Development and Comparison of Quantitative Methods Using Orthogonal Chromatographic Techniques for the Analysis of Potential Mutagenic Impurities

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There are many steps during the manufacturing process of an active pharmaceutical ingredient (API) where impurities can be introduced, whether as reagents, byproducts, intermediates, etc [1]. Some of these impurities may be mutagenic, or have the potential to interact with DNA and ultimately cause carcinogenicity. Methodologies associated with monitoring API purity levels are often HPLC-UV based [2], which frequently do not provide the sensitivity levels needed to detect potentially mutagenic impurities (PMIs) at the levels required by regulatory agencies [3]. For example, ondansetron is a pharmaceutical used in the prevention of nausea and vomiting and may contain one potential mutagenic impurity, 2-methylimidazole, as well as a second impurity very closely related in structure, imidazole.

Similar to 2-methylimidazole and imidazole, many mutagenic impurities are small, highly polar compounds that are poorly retained under typical reversed phase liquid chromatography (RPLC) conditions. Alternate forms of chromatography, such as hydrophilic interaction chromatography (HILIC), or the use of ion-pairing reagents can be employed, but these often result in tedious method development or non-MS friendly mobile phases. Supercritical fluid chromatography (SFC) is known to be orthogonal to RPLC, and employs reagents which are suitable for MS detection. In this study, methods for the analysis of ondansetron and five organic impurities were developed using both liquid and supercritical fluid chromatographic methods. Both chromatographic techniques generated high sensitivity methods that met the required limits of detection and both techniques showed good accuracy and reproducibility.

Experimental:

Ammonium acetate (≥99%), ammonium formate (≥99.99%), formic acid (~98%), acetic acid (≥99.7%) and ammonium hydroxide (28.0-30.0%) were purchased from Sigma-Aldrich (St Louis, MO). Acetonitrile and methanol were Optima Grade and purchased from Fisher Chemical (Fair Lawn, NJ). Ondansetron hydrochloride and ondansetron impurities A, C, and D were purchased from the United States Pharmacopeia (Frederick, MD). Impurity E (imidazole) and impurity F (2-methylimidazole) were purchased from Sigma-Aldrich.

All Ultra High Performance Liquid Chromatography (UHPLC) studies were conducted on a Waters ACQUITY I-Class system and all SFC studies were conducted on a Waters ACQUITY Ultra Performance Convergence Chromatography (UPC^2) system which utilises compressed or supercritical CO_2. Both systems were connected to a Waters Xevo TQ-S micro tandem quadrupole mass spectrometer. MS source conditions were optimised separately for UHPLC and SFC experiments.

Calibrator and quality control (QC) samples for impurities A, C, D, E and F were prepared in diluent containing 0.125 mg/mL API (ondansetron) in methanol (SFC/HILIC) or water (RPLC) at the following concentrations: calibrators at 15, 20, 25, 50, 75, 100, 125, 200, 300, and 500 ng/mL and QCs at 17.5, 95, and 350 ng/mL. This is equivalent to impurity A, C, D, E, and F concentrations of 120, 160, 200, 400, 600, 800, 1000, 1600, 2400, and 4000 ppm for the calibrators and 140, 760, and 2800 ppm for the QCs, where ppm is in reference to the API.

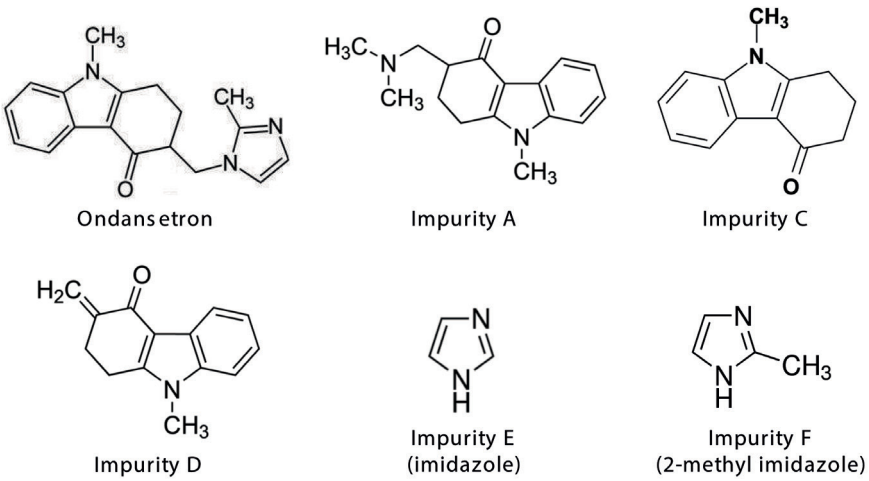

Figure 1. Structures of ondansetron and related impurities A, C-F.

Results and Discussion:

The USP monograph for ondansetron hydrochloride [2], which is an HPLC-UV method, includes identification and quantification of 5 related organic impurities (Figure 1), including two process impurities [4] E and F, which are imidazole and 2-methylimidazole respectively. Studies done by the National Toxicology Program (NTP) on 2-methylimidazole show exposure-related increases of micronucleated normochromatic erythrocytes in peripheral blood samples of male and female mice, which is an indicator of chromosomal damage. Additionally, the amount of damage increases with increasing duration of exposure [5]. In light of this information, 2-methylimidazole can be considered to be a potentially mutagenic impurity, and due to its closely related structure for this example imidazole will also be considered a PMI.

Due to the dangers posed by PMIs, the maximum acceptable daily intake for PMIs is set to specific levels according to ICH M7: Assessment and Control of DNA Reactive (Mutagenic) Impurities in Pharmaceuticals to Limit Potential Carcinogenic Risk guidelines [3]. Depending on the treatment it is prescribed for, ondansetron may be taken for more than 30 non-consecutive days, thus the allowable PMI daily intake limit is 20 µg/day per ICH M7. The highest possible daily dosing for ondansetron is 48 mg/day [6], which means that PMIs are allowable at 417 ppm with reference to the API, which is five times lower than the USP monograph limit of 0.2% (2000 ppm). For this reason, the use of a higher sensitivity detector, specifically a tandem quadrupole MS, was employed to provide detection levels much lower than can be achieved by UV. To mimic a true test sample, all calibrators and QCs were prepared in diluent containing 0.125 mg/mL of the ondansetron API. When using MS detection, it is important to separate all impurity peaks from the main API peak to avoid any potential matrix effects, i.e. signal suppression or enhancement, due to the presence of API at such a high concentration which may affect the ionisation process within the MS source. Additionally, the original USP monograph utilises ion-pairing reagents to facilitate retention of the polar impurities E & F, however, the use of ion-pairing reagents with MS is known to cause signal suppression so they are often avoided. Therefore, a new higher sensitivity methodology was developed for the detection of 5 organic impurities of ondansetron utilising MS detection.

Liquid Chromatography Method Development:

For the reversed phase method development, numerous variables were evaluated to facilitate retention of the polar impurities and overall separation of the API and all 5 impurities. Multiple column chemistries were examined (including CSH fluoro-phenyl, BEH amide, HSS T3 and HSS cyano chemistries), along with various mobile phases over a range of pH. The column chemistries were chosen because of their ability to increase retention for polar compounds; however, under generic RPLC conditions of formic acid in water/acetonitrile, both polar impurities E & F were unretained under all conditions tested. Figure 2a shows an example chromatogram generated on an HSS T3 column. The same general trend was seen for all column and mobile phase combinations tested. For these reasons, it was determined that an alternate approach was required, specifically HILIC.

HILIC chromatography is a technique that uses hydrophilic stationary phases with typical reversed phase mobile phases, with the notable difference that the aqueous mobile phase is the strong solvent which facilitates elution. The separation mode is based on a combination of partitioning, ion-exchange and hydrogen bonding with a layer of water on the surface of the particles. Common method development strategies for HILIC chromatography include screening different columns and changing the buffer and additive concentration of the mobile phases. For this example, an existing method for 2-methylimidazole using a CORTECS HILIC column along with ammonium formate in the mobile phase was evaluated [7]. Under the prescribed HILIC conditions, impurities E & F were well retained, however, impurities C & D were not retained (data not shown). An alternate mobile phase combination was examined, specifically the use of ammonium acetate and acetic acid as the buffering system. Under these conditions, there was still no retention for impurities C & D, however, there was better separation of the API from impurities A, E and F (Figure 2b). The same gradient profile was used for both mobile phase combinations with initial LC conditions at 98% organic solvent containing either formic or acetic acid at 0.1% by volume. Because acetic acid is a weaker acid, the pH of the starting conditions and the subsequent gradient will be slightly higher for the acetic acid mobile phase combination. The use of the higher pH mobile phases resulted in increased retention of all compounds and an alternate selectivity which ultimately provided a better separation.

The final HILIC method developed was used for the quantification of impurities A, E, and F (Figure 2b). It utilised a CORTECS UPLC HILIC column (2.1 x 100 mm, 1.6 µm) maintained at 30°C. The mobile phases consisted of 0.1% (v:v) acetic acid in acetonitrile and 10mM ammonium acetate at pH 4. The flow rate was 0.6 mL/min and used an injection volume of 2 µL.

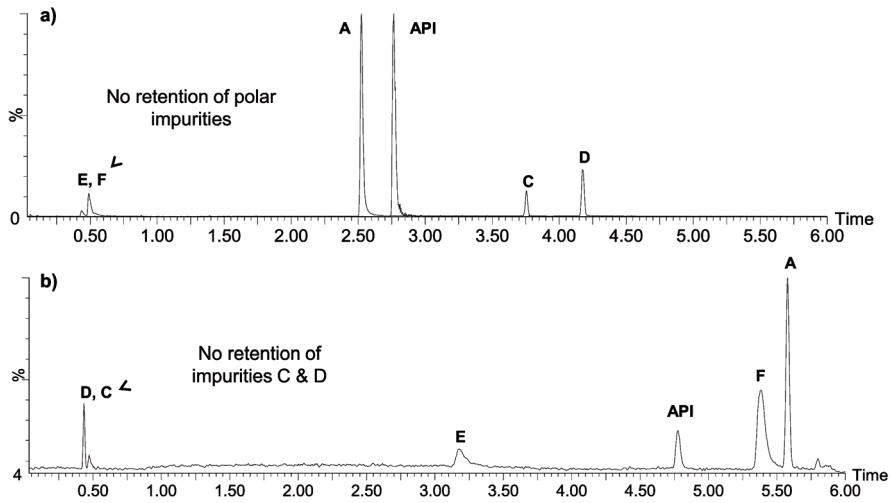

Figure 2. a) Chromatogram showing ondansetron (API) and related impurities A, C, D, E and F separated under generic reversed phase LC conditions. An ACQUITY HSS T3 column (2.1 x 100 mm, 1.7 µm) was used with a linear gradient over 4 minutes going from 2 - 50% B where mobile phase A = 0.1% formic acid in water and mobile phase B = 0.1% formic acid in acetonitrile. b) Chromatogram showing ondansetron (API) and related impurities A, C, D, E and F separated under HILIC conditions. A CORTECS HILIC column (2.1 x 100 mm, 1.6 µm) was used with a linear gradient over 6 minutes going from 2 - 16% B where mobile phase A = 0.1% acetic acid in acetonitrile and mobile phase B = 10mM ammonium acetate in water at pH 4.

Table 1. Experimental results obtained using LC methodologies for ondansetron impurities A, C – F in the presence of 125 µg/mL API (ondansetron).

	Calibrator Results			
	Methodology	Fit	R^2	LLOQ s/n
Impurity A	HILIC	Linear; log, log	0.994	3050
Impurity C	RP	Quadratic; 1/x	0.997	300
Impurity D	RP	Quadratic; 1/x	0.994	180
Impurity E (imidazole)	HILIC	Linear; log, log	0.998	209
Impurity F (2-methylimidazole)	HILIC	Linear; log, log	0.997	13

	Quality Control Results									
		Mean			% Bias			% RSD		
	Methodology	140 ppm	760 ppm	2800 ppm	140 ppm	760 ppm	2800 ppm	140 ppm	760 ppm	2800 ppm
Impurity A	HILIC	131	749	2718	-2.6	-4.2	-2.8	1.3	1.3	1.5
Impurity C	RP	164	819	2761	-4.5	1.0	-1.4	1.0	1.0	1.4
Impurity D	RP	139	760	2766	-5.0	1.7	-1.5	2.3	1.4	1.5
Impurity E (imidazole)	HILIC	125	669	2879	1.9	3.1	4.6	3.6	4.0	3.2
Impurity F										
(2-methylimidazole	HILIC	135	811	2663	6.1	-4.5	2.2	1.5	0.7	0.8

The gradient was relatively shallow, with a gradient of 2 - 16% aqueous over 6 min. The separation shows the API was well separated from all impurities, thus minimising the risk of any matrix effects.

Since the HILIC method could not be used for the quantification of unretained impurities C & D, a reversed phase method was developed to quantify the two remaining impurities. The method used a BEH C18 column (2.1 x 50 mm, 1.7 µm) maintained at 30°C along with mobile phases consisting of 0.1% (v:v) formic acid in water and 0.1% (v:v) formic acid in acetonitrile. Again, the flow rate was 0.6 mL/min and used an injection volume of 2 µL. Table 1 shows the overall results for the LC methods developed to quantify the impurities of ondansetron. All compounds showed good R^2 values of >0.994 for the calibration curves along with acceptable s/n values for the lower limits of quantitation (LLOQ). In addition, QCs run in replicates of 6 at three different concentration levels gave mean calculated concentrations within 8.7% of nominal, and all RSD values were ≤ 7.3% which shows good accuracy and precision for the methods developed.

In this analysis, because of the structural similarity between the API and impurities, there is also potential that if any in-source fragmentation occurs, it could lead to erroneous identification or quantification of compounds. In source fragmentation occurs when a precursor compound is fragmented in the source and is then seen in Q1 at a different mass than expected. In this case, ondansetron (294 m/z) fragments in the source to form impurity D (212 m/z). Although the mass spectrometer cannot distinguish between the in-source fragmented ondansetron and native impurity D, the two compounds are separated chromatographically. The ondansetron peak in channel 212 > 184 is easily identified as ondansetron by retention time (Figure 3). Since ondansetron is not being quantified in this example, the in-source fragmentation is not problematic, however, in addition to potential matrix interferences, in-source fragmentation is another example of why a good chromatographic method where all analytes are well separated is important not just for UV but also MS methods.

Supercritical Fluid Chromatography Method Development:

Supercritical fluid chromatography is a viable alternative approach to solving this analytical challenge. In SFC, the retention mechanism is most comparable to normal phase chromatography, but uses mobile phases (modifiers and additives) which are readily compatible with the source design employed for LC-MS. Supercritical fluid chromatography is known to be well suited for the retention of small polar compounds, such as imidazole and 2-methylimidazole [8]. Additionally, the low viscosity and high diffusivity of CO_2 produces high efficiency separations in relatively short run times, which can significantly increase throughput compared to traditional LC methods.

Method development using supercritical fluid on the previously described system is analogous to LC method development, however, in place of varying the gradient and composition of aqueous and organic solvent, the gradient and composition of compressed CO_2 and organic co-solvent is varied. Methanol is the most common co-solvent used and similar to LC acidic or basic additives are often required to minimise secondary interactions and produce symmetrical peak shapes.

The final method developed for ondansetron & impurities utilised a Torus 2-PIC column (3x100 mm, 1.7 µm), maintained at 30°C (Figure 4). The co-solvent used was 0.2% ammonium hydroxide in methanol and the gradient went from 5 - 15% co-solvent over 6 minutes. The flow

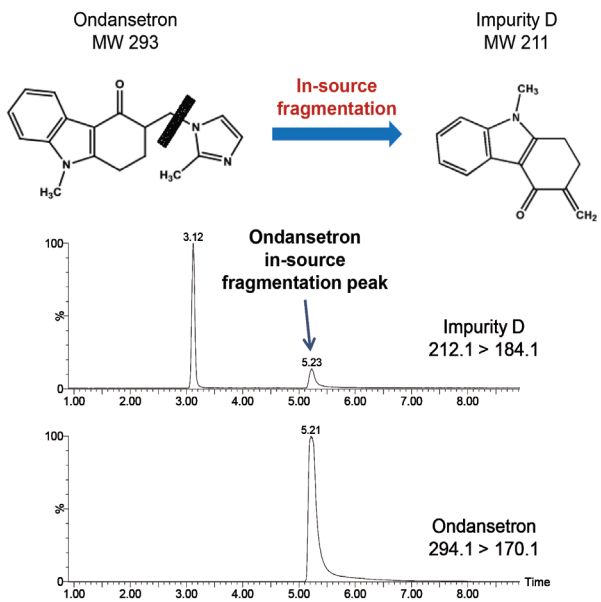

Figure 3. Ondansetron in-source fragmentation, where ondansetron fragments to the same precursor mass as impurity D and thus shows up in the MRM channel. It can easily be identified by its retention time as ondansetron.

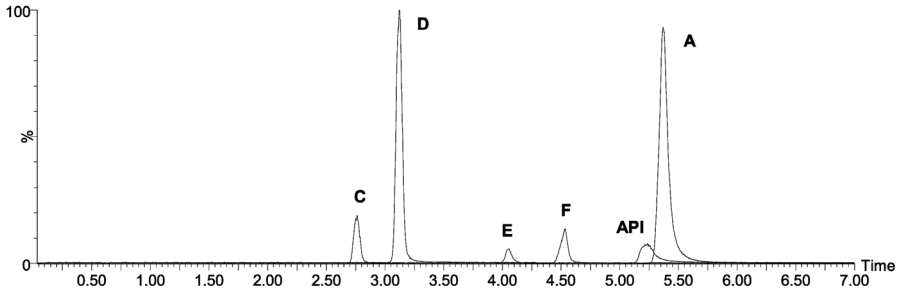

Figure 4. Chromatogram showing ondansetron (API) and related impurities A, C, D, E and F separated using SFC. A Torus 2-PIC column (3 x 100 mm, 1.7 µm) was used with 0.2% NH4OH in methanol as co-solvent and make-up flow.

rate was 1 mL/min, and the injection volume used was 2 µL. Because the relative amount of organic co-solvent is low (a gradient of 5 - 15% at 1 mL/min is equal to 50 - 150 µL/min of organic solvent reaching the MS probe), a make-up solvent was teed in post column to aid ionisation. The make-up solvent also consisted of 0.2% ammonium hydroxide in methanol and was added at 0.5 mL/min.

Table 2 shows the overall results for the single SFC method developed to quantify the impurities of ondansetron. All compounds showed good R^2 values of >/= 0.998 for the calibration curves along with acceptable s/n values for the lower limits of quantitation (LLOQ). In addition, QCs run in replicates of 6 at three different concentration levels gave mean calculated concentrations within 6.1% of nominal, and all RSD values were ≤ 4.0% which shows good accuracy and precision of the method developed.

Conclusions:

The development of a high sensitivity method for the analysis of impurities of ondansetron was challenging due to a number of factors including retention of small polar impurities, required detection levels, potential matrix interferences, and in-source fragmentation. With liquid chromatography, it was necessary to develop two methods: a HILIC method for the quantitation of highly polar impurities imidazole and 2-methylimidazole, and a reversed phase method for quantitation of the less polar impurities. However, using SFC, it was possible to analyse all five impurities in a single method. Both the UHPLC and SFC methodologies were amenable with MS detection, which facilitated detection at the levels required for potential mutagenic impurities set forth by ICH M7. In addition, all methods developed for the quantification of PMIs of ondansetron met the general requirements of an accurate and precise method. Finally, any possibility for matrix effects or negative effects due to in-source fragmentation was eliminated by adequate separation of the peaks of interest from the main API peak.

Table 2. Experimental results obtained using SFC methodology for ondansetron impurities A, C-F in the presence of 125µg/mL API (ondansetron).

	Calibrator Results		
	Fit	R^2	LLOQ s/n
Impurity A	Linear; 1/x	0.998	2500
Impurity C	Linear; 1/x	0.999	2000
Impurity D	Linear; 1/x	0.999	2000
Impurity E	Quadratic; 1/x	0.999	15
Impurity F (2-methylimidazole)	Linear; 1/x	0.998	75

	Quality Control Results								
	Mean			% Bias			% RSD		
	140 ppm	760 ppm	2800 ppm	140 ppm	760 ppm	2800 ppm	140 ppm	760 ppm	2800 ppm
Impurity A	131	749	2718	-2.6	-4.2	-2.8	1.3	1.3	1.5
Impurity C	164	819	2761	-4.5	1.0	-1.4	1.0	1.0	1.4
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Impurity E (imidazole)	125	669	2879	1.9	3.1	4.6	3.6	4.0	3.2
Impurity F (2-methylimidazole)	135	811	2663	6.1	-4.5	2.2	1.5	0.7	0.8

As stated previously, SFC shares the same selectivity as normal phase LC, thus providing a high degree of orthogonality to RPLC when utilising polar stationary phases. However, the flexibility of SFC also allows the use of conventional RP stationary phases, such as C18, yielding similar retention characteristics to RPLC when hydrophobic stationary phases are used. Combining the miscibility of CO_2 with both polar and non-polar organic solvents, SFC is a technique widely applicable to a diverse range of compounds. SFC is especially useful for separating mixtures containing polar compounds, as in the impurity example outlined above, and is also ideally suited for positional isomers, stereoisomers, diastereomers and chiral compounds. Finally, SFC is compatible with many popular detection techniques such as photodiode array, evaporative light scattering, and mass detection, making it a beneficial addition to any analytical laboratory.

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