APPLICATION NOTE 73365

Forced-degradation evaluation of erythromycin by HPLC and single quadrupole mass spectrometry

Authors: Mauro De Pra, Stephan Meding

Thermo Fisher Scientific, Germering, Germany

Keywords: Vanquish Core, ISQ EC, ISQ EM, antibiotics, pharmaceutical analysis, stability studies

Goal

Develop a simple, stability-indicating LC-MS method for erythromycin. Compare the impurity profiles of an erythromycin reference standard with stressed analyte.

Application benefits

- Simple fit-for-purpose method development
- Peak assignment based on *m/z*
- Sensitive detection of analytes with low UV absorption

Introduction

Forced degradation studies are common during early development of drugs. Typically, LC-UV is the technique of choice to profile drug impurities both prior to and after the stress procedure. At this point, some information on the related impurities may be available, such as a list of intermediate products or side-products based on the known synthesis pathway. During early development, standards of such impurities are usually not available, so identification by LC-UV based on their respective retention times is not possible. The LC-UV method developed for a forced degradation study is usually fit-for-purpose, and the effort required for method development should be reasonable. Since the method is usually not validated, or transferred for validation, it is preferred to use generic methods, possibly slightly modified to accommodate the specificity of the analytical target. The method suitability is normally assessed by injecting the active pharmaceutical ingredient (API). If the API peak is resolved from the rest of the impurities the method is considered suitable. However, after a forced degradation, the impurity profile may be substantially different than that of the API reference sample. Therefore, the purity method may no longer be sufficient to resolve all impurities from the API. In this case the method needs to be adapted to the new impurity profile observed for the stressed samples.

Mass spectrometric (MS) detection can address the limitations of UV-based LC purity analysis in forced degradation studies. Since the molecular mass of the expected synthesis-related impurities is known, the hyphenation of mass detection with LC enables putative identification of peaks without the need for standards. When mass detection is used, the method development effort is considerably lower than UV-detection based methods, since co-elution can be tolerated as long as the co-eluting substances have different molecular masses and thus can be resolved based on their respective mass-to-charge ratios. Thanks to this, even if the impurity population of the stressed sample is considerably larger than the reference sample, resulting in multiple co-elutions, it will not be required to optimize and improve the initial method.

Another scenario in which MS detection should be considered as an alternative to UV detection in forced degradation studies is when the APIs and/or related impurities have poor UV absorption. In this case, LC-UV methods require injections of large amounts of samples to be able to detect the API and related impurities with satisfactory sensitivity. In a situation when sample availability is limited, a common scenario during early development, high sample consumption is undesirable. Additionally, due to the large amounts injected, there is a risk of column overloading, which results in loss of efficiency, poor peak shape, and retention time shifts.

Erythromycin is an antibiotic produced by bacterial biosynthesis. Even though erythromycin can be analyzed by LC-UV, the UV absorption of the API and related impurities is low. Thus, injections of large sample amounts are required to obtain sufficient sensitivity. For instance, the European Pharmacopeia method recommends the injection of 400 µg of sample in 4.6 mm ID columns.[1] Additionally complexity arises since erythromycin is a biological product and contains five additional variants alongside the principal ingredient erythromycin A and other biosynthetic impurities. In this work, erythromycin is used to showcase a situation where MS detection is preferred over UV detection in forced degradation studies.

The Thermo Scientific™ Vanquish™ Core Binary HPLC System combined with the Thermo Scientific™ ISQ™ EM Single Quadrupole Mass Spectrometer was used. The Vanquish Core HPLC System is ideal for laboratories running routine and general purpose methods. At the same time it can be used for method development. The ISQ EM single quadrupole MS is a robust and versatile mass detector designed for chromatographers. It can be operated like any other LC detector through Thermo Scientific™ Chromeleon™ Chromatography Data Systems (CDS). Its Autospray source settings use a patented algorithm to select source parameters based on the flow rate and minimal user input. As a result chromatographers can focus on results instead of method optimization.

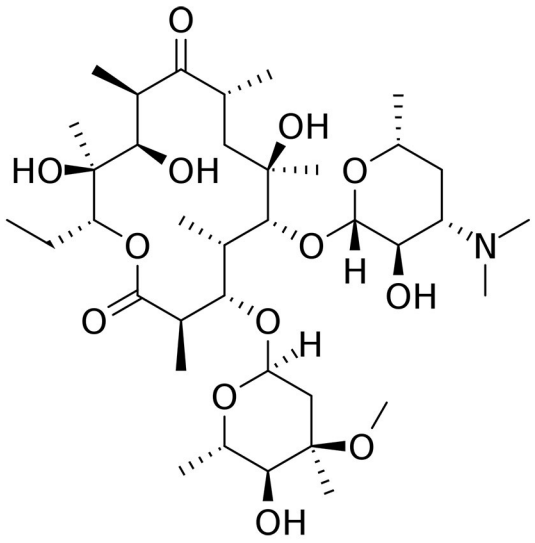

Figure 1. Chemical structure of erythromycin A

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™ Formic acid, Optima™ LC/MS grade (P/N A117-50)
- Erythromycin for system suitability CRS 1 (from EDQM) (P/N Y0001847)
- Erythromycin pharmaceutical secondary standard (from Sigma-Aldrich) (P/N PHR1039)

Sample handling

- Fisher Scientific™ Fisherbrand™ Mini Vortex Mixer (P/N 14-955-152)
- Glass Vials (amber, 2 mL), Fisher Scientific (P/N 03-391-6)
- Vial Caps with Septum (Silicone/PTFE), Fisher Scientific (P/N 13-622-292)

Instrumentation

- Thermo Scientific Vanquish Core HPLC system consisting of:
  - Vanquish System Base Core (P/N VC-S01-A)
  - Vanquish Binary Pump C (P/N VC-P10-A)
  - Vanquish Split Sampler CT (P/N VC-A12-A)
  - Vanquish Column Compartment C (P/N VC-C10-A)
  - Vanquish Solvent Monitor (P/N 6230.1310-01)
  - Thermo Scientific™ ISQ™ EM single quadrupole mass spectrometer (P/N ISQEM-ESI)

Sample preparation

Preparation of SST standard for injection

- 15.0 mg erythromycin for system suitability CRS were dissolved in 7.0 mL 8/2 = water/(acetonitrile + 0.1% formic acid) (volume/volume). Final concentration was 2.14 µg/µL.

Preparation of erythromycin reference sample for injection

- 2.8 mg erythromycin pharmaceutical secondary standard was dissolved in 1.0 mL 8/2 = water/(acetonitrile + 0.1% formic acid) (volume/volume). Final concentration was 2.8 µg/µL.

Preparation of stressed erythromycin samples

- Degraded erythromycin: Erythromycin pharmaceutical secondary standard 5.9 mg was dissolved in 2.90 mL HCl 1N and stored at room temperature for one week. Afterwards the sample was diluted with 3.00 mL of saturated $NaHCO_3$ solution to quench further degradation. Final concentration was 0.99 µg/µL.
- Erythromycin stressed sample for injection: 100 µL of degraded erythromycin (see above) were added to 900 µL of erythromycin reference solution. Final concentration was 2.71 µg/µL.

Table 1. HPLC conditions

Parameter	Value
Column	Thermo Scientific™ Acclaim™ PolarAdvantage II, 3 µm, 120 Å, (3 × 150) mm (P/N 063705)
Mobile phase	A – Water with 0.1% formic acid B – Methanol / water = 9 / 1 (v/v) with 0.1% formic acid
Gradient	Time (min) %B 0 50 15 100 20 100 20.001 50 30 50
Flow rate	425 µL/min
Autosampler temperature	4 °C
Column temperature	40 °C, forced air mode passive pre-heater
Injection volume	0.1–10 µL

Table 2. MS settings for European Pharmacopeia System Suitability CRS 1 sample

Parameter	Value
Source settings	Easy mode. Default settings (3) for sensitivity and mobile phase volatility. Setting for thermal lability of analyte was 2. Ion transfer temperature was 250 °C.
Component mode	
Time	2–20 min
Mass range full scan	350–1050 *m/z*
Acquisition rate	Minimum baseline peak width: 3 s; Desired scans per peak: 20
Polarity	Positive
Source CID voltage	0 V
SIM scan width	0.1 amu
SIMs	734.47; 718.47; 720.45; 748.36; 750.46; 716.44

Table 3. MS settings for full scan acquisition of erythromycin reference and stressed samples

Parameter	Value
Source settings	Easy mode. Default settings (3) for sensitivity and mobile phase volatility. Setting for thermal lability of analyte was 2. Ion transfer temperature was 250 °C.
Component mode	
Time	1–20 min
Mass range full scan	350–1050 *m/z*
Dwell time	0.0729 s
Polarity	Positive
Source CID voltage	0 V

Table 4. MS settings for SIM and full scan acquisition of erythromycin reference and stressed samples

Parameter	Value
Source settings	Easy mode. Default settings (3) for sensitivity and mobile phase volatility. Setting for thermal lability of analyte was 2. Ion transfer temperature was 250 °C.
Component mode	
Time	1–30 min
Mass range full scan	350–1050 *m/z*
Polarity	Positive
Source CID voltage	0 V
SIM scan width	0.2 amu
SIM	734.47; 718.47 ; 720.45; 748.36; 750.46; 716.44; 576.29; 558.30; 556.61; 560.36; 540.20; 540.41; 584.29; 750.17
Dwell time	0.0032 s

Chromatography Data System

The Chromeleon software, version 7.3 was used for data acquisition and analysis.

Results and discussion

Method development highlights

The method was developed using the European Pharmacopeia SST CRS-1 standard. With the aim of keeping HPLC method development effort to a minimum, only one column and few elution conditions were tested. The reversed-phase polar embedded Acclaim PolarAdvantage II stationary phase was selected based on its excellent retention properties for charged analytes. The tested methods were: gradient from 0 to 50% acetonitrile, 0 to 100% methanol, 50 to 100% methanol, isocratic with 35, 30, 25, 20% acetonitrile. In all cases, 0.1% formic acid was used as additive for aqueous and organic eluents.

During the first steps of the development, the Autospray source parameters were kept at default. However, the spectrum associated with the erythromycin A peak always showed a characteristic series of erythromycin fragments (*m/z* 576.3, 558.3, 540.4). Regardless of the mobile phase conditions these ions were always present at an intensity level of about 1% of the main ion (*m/z* 734.46) (data not shown). Since the detected ions eluted at the identical retention times as erythromycin A they were considered fragments ions, most likely generated by thermal degradation within the MS ion source. This hypothesis was supported by a manuscript on LC-MS/MS analysis on erythromycin variants and impurities, which indicated the same ions were generated during fragmentation of the erythromycin A parent ion.[3] Following the assumption of thermal degradation, the Autospray source setting was adjusted for a more thermally labile analyte. This corresponded to a lower ion transfer tube temperature setting. The new setting eliminated the generation of fragment ions, and the quantitation of erythromycin A was not negatively affected by fragmentation (Figure 2).

Another requirement of the method was the chromatographic resolution of the erythromycin B (*m/z* 718.4) and the impurities with *m/z* 716.4 (Impurities D, E, F). The isotopic pattern of the *m/z* 716.4 impurities includes a signal of *m/z* 718.4 which has an abundance of ~8% of the monoisotopic mass. In case of co-elution of erythromycin B and one of the *m/z* 716.4 impurities, the quantitation of the erythromycin would be biased by the impurity isotope *m/z* 718.4 (Figure 3). Since the impurities with *m/z* 716.4 were rather abundant in the SST sample, it was deemed critical to chromatographically resolve erythromycin B from these impurities.

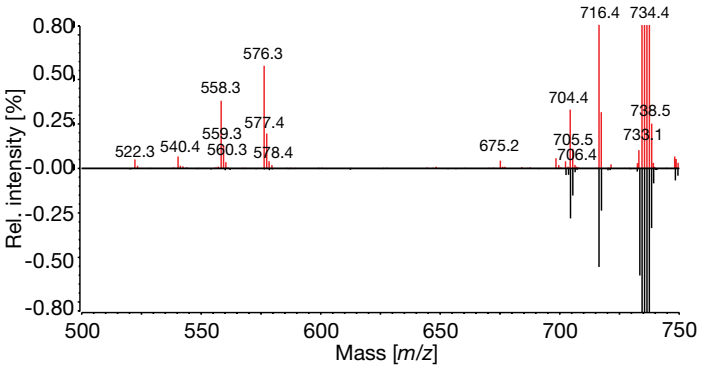

Figure 2. Mass spectrum of erythromycin A, and its thermal degradation products. Top red spectrum: ion transfer tube at 300 °C; bottom black spectrum: at 250 °C. Injected sample: 0.28 µg erythromycin SST standard. Mobile phase 25% ACN in water + 0.1% formic acid. Rest of conditions as in Table 1. The erythromycin fragments caused by thermal degradation are eliminated by reducing the ion transfer tube temperature.

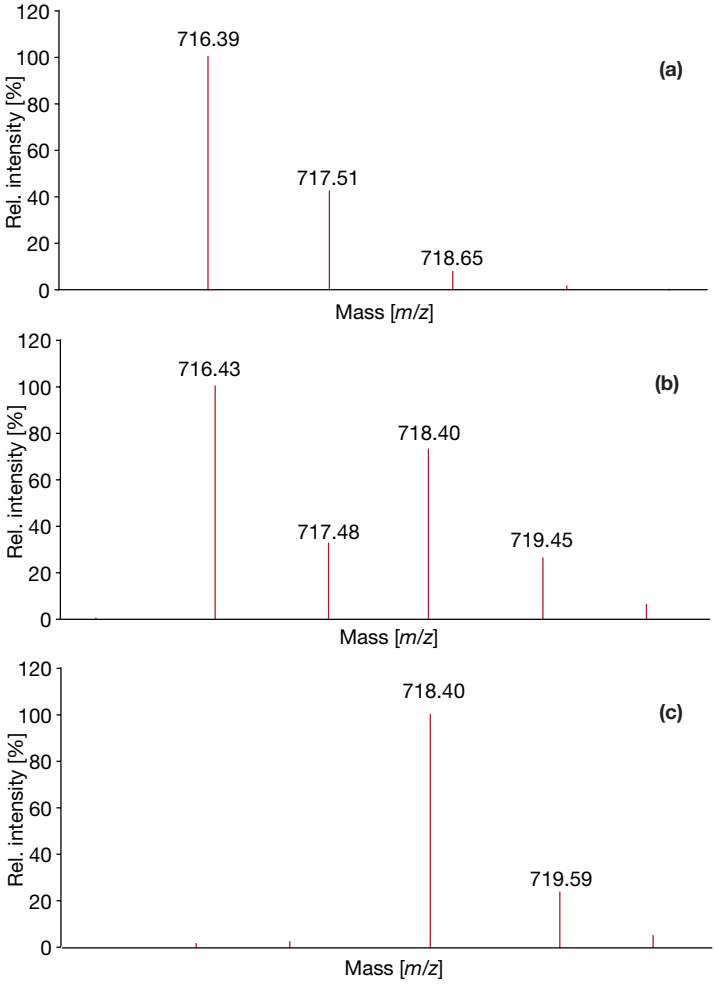

Figure 3. Comparison between spectra of Impurity D (a), impurity D and erythromycin B co-eluting (b), and erythromycin B (c). The quantitation of erythromycin B based on XIC *m/z* 718.4 is impacted by the isotope of impurity D containing two C13 atoms, in case of coelution.

The final HPLC method is described in Table 1. In Figure 4, the final method applied to the SST standard shows good separation of the main components of the mixture. Erythromycin B could be resolved from the impurities with *m/z* 716.4 (Impurity D, E, F). Based on this, the method was considered fit-for-purpose to assess the purity of erythromycin drug substance. Beside the species assigned to the TIC peaks, additional erythromycin variants known to be present in the SST standard (Table 5) could be confirmed; these additional components were erythromycin C, E, and F (data not shown).

Purity analysis of stressed sample erythromycin A

After the stress procedure was completed, the original erythromycin sample, and the one modified by degradation, were run in full scan mode, and combined SIM scan / full scan mode. SIM scan targeted the known erythromycin variants and the impurities detected in the SST sample. The TIC of the full scan showed that the erythromycin sample used in the forced degradation study is more complex than the SST standard used in the development of the method (Figure 5). The TIC confirms the presence of more impurities in the stressed sample, compared to the original one. Two signals in the TIC (~5.7 min, ~8.0 min) were more intense for the reference material. The retention time of these peaks, corresponded to the retention time of the two impurities with *m/z* 716.4 found in the SST standard.

The peaks detected in full scan were analyzed to determine the related spectra, and additional species not determined in the SST sample were added to the SIM list of the instrument method. The complete list of SIMs corresponds to the extracted ions listed in Table 6. Finally, both reference and stressed samples were analyzed by LC-MS with the complete set of SIMs.

The data resulting from the full set of SIMs were further processed in Chromeleon CDS 7.3 software. A list of MS components was obtained by generating extracted ion chromatograms (XIC) for each SIM channel. The apex, front, and tail (at 10% signal intensity of the apex) spectra of each component were reviewed to assess possible co-elutions.

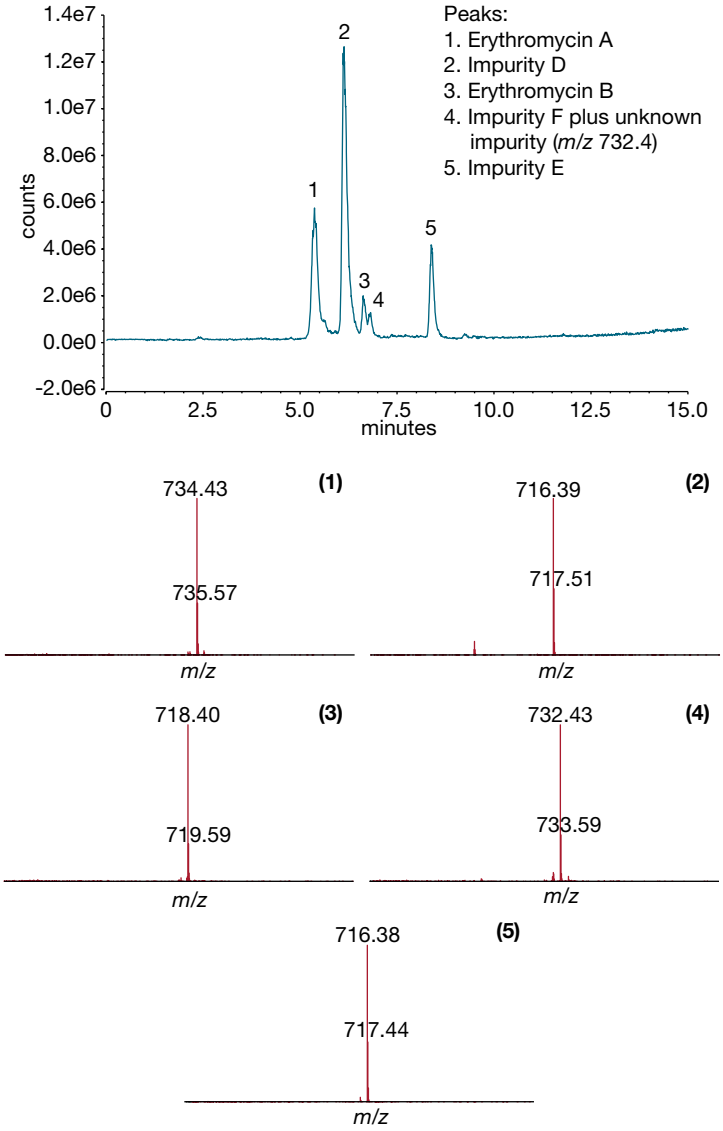

Figure 4. Injection of SST standard 0.28 µg. HPLC method as in Table 1, MS settings as in Table 2. Five peaks are detected in the TIC; the apex peak spectra shows the main component of the peaks. Peak assignment: 1) erythromycin A; 2) Impurity D; 3) erythromycin B; 4) Impurity F plus unknown impurity (*m/z* 732.4) ; 5) Impurity E. Impurity D, E, F were tentatively assigned assuming the elution order is the same as in reference 2.

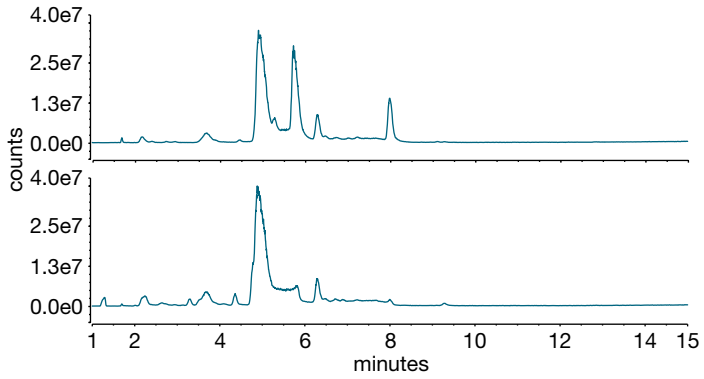

Figure 5. Full scan TIC of erythromycin reference sample (top) and erythromycin stressed sample (bottom). HPLC conditions as in Table 1, MS settings as in Table 3.

Table 5. List of expected impurities in the sample "Erythromycin for system suitability CRS 1" (see reference 2). Note that the protonated species $[M+H]^+$ is detected by MS which is 1.01 Da heavier than the monoisotopic mass.

Compound	Molecular formula	Molecular mass	Monoisotopic mass [M]
Erythromycin A	$C_{37}H_{67}NO_{13}$	733.92	733.46
Erythromycin B	$C_{37}H_{67}NO_{12}$	717.92	717.47
Erythromycin C	$C_{36}H_{65}NO_{13}$	719.9	719.45
Impurity A (Erythromycin F)	$C_{37}H_{67}NO_{14}$	749.92	749.46
Impurity B	$C_{36}H_{65}NO_{13}$	719.9	719.45
Impurity C (Erythromycin E)	$C_{37}H_{65}NO_{4}$	747.91	747.44
Impurity D	$C_{37}H_{65}NO_{12}$	715.91	715.45
Impurity E	$C_{37}H_{65}NO_{12}$	715.91	715.45
Impurity F	$C_{37}H_{65}NO_{12}$	715.91	715.45
Impurity H	$C_{37}H_{67}NO_{4}$	749.92	749.46
Impurity L	$C_{37}H_{66}NO_{4}$	748.92	748.44

Unlike the SST standard, in both reference and stressed samples, the peaks with *m/z* 716.4 could not be identified as a well-resolved pair; instead they were detected as a broad band eluting over 5.5–8 min. Most likely, this is due to the presence of additional unresolved species with the same mass-to-charge ratio (*m/z* 716.4). Therefore, in order to simplify integration and data interpretation, the signal of the impurities with *m/z* 716.4 was combined and reported as a single impurity. The peak assigned to the main API component, i.e. erythromycin A, was not pure, and low level co-elution from species with *m/z* 716.4 and 748.4 was observed; the abundance relative to erythromycin A of the co-eluting impurities was approximately 1% and 5%, respectively (based on signal intensity of monoisotopic ions). Unlike for a LC-UV method, the fact that the main peak was not pure was not considered a limitation, since the MS allowed the mass selective quantification of erythromycin A.

Comparison between the stressed and reference sample indicated an increase of the number and relative peak area of all lower-molecular weight impurities, with the exception of Impurity 2, which decreased from 3.47% in the reference material to 2.99% in the stressed sample. Five impurities not detected in the reference material were present in the stressed sample. A substantial decrease of the peak area for the impurities with *m/z* 716.4, which mainly consist of Impurity D and F, was observed.

Table 6. Overview of the impurity profile of reference and erythromycin sample stressed with acidic conditions. Relative areas are normalized to the main pharmaceutical ingredient erythromycin A. HPLC conditions as in Table 1, and MS conditions as in Table 4.

	Reference (2.89 µg injected)			Stressed (2.71 µg injected)			
Peak name	Ret. time (min)	Area (counts*min)	Area relative to erythromycin A (%)	Ret. time (min)	Area (counts*min)	Area relative to erythromycin A (%)	Extracted ion
Impurity A (Erythromycin F)	2.18	3.63E+05	2.45	2.20	2.88E+05	2.13	750.46
Degradation impurity 1	2.28	2.42E+04	0.16	2.28	7.39E+05	5.47	576.29
Degradation impurity 3	n.a.	n.a.	n.a.	3.56	3.25E+04	0.24	716.41
Degradation impurity 4	n.a.	n.a.	n.a.	3.56	7.21E+04	0.53	560.36
Erythromycin C	3.73	1.48E+06	10.03	3.71	1.33E+06	9.86	720.45
Degradation impurity 8	n.a.	n.a.	n.a.	4.82	1.05E+06	7.8	540.33
Erythromycin A	4.95	1.48E+07	100	4.98	1.35E+07	100	734.47
Combined impurities with *m/z* 716.4	5.86*	8.70E+06	58.79	5.86*	6.71E+06	49.66	716.45
Degradation impurity 2	5.86	5.13E+05	3.47	5.86	4.05E+05	2.99	558.28
Erythromycin B	6.35	1.27E+06	8.59	6.36	1.12E+06	8.31	718.47
Impurity C (Erythromycin E)	6.50	1.14E+06	7.7	6.51	1.09E+06	8.07	748.36
Degradation impurity 6	n.a.	n.a.	n.a.	6.77	9.68E+04	0.72	540.24
Degradation impurity 9	n.a.	n.a.	n.a.	6.95	3.27E+04	0.24	584.29
Unknown impurity -SST	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	732,43

*Retention time of the first-eluting impurity with *m/z* 716.4 (Impurity D)

Conclusion

- A fit-for-purpose LC-MS method to assess the purity profile of erythromycin stressed samples was developed with low effort.
- Mass detection enabled assessment of the relative impurity content in spite of erythromycin A co-eluting with two impurities.
- Injection of less than 3 µg sample was sufficient to obtain satisfactory sensitivity. The amount injected is more than 100 times lower than that recommended by the EP method.

References

1. European Directorate for the Quality of Medicines & HealthCare; European Pharmacopoeia (Ph.Eur.), monograph 0179; erythromycin.
2. European Directorate for the Quality of Medicines & HealthCare European Pharmacopoeia (Ph. Eur.). INFORMATION LEAFLET Ph. Eur. Reference Standard. Erythromycin for system suitability CRS batch 1.
3. Chitneni, S. K., *et al.*, Identification of impurities in erythromycin by liquid chromatography–mass spectrometric detection, *J. Chromatogr. A* **2004,** *1056,* 111–120.

Find out more at **thermofisher.com**

ThermoFisher SCIENTIFIC