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Identity confirmation and accurate quantitation of a genotoxic impurity in an active pharmaceutical ingredient by UHPLC-UV coupled to a single quadrupole mass detector

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Keywords

Genotoxic impurities, methyl-*p*-toluenesulfonate, aprepitant, ISQ EM, single quadrupole, mass detection, UV detection, Autospray

Application benefits

- Mass detection delivers easy and reliable peak identity confirmation
- Sensitive UV detection provides quantitation of a genotoxic impurity at low ng/mL level
- Easy and user-friendly adjustment of the ion source parameter settings with Autospray

Goal

Confirm the identity of related impurities and a genotoxic impurity in a drug substance by single quadrupole mass detector. Accurately quantify the genotoxic impurity by UV detection.

Introduction

The analysis of process- and product-related impurities is an essential step throughout the lifecycle of a drug. High performance liquid chromatography (HPLC) with ultraviolet light (UV) detection is by far the most common technique used to assess the purity of an active pharmaceutical ingredient (API). Identification is typically assessed based on retention time. For this purpose, a single impurity standard has to be run separately. During the early

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drug development stages, impurity standards are often not available, and the identity of peaks cannot easily be determined.

Genotoxic impurities are a special group of impurities that pose a greater risk to patient health, since they are carcinogenic.[1] The genotoxic impurities identified as potential contaminants of the drug must be monitored and accurately quantified according to rules which are stricter than for other impurities. The United States Food and Drug Administration (U.S. FDA) as well as the European Medicines Agency (EMEA) have established a threshold of toxicological concern (TTC) of 1.5 µg/day for long-term treatments with the drug product.[2,1] Additionally, the international conference on harmonization (ICH) M7 suggested a staged TTC based on the duration of drug exposure as described in Table 1.[3]

From the TTC value, the concentration limit can be calculated based on the expected daily dose of the drug administered to the patient using the following equation[1]:

Concentration limit (ppm) = TTC (µg/day) / dose (g/day)

P-toluenesulfonates, are an example of genotoxic impurities. They can occur as by-products of the drug synthesis where *p*-toluenesulfonic acid reacts with alcohols such as methanol, ethanol or isopropanol. Since *p*-toluenesulfonic acid is frequently used as a counterion for salt formation, *p*-toluenesulfonates are common genotoxic impurities.[4] The identification of a *p*-toluenesulfonate is straightforward as standards are readily available. However, it is practical and desirable that HPLC methods, during the early stage of drug development, are capable of identifying both genotoxic and other related impurities in a single run without the need for separate injections of standard. This can be achieved by coupling the HPLC to UV and a mass detector, where the UV detection provides the quantitative information and the mass detector the identity information. The understanding of the synthetic pathways is typically sufficient to deduct many expected impurities, which can be compiled in a list of intact masses. These can then be screened in SIM mode by a single quadrupole MS, allowing for putative identity assignments.

The aim of this study was to develop a method for monitoring and quantifying methyl-*p*-toluenesulfonate along with other related impurities in a drug substance. The API chosen in this study is aprepitant. Aprepitant is an antiemetic administered for the prevention of nausea and vomiting during chemotherapy. Contamination of aprepitant by genotoxic methyl-*p*-toluenesulfonate may occur, since *p*-toluenesulfonic acid and methanol are used in different steps of the synthesis.[4,5]

Experimental

Chemicals

- Deionized water, 18.2 MΩ·cm resistivity or higher
- Fisher Scientific Acetonitrile, Optima™ LC/MS grade (P/N A955-212)
- Fisher Scientific Methanol, Optima™ LC/MS grade (P/N A456-212)
- Fisher Scientific™ Ammonium acetate, LC/MS grade (P/N A114-50)
- Fisher Scientific™ Methyl-*p*-toluenesulfonate (P/N AAA11088130)

Two aprepitant samples were purchased from commercial vendors.

Equipment

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

Table 1. Acceptable daily intakes for individual and multiple impurities

	Duration of exposure			
	<1 month	>1–12 months	>1–10 years	>10 years
Acceptable daily intakes for an individual impurity (µg/day)	120	20	10	1.5
Acceptable total daily intakes for multiple impurities (µg/day)	120	60	30	5

Preparation of standards

A stock solution of methyl-*p*-toluenesulfonate (methyl-TSF) was prepared in acetonitrile at a concentration of 1 mg/mL.

A standard solution with 10 µg/mL was prepared by transferring 100 µL of the stock solution into a 10 mL volumetric flask and filling up to volume with water/acetonitrile 50/50 (v/v). Based on this solution calibration standards were prepared with concentrations of 0.01 µg/mL, 0.025 µg/mL, 0.05 µg/mL, 0.075 µg/mL, 0.1 µg/mL, 0.25 µg/mL, 0.5 µg/mL, 0.75 µg/mL, 1 µg/mL, and 2.5 µg/mL.

Additionally, a standard solution at 0.005 µg/mL was prepared for the determination of the limit of detection (LOD).

Preparation of samples

Solutions of 1 mg/mL of each aprepitant sample (aprepitant I and aprepitant II) were prepared in water/acetonitrile 50/50 (v/v).

Aprepitant I sample contained methyl-TSF as an impurity and was used for the impurity screening experiments and quantitation.

Aprepitant II sample was found to be a methyl-TSF free sample and was therefore used for the determination of recovery rates by spiking methyl-TSF into the aprepitant II sample (1 mg/mL) with 0.01 µg/mL, 1 µg/mL and 2.5 µg/mL.

Instrumentation

A Thermo Scientific™ Vanquish™ Flex Quaternary UHPLC system equipped with a Thermo Scientific™ ISQ™ EM single quadrupole mass spectrometer was used for the analysis:

- Thermo Scientific™ Vanquish™ System Base Vanquish Horizon/Flex (P/N VH-S01-A)
- Thermo Scientific™ Vanquish™ Quaternary Pump F (P/N VF-P20-A)
- Thermo Scientific™ Vanquish™ Sampler FT (P/N VF-A10-A)
- Thermo Scientific™ Vanquish™ Column Compartment H (P/N VH-C10-A)
- Thermo Scientific™ Vanquish™ Diode Array Detector F (P/N VF-D11-A) with semi-micro flow cell, 2.5 µL (P/N 6083.0550)
- Thermo Scientific ISQ EM mass spectrometer (P/N ISQEM-ESI)

Table 2. LC conditions used for impurity analysis next to the API

Column:	Thermo Scientific™ Acclaim™ Polar Advantage II, 150 × 2.1 mm, 2.2 µm (P/N 071401)	
Mobile phase:	A: 15 mM ammonium acetate B: methanol	
Flow rate:	0.3 mL/min	
Gradient:	*Time [min]*	*% B*
	0	60
	4.5	60
	6.0	73
	21.0	73
	21.5	60
	35.0	60
Active pre-column heater temp.:	35 °C	
Column temp.:	35 °C (forced air mode, fan speed 5)	
Autosampler temp:.	4 °C	
UV wavelength:	225 nm	
3D scan:	190–280 nm	
UV data collection rate:	10 Hz	
UV response time:	0.5 s	
Injection volume:	10 µL	
Needle wash:	50% methanol	

Adjustment of ion source parameters of the mass detector

With a feature implemented in the Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) software, an automated adjustment of the ion source parameters of the ISQ EM mass detector can be carried out.

The software allows for an automatic optimization of source CID voltage by using sequence custom variables in the injection list (see Chromeleon 7 Help – Custom Variables). Only one instrument method needs to be created and is automatically run with all CID voltage values defined in custom variable window within the injection sequence (Figure 1).

The signal intensities were compared to determine the best CID voltage for methyl-TSF. A CID voltage of 10 V was found to be the optimal setting.

Moreover, Autospray intelligent ion source settings allow for an easy adjustment of the gas and temperature settings of the ion source based on the flow rate used in the chromatographic method. Three aspects are given special consideration: robustness/sensitivity, volatility of mobile phase, and thermal stability of analytes.

Figure 2 demonstrates a selection of Autospray ion source settings and the effect on gas and temperature values when using different slider positions.

The best results are then chosen, e.g., by overlaying the extracted ion chromatograms (XICs) as shown in Figure 2. The highest signal intensity was achieved using the settings of Figure 2-C. If complete optimization of the ion source parameters is required for the method, e.g., due to sensitivity issues, the Autospray settings can serve as a starting point.

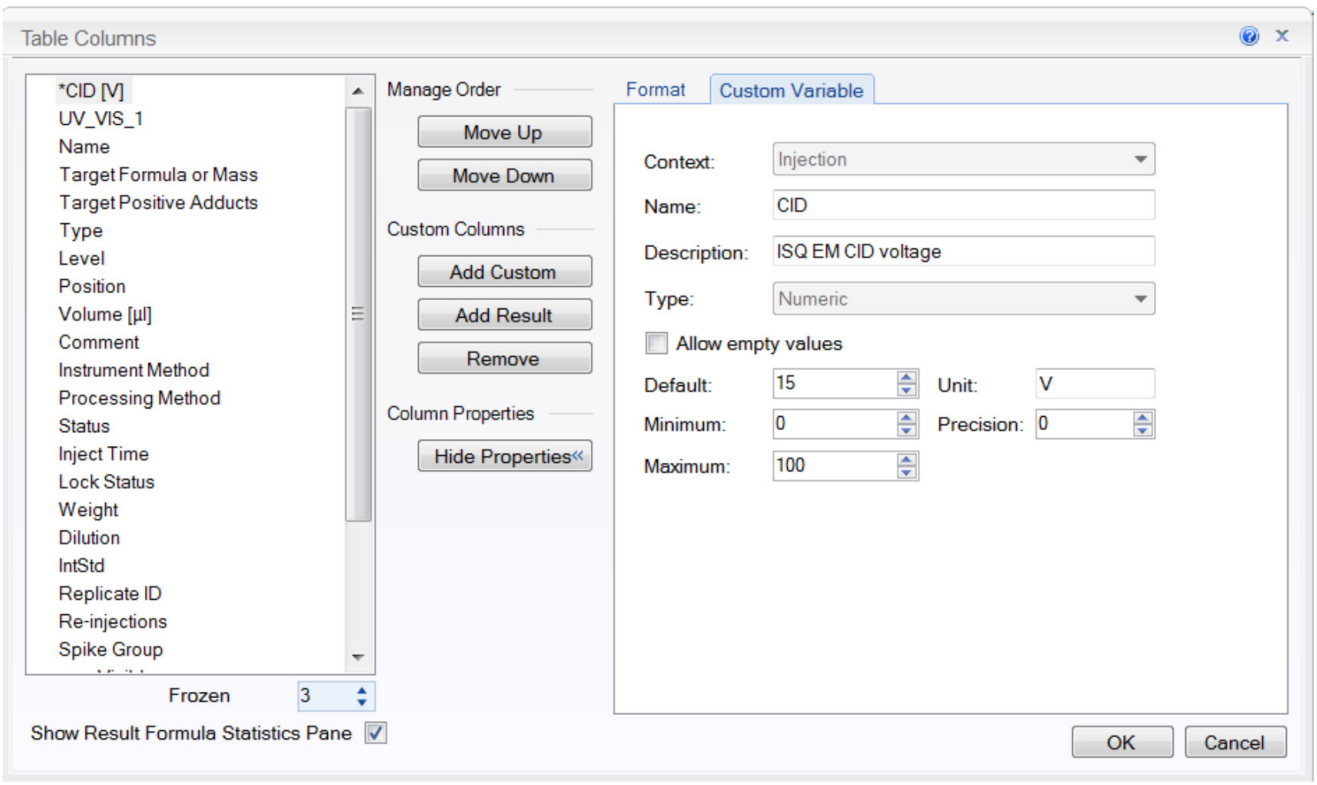
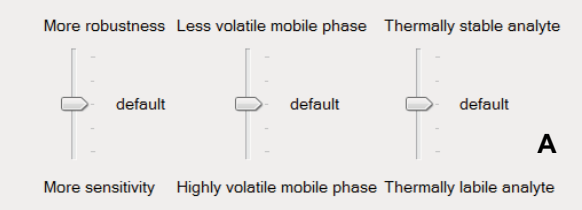
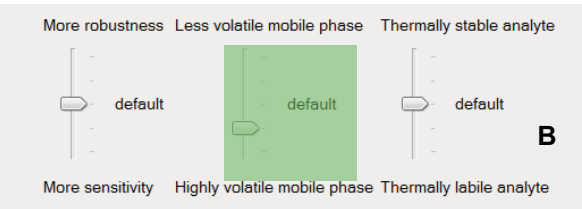


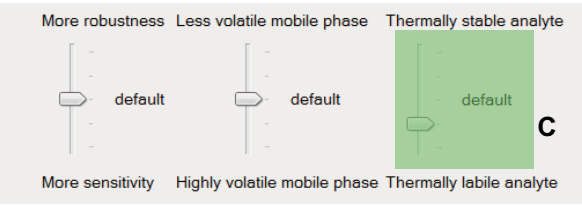
Figure 1. Screenshot of CID custom variable window



Parameter	Value
Sheath Gas	35.8 psig
Aux Gas	4 psig
Sweep Gas	0.5 psig
Ion Transfer Tube Temp	300 °C
Vaporizer Temp	172 °C



Parameter	Value
Sheath Gas	20.8 psig
Aux Gas	3 psig
Sweep Gas	0.5 psig
Ion Transfer Tube Temp	300 °C
Vaporizer Temp	172 °C



Parameter	Value
Sheath Gas	35.8 psig
Aux Gas	4 psig
Sweep Gas	0.5 psig
Ion Transfer Tube Temp	250 °C
Vaporizer Temp	172 °C

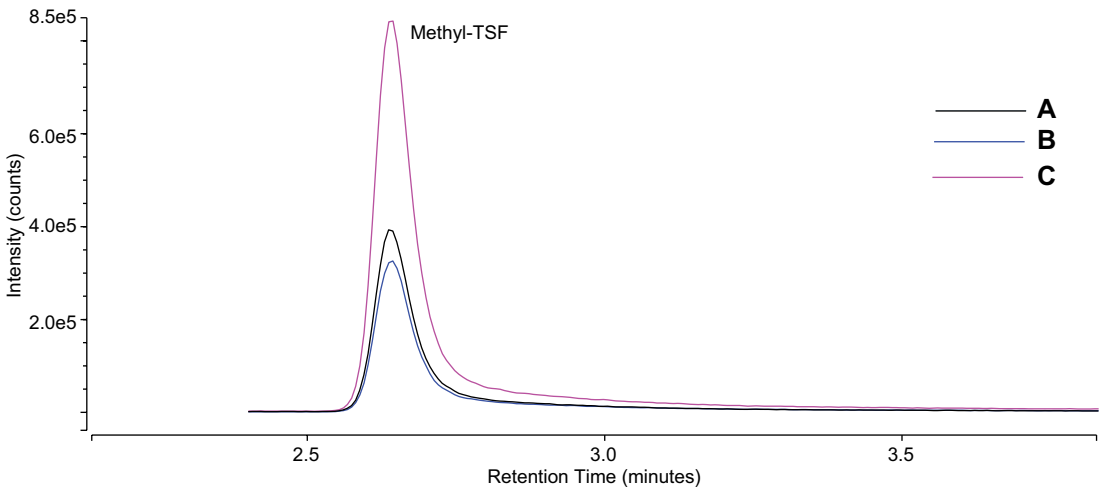


Figure 2. Overlay of extracted ion chromatograms obtained for methyl-TSF with different MS ion source parameter settings based on adjustments with Autospray intelligent ion source settings concept; A- Default position; B- varied position on volatility of mobile phase, C- varied position on thermal analyte stability

Table 3 shows the MS conditions used for impurity analysis after adjustments of the ion source parameters based on methyl-TSF.

Impurity screening

Genotoxic impurity methyl-TSF, and several known non-genotoxic impurities of aprepitant[6,7] were investigated in the study and qualitatively monitored (Table 4). Chemical structures of the analytes are presented in Figure 3. Data were acquired in SIM scan mode to allow for a more sensitive detection. Methyl-TSF exclusively forms the ammonium adduct $[M+NH_4]^+$, when ammonium acetate as aqueous mobile phase is used, while the API and its non-genotoxic related impurities mainly form $[M+H]^+$ ions.

Additionally, data was recorded in full scan mode to detect possible unknown impurities.

Data processing and software

Analysis was performed with Chromeleon 7.2.9 CDS software, which fully integrates the ISQ EM mass detector, enabling instrument control, data acquisition, data processing and reporting on a single software platform.

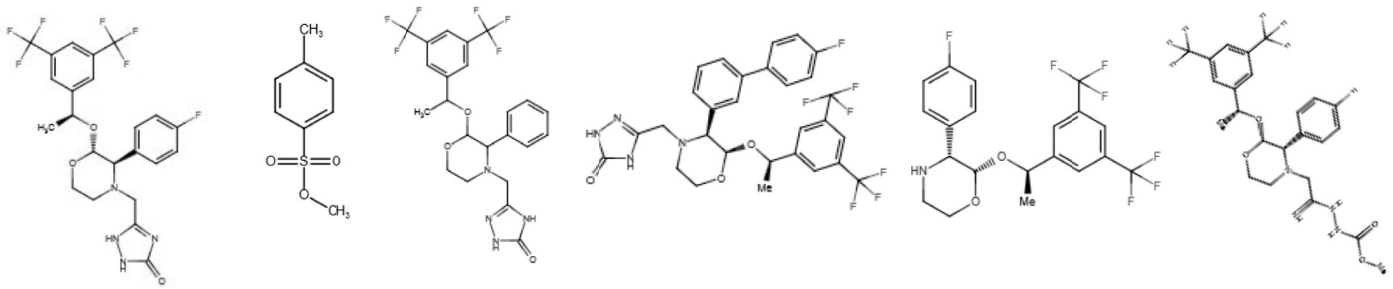
Table 3. MS conditions used for impurity analysis in API

MS source parameters	
Sheath gas pressure:	35.8 psig
Aux gas pressure:	4 psig
Sweep gas pressure:	0.5 psig
Vaporizer temperature:	172 °C
Ion transfer tube temp.:	250 °C
Source voltage:	3000 V
MS method parameters	
Ionization mode:	HESI
Ion polarity:	Positive
Scan type:	Full scan with six targeted SIM scans
Spectrum data type:	Profile (full MS)
Full scan mass range:	*m/z* 100–650
SIM width:	0.5 amu
Dwell time:	0.1 s
Source CID voltage:	10 V

Table 4. Aprepitant and some of its related impurities with chemical formula, *m/z* used for SIM scans, and detected ion species

Compound	Aprepitant	Methyl- TSF	Impurity I	Impurity II	Impurity III	Impurity IV
Chemical formula	$C_{23}H_{21}F_7N_4O_3$	$C_8H_{10}O_3S$	$C_{23}H_{22}F_6N_4O_3$	$C_{29}H_{25}F_7N_4O_3$	$C_{20}H_{18}F_7NO_2$	$C_{24}H_{25}F_7N_4O_4$
SIM Scan *m/z*	535.2	204.1	517.2	611.2	438.1	567.2
Ion species	$[M+H]^+$	$[M+NH_4]^+$	$[M+H]^+$	$[M+H]^+$	$[M+H]^+$	$[M+H]^+$

Chemical names of impurities:
Impurity I = Defluoro Aprepitant
Impurity II = 4-Defluoro-4-(*p*-fluorophenyl)aprepitant
Impurity III = Des-1,2,4-triazol-3-one-5-methyl –aprepitant
Impurity IV = *N*-(Destriazolonomethyl) *N*-(Methylcarboxyacetamidohydrazono)aprepitant)



Aprepitant | Methyl-TSF | Impurity I | Impurity II | Impurity III | Impurity IV

Figure 3. Chemical structures of API and its related impurities investigated in the study

Results and discussion

Impurity screening

The analysis of the API sample (aprepitant I) showed several peaks in the UV chromatogram (Figure 4). SIM scans according to Table 4 were performed to confirm the presence of expected impurities (Figure 5). Four impurities were confirmed by SIM scans including the genotoxic one, methyl-TSF. For impurity II, the SIM scan chromatogram provides only a very weak signal with a strong baseline effect and the UV trace did not show any peak at the retention time of the MS detected weak signals. Thus, the presence of impurity II in the sample could not be confirmed. Figure 6 demonstrates the full scan mass spectra of the methyl-TSF peak in the aprepitant sample compared with the spectra in the standard. Both spectra look similar, which indicates a methyl-TSF peak without co-elution of other compounds in the sample. Chromeleon CDS allows the accounting for the time delay between the UV and MS signals, which greatly facilitates mass assignment of peaks in the UV chromatogram. Furthermore, the acquired full scan spectra provided information about possible unexpected impurities. One additional impurity with *m/z* 593.1 at RT 11.7 min could be assigned, however no further investigation on the identity of this impurity was performed.

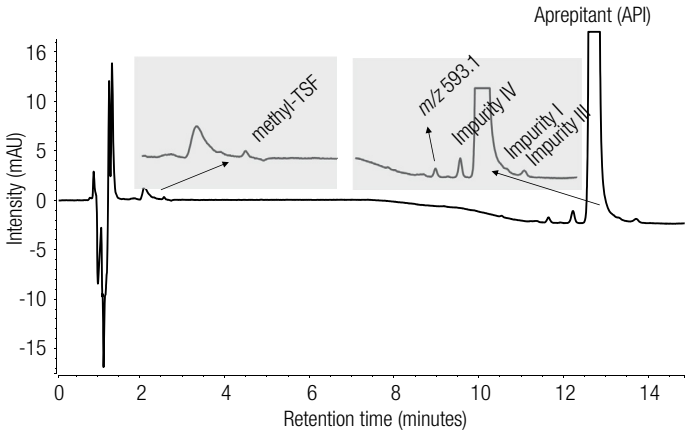


Figure 4. UV chromatogram acquired at 225 nm of aprepitant I sample with peak assignments based on MS data; grey boxes: zoom into baseline of API and related impurities; only peaks with >0.03% relative area were considered

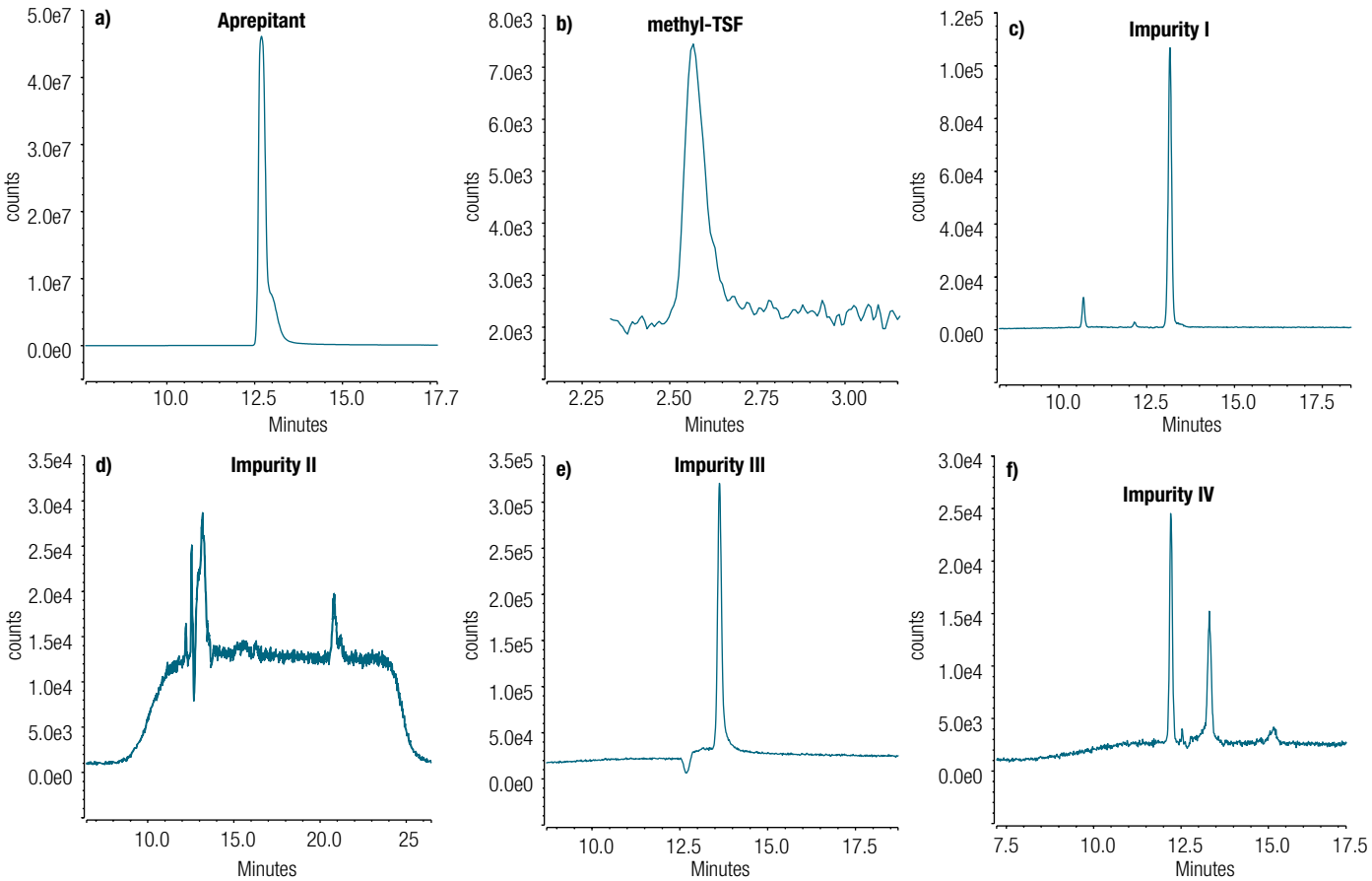


Figure 5. SIM scan chromatograms of aprepitant I (a) and related impurities (b-f) according to Table 4

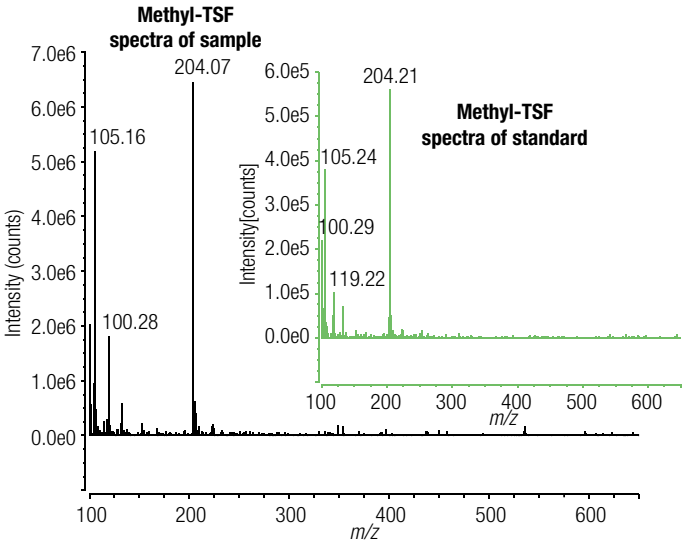


Figure 6. Full scan spectra of methyl-TSF Peak in the sample (black) compared to the spectra of the methyl-TSF standard injection (green)

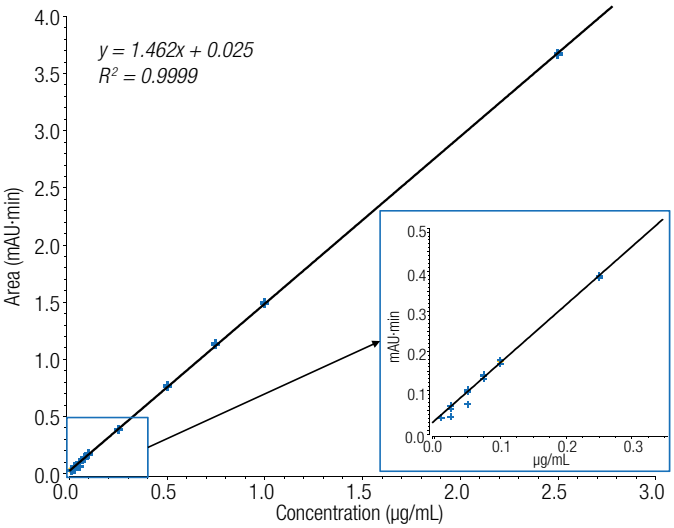


Figure 8. UV calibration curve of methyl-*p*-toluenesulfonate over the concentration range of 0.01 µg/mL to 2.5 µg/mL

To unequivocally confirm the presence of the genotoxic impurity methyl-TSF, it was spiked into the sample and measured under the same conditions. The overlaid chromatograms in Figure 7 show an exact match of the methyl-TSF peak in the spiked and unspiked sample.

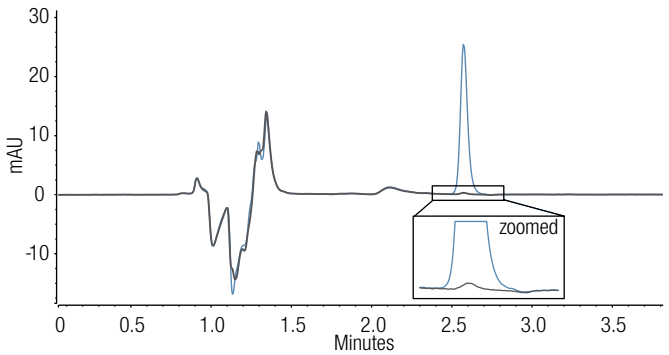


Figure 7. Overlaid UV chromatograms of spiked API sample (blue) and unspiked sample (gray)

Linearity, limit of quantification and recovery of methyl-*p*-toluenesulfonate using UV detection

The calibration curve for methyl-*p*-toluenesulfonate was obtained by triplicate injections of ten concentration levels (0.01 µg/mL–2.5 µg/mL). As shown in Figure 8, excellent linearity was obtained with a correlation coefficient (R^2) = 0.9999.

The limit of detection (LOD) and limit of quantification (LOQ) was determined by diluting the standard solution until a S/N ratio of ≥3 for LOD and ≥10 for LOQ, was observed. Five replicate injections were evaluated to examine LOD and LOQ values. The LOD was found to be 3.3 ng/mL (with standard deviation ±0.7 ng/mL) and LOQ 9.4 ng/mL (with standard deviation ±1.9 ng/mL).

The recovery rates were estimated by spiking the aprepitant II sample (methyl-TSF free sample) with 0.01 µg/mL (LOQ level), 1 µg/mL (concentration limit based on TTC value), and 2.5 µg/mL (highest calibration level) with methyl-*p*-toluenesulfonate standard solution. With 93–99%, excellent recovery could be achieved throughout the measurement range allowing for accurate quantification over a wide concentration range (Table 5).

Table 5. Recovery rates at different spike levels for methyl-*p*-toluenesulfonate

Spike Level	Recovery (%)
0.01 µg/mL	93
1 µg/mL	98
2.5 µg/mL	99

Quantitation of the genotoxic impurity methyl-TSF in the sample using UV detection

According to ICH M7 guidelines a TTC of 120 µg/day is allowed for aprepitant because the duration of medication is less than 1 month.[3]

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Based on a 125 mg tablet dosage the calculated concentration limit is 960 ppm. With 1 mg/mL aprepitant sample this results in a concentration of 0.96 µg/mL using the described method.

Quantitative analysis was carried out with injecting the API sample (aprepitant I) three times. As shown in Table 6 the calculated amount corrected by the recovery rate resulted in 0.010 µg/mL with a relative standard deviation (RSD) of 5.05%. As a result, the genotoxic impurity found in the sample is far below the concentration limit based on the TTC value (0.96 µg/mL) and close to the LOQ of the method.

Table 6. Quantitation result of methyl-*p*-toluenesulfonate in aprepitant I sample

Measured amount (µg/mL)	Recovery (%)	Calculated amount (µg/mL)	RSD (%)
0.009	93	0.010	5.05

Conclusion

- The combination of UV and a single quadrupole mass detector is a powerful tool for API analysis in early stages of development, enabling confirmation of expected impurities
- The Autospray intelligent source settings allow a non-experienced MS user an easy and fast adjustment of the MS ion source parameters
- With a 10-fold lower LOQ value compared to the concentration limit based on the TTC value of 960 ng/mL, the UV method proved to be very sensitive to the determination of methyl-*p*-toluenesulfonate
- Excellent linearity and recovery rates are demonstrated for the genotoxic impurity

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