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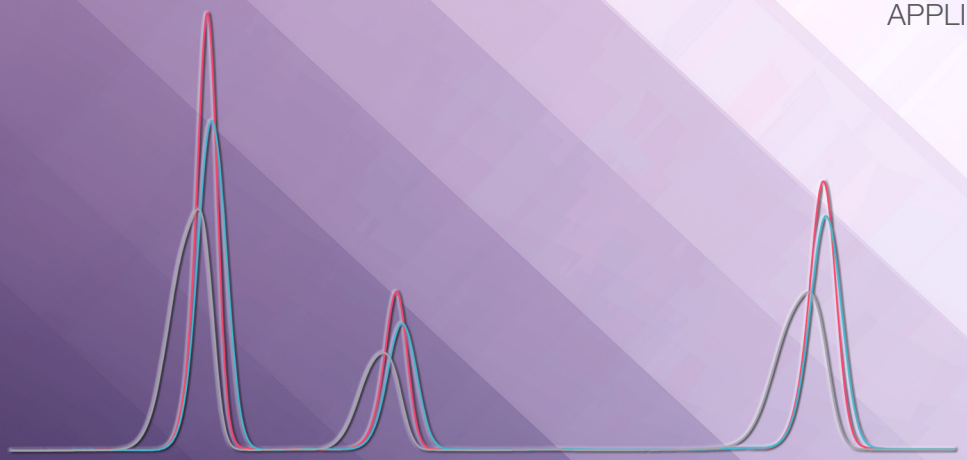

Improving peak results using a custom injection program to reduce solvent strength prior to sample injection

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Keywords

Mebendazole, system suitability, Vanquish, European Pharmacopoeia, custom injection program, sandwich injection, autosampler automation, automated liquid handling, peak results, resolution, asymmetry, peak width, efficiency

Application benefits

- Easy and flexible custom injection programs expand the possibilities of liquid handling in the Thermo Scientific™ Vanquish™ Split Sampler
- Custom injection provides the ability to improve certain system suitability parameters of an EP method without modifying chromatographic conditions

Goal

To demonstrate how custom injection programs affect chromatographic parameters such as asymmetry, resolution, and efficiency of a selected EP method.

Introduction

Pharmacopoeia monographs contain standardized analytical methods including acceptance criteria for the quality control of drug substances. Provided the regulations are strictly followed, the methods can be adopted without full validation.[1,2] To ensure adequate performance of the

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chromatographic system, a reference standard for the system suitability test (SST) needs to be injected and evaluated as indicated in the individual monograph. The general chapter of the European Pharmacopoeia (EP) (Chromatographic Separation Techniques 2.2.46) defines the permissible deviations from the EP method.[2] In particular, for gradient methods only a few adjustments are allowed, which can be critical to a method's success if poor chromatographic results are obtained with the original method.

Several monographs stipulate dissolution of the analytes in a solvent that is not the initial mobile phase composition of the method. In some cases, the recommended solvent has much higher elution strength. As a result, serious peak shape problems may occur. The reason for these anomalies lies in the fact that the analytes are transported to the column in a plug of strong solvent. Analyte molecules on the outside of the injection plug will mix with the mobile phase and will be retained. In contrast, molecules in the interior of the injection plug will not be retained and will migrate further into the column. These retention differences will cause peak distortion.[3]

With modern high-performance liquid chromatography (HPLC) instruments, system volumes are minimized so that a mismatch between sample solvent and initial gradient composition can result in insufficient mixing, which causes fronting or split peaks. A custom injection program that does not change the injection volume or solvent can be used to reduce the solvent strength of the solution to be injected. Thus, in this study, the EP method of mebendazole was not modified, except that a custom injection program was used to demonstrate the effect on system suitability criteria such as asymmetry, resolution, and efficiency.

Experimental

Chemicals

- Deionized water, 18.2 MΩ·cm resistivity or higher
- Fisher Scientific™ Acetonitrile, Optima™ LC/MS grade (P/N A955-212)
- Fisher Scientific™ Ammonium acetate, LC/MS grade (P/N A114-50)
- Fisher Scientific™ N,N-Dimethylformamide, Acros Organics™, ACS reagent (P/N 10567942)
- EP reference standard: Mebendazole for system suitability CRS batch 1[4] (P/N Catalog code Y0000144)

Equipment

- Vials (amber, 2 mL), Fisher Scientific (P/N 11545884)
- Snap Cap with Septum (Silicone/PTFE), Fisher Scientific (P/N 10547445)

Preparation of standards

The mebendazole SST reference standard contains the API and seven impurities (impurity A–G). According to the EP monograph, 5 mg of mebendazole SST reference material was weighed into a 5 mL volumetric flask and filled to the line with dimethylformamide (DMF).

Instrumentation

A Thermo Scientific™ Vanquish™ Core Quaternary HPLC system equipped with the following was used for the analysis:

- Thermo Scientific™ Vanquish™ System Base Vanquish (VC-S01)
- Thermo Scientific™ Vanquish™ Quaternary Pump C (VC-P20)
- Thermo Scientific™ Vanquish™ Sampler CT (VC-A12)
- Thermo Scientific™ Vanquish™ Column Compartment C (VC-C10-A-03)
- Thermo Scientific™ Vanquish™ Diode Array Detector CG (VC-D11) with standard flow cell, 13 µL (P/N 6083.0510)

Table 1. Chromatographic conditions

Column:	Thermo Scientific™ Hypersil GOLD™, 100 × 4.6 mm, 3 µm (P/N 25003-104630)	
Mobile phase:	A: 7.5 g/L ammonium acetate in water B: acetonitrile	
Flow rate:	1.2 mL/min	
Gradient:	*Time (min)*	*Mobile Phase B (%)*
	0	20
	15.0	30
	20.0	90
	25.0	90
	25.1	20
	30.0	20
Mixer volume:	350 µL + 50 µL	
Column temp.:	40°C with passive pre-heater	
Sampler temp.:	4 °C	
UV wavelength:	250 nm	
UV data collection rate:	10 Hz	
UV response time:	0.5 s	
Injection volume:	10 µL	

Custom injection program

In the present study, the custom injection program is used to reduce the solvent strength of the sample prior to injection. Various custom injection programs were tested, and two were selected as being the most effective. Details about the individual programs can be found in Tables 2 and 3.

If the custom injection mode is selected, predefined commands can be chosen from a drop-down menu when creating a new instrument method with the Thermo Scientific™ Chromeleon™ Chromatography Data System wizard. This allows quick and flexible programming of several pre-injection liquid handling steps. These commands are named as UDP (user-defined program) in Chromeleon software to distinguish from standard commands.

Data processing and software

The data acquisition and processing were done with Thermo Scientific™ Chromeleon™ 7.3 Chromatography Data System software.

Table 2. Custom injection A

Custom Injection A		
Command	**Parameter**	**Description**
UDP_PrepareLiquidHandling	Volume = 100 µL	Define the total liquid handling volume
UDP_Draw	Position = R:A2 Volume 10 µL Speed = 5 µL/s Needle Height = 2000	Draw 10 µL sample from the specified vial position (e.g., R:A2) with a draw speed of 5 µL/s, at needle height of 2000 µm
UDP_Draw	Position = R:A4 Volume = 90 µL Draw Speed = 20 µL/s Needle Height = 2000	Draw 90 µL initial mobile phase (20:80 acetonitrile:ammonium acetate buffer) from the specified vial position (e.g., R:A4) with a draw speed of 20 µL/s, at needle height of 2000 µm
UDP_Wait	10 s	Move needle to injection port and wait 10 s before injection
UDP_PrepareInject		End of liquid handling

Table 3. Custom injection B

Custom Injection B		
Command	**Parameter**	**Description**
UDP_PrepareLiquidHandling	Volume = 100 µL	Define the total liquid handling volume
UDP_Draw	Position = R:A3 Volume = 45 µL Draw Speed = 5 µL/s Needle Height = 2000	Draw 45 µL mobile phase A (7.5 g/L ammonium acetate) from the specified vial position (e.g., R:A3) with a draw speed of 5 µL/s, at needle height of 2000 µm
UDP_Draw	Position = R:A2 Volume 10 µL Speed = 5 µL/s Needle Height = 2000	Draw 10 µL sample from the specified vial position (e.g., R:A2) with a draw speed of 5 µL/s, at needle height of 2000 µm
UDP_Draw	Position = R:A3 Volume = 45 µL, Draw Speed = 20 µL/s Needle Height = 2000	Draw 45 µL mobile phase A (7.5 g/L ammonium acetate) from the specified vial position (e.g., R:A3) with a draw speed of 20 µL/s, at needle height of 2000 µm
UDP_Wait	10 s	Move needle to injection port and wait 10 s before injection
UDP_PrepareInject		End of liquid handling

Results and discussion

System suitability (SST)

The mebendazole EP monograph requires that the reference standard for system suitability is dissolved and injected in DMF, probably to dissolve all seven impurities contained in the standard.[5] Literature data on the eluotropic strength of common HPLC solvents on stationary C18 phases suggest that DMF is a stronger solvent than acetonitrile.[6]

As can be seen in Figure 1, injection of the mebendazole SST standard into the initial mobile phase condition of the gradient method (20:80 acetonitrile:ammonium acetate buffer) causes peak distortion for the early eluting impurity peaks A–C. These peaks are detected with shoulders or show fronting, while the later eluting impurities D–G are less affected and elute as more symmetrical peaks. Peak distortion is typically observed if the injection solvent is stronger than the initial mobile phase composition, as is likely the case for impurities A–C. Another cause of peak fronting is overloading the column, as can be inferred for mebendazole, which is contained in the standard at a much higher concentration than the impurities.

In the following, impurities A–C are discussed in more detail to demonstrate the effect of using custom injection programs on various system suitability criteria.

In EP 9.2, chapter 2.2.46, section system suitability, the parameters usually employed for a system suitability check are listed:[1]

- Efficiency
- Retention factor
- Resolution
- Symmetry factor

A symmetry factor of 0.8–1.5 needs to be achieved. The asymmetry factors of the early eluting impurities A–C with normal injection are very close to the lower end of this requirement. Impurities A and C are even slightly outside at 0.76 and 0.77, respectively, as shown in Table 4.

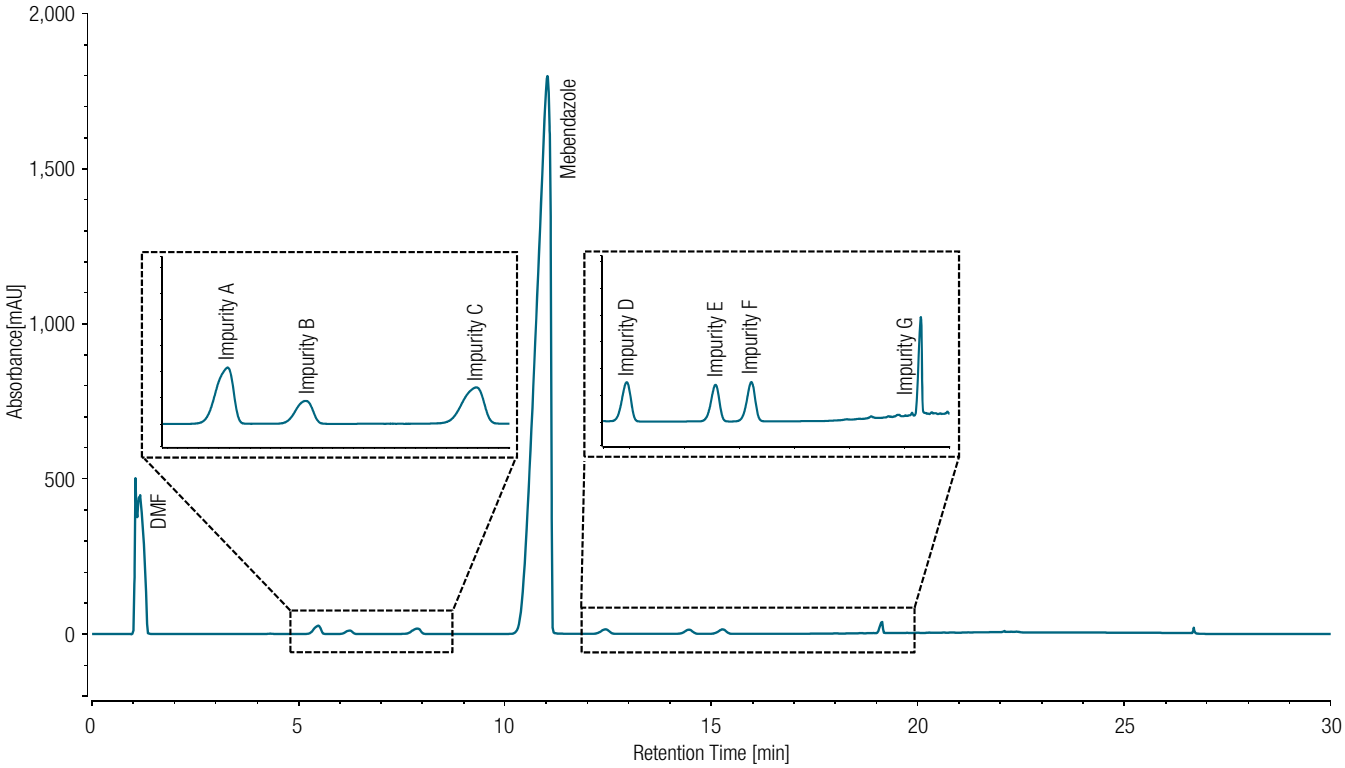

Figure 1. Chromatogram of a normal injection with enlargements of early eluting impurities A–C and later eluting impurities D–G. Sample: 1 mg/mL mebendazole SST in DMF; separation on a Hypersil GOLD column with 100 × 4.6 mm and 3 µm particle size; injection volume: 10 µL; for full details on the chromatographic method refer to Table 1.

Table 4. Asymmetry factors (EP) obtained for normal injection, custom injection A, and custom injection B. Averaged values of five consecutive injections; peak asymmetry improves with custom injections.

Compound	Asymmetry (EP)		
	Normal injection	Custom injection A	Custom injection B
Impurity A	0.76	0.98	0.89
Impurity B	0.83	0.95	0.87
Impurity C	0.77	0.93	0.89

Without adjusting the chromatographic conditions of the EP method, custom injection programs can be used to reduce the solvent strength in the sample prior to injection. Figure 2 illustrates the sample loop schematically. With the normal injection (I), after switching the autosampler valve to bypass, the loop is filled with initial mobile phase (20:80 acetonitrile:7.5 g/L ammonium acetate in water) from the column equilibration step and the sample is drawn. With the custom injection A program (II), first the sample is drawn and then the

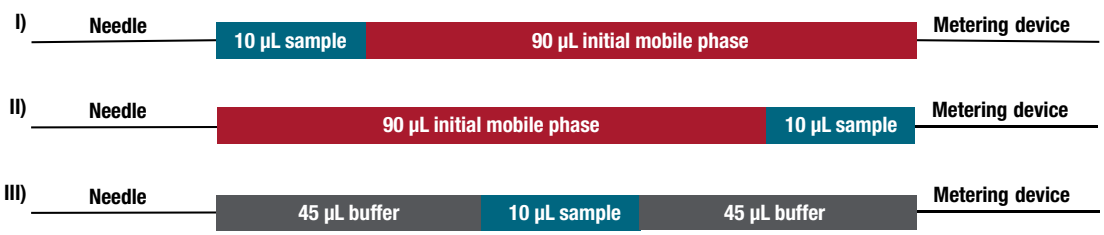

Figure 2. Schematic drawing of the sample loop on the three different injection methods. I = normal injection, II = custom injection A, III = custom injection B; sample = 1 mg/mL mebendazole SST standard in DMF; initial mobile phase = 20:80 acetonitrile:7.5 g/L ammonium acetate in water; buffer = 7.5 g/L ammonium acetate in water (refer to Tables 1–3 for more details)

remaining loop volume is filled with the initial mobile phase. With the custom injection B program (III), the solvent strength is reduced even more by using a sandwich injection with the mobile phase A (7.5 g/L ammonium acetate in water). The sample is drawn between two aqueous buffer regions, which results in a lower solvent strength than the initial mobile phase and should result in a focusing effect of the analytes on the column head. Figure 3 demonstrates the effect of both customized injection programs on the peak shapes of impurities A–C compared to the normal injection. Both custom injection programs result in peaks with asymmetry values between 0.87 and 0.98 (Table 4). The closer the value is to 1, the more symmetric the peak is. Asymmetry values below 1 indicate a fronting peak shape, while values above 1 indicating peak tailing.

Overall, the peak area remains the same, while the peak height increases with an increase of the aqueous buffer content used in the custom injections (data not shown). This observation is in accordance with literature data published on the same approach but using different injection solvents instead of custom injection programs.[3]

The SST criteria of resolution and efficiency are not part of this particular EP monograph, but it can be demonstrated that custom injection programs improve both, compared to the normal injection (Figure 4). A better peak height, and thus narrower peaks, have a direct effect on resolution. The plate number increased up to 2 times with custom injection A and by a factor of 3–4 for custom injection B.

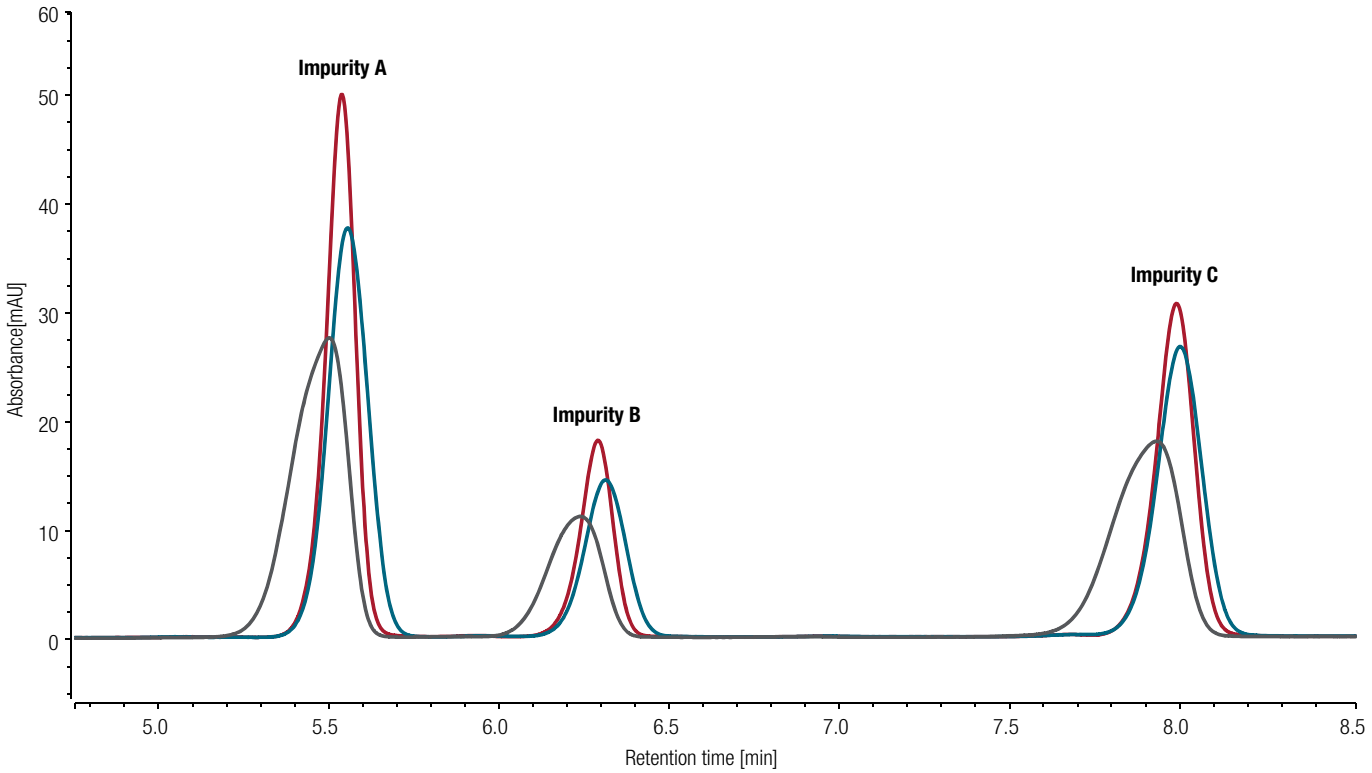

Figure 3. Overlaid chromatograms of normal injection (gray), custom injection A (blue), and custom injection B (red) of the impurities A–C. Sample: 1 mg/mL mebendazole SST in DMF; separation on a Hypersil GOLD column with 100 x 4.6 mm and 3 µm particle size; injection volume: 10 µL. For full details on the chromatographic method refer to Table 1; for full details on the custom injection programs refer to Table 2 and Table 3.

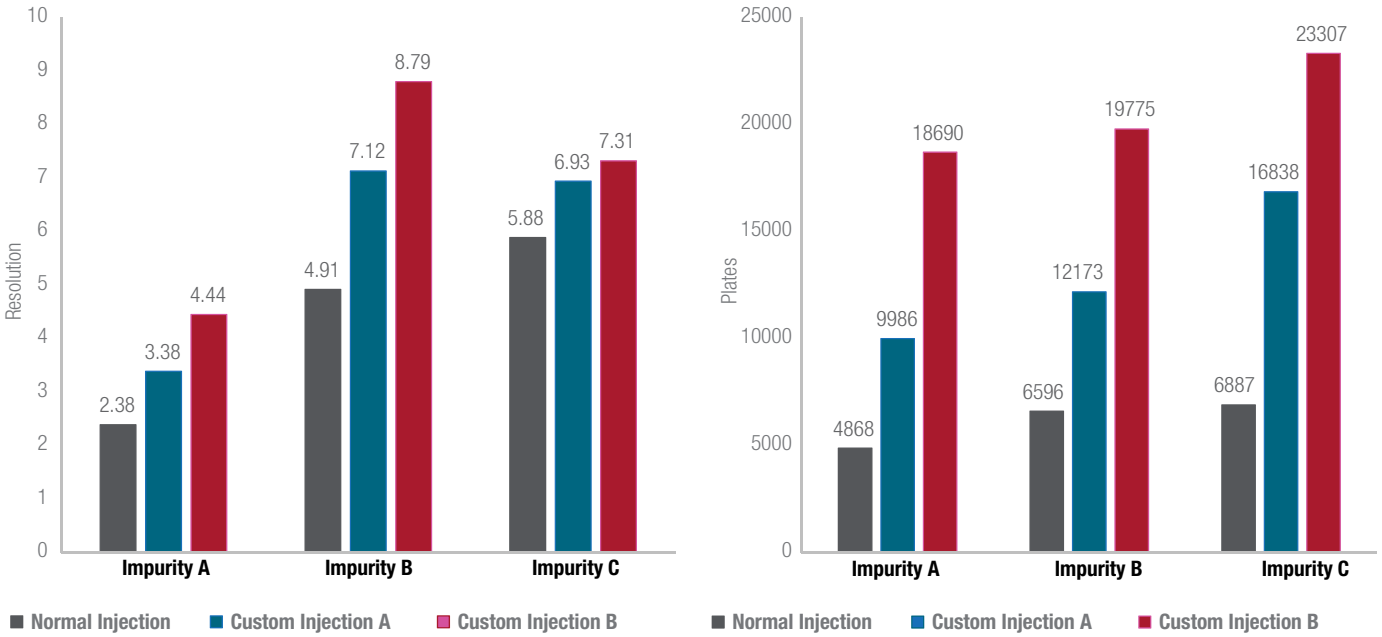

Figure 4. Peak resolution and plate number obtained for normal injection, custom injection A, and custom injection B. Resolution improved with custom injections; highest resolution was achieved for custom injection B; plate number increased with custom injections.

Instead of resolution, the EP monograph requires a peak-to-valley ratio for impurity D (relative to the API mebendazole) of at least 4 for system suitability.[5] This parameter is only used if two peak pairs are not fully baseline resolved. In the present study, baseline resolution was easily achieved with the Vanquish Core instrument and the Hypersil GOLD column. The peak-to-valley ratio is 137 for the normal injection mode, which is already far above the required limit.

Method performance

Method performance data on retention time and area precision were evaluated for five consecutive injections for each injection method (normal injection, custom injection A, custom injection B). The relative standard deviation (%RSD) of retention time (RT) and area are summarized in Table 5. The obtained %RSD RT values are extremely low (<0.1%) regardless of which method was applied.

In general, the %RSD area values are below 0.5%, or even lower (<0.2%) for the majority of impurities. Custom injection B shows slightly higher values in the range of 0.3%.

Table 5. Chromatographic results (%RSD RT and %RSD area) obtained for the different injection methods (n= 5). Excellent %RSD RT precision with <0.1% could be achieved; good area precision is obtained for impurities A–F; impurity G shows higher values up to 3.3%.

Compound	Normal injection		Custom injection A		Custom injection B	
	% RSD RT	%RSD area	%RSD RT	%RSD area	%RSD RT	%RSD area
Impurity A	0.06	0.06	0.09	0.06	0.08	0.26
Impurity B	0.03	0.10	0.08	0.09	0.06	0.27
Impurity C	0.06	0.14	0.08	0.06	0.05	0.23
Impurity D	0.02	0.17	0.04	0.10	0.02	0.34
Impurity E	0.02	0.47	0.04	0.12	0.02	0.23
Impurity F	0.01	0.03	0.04	0.09	0.02	0.25
Impurity G	0.02	3.31	0.01	1.90	0.01	3.03

Only impurity G shows much higher %RSDs for peak area for all three injection methods up to 3.3%. Impurity G is structurally very different from the API and the other impurities and is therefore much more hydrophobic. This causes impurity G to elute during the steeper gradient region. As the substitution reaction product of impurity A and mebendazole (Figure 5), it is multiply positively charged with the mobile phase indicated for the EP method. Complex ionization behavior likely contributes to a worse peak shape and non-reproducible results for this basic molecule. To accurately measure impurity G, it may be necessary to adjust the pH of the mobile phase in order to reduce the degree of protonation.

Note, adjusting the pH is not permitted under 2.2.46 of the EP general chapter for gradient elution.

Custom injection programs are available for the entire Vanquish platform line (Horizon, Flex, and Core) and can be extremely useful in many applications. As demonstrated in this study, custom injections can be used to improve peak results to achieve system suitability criteria for critical applications. They can also be used to automate sample preparation steps, such as derivatization, generation of calibration standards for quantitative analysis, or addition of internal standards to the sample prior to injection.

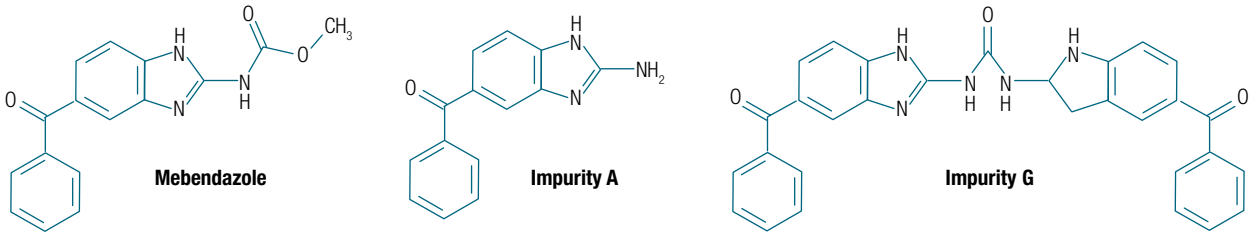

Figure 5. Chemical structures of mebendazole and its related impurities A and G

Conclusion

Custom injection programs provide the following benefits:

- Improved asymmetry factors, particularly for early eluting impurities A–C contained in the mebendazole system suitability standard, by reducing the solvent strength prior to injection.
- Increased column efficiency and resolution by producing narrower peaks.
- A practical and easy solution in cases where modifications of chromatographic conditions are not permitted.

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