Statistic evaluation of mass accuracy measurements by ESI TOF with a sample of degradation products from the antibiotic drug amoxicillin

Application Note

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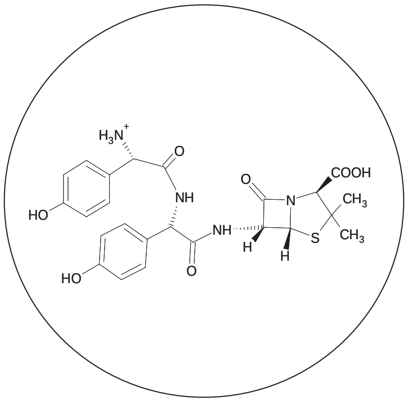

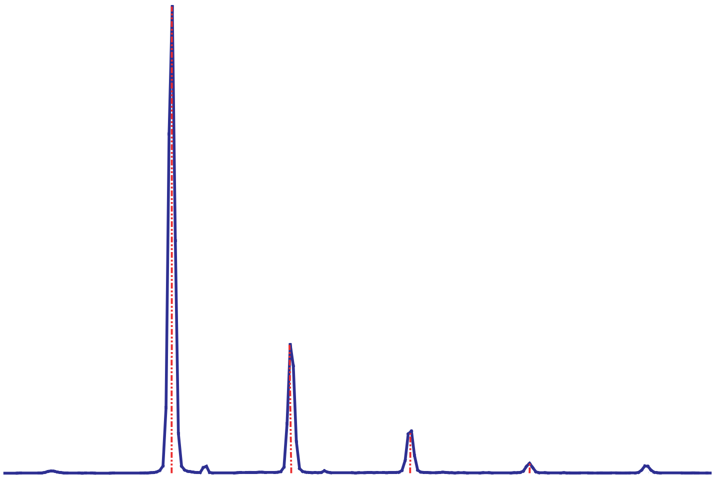

Abstract

The measurement of accurate molecular mass by mass spectrometry and calculation of the corresponding empirical formula is an important step in the identification process of small molecules in a range of application fields. In order to rely on the measured values it is important to know the performance of the mass spectrometer for accurate mass measurement. In this Application Note the mean and standard deviation of repeatedly measured mass accuracies from a real-life pharmaceutical sample will be presented.

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Introduction

For a reliable empirical formula confirmation it is necessary to set a mass accuracy limit which takes the acceptable uncertainty of the accurate molecular mass measurement into consideration. For instance, for a mass measurement of *m/z* 118 (where C_{0-100}, H_{3-74}, O_{0-4} and N_{0-15}) there must be no alternative formulas within 34 ppm before such a claim is made. Increasing the mass measurement to *m/z* 750 (where C_{0-100}, H_{25-110}, O_{0-15} and N_{0-15}) there are 626 alternative formulas within 5 ppm. The error measurement acceptable at *m/z* 750 must be 0.018 ppm to eliminate all alternative formulas[1]. Therefore, it is necessary to know the instrument performance for the determination of accurate molecular mass and the empirical formula. Knowing mean and the standard deviation of measured accurate masses over a certain mass range is of crucial interest to exclude possible empirical formulas, which are outside of the statistic confidence interval[2]. For that purpose there are some methods for the statistical evaluation of accurate mass measurement quality by a mass spectrometer instrument described in applicable literature[3]. Several years ago only operation intensive magnetic sector field and FT mass spectrometers were able to perform these measurements with sufficient mass accuracy. Nowadays, comparably easy-to-use and inexpensive ESI orthogonal acceleration TOF (oaTOF) instruments are also capable of handling this task. This is clearly demonstrated by a comparison study of different types of mass spectrometer instruments for the determination of accurate mass of small molecules[4]. This was made possible by some technical innovations in TOF technology introduced during the past years. One of the main technical innovations is the development of orthogonal acceleration TOF technology, which decouples the ion beam velocity spread from the TOF axis, which provides better resolution of the TOF mass spectrometers[5]. In this environment the coupling of continuous ionization sources such as the electrospray ionization (ESI) source with orthogonal acceleration TOF mass analyzers is of special importance for LC-ESI TOF applications. A high mass accuracy is only achieved when a reference compound, a reference mass, is simultaneously introduced into the mass spectrometer with the analyte itself. Mixing the LC column effluent with a stream of reference material can result in ion suppression, discrimination or adduct formation. To prevent mixing the analyte and the reference compound prior to spray ionization, an innovation which applies a dual ESI sprayer interface is used[6,7]. This instrument is capable of achieving resolutions above 15,000 and mass accuracies in the single digit ppm range for small molecules[4]. In this Application Note the mean and standard deviation of repeatedly measured mass accuracies from a real life pharmaceutical sample of degraded antibiotic amoxicillin will be presented.

Experimental

Equipment

- The ESI TOF MS analysis was performed with the Agilent LC/MSD TOF equipped with a dual sprayer source for the simultaneous infusion of the reference mass solution.
- The LC system used was an Agilent 1100 Series capillary LC system containing a capillary pump with a micro vacuum degasser, a micro well-plate autosampler with a thermostat and a column compartment.
- The column used was a ZORBAX SB Aq, 0.3 mm x 150 mm, 3.5 µm.
- The software used for instrument control was TOF software A01.01 and for data analysis Analyst QS software.

Methods

- The Agilent 1100 Series capillary pump was operated under the following conditions:
  Solvent A: water, 10 mM ammonium formate, pH 4.1;
  Solvent B: ACN
  Column flow: 8 µL/min,
  Primary flow: 500-800 µL/min
  Gradient: 0 min 0 % B, 1 min 0 % B, 13 min 25 % B, 23 min 25 % B
  Stop time: 23 min
  Post time: 15 min.
- The Agilent 1100 Series autosampler was used to make injections of 1 µL sample and the samples were cooled. The sample loop was switched to bypass after 1 minute to reduce delay volume.
- The mass spectrometer was operated under the following conditions:
  Source: ESI in
  positive mode with dual spray for reference mass.
  Dry gas: 7.0 L/min
  Dry Temp.: 300 °C
  Nebulizer: 15 psi
  Scan: 50-1000
  Fragmentor: 300 V for CID
  Skimmer: 60 V
  Capillary: 5000 V
- Sample preparation: The antibi-

otic amoxicillin was stressed under acidic conditions. Approximately 1 mL of amoxicillin solution (25 mg/mL in DMSO) was added to 1 mL 0.1 M HCl solution. The sample was stirred for 1 hour at room temperature (RT = 25 °C) and then diluted (1:10 with DMSO).

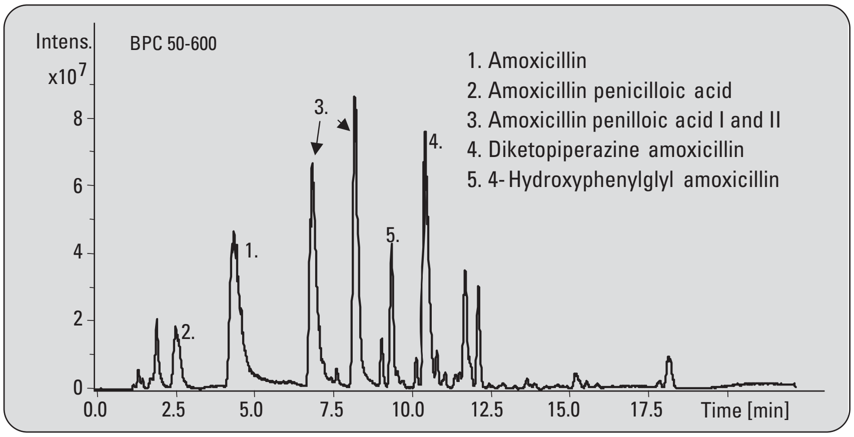


Figure 1
BPC of amoxicillin (1) and its degradation products after acid exposure..

Results and discussion

The complex real life pharmaceutical sample, which was used for this instrument performance evaluation under real application conditions, is a mixture of degradation products of the antibiotic drug amoxicillin obtained by acid treatment of the pharmaceutical drug substance. Five compounds, which were identified in an earlier examination of this sample[8,9], were used for the performance evaluation of accurate mass measurement by the ESI oaTOF (figure 1). In this earlier work the structure elucidation of the degradation products was done by ion trap mass spectrometry[8] and the final identity confirmation was performed by accurate mass measurement and empirical formula calculation using the ESI oaTOF[9]. Herein, the sample was measured once and the mass accuracy of each compound was determined. To also confirm the molecular identity of the fragments obtained earlier in the ion trap experiment, the ESI oaTOF experiment was repeated at a higher capillary voltage to induce CID fragmentation. With the obtained data of high mass accuracy the compounds could be identified with sufficient confidence (figure 2A - E).
The obtained set of data, consisting of the accurate mass of the molecular ions and the corresponding fragments for each compound, was not enough to make a reliable statistical analysis. Therefore, the measurement was repeated five times to obtain enough data points to make a legitimate statistical statement. Altogether, the five experiments provided 135 data points between *m/z* 114 and *m/z* 515 comprising the molecular ions and the fragments of the compounds (table 2A – E). The tables show the individually measured masses of the molecular ions and their fragments as well as the individual mean mass and standard deviation thereof. For each measured mass the mass accuracy was calculated in mDa and ppm. The calculated mean and its standard deviation of the mDa and ppm values are shown in table 2A – E. To obtain the value and the confidence interval of the accuracy performance over the used mass range the mean and the standard deviation of all mDa and ppm accuracy data was calculated. The overall mass accuracy was calculated as 1.73 ppm with a standard deviation of 0.97 respective to 0.39 mDa with a standard deviation of 0.21. The standard deviation (s) gives the confidence interval with a probability to find the measured value around the mean. The confidence interval of 3s contains the value with 99.7 % probability. Therefore, for one of the measured masses of 4-hydroxy-phenylglycyl amoxicillin **5** all possible empirical formulas within a window of 3 ppm around the measured mass at *m/z* 515.1596 were calculated. Within this mass accuracy window and a possible formula in the range of $C_{0-100}H_{0-200}N_{0-10}O_{0-10}S_{0-5}$ there are 12 possible empirical formulas. To find the right empirical formula out of this set of possible formulas an isotopic intensity analysis of the mass spectrum by comparison to a calculated isotopic ratio was done (figure 3). The isotopic ratio analysis showed clearly that only the calculated empirical formula for 4-hydroxyphenylglycyl amoxicillin **5**, which contains one sulfur atom, exactly matches the measured isotopic ratios. All other empirical formulas in the 3 ppm mass accuracy window contained none or more than one sulfur atom, which resulted in an isotopic ratio easily distinguished from the measured isotopic ratio.

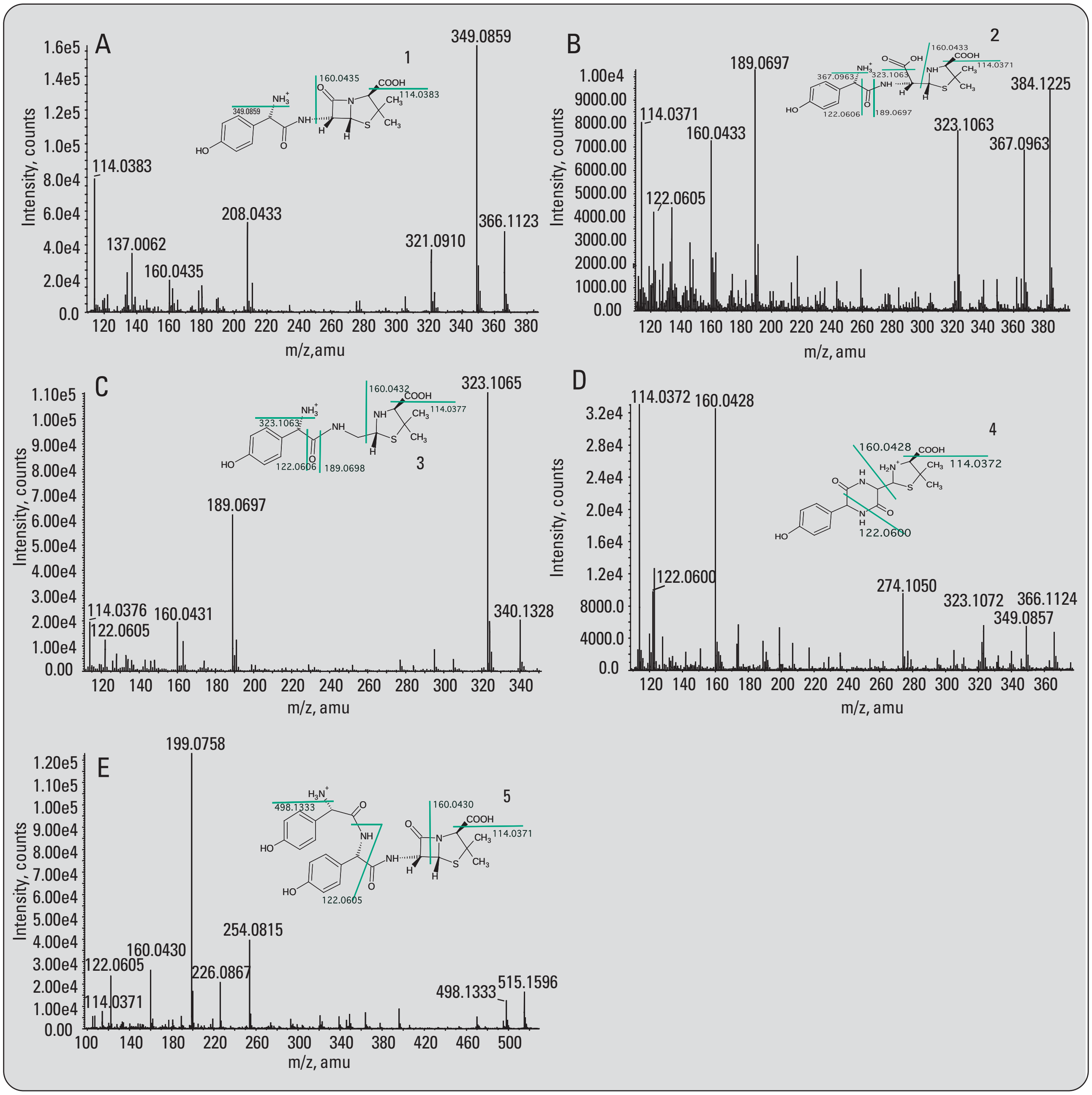


Figure 2

ESI oaTOF analysis of amoxicillin (1) and its degradation products after acidic exposure for accurate mass measurement and compound confirmation

A) amoxicillin (1) ($C_{16}H_{19}N_3O_5S$), $[M+H^+]^+$ = *m/z* 366.1124

B) amoxicillin penicilloic acid (2) ($C_{16}H_{21}N_3O_6S$), $[M+H]^+$ = *m/z* 384.1229

C) amoxicillin penilloic acid I and II (3) ($C_{15}H_{21}N_3O_4S$), $[M+H]^+$ = *m/z* 340.1331

D) diketopiperazine amoxicillin (4) ($C_{16}H_{19}N_3O_5S$), $[M+H]^+$ = *m/z* 366.1124

E) 4-hydroxyphenylglycyl amoxcillin (5) ($C_{24}H_{26}N_4O_7S$), $[M+H]^+$= *m/z* 515.1600

Measured mass	Mean SD [x10^{-4}]	Calculated mass	Formula	Mass accuracy [mDa]	Mean [mDa] SD [mDa]	Mass accuracy [ppm]	Mean [ppm] SD [ppm]
366.1123				-0.10		0.27	
366.1125	366.1125			0.10	0.14	-0.27	0.41
366.1127		366.1124	$C_{16}H_{20}N_3O_5S$	0.30		-0.82	
366.1126	1.80			0.20	0.11	-0.55	0.33
366.1123				-0.10		0.27	
349.0859				0.10		-0.28	
349.0862	349.0859			0.40	0.30	-1.14	0.86
349.0865		349.0858	$C_{16}H_{17}N_2O_5S$	0.70		-2.00	
349.0861	2.50			0.30	0.27	-0.86	0.71
349.0859				0.10		-0.28	
160.0435				0.30		-1.87	
160.0434	160.0435			0.20	0.26	-1.25	1.46
160.0434		160.0432	$C_6H_{10}NO_2S$	0.20		-1.25	
160.0435	0.50			0.30	0.05	-1.87	0.34
160.0435				0.30		-1.87	
114.0383				0.60		-5.26	
114.0372	114.0378			-0.50	0.42	4.38	3.60
114.0374		114.0377	C_5H_8NS	-0.30		2.63	
114.0382	4.80			0.50	0.16	-4.38	1.45
114.0379				0.20		-1.75	

Table 1A

Summary of the mass accuracy and precession data measurement for the Amoxicillin (1) and its degradation products and their CID fragments measured with the ESI TOF (mean and standard deviation SD of the ppm values)

A) amoxicillin (1) ($C_{16}H_{19}N_3O_5S$), [M+H$^+$]$^+$ = *m/z* 366.1124

Measured mass	Mean SD [x10^{-4}]	Calculated mass	Formula	Mass accuracy [mDa]	Mean [mDa] SD [mDa]	Mass accuracy [ppm]	Mean [ppm] SD [ppm]
384.1225				-0.40		1.04	
384.1225	384.1226			-0.40	0.38	1.04	0.98
384.1230		384.1229	$C_{16}H_{22}N_3O_6S$	0.10		-0.26	
384.1221	4.10			0.80	0.26	-2.08	0.69
384.1231				0.20		-0.52	
367.0963				-0.10		0.27	
367.0961	367.0965			-0.30	0.38	0.82	1.03
367.0969		367.0964	$C_{16}H_{19}N_2O_6S$	0.50		-1.36	
367.0960	4.61			-0.40	0.19	1.09	0.53
367.0970				0.60		-1.63	
323.1063				-0.30		0.93	
323.1069	323.1068			0.30	0.34	-0.93	1.05
323.1071		323.1066	$C_{15}H_{19}N_2O_2S$	0.50		-1.55	
323.1065	3.63			-0.10	0.16	0.31	0.51
323.1071				0.50		-1.55	
189.0697				-0.10		0.53	
189.0699	189.0700			0.10	0.18	-0.53	0.95
189.0699		189.0698	$C_7H_{13}N_2O_2S$	0.10		-0.53	
189.0700	1.81			0.20	0.13	1.05	0.68
189.0702				0.40		2.11	
160.0433				0.10		-0.62	
160.0434	160.0435			0.20	0.26	-1.25	1.62
160.0434		160.0432	$C_6H_{10}NO_2S$	0.20		-1.25	
160.0438	1.95			0.60	0.19	-3.75	1.21
160.0434				0.20		-1.25	
122.0605				-0.10		0.82	
122.0605	122.0605			-0.10	0.20	0.82	1.36
122.0605		122.0606	C_7H_8NO	-0.10		0.82	
122.0610	2.60			0.40	0.14	-2.50	0.78
122.0603				-0.30		1.87	
114.0371				-0.60		5.26	
114.0375	114.0375			-0.20	0.30	1.75	1.57
114.0380		114.0377	C_5H_8NS	0.30		-2.63	
114.0375	3.19			-0.20	0.17	1.75	0.95
114.0375				-0.20		1.75	

Table 1B

Summary of the mass accuracy and precession data measurement for the Amoxicillin (1) and its degradation products and their CID fragments measured with the ESI TOF (mean and standard deviation SD of the ppm values)

B) amoxicillin penicilloic acid (2) ($C_{16}H_{21}N_3O_6S$), [M+H]$^+$ = *m/z* 384.1229

Measured mass	Mean SD [x10^{-4}]	Calculated mass	Formula	Mass accuracy [mDa]	Mean [mDa] SD [mDa]	Mass accuracy [ppm]	Mean [ppm] SD [ppm]
340.1328				-0.30		0.88	
340.1334	340.1333			0.30	0.36	-0.88	1.05
340.1332		340.1331	$C_{16}H_{22}N_3O_4S$	0.10		-0.29	
340.1338	3.71			0.70	0.22	-2.06	0.44
340.1335				0.40		-1.17	
323.1065				-0.10		0.31	
323.1065	232.1065			-0.10	0.28	0.31	0.86
323.1062		323.1066	$C_{15}H_{19}N_2O_4S$	-0.40		1.24	
323.1062	3.27			-0.40	0.16	1.24	0.50
323.1070				0.40		-1.24	
189.0697				-0.10		0.53	
189.0699	189.0699			0.10	0.20	-0.53	1.06
189.0696		189.0698	$C_7H_{13}N_2O_2S$	-0.20		1.05	
189.0703	2.68			0.50	0.17	-2.64	0.91
189.0699				0.10		-0.53	
160.0431				-0.10		0.62	
160.0433	160.0433			0.10	0.24	-0.62	1.49
160.0429		160.0432	$C_6H_{10}NO_2S$	-0.30		1.87	
160.0438	3.36			0.60	0.22	-3.75	1.37
160.0432				-0.10		0.62	
122.0605				-0.10		0.82	
122.0605	122.0605			-0.10	0.42	0.82	3.43
122.0598		122.0606	C_7H_8NO	-0.80		6.55	
122.0613	5.50			0.70	0.32	-5.73	2.67
122.0602				-0.40		3.27	
114.0376				-0.10		0.87	
114.0373	114.0374			-0.40	0.30	3.50	2.62
114.0372		114.0377	C_5H_8NS	-0.50		4.38	
114.0378	2.51			0.10	0.18	-0.87	1.64
114.0373				-0.40		3.50	

Table 1C
Summary of the mass accuracy and precession data measurement for the amoxicillin (1) and its degradation products and their CID fragments measured with the ESI TOF (mean and standard deviation SD of the ppm values)
C) amoxicillin penilloic acid I and II (3) ($C_{15}H_{21}N_3O_4S$), $[M+H]^+$ = *m/z* 340.1331

Measured mass	Mean SD [x10^{-4}]	Calculated mass	Formula	Mass accuracy [mDa]	Mean [mDa] SD [mDa]	Mass accuracy [ppm]	Mean [ppm] SD [ppm]
366.1124				-0.10		0.27	
366.1127	366.1126			0.20	0.16	-0.54	0.42
366.1126		366.1125	$C_{16}H_{20}N_3O_5S$	0.10		-0.27	
366.1127	1.30			0.20	0.05	-0.54	0.14
366.1127				0.20		-0.54	
349.0857				-0.10		0.22	
349.0870	349.0860			1.20	0.68	-3.44	1.92
349.0848		349.0858	$C_{16}H_{17}N_2O_5S$	-1.00		2.86	
349.0868	8.90			1.00	0.53	-2.86	1.56
349.0859				0.10		-0.22	
160.0428				-0.40		2.50	
160.0434	160.0433			0.20	0.32	-1.25	1.99
160.0431		160.0432	$C_6H_{10}NO_2S$	-0.10		0.62	
160.0434	4.08			0.20	0.24	-1.25	1.49
160.0439				0.70		-4.37	
122.0600				-0.60		4.92	
122.0605	122.0604			-0.10	0.24	0.82	1.96
122.0604		122.0606	C_7H_8NO	-0.20		1.64	
122.0605	2.10			-0.10	0.20	0.82	1.70
122.0604				-0.20		1.64	
114.0372				-0.50		4.38	
114.0372	114.0376			-0.10	0.18	0.88	1.58
114.0372		114.0377	C_5H_8NS	-0.10		0.88	
114.0372	2.45			0.10	0.17	-0.88	1.56
114.0372				0.10		-0.88	

Table 1D
Summary of the mass accuracy and precession data measurement for the amoxicillin (1) and its degradation products and their CID fragments measured with the ESI TOF (mean and standard deviation SD of the ppm values)
D) diketopiperazine amoxicillin (4) ($C_{16}H_{19}N_3O_5S$), $[M+H]^+$ = *m/z* 366.1124

Measured mass	Mean SD $[x10^{-4}]$	Calculated mass	Formula	Mass accuracy [mDa]	Mean [mDa] SD [mDa]	Mass accuracy [ppm]	Mean [ppm] SD [ppm]
515.1596				-0.40		0.76	
515.1605	515.1607			0.50	0.84	-0.97	1.63
515.1608		515.1600	$C_{24}H_{27}N_4O_7S$	0.80		-1.55	
515.1613	6.83			1.30	0.40	-2.52	0.80
515.1612				1.20		-2.39	
498.1333				-0.20		0.40	
498.1342	498.1343			0.70	0.88	-1.40	1.76
498.1338		498.1335	$C_{24}H_{24}N_3O_7S$	0.30		-0.60	
498.1349	8.09			1.40	0.69	-2.81	1.40
498.1353				1.80		-3.61	
160.0430				-0.20		1.25	
160.0434	160.0436			0.20	0.50	-1.25	3.25
160.0434		160.0432	$C_6H_{10}NO_2S$	0.20		-1.25	
160.0440	5.55			0.80	0.43	-4.99	2.87
160.0444				1.12		-7.50	
122.0605				-0.10		0.82	
122.0605	122.0608			-0.10	0.34	0.82	2.79
122.0605		122.0606	C_7H_8NO	-0.10		0.82	
122.0611	4.60			0.50	0.35	-4.10	2.94
122.0615				0.90		-7.40	
114.0371				-0.60		5.46	
114.0369	114.0372			-0.80	0.48	7.01	4.03
114.0374		114.0377	C_5H_8NS	-0.30		2.63	
114.0371	2.77			-0.60	0.27	5.26	2.43
114.0376				-0.10		0.87	

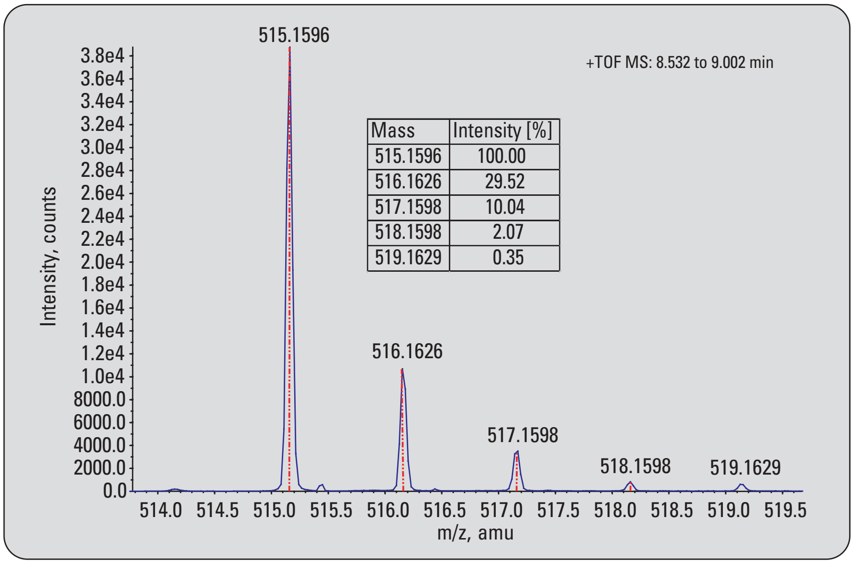
Table 1E

Summary of the mass accuracy and precession data measurement for the amoxicillin (1) and its degradation products and their CID fragments measured with the ESI TOF (mean and standard deviation SD of the ppm values)

E) 4-hydroxyphenylglycyl amoxcillin (5) ($C_{24}H_{26}N_4O_7S$), $[M+H]^+$= *m/z* 515.1600

Conclusion

For the determination of an empirical formula it is of crucial importance to work with a mass spectrometer, which can measure accurate molecular masses with the highest possible accuracy to minimize the number of possible formulas in a given mass accuracy window around the measured mass value. This Application Note evaluates the mean mass accuracy and its standard deviation achievable by means of the ESI oaTOF instrument under real life conditions with a pharmaceutical sample of the degraded antibiotic drug amoxicillin. The statistic evaluation of the obtained data showed that an empirical formula of an unknown compound could be expected in a mass accuracy window of 3 ppm around the measured mass value with a reliability of 99.7% (3s). It is common for higher molecular weight compounds to have more than one possible empirical formula within this mass accuracy window. To determine the right formula an additional analysis of the measured isotopic ratio of the molecular ion by comparing it to a calculated isotopic ratio is outlined.



Mass	Intensity [%]
515.1596	100.00
516.1626	29.52
517.1598	10.04
518.1598	2.07
519.1629	0.35

Figure 3

Measured isotopic ratio of 4-hydroxyphenylglycyl amoxicillin overlaid by the calculated isotopic ratio (red line) and calculated intensities for the isotopic mass peaks.

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