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APPLICATION NOTE 72391

Achieve confident impurity detection with the Thermo Scientific ISQ EC single quadrupole mass spectrometer

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Keywords

Single quadrupole mass spectrometer, Impurity analysis, Peak purity, Quantitative LC MS, Tenofovir disoproxil fumarate

Goal

Demonstrate quantitative impurity analysis with the Thermo Scientific™ ISQ™ EC™ single quadrupole mass spectrometer and show its benefit for pharmaceutical development and quality control.

Introduction

Impurity analysis of produced chemicals is essential for small molecule pharmaceutical development or quality control. According to guidelines from the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH), all side products above a certain threshold need to be first characterized and later monitored. Identification and qualification thresholds depend on the daily dose and range between 0.1% and 1.0%.[1] Analysis is often done by an array of different detection methods. In quality control, UV-based detection is still the standard, but MS detection is gaining acceptance because of its clear advantages. Besides its lower detection limit, it also allows immediate analyte identification based on its respective mass and straightforward peak purity analysis based on its mass spectrum. UV-based identification, on the other hand, is often ambiguous since analyte identities are inferred based on their retention time and UV absorption.

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Modern single quadrupole mass spectrometers, such as the ISQ EC single quadrupole mass spectrometer (ISQ EC MS), are reliable workhorses designed for routine applications. The ISQ EC MS can operate in Full Scan or Single Ion Monitoring (SIM) mode, to either scan a mass range for all detectable analytes or focus on a specific compound. It can run at scan rates suitable for fast UHPLC applications while delivering picogram detection limits. The new orthogonal source design provides high levels of instrument robustness, even with challenging matrices. Full integration into the Thermo Scientific™ Chromeleon™ 7.2 chromatography data system (CDS) and the Thermo Scientific™ AutoSpray™ smart method set-up make LC-MS operation and data analysis straightforward and intuitive.

In the current work, the advantages of ISQ EC MS based impurity profiling are exemplified using tenofovir disoproxil fumarate. This drug is used for HIV treatment, often in combination with other anti-retroviral drugs. In combination with emtricitabine it is marketed as Truvada® by Gilead. Several impurities are described by the United States Pharmacopeia (USP).[2] Two of them, adenine and tenofovir, were selected for showcasing an ISQ EC MS based impurity analysis workflow. Both of them are structurally related to tenofovir disoproxil (Figure 1). The upper impurity limit for each of them is 0.15% in relation to the amount of tenofovir disoproxil. The challenging chromatographic separation was developed in previously published work.[3]

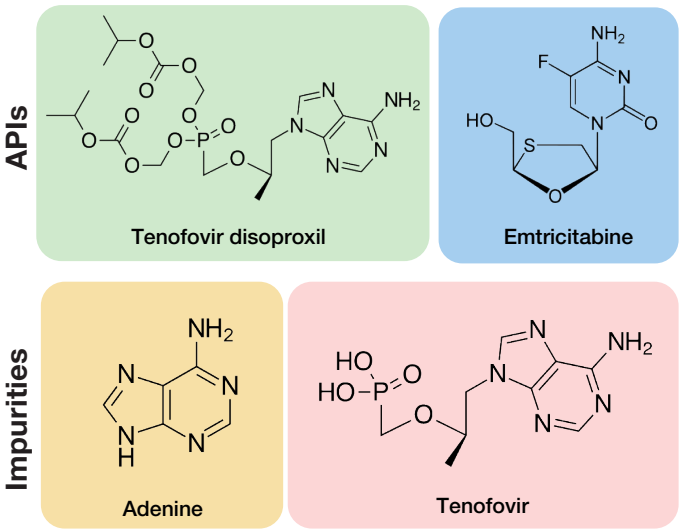

Figure 1. Chemical structures of tenofovir disoproxil, emtricitabine, adenine, and tenofovir. The first two are the active pharmacological ingredients (APIs) in Truvada while the latter two are structurally related impurities of tenofovir disoproxil.

Experimental

Fisher Scientific™ ACROS Organics™ adenine was used. Other sample reagents were purchased as USP reference standards.

Table 1. Overview of analytes. Tenofovir disoproxil is 1:1 complexed with fumarate in the formulation. During chromatographic analysis the complex separates and tenofovir disoproxil is detected. Therefore, only tenofovir disoproxil is mentioned here and in the following.

Analyte	CAS	Chemical Formula	Molecular Weight	Monoisotopic Mass [M]	$[M+H]^+$
Tenofovir disoproxil (fumarate)	201341-05-1	$C_{19}H_{30}N_5O_{10}P$	519.44	519.17	520.18
Emtricitabine	143491-57-0	$C_8H_{10}FN_3O_3S$	247.25	247.04	248.05
Adenine	73-24-5	$C_5H_5N_5$	135.13	135.05	136.06
Tenofovir	147127-20-6	$C_9H_{14}N_5O_4P$	287.21	287.08	288.09

Table 2. Solvents and additives.

Reagent	Grade	Supplier	Part number
Acetonitrile	Optima™ LC-MS	Fisher Chemical™	A955-212
Acetic acid	Optima LC-MS	Fisher Chemical	A113-50
Methanol	Optima LC-MS	Fisher Chemical	A456-212
Water	Ultra-Pure, 18.2 MΩ at 25 °C	Thermo Scientific™ Barnstead™ GenPure™ xCAD Plus Ultrapure Water Purification System	

Chromatographic separation was performed on a Thermo Scientific™ Vanquish™ Flex Quaternary UHPLC system (Table 3). A 75 cm long MP35N capillary with 100 µm inner diameter (P/N 6042.2390) was used for connecting to the ISQ EC MS. LC and MS conditions are given in Tables 4 and 5, respectively.

Table 3. Vanquish Flex Quaternary UHPLC system modules.

Module	Part Number
Vanquish System Base F	VF-S01-A
Vanquish Quaternary Pump F (with 200 µL mixer)	VF-P20-A (6044.5110 and 6044.5026)
Vanquish Split Sampler FT	VF-A10-A
Vanquish Column Compartment H	VH-C10-A
Vanquish Variable Wavelength Detector F (2.5 µL SST flow cell)	VF-D40-A (6074.0360)

Table 4. HPLC conditions.

Parameter	Value
Column	Thermo Scientific™ Accucore™ aQ, 2.6 µm, 2.1 x 100 mm (P/N 17326-102130)
Mobile phase	A: Water with 0.1% acetic acid B: Methanol with 0.1% acetic acid C: Acetonitrile with 0.1% acetic acid
Gradient	0–4 min: 0–70% B, 0–15% C 4–4.5 min: 70% B, 15% C 4.5–5 min: 70–25% B, 15–70% C 5–6 min: 25% B, 70% C 6–6.1 min: 25–0% B, 70–0% C 6.1–15 min: 0% B, 0% C
Flow rate	0.6 mL/min
Column temperature	Still air, 40 °C Active pre-heater, 40 °C
Injection volume	1 µL or 10 µL
UV detection	260 nm, 100 Hz, easy mode

Table 5. MS conditions.

Parameter	Value
Vaporizer temperature	450 °C
Ion transfer tube temperature	350 °C
Source voltage	+750 V
SIM scan	
Compound Time Mass Source CID voltage	Adenine 0–1.5 min 136.1 *m/z* 20 V
Compound Time Mass Source CID voltage	Tenofovir 0–1.5 min 288.1 *m/z* 25 V
Compound Time Mass Source CID voltage	Emtricitabine 1.5–3.0 min 248.1 *m/z* 10 V
Compound Time Mass Source CID voltage	Tenofovir disoproxil 3.0–4.0 min 520.2 *m/z* 10 V
Full Scan	
Time Mass range Source CID voltage	0–15 min 120–600 *m/z* 10 V

The ISQ EC MS was fully integrated into the Chromeleon 7.2 CDS, which was used for system operation and subsequent data analysis.

Calibration standards (10 ppb–10 ppm) were prepared by serially diluting 10 ppm adenine and tenofovir in 5% methanol in water. Samples for measuring the impurity levels were prepared diluting 1000 ppm tenofovir disoproxil, 1000 ppm emtricitabine, and 10 ppm adenine/tenofovir solutions in 5% methanol in water. Prepared samples are listed in Table 6.

Table 6. Impurity samples and used sample concentrations (1 ppm = 1 ng/µL).

Impurity Level	Adenine (ppm)	Tenofovir (ppm)	Emtricitabine (ppm)	Tenofovir Disoproxil (ppm)
1%	1	1	66.7	100
0.2%	0.2	0.2	66.7	100
0.1%	0.1	0.1	66.7	100
0.02%	0.02	0.02	66.7	100
0.01%	0.01	0.01	66.7	100

Results and discussion

First, system suitability for the impurity analysis was assessed. The USP reference method was adapted in a previous publication to reduce cycle time and transfer from 4.6 to 2.1 mm columns.[3] The method in the presented work was further adapted. Solvent A was water with 0.1% acetic acid, solvent B methanol with 0.1% acetic acid, and solvent C acetonitrile with 0.1% acetic acid. Due to the modifications, the method is not equivalent to the USP method. Nevertheless, the developed method is expected to meet the chromatographic requirements stated by the USP, namely peak tailing of tenofovir disoproxil ≤ 2.0 with a relative standard deviation of ≤ 10%, and a resolution between adenine and tenofovir ≥ 1.5. The USP suitability requirements were determined using mass spectrometric detection doing quintuplicate injections of 10 ng adenine and tenofovir on column, and 100 ng tenofovir disoproxil and 66.7 ng emtricitabine on column (Figure 2).

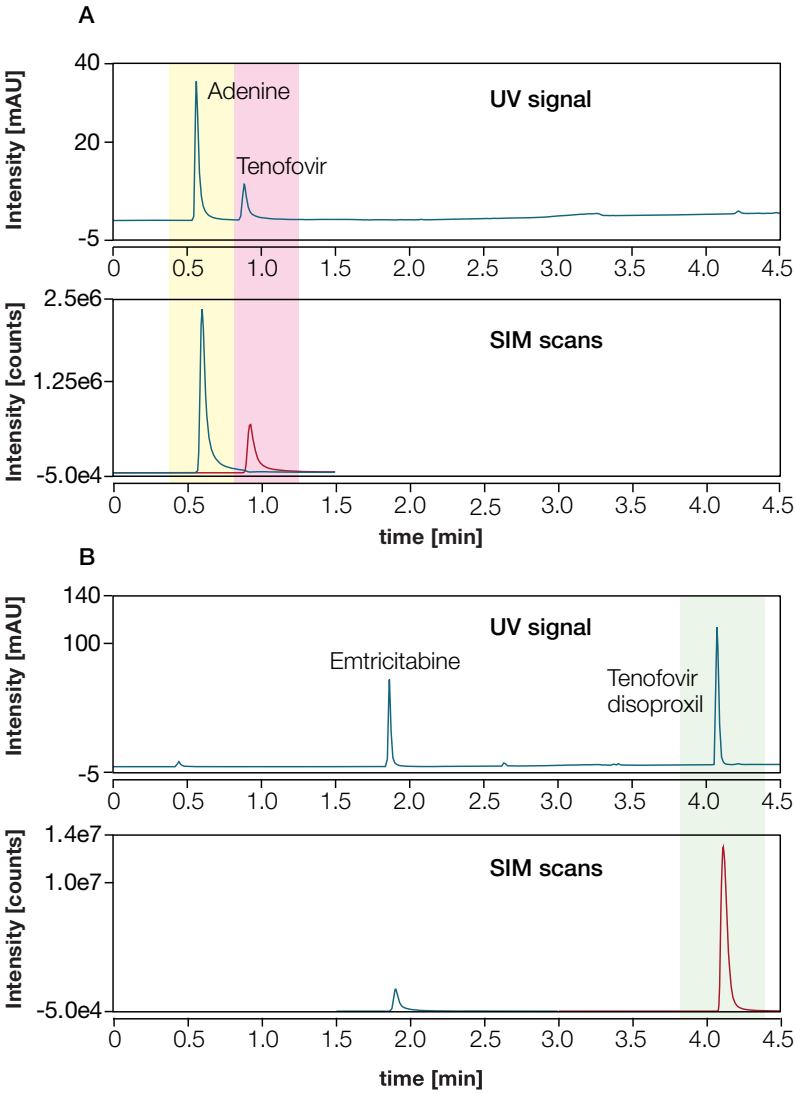

Figure 2. Chromatography of adenine, tenofovir, emtricitabine and tenofovir disoproxil. A) UV chromatogram (top) and SIM scans (bottom) of 10 ng adenine and tenofovir. B) UV chromatogram (top) and SIM scans (bottom) of 100 ng tenofovir disoproxil fumarate and 66.7 ng emtricitabine.

The tailing factor of tenofovir disoproxil was 1.7 with 4.0% RSD. The resolution of adenine and tenofovir was 4.0 (calculation based on Formula 1). Thus, the required suitability thresholds were met. Therefore, the method was considered suitable for impurity analysis.

Formula 1. Resolution according to US Pharmacopeia (USP) (t: time, W: peak width at base).

$$Resolution_{USP} = 2 * \frac{t_{Tenofovir} - t_{Adenine}}{W_{Tenofovir} + W_{Adenine}}$$

Next, the detection limits between UV detection and mass spectrometric detection were compared. Mass spectrometric detectors usually outperform UV detectors in terms of detection limits. Thus, the detection limits of the ISQ EC MS and the Vanquish Flex variable wavelength detector (VWD) were compared. Looking at the signal response of the ISQ EC MS and the VWD revealed differences in detection limits of up to three orders of magnitude (Figure 3). With the VWD, 1 ng of tenofovir on column was measured with a signal-to-noise ratio (S/N; peak to peak) of 10, and 100 pg adenine were detected with S/N 9. So, the limits of detection can be assumed to be 2 to 3 times lower (S/N 3). In single ion monitoring (SIM) mode, 1 pg adenine on column with S/N 10 and 10 pg tenofovir with S/N 7 were measured. Therefore, detection limits are probably 2 to 3 times lower S/N 3.

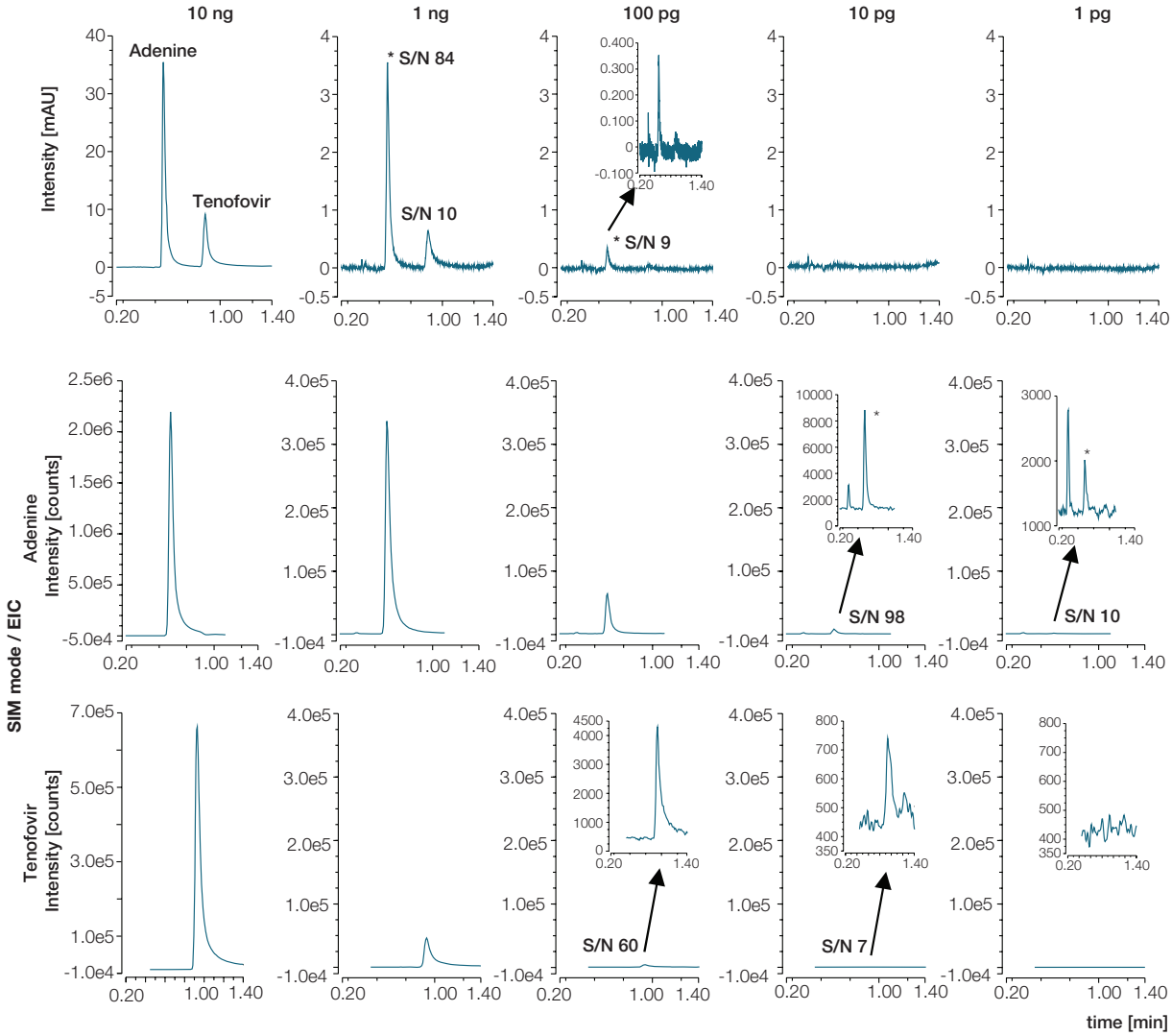

Figure 3. Comparison of signal response between the UV detector and the ISQ EC MS for adenine and tenofovir (EIC: Extracted ion chromatogram; S/N: signal-to-noise calculated by peak-to-peak method).

To prove that the ISQ EC MS can deliver accurate quantification of impurities at a low level, such as adenine and tenofovir levels between 0.01% and 0.2% of tenofovir disoproxil were analyzed. Timed-SIM mode was used for the targeted analysis of adenine, tenofovir, emtricitabine, and tenofovir disoproxil (Figure 4). SIM window (0.6 amu) and dwell time (0.2 s) parameters were selected to increase signal intensity and to assure at least 15 MS scans over the peak for good quantitation results. Full Scan (0.05 s dwell time) was used for determining peak purity and for untargeted background screening.

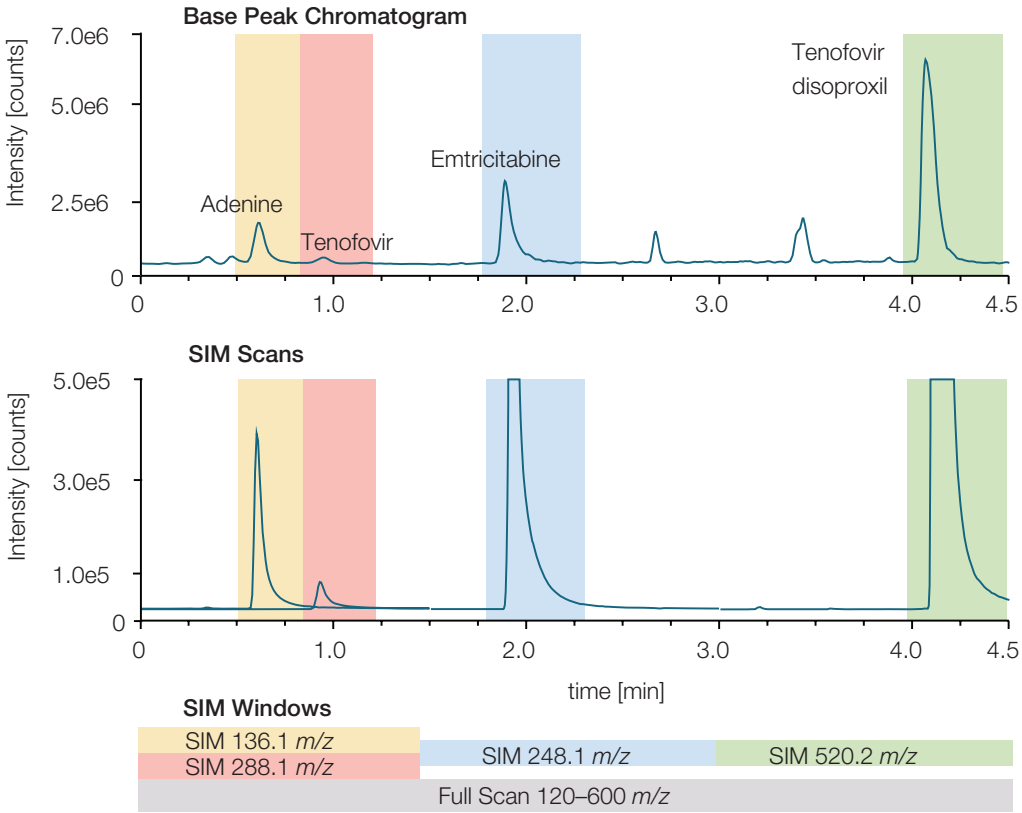

Figure 4. Chromatograms of 1% adenine and tenofovir (1 ppm each) in tenofovir disoproxil fumarate/emtricitabine (100 ppm / 67 ppm) analysis. Top: Base peak chromatogram. Middle: SIM scans. Bottom: SIM Windows - Acquisition windows for SIM scans.

Calibration curves for adenine and tenofovir spanning the relevant sample concentrations were generated (Figure 5). All injections were done in quintuplicate. Afterwards, reinjections of calibrants were done in triplicate to verify the accuracy of the calibration. Adenine and tenofovir showed good recovery rates, deviating by less than 10% at the lowest concentration and less than 5% at all other concentrations. The standard deviation between the reinjection replicates was below 10% indicating high precision (data not shown).

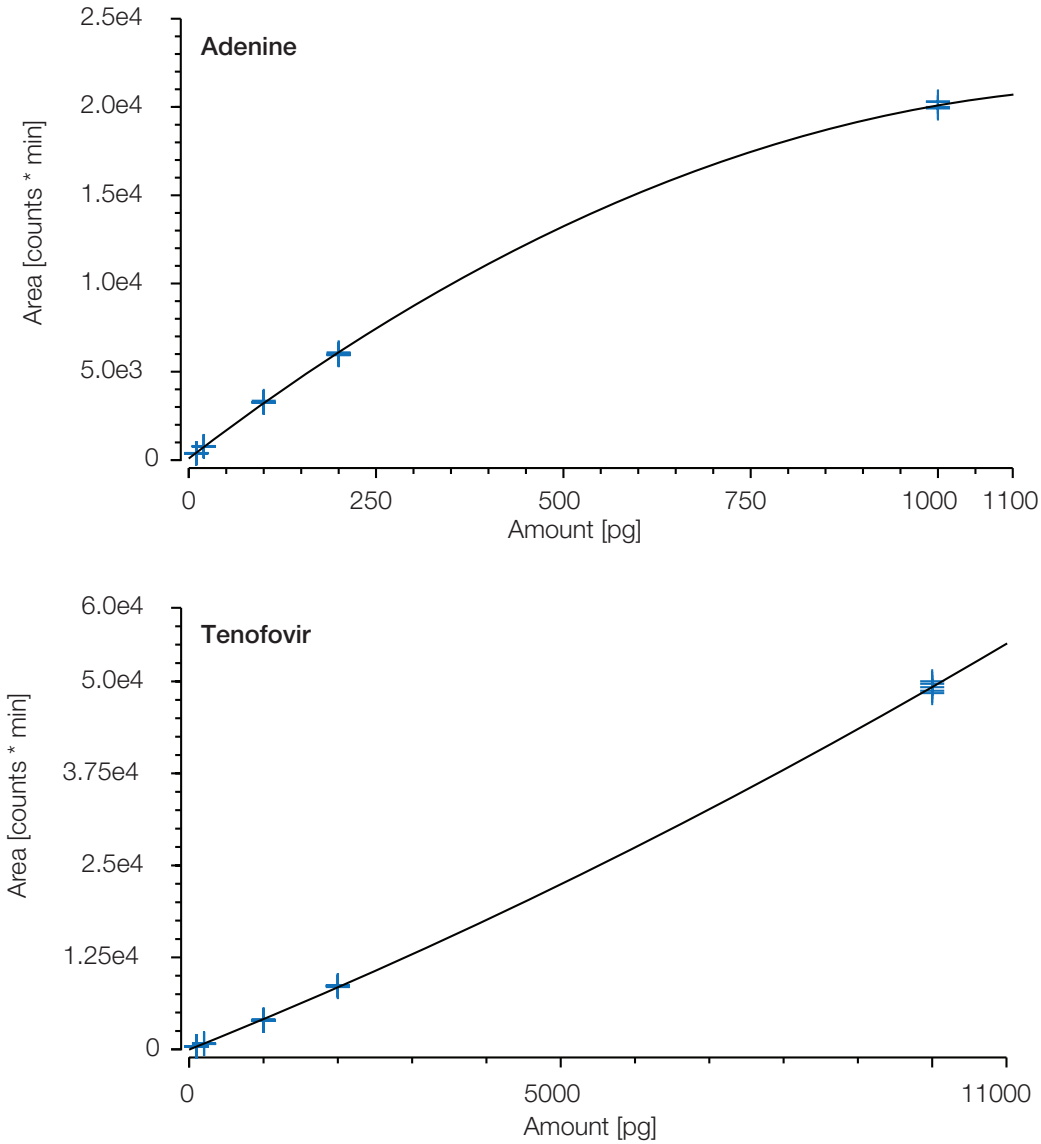

Figure 5. Calibration curves for adenine and tenofovir. Quadratic fit with 1/x weighting was applied. Adenine: 80.7655 + 32.6257x - 0.0126x^2, R^2 = 0.9994; tenofovir: -43.5406 + 4.0614x + 0.0001x^2, R^2 = 0.9996.

Quintuplicate analysis was done for impurity analysis. Adenine and tenofovir were confidently quantified down to an impurity level of 0.01% (Figure 6). Good accuracy was achieved for both compounds (Table 7). High precision was achieved with standard deviations smaller than 5% for most impurity levels. The lowest one showed a standard deviation smaller than 10%.

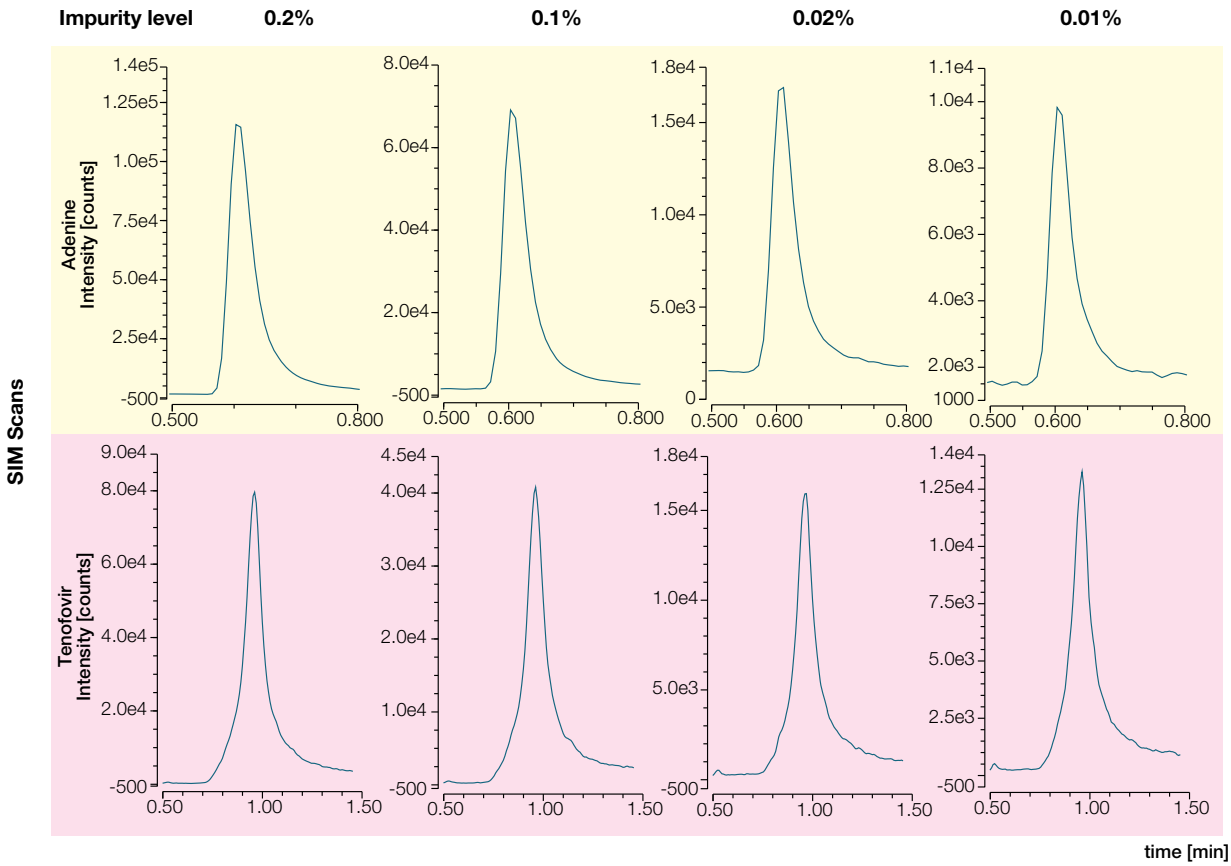

Figure 6 – Extracted ion chromatograms of adenine and tenofovir SIM scans for quantified impurity levels.

Table 7. Recovery rates and standard deviations (SD) for adenine and tenofovir at different impurity levels. Adenine was quantified using 1 µL injections, tenofovir using 10 µL injections to allow sufficient signal response for accurate quantification.

Impurity Level	Adenine Recovery ± SD	Tenofovir Recovery ± SD
0.2%	105.1% ± 0.8%	100.5% ± 2.0%
0.1%	111.3% ± 2.2%	93.7% ± 2.4%
0.02%	111.7% ± 3.7%	92.9% ± 4.4%
0.01%	103.7% ± 6.2%	100.0% ± 9.4%

The existence of additional components co-eluting with the API can be assessed using the Full Scan data which was acquired in parallel to the SIM data. The mass spectra of the front, apex, and tail of the tenofovir disoproxil peak were checked for the presence of additional masses (Figure 7). $[M+H]^+$ of tenofovir disoproxil was the dominant peak. Additionally, the sodium adduct $[M+Na]^+$ was detected (*m/z* 542.0). No other peaks were detected indicating peak purity.

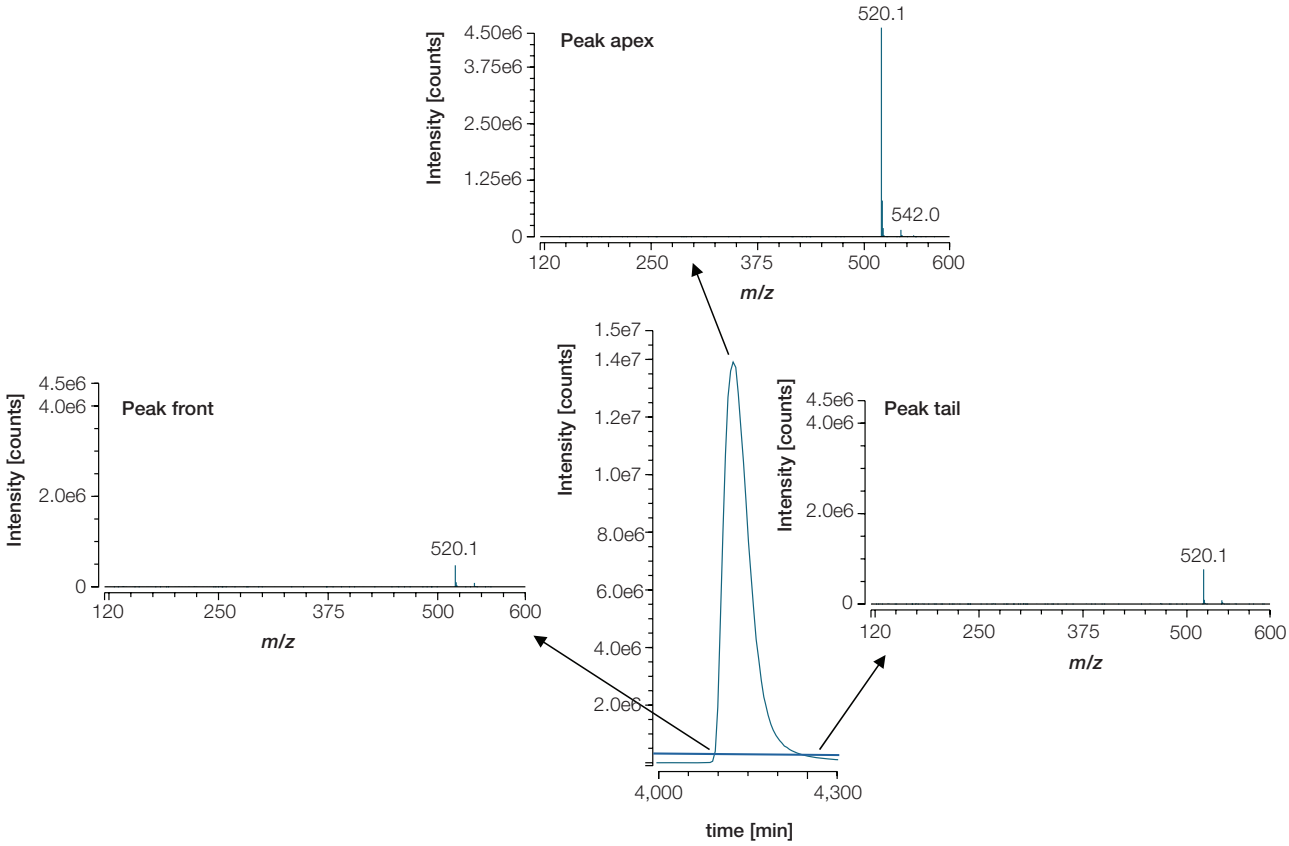

Figure 7. Peak purity analysis of tenofovir disoproxil. Mass spectra of the peak front, peak apex and peak tail of tenofovir disoproxil are shown. 15% peak height was used for the peak front and the peak tail. 1% of the main peak was used as detection threshold.

Another important aspect of impurity analysis is checking whether additional unexpected or unknown impurities are present in the sample. This is done by reviewing the Full Scan data. In the presented work, additional impurities eluting between emtricitabine and tenofovir disoproxil were detected (Figure 8). Combining the mass spectrometric information with impurity information provided by the USP allowed mass confirmation of two impurities: tenofovir isoproxil monoester and tenofovir isopropyl isoproxil. A third one could be identified as tenofovir methyl isoproxil, which is a degradation product formed by the replacement of one of the isoproxils with methanol. The observed masses for all three compound deviated 0.1 amu from the theoretical ones (Table 8).

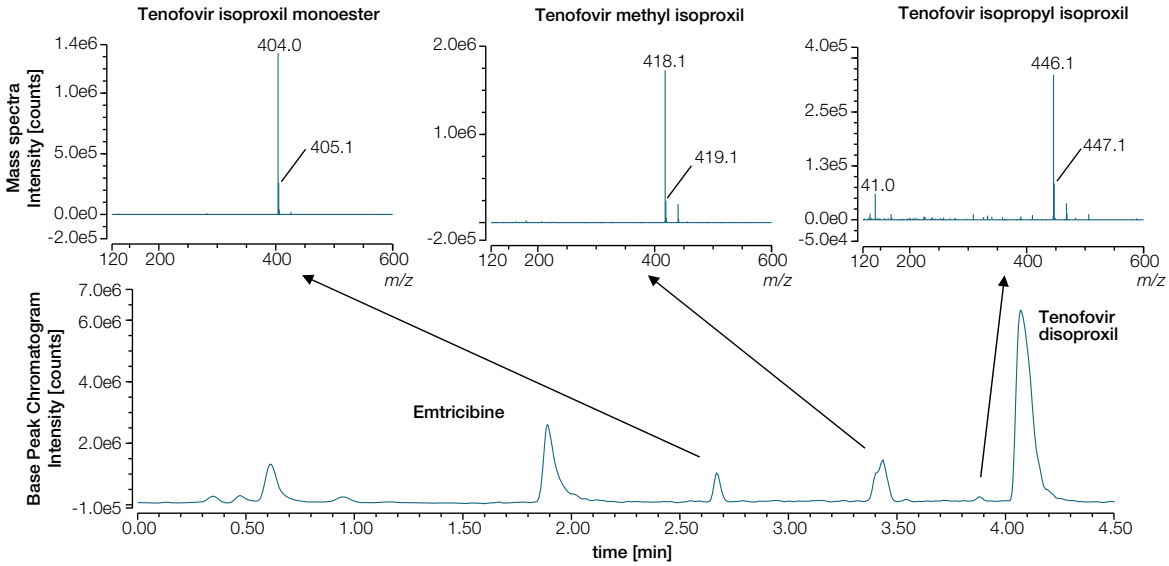

Figure 8. Identification of unknown impurities by mass spectrometric confirmation. Bottom: Base peak chromatogram of 1% adenine and tenofovir in tenofovir disoproxil/emtricitabine. Three additional peaks eluting between emtricitabine and tenofovir disoproxil were detected. Top: Mass spectra of these peaks identifying them as tenofovir isoproxil monoester, tenofovir methyl isoproxil, and tenofovir isopropyl isoproxil.

Table 8. Masses of discovered impurities.

Impurity	Chemical Formula	Theoretical Mass $[M+H]^+$	Observed Mass $[M+H]^+$	Mass Deviation (amu)
Tenofovir isoproxil monoester	$C_{14}H_{22}N_5O_7P$	404.1	404.0	0.1
Tenofovir methyl isoproxil	$C_{15}H_{24}N_5O_7P$	418.2	418.1	0.1
Tenofovir isopropyl isoproxil	$C_{17}H_{28}N_5O_7P$	446.2	446.1	0.1

Conclusion

- Quantitative impurity detection can be done with the ISQ EC single quadrupole mass spectrometer.
- SIM mode greatly increases sensitivity over UV detection and can be used for targeted quantification.
- Full Scan mode results in general detection of present analytes and provides their mass information. This facilitates determination of peak purity and detection of unknown impurities.

References

1. ICH Harmonised Tripartite Guideline – Impurities in New Drug Products, Q3B(R2), 2 June 2006.
2. USP Pending Monograph Version 1 – Tenofovir http://www.usp.org/sites/default/files/usp_pdf/EN/USPNF/pendingStandards/m3429_authorized.pdf (accessed May 26, 2017)
3. Thermo Scientific Application Note 1129: Ternary Gradient for Tenofovir Disoproxil Fumarate Impurity Profiling, Germering, Germany, 2016. https://tools.thermofisher.com/content/sfs/brochures/AN-1129-LC-Ternary-Gradient-Tenofovir-AN71676-EN.pdf (accessed May 26, 2017)

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