mobile phases and pH	• 0.1% (v/v) formic acid in water • 20 mM aqueous ammonium acetate buffer, pH 4.7 • 20 mM ammonium bicarbonate in water, pH 6.7 • 20 mM ammonium bicarbonate in water at pH 7.7
Organic solvents	90/10 (v/v) acetonitrile/water, 90/10 (v/v) methanol/water
Gradient	**Time (min)** / **%B** 0 / 6 25 / 100
Flow rate	0.3 mL/min
Column temperature	40 °C (still air)
Sampler temperature	10 °C
Injection volume	2 µL
UV detector parameters	Detection at 250 nm 3D scan: 190–350 nm Data collection rate: 10 Hz

Chromatography software

ChromSword Chromeleon Connect complete (P/N 7200.0165), integrated with Thermo Scientific™ Chromeleon™ 7.3 Chromatography Data System (CDS), was used for data acquisition during method scouting, rapid and fine optimization, and robustness testing. The ReportViewer module of ChromSword Chromeleon Connect was used for data analysis and evaluation. The ChromSword Chromeleon Connect complete consists of ChromSwordAuto Chromeleon Connect and ChromSword AutoRobust Chromeleon Connect, which requires a fully licensed version of Chromeleon software.

Results and discussion

Vanquish Method Development systems with ChromSword Chromeleon Connect support the automation of method scouting, optimization, and robustness testing, resulting in significantly reduced development time and associated costs. Users can easily start each of the four modules of the software (Scout, Developer, AutoRobust, ReportViewer) under the 'Tools' menu button in Chromeleon CDS. In addition, all data is stored securely in a Chromeleon Data Vault, ensuring digital regulatory compliance. An overall workflow for automated method development using ChromSword Chromeleon Connect is shown in Figure 2. First, key chromatographic parameters such as columns, solvents, and mobile phase buffer composition and pH are screened (**step 1**). The gradient profile is then optimized with method parameters selected in step 1 (**step 2**). Robustness testing is performed to verify that the final optimized method remains unaffected by small variations in operating parameters, such as buffer pH, concentration of organic solvent B (%), and column temperature (**step 3**). Data obtained from steps 1 to 3 were analyzed and evaluated using the ReportViewer module of ChromSword Chromeleon Connect.

Step 1: Method scouting

- ChromSword Chromeleon Connect software module: Scout
- Screen for a promising combination of column, solvent and mobile phase pH

Step 2: Rapid and fine optimization

- ChromSword Chromeleon Connect software module: Developer
- Optimize the gradient profile with the column, mobile phase buffer, and organic solvent chosen in method scouting

Step 3: Method robustness test

- ChromSword Chromeleon Connect software module: AutoRobust
- Create design space (or robust region) by multivariate study

Figure 2. Workflow for automated method development using ChromSword Chromeleon Connect

Table 2. Columns, aqueous mobile phases, and organic solvents used in scouting conditions delivering number of peaks ≥8 and $R_{s,min}$ >1.5

Method number	Column	Aqueous mobile phase	Organic solvent	Number of peaks	$R_{s,min}$
1	Hypersil GOLD	0.1% Formic acid	90% ACN	9	2.8
2	Hypersil GOLD	Amm. acetate pH 4.7	90% ACN	10	1.7
3	Hypersil GOLD	Amm. bicarbonate pH 6.7	90% ACN	8	1.7
4	Hypersil GOLD	Amm. bicarbonate pH 7.7	90% ACN	8	1.7
5	Hypersil GOLD	Amm. acetate pH 4.7	90% MeOH	8	2.4
6	Acclaim PA2	0.1% Formic acid	90% MeOH	8	1.6
7	Accucore Phenyl Hexyl	Amm. bicarbonate pH 6.7	90% ACN	8	1.6
8	Accucore Phenyl Hexyl	Amm. bicarbonate pH 7.7	90% ACN	8	1.7

Step 1: Method scouting

Column and mobile phase screening are performed early in the method development process to identify promising candidate methods for further optimization. A total of four columns, four aqueous mobile phases, and two organic solvent types were chosen for screening (Table 1). From these, a sequence with all candidate column-aqueous mobile phase-organic solvent combinations was rapidly generated using the Scout module of ChromSword Chromeleon Connect, yielding 32 individual methods and 64 duplicate injections of mebendazole standard. A generic linear gradient profile of 6 to 100% organic solvent B was used for the scouting experiments, along with the other parameters such as flow rate of 0.3 mL/min, column temperature of 40 °C, UV wavelength of 250 nm.[6] In total, method scouting required 51 h of instrument operation and 1.5 h of analyst work time, including data evaluation and the choice of method parameters (i.e., column, aqueous mobile phase, and organic solvent).

Method scouting was done by evaluating performance criteria related to column selectivity and peak shape, namely total number of peaks, minimum peak resolution, peak asymmetry, and peak width. The eight conditions out of a total of 32 were first filtered by applying two criteria: the number of peaks greater than 8 and a minimum resolution of 1.5 (Table 2).

Three conditions out of the eight listed in Table 2—one Hypersil GOLD (method 2), one Acclaim PA2 (method 6), and one Accucore Phenyl-Hexyl (method 7)—were selected for further screening. The best method with the Hypersil GOLD column was found to be method 2. Methods 6 and 7 using the Accucore Phenyl-Hexyl columns showed similar separations, and method 7 using an aqueous mobile phase pH of 6.7 was selected (refer to Table 2 for the method number). Figure 3 compares separations of mebendazole and related impurities using the selected three methods (methods 2, 6, and 7). Table 3 summarizes the number of peaks, resolution, asymmetry, and peak width at half-height for the three separations shown in Figure 3. Method 2 using the Hypersil GOLD column yielded the largest number of peaks with R_s greater than 1.5 and was ultimately selected. Using this method, mebendazole and all related impurities peaks were baseline separated ($R_{s,min}$ >1.7). Overall, it required approximately 1.5 h of total analyst work time to identify the most promising method from the scouting runs.

Table 3. Number of peaks, resolution (R_s), asymmetry (Asym.), and peak width at half-height (Width) of mebendazole and related impurities for the three separations in Figure 3a-c

Peak #	Hypersil GOLD (Figure 3a)			Acclaim PA2 (Figure 3b)			Accucore Phenyl-Hexyl (Figure 3c)		
	Number of peaks: 10			Number of peaks: 8			Number of peaks: 8		
	R_s	Asym.	Width (min)	R_s	Asym.	Width (min)	R_s	Asym.	Width (min)
1		1.41	0.05		1.10	0.09		1.31	0.05
2	6.0	1.36	0.05	2.4	1.56	0.09	2.7	1.24	0.05
3	8.0	1.20	0.05	48.4	1.05	0.08	10.5	1.30	0.05
4	9.8	2.65	0.17	3.7	1.06	0.08	5.6	2.55	0.17
5	2.4	1.14	0.06	2.1	2.55	0.18	2.2	1.18	0.05
6	5.8	1.15	0.06	6.1	1.12	0.08	5.6	1.09	0.05
7	1.7	1.17	0.06	1.6	1.03	0.08	1.6	1.02	0.05
8	18.3	1.86	0.13	22.4	2.37	0.15	20.4	1.16	0.12
9	2.9	1.48	0.08						
10	27.6	1.21	0.07						

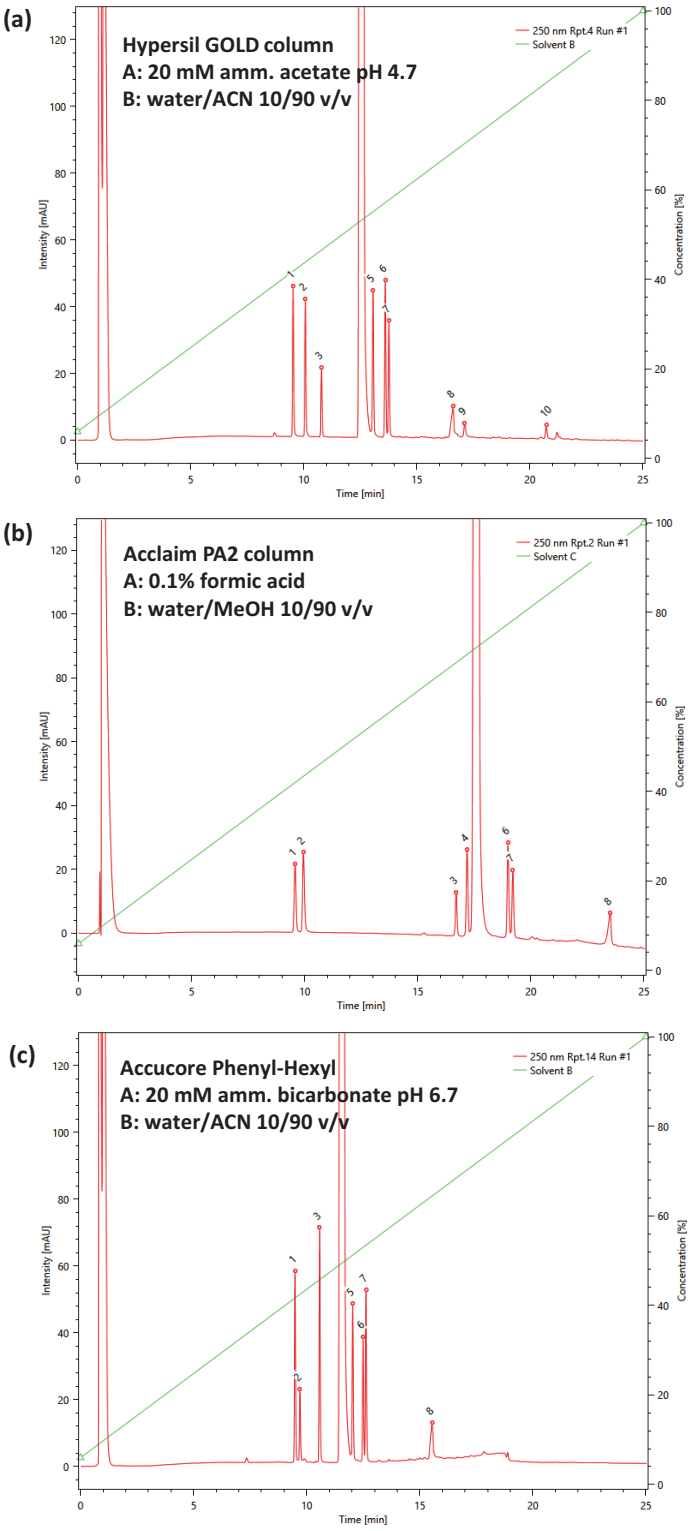

Figure 3. Three chromatograms selected from method scouting as candidates for further optimization. (a) Hypersil GOLD with 20 mM ammonium acetate pH 4.7 and 90% (v/v) acetonitrile in water, (b) Acclaim Polar Advantage II with 0.1% (v/v) formic acid in water and 90% (v/v) methanol in water, (c) Accucore Phenyl-Hexyl with 20 mM ammonium bicarbonate pH 6.7 and 90% (v/v) acetonitrile in water.

Step 2: Method optimization

The Developer module of ChromSword Chromeleon Connect supports the automation of different types of method optimization tasks, such as rapid optimization, rapid optimization for large molecules, separation of the largest peak, sample profiling for isocratic optimization, sample profiling for isocratic and gradient optimization, sample profiling for gradient optimization for large molecules, and retention optimization for the largest peak. Here, we performed two different optimization tasks: rapid optimization and sample profiling (or fine optimization) for isocratic and gradient optimization. The rapid optimization algorithm automatically performs three or four runs to find a good separation for target analytes. Based on the first run, rapid optimization is generally achieved in the second or subsequent runs. The sample profiling/fine optimization algorithm executes comprehensive separation optimization through detailed exploration of analyte retention. Multiple isocratic runs are first performed to build elution models for every component in a sample. Multiple linear and/or step gradient runs are then carried out to identify the best method. Similar to rapid optimization, promising conditions are calculated by the “intelligent” algorithm for subsequent runs based on the result of previous runs. Therefore, fine optimization is recommended especially for challenging analyses (e.g., comprehensive impurity profiling) that require full baseline separation of sample components. In this work, both rapid and fine optimization are described to help guide users with choosing the most suitable approach for their scope. If an adequate method is obtained from rapid optimization, fine optimization can be omitted. Here, fine optimization was performed to identify a superior method. Table 4 shows the chromatographic conditions used for method optimization including the above selected column, aqueous mobile phase pH, and organic solvent. It should be noted that the flow rate was increased to 0.8 mL/min both to reduce overall development time and improve final method throughput.

Table 4. Chromatographic conditions for method optimization

Column	Hypersil GOLD (100 × 2.1 mm, 1.9 µm)
Aqueous mobile phase	20 mM aqueous ammonium acetate buffer, pH 4.7
Organic solvent	90/10 (v/v) acetonitrile/water
Flow rate	0.8 mL/min
Column temperature	40 °C (still air)
Sampler temperature	10 °C
Injection volume	2 µL
UV detector parameters	Detection at 250 nm
	3D scan: 190–350 nm
	Data collection rate: 10 Hz

A total of four runs were performed for rapid optimization, taking around 1.5 h. The best method was selected with an analyst work time of around 15 min by evaluating parameters such as the number of peaks, the resolution of critical peak pair, and run time. Figure 4 shows the result of rapid optimization, where all related impurities were fully resolved from mebendazole within 13 minutes using a multi-step gradient profile. Mebendazole eluted at 7.2 min and peaks 1–3, 5–7, and 9 are due to mebendazole-related impurities. The resolution of the critical peak pair 6 and 7 was observed to be 3.15, and EP peak tailing factors for all mebendazole-related impurities were less than 1.2 (apart from peaks due to solvent matrix and API). The initial peaks eluting before 0.5 min, peaks 8 and 10, were seen as a result of sample matrix (data not shown).

For fine optimization, a total of 30 runs consisting of 18 isocratic and 22 gradient methods were performed to maximize resolution of mebendazole and related impurities. The sequence required 12.5 h of unattended instrument operation. The 22 gradient runs were then evaluated quickly (around 1 h of analyst work time) using method attributes such as number of peaks, resolution of critical peak pair (≥2), and run time. Figure 5 shows the best separation of mebendazole and related impurities, with the analysis time less than 11 min. Mebendazole eluted at 7.2 min and peaks 1-3, 5-7, and 9 are due to mebendazole-related impurities. The gradient provided by the fine optimization was linear. Linear gradients are preferred compared to a step-gradient when method robustness is a critical method requirement. In addition, methods that require full portability and are expected to have a long life-cycle spanning (i.e., final batch-release method for commercial and soon-to-be commercial drugs) benefit from

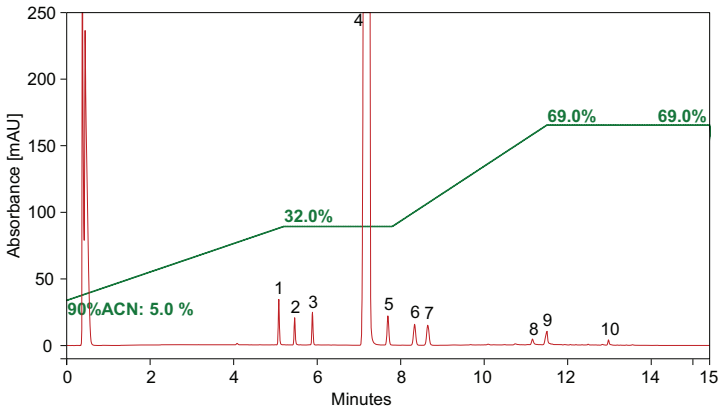

Figure 4. Chromatogram selected during rapid optimization, showing a good separation of mebendazole and related impurities with a multi-step gradient method. The Hypersil GOLD column with 20 mM ammonium acetate pH 4.7 and 90% (v/v) acetonitrile in water was used. Mebendazole eluted at 7.2 min (Peak 4). Peaks 1–3, 5–7, and 9 are due to mebendazole-related impurities and peaks 8 and 10 are due to solvent matrix. The green line represents the gradient.

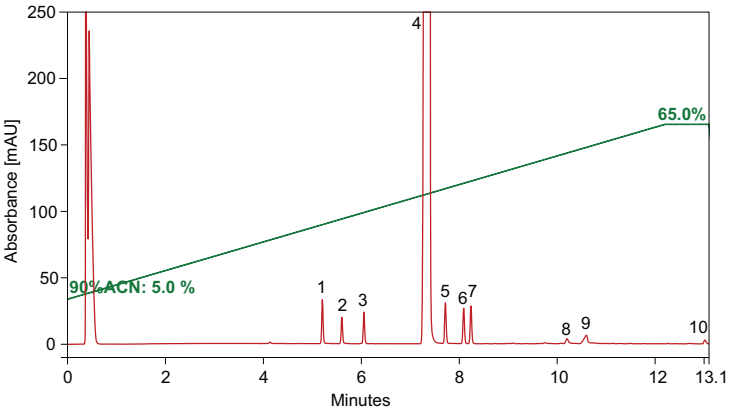

Figure 5. Chromatogram with best method from fine optimization, showing the best separation of mebendazole and related impurities with a simple linear gradient method. The Hypersil GOLD column with 20 mM ammonium acetate pH 4.7 and 90% (v/v) acetonitrile in water was used. Mebendazole eluted at 7.4 min (Peak 4). Peaks 1–3, 5–7, and 9 are due to mebendazole-related impurities and peaks 8 and 10 are due to solvent matrix. The green line represents the gradient.

the simpler elution method. Therefore, the linear gradient method was chosen as the final method for further method robustness testing. In this method, all impurities were fully resolved from the API, and the resolution of critical peaks 6 and 7 was 2.6. The EP peak tailing factors for the API and all related impurities are less than 1.62.

Step 3: Method robustness test

Method robustness testing was performed automatically using the AutoRobust module of ChromSword Chromeleon Connect to create a design space and determine robust operating ranges of method parameters. The software supports design of experiments (DoE) through one of three different design principles: one-by one (or one parameter at a time), Plackett-Burman, and full factorial design. The Plackett-Burman experimental design can determine the main effects with the smallest number of experiments but ignores parameter interactions. The full factorial design performs an experimental run for every parameter combination to explore both main effects and parameter interactions.[4] The Plackett-Burman design has been often used for HPLC robustness testing because it is more efficient and generally yields a good understanding of method robustness.[4] Thus, the Plackett-Burman design was used in this work. The final method selected above was input into the software along with the relevant method parameters: column temperature, mobile phase buffer pH and concentration of organic solvent B (%), and gradient slope (adjusted by break point time) (Table 5). The AutoRobust module automatically created the selected design plans and performed 17 test runs, taking around 5.6 h of instrument time. The test results were analyzed by the ReportViewer module of ChromSword Chromeleon Connect, taking around 1 h of analyst work time. The two-dimensional (2D) design spaces for the final method showed the resolution maps based on the method parameters tested. Figure 6 shows the 2D resolution map for the effect of column temperature and concentration of organic solvent at pH 4.7. The light green region indicates resolution greater than 1.8. The blue box represents the robust region with resolution >1.8 for temperature, % B, and ±0.1 pH units. With full pH ranges with ±0.2 pH units, the design space with other parameters could not be determined. Therefore, design space at pH 4.6 and pH 4.8 were extracted from the models built by the AutoRobust module. The experimental measurements in the 2D space are marked by circles and the final method a square (Figure 6a) in the blue box.

Table 5. Method parameters and buffer pH range varied for robustness testing. The break point time represents time points where the concentration of organic solvent B is changed in the gradient profile. Changes in break point time imply the change in gradient slope.

Parameters	Test ranges
Column temperature	± 5 °C
Concentration of organic solvent B (%)	± 5%
Break point time	± 0.25 min
Mobile phase buffer pH	± 0.2 pH units

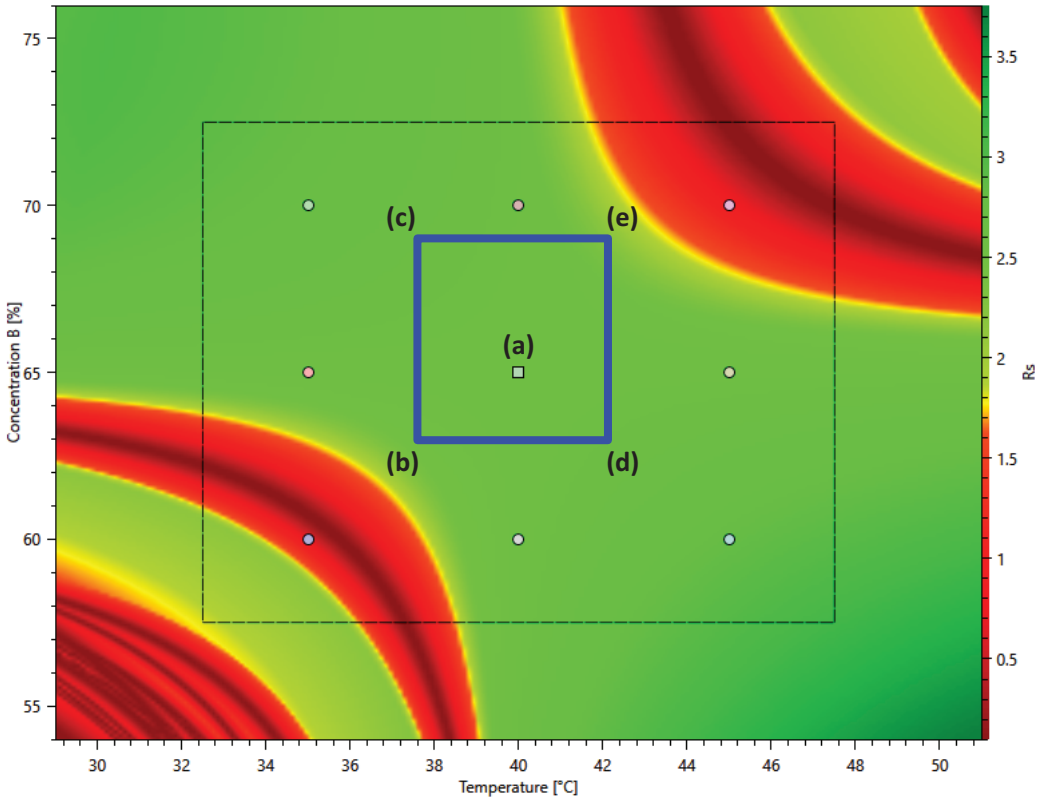

Figure 6. A 2D resolution map illustrating the effect of temperature and concentration of organic solvent B (%), with the design space (or robust region) indicated by a blue box. (a) Final method, and (b) to (e) – four corners of the blue box. Eight circles and a square (i.e., the final method) in the 2D space represent the experimental measurements. The region of the blue box was determined by identifying common robust green regions, obtained at pH 4.6, pH 4.7, and pH 4.8.

To demonstrate robust method operation throughout the design space (i.e., inside the blue square), separations were performed at four corners of the region (i.e. Figure 6b-e) and at the final method indicated by the square point (i.e. Figure 6a), as shown in Figure 7. This represents +2 %B and -1.5 % B, and ± 2°C. The critical peak pair resolution within the robust region remained above 2.4. Specifically, the values of critical peak pair resolution were found to be 2.53 (peaks 6 and 7 in Figure 7a), 2.43 (peaks 4 and 5 in Figure 7b), 2.44 (peaks 4 and 5 in Figure 7c), 2.59 (peaks 6 and 7 in Figure 7d), and 2.50 (peaks 6 and 7 in Figure 7e). In summary, a fast, robust method for mebendazole and its related impurities was developed within five days, including the preparation of mobile phases, sample, and setting up of the instrument. Table 6 breaks down the total amount of instrument and analyst work time (70. 6 h and 3.75 h, respectively) required during the entire process. It can be concluded that it would take much longer to develop a method manually and to test for robustness. For example, a considerable amount of analyst work time is required to analyze the data and then decide what the next step should be. This is only possible during working hours. A more challenging analysis would require a greater amount of time, analytical knowledge, and experience. The Vanquish Method Development system with ChromSword Chromeleon Connect software enabled comprehensive method scouting, optimization, and robustness testing in a short time with minimal analyst-instrument interaction.

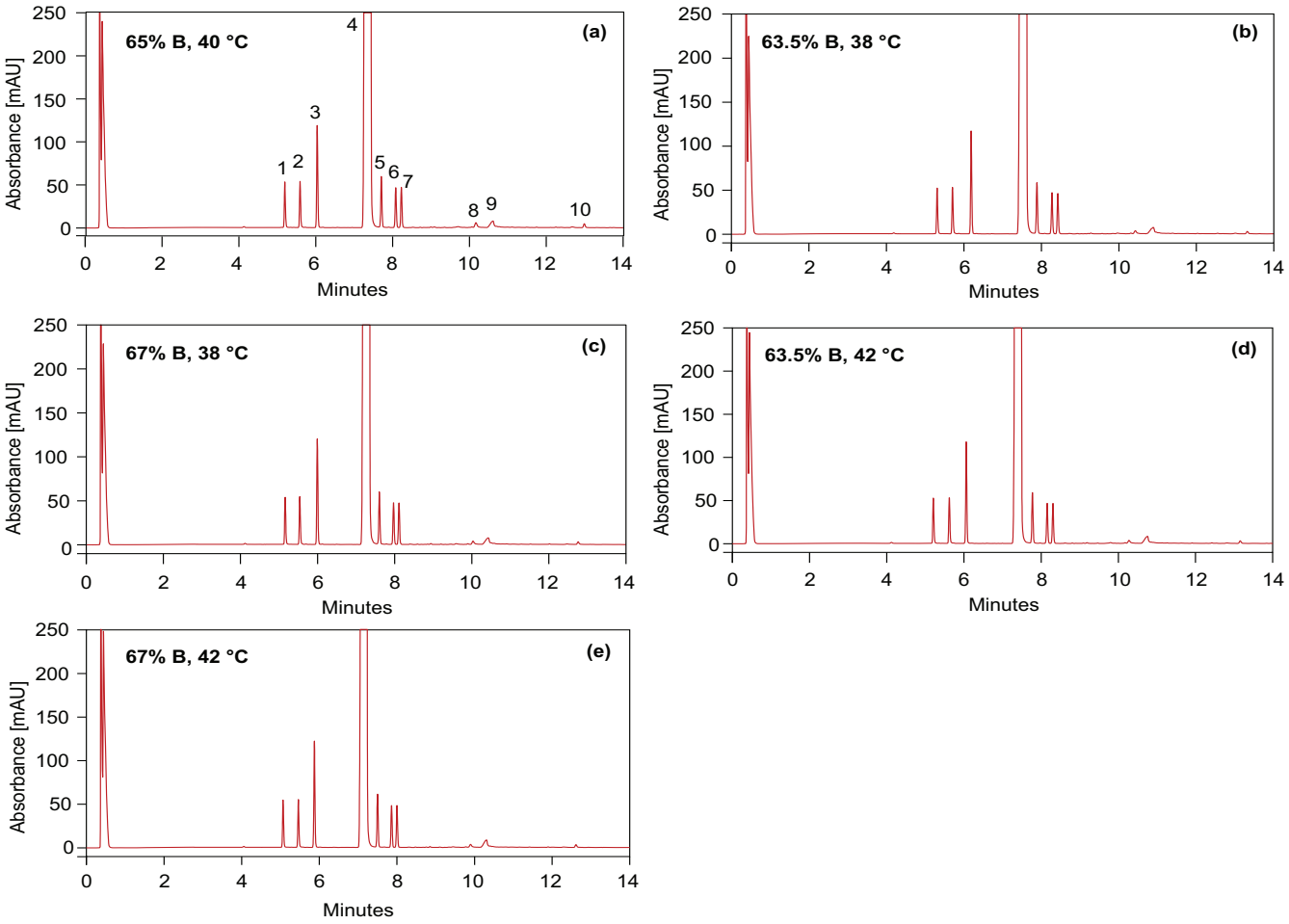

Figure 7. Chromatograms, measured at four edges in the 2D design space and a square point (i.e. final method), as displayed in Figure 6. The Hypersil GOLD column with 20 mM ammonium acetate pH 4.7 and 90% (v/v) acetonitrile in water was used. The chromatogram with the final method (Figure 7a) shows a good separation of mebendazole and related impurities with $R_{s,min}$ >2.5. The critical peak pair resolution within the robust region remained above 2.4.

Table 6. Summary of the time required during automated method development for mebendazole and related impurities, using ChromSword Chromeleon Connect software

	Step	Instrument time [h]	Analyst time [h]
1	Method scouting	51	1.5
2	Rapid optimization	1.5	0.25
3	Fine optimization	12.5	1
4	Robustness testing	5.6	1

Conclusion

- The Vanquish Method Development System, consisting of a combination of ChromSword Chromeleon Connect and the Vanquish Flex UHPLC system, enabled rapid, unattended development of a fast and robust method for the analysis of mebendazole and related impurities.
- The proposed workflow substantially reduced both development time and user intervention.
- By a systematic approach using method development software, the Thermo Scientific Hypersil GOLD column was rapidly selected as the most promising column for separating mebendazole and related impurities.

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