Structure elucidation of degradation products of the antibiotic drug amoxicillin

Part II: Identification and confirmation by accurate mass measurement with ESI TOF of the compound ions and the fragments after CID

Application Note

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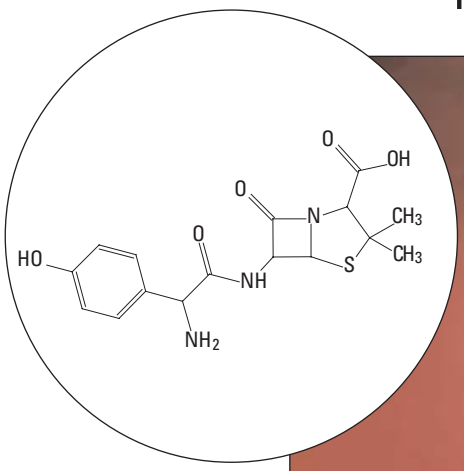

Abstract

Today, it is necessary to identify and confirm the identity of all compounds appearing in the process of the discovery and the development of a new drug substance in the pharmaceutical industry.
In this Application Note the confirmation of degradation products from a final dosage of the antibiotic drug amoxicillin obtained under stress conditions, whose structures were elucidated by ion trap MS/MS and MS^3 in a former work (Part I[1]), will be demonstrated by accurate mass measurement and empirical formula confirmation for the molecular ions and for the CID fragments by LC/ESI oaTOF.

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Introduction

In modern pharmaceutical drug discovery and development it is of crucial importance to identify an unknown compound with the highest possible confidence because of its potential toxic effects on humans. Such a compound could be, for instance, the pharmaceutical active substance itself, a minor byproduct out of the production process, a secondary substance in a drug isolated from a natural source, a metabolite created in the human body or a degradation product of the pharmaceutical agent created under storage conditions. In addition to the repertoire of analytical methods for structure elucidation, the mass spectrometric measurement of accurate molecular mass and consequently the calculation of the empirical formula is a common method for the identification and identity confirmation of an unknown compound. Several years ago only operation intensive magnetic sector field and FT mass spectrometers were able to perform these measurements with sufficient 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[2]. 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[3]. In this environment the possibility of coupling continuous ionization sources like 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 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, a innovation which applies a dual ESI sprayer interface is used[4,5]. This instrument is capable of achieving resolutions above 15,000 and mass accuracies below 1 ppm for small molecules[2]. Recently, the implementation of oaTOF instruments for the measurements of accurate molecular mass and the calculation of the total formula and consequently the identity confirmation of an unknown compound was impressively demonstrated in a considerably high number of published applications[6-11]. For instance, LC-ESI oaTOF was used for the characterization of in vitro drug metabolites by accurate mass determination of the molecular ion and CID fragments[6,7], for the quantization and accurate mass measurement of pharmaceutical drugs in plasma[8], for the characterization of trace level impurities in a drug substance[9], with an additional ion trap instrument for MS/MS and MS^3 experiments to gain structural information for the identification of a photooxygenation product of a broadspectrum antigiotic used for live stock[10] and for the identification of highly complex polyene macrolides isolated from *Streptomyces noursei* by means of an ion trap monitored purification process[11]. In this Application Note the confirmation of formerly identified degradation products from the antibiotic drug amoxicillin obtained under harsh conditions will be demonstrated by means of accurate mass measurement of the molecular ions and CID fragments using an LC/ESI oaTOF for the confirmation of the molecular formula and the fragment formulas. The structure elucidation with an LC ion trap MS/MS and MS^3 is discussed in Part I of this work[1]. Both sets of data are combined and used to build up a degradation pathway of amoxicillin.

Experimental

Equipment

- The ESI ion trap (Part I of this work) and the ESI TOF MS analysis (Part II of this work) was performed with the Agilent 1100 Series LC/MSD ion trap XCT plus and 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 ChemStation A10.02, ion trap software 4.2, TOF software A.01 and for data analysis the ion trap data analysis software 3.2 and 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. The sample loop was switched to bypass after 1 minute to reduce delay volume.
- The mass spectrometers were operated under the following conditions:

  Part I – Ion Trap MS:
  Source: ESI in positive mode.
  Dry gas: 5.0 L/min
  Dry temp.: 300 °C
  Nebulizer: 15 psi
  Target: 150,000
  Max. accum. time: 50 ms
  Scan: 200-600
  Averages: 2
  Automated MS/MS and MS^3

  Part II – ESI TOF MS:
  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: 150 V or 300 V for CID
  Skimmer: 60 V
  Capillary: 5000 V

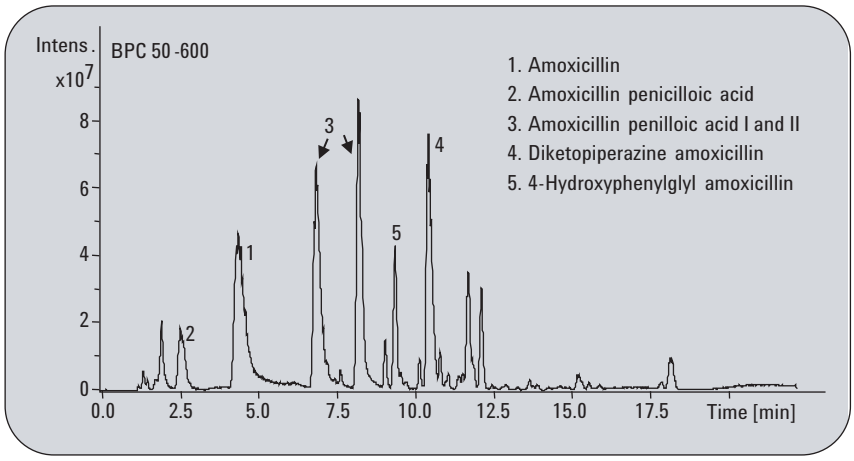


Figure 1
BPC of amoxicillin (1) and its degradation products

- Sample preparation: The antibiotic 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).

Results and discussion

The degradation of amoxicillin (1) was induced by subjecting the pure drug substance to harsh conditions, as described in the

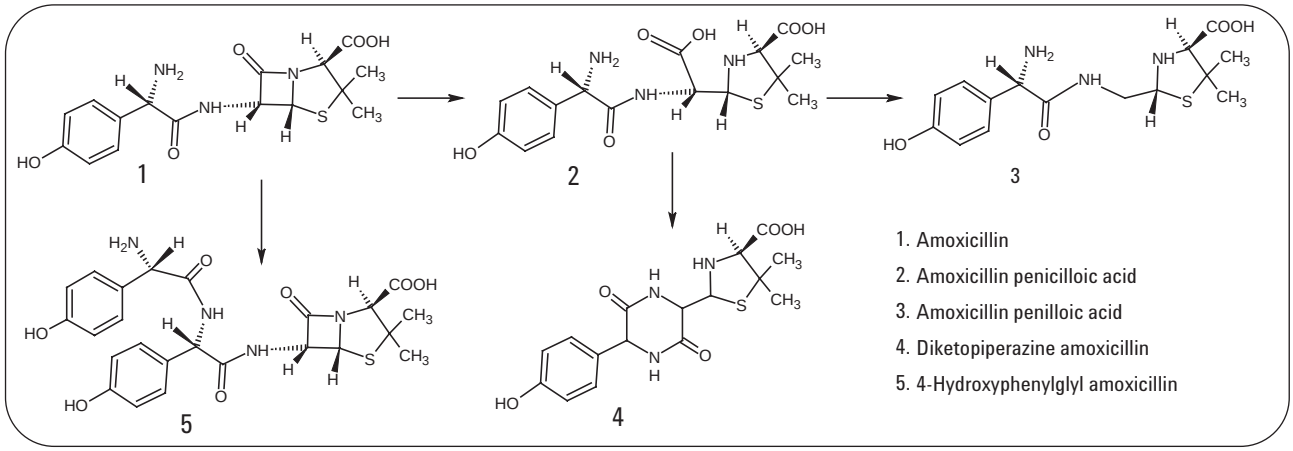
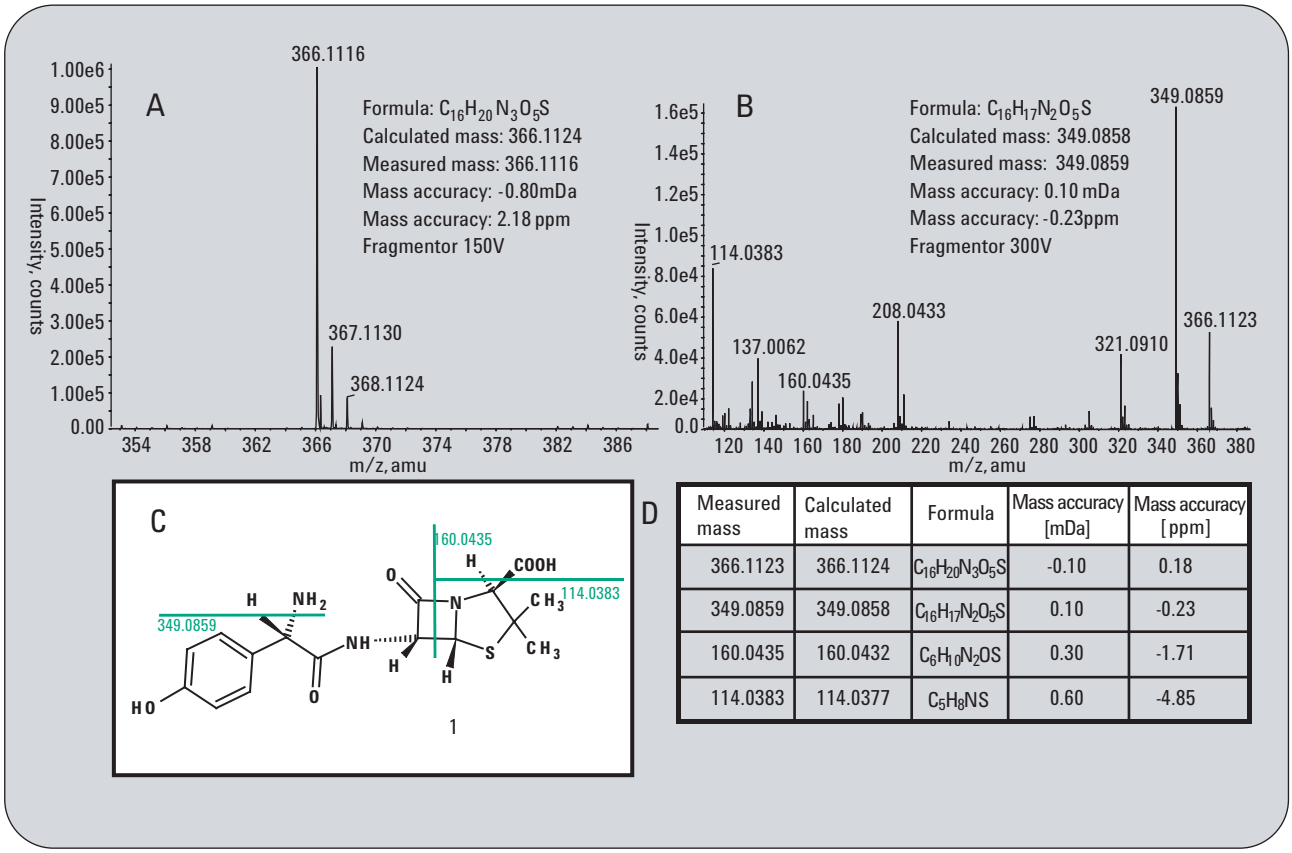


Figure 2
Degradation pathway of amoxicillin

experimental section. Aliquots of this solution were collected at various timeframes and subjected to capillary chromatography to separate the accumulated degradation products. The base peak chromatogram (BPC) obtained clearly shows the degradation of amoxicillin into various products (figure 1). The chromatogram assigns the degradation products, which were identified by structure elucidation with ion trap MS/MS and MS^3 experiments (see Part I of this work[1]). The final degradation pathway of amoxicillin (1) created from the results obtained in Part I with the identified degradation products is summarized in figure 2. For the identity confirmation by accurate mass measurement and formula calculation of the proposed intermediates (figure 2), which are involved in the degradation pathway of amoxicillin (1) the analysis was performed on an ESI oaTOF instrument connected to a capillary LC for separation of the amoxicillin degradation products. The experiment was performed twice using different fragmentor voltages of 150 V and 300 V in the TOF MS. At a fragmentor voltage of 150 V there is no collision induced dissociation (CID) observed whereas a good fragmentation for the molecular ion from the separated degradation products are observed at a voltage of 300 V. The measured molecular mass for amoxicillin (1) at *m/z* 366.1116 has a deviance of 0.08 mDa or 2.18 ppm to the calculated molecular mono isotopic mass (figure 3A). For structural confirmation a useful fragmentation for amoxicillin (1) besides the molecular

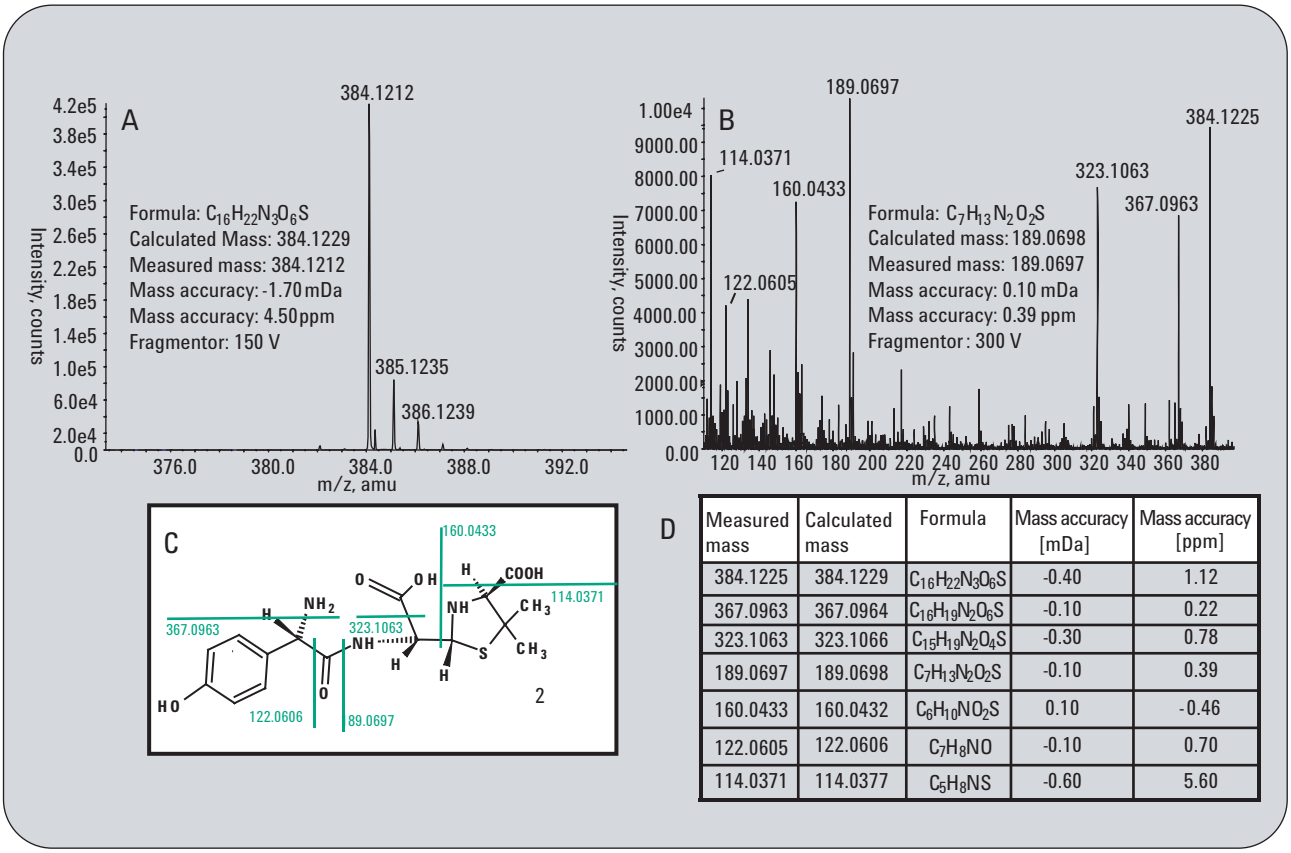


Measured mass	Calculated mass	Formula	Mass accuracy [mDa]	Mass accuracy [ppm]
366.1123	366.1124	$C_{16}H_{20}N_3O_5S$	-0.10	0.18
349.0859	349.0858	$C_{16}H_{17}N_2O_5S$	0.10	-0.23
160.0435	160.0432	$C_6H_{10}N_2OS$	0.30	-1.71
114.0383	114.0377	C_5H_8NS	0.60	-4.85

Figure 3
Amoxicillin (1), ($C_{16}H_{19}N_3O_5S$), $[M+H]^+$ = 366.1124 *m/z*
A) Accurate mass measurement of the molecular ion of amoxicillin.
B) Accurate mass measurement of the CID fragment ions of amoxicillin.
C) CID fragmentation pattern of amoxicillin.
D) Mass accuracy of all CID fragments of amoxicillin.

ion is obtained by the application of an increased fragmentor voltage up to 300 V (figure 3B). The main fragment shown in the mass spectrum is the product of a loss of ammonium at *m/z* 349.0859, which has a deviance to the calculated mass of 0.1 mDa and 0.23 ppm. The formula $C_6H_{10}NO_2S$ of the fragment at *m/z* 160.0435 obtained by the cleavage of the five membered thiazolidine ring from the molecule ion is calculated with a mass accuracy of 0.3 mDa and 1.71 ppm. This is the only elemental formula calculated given the measured mass and a 5 ppm mass accuracy window. This gives undoubted evidence of the chemical structure. The complete fragmentation is shown in figure 3C and the mass accuracy of each fragment is outlined in table 3D.

The first degradation product of amoxicillin (1) obtained after breaking the four membered beta lactam ring amoxicillin penicilloic acid (2) was confirmed by accurate mass measured at *m/z* 384.1212, with 4.50 ppm mass accuracy and empirical formula calculation with the ESI TOF at a fragmentor voltage of 150 V (figure 4A). The structure of this degradation product was confirmed by the appearance of the special fragment at *m/z* 323.1063 with 0.78 ppm mass accuracy, which is the product of a decarboxylation reaction (figure 4B). The complete fragmentation pattern of amoxicillin penicilloic acid (2) also reveals fragments at

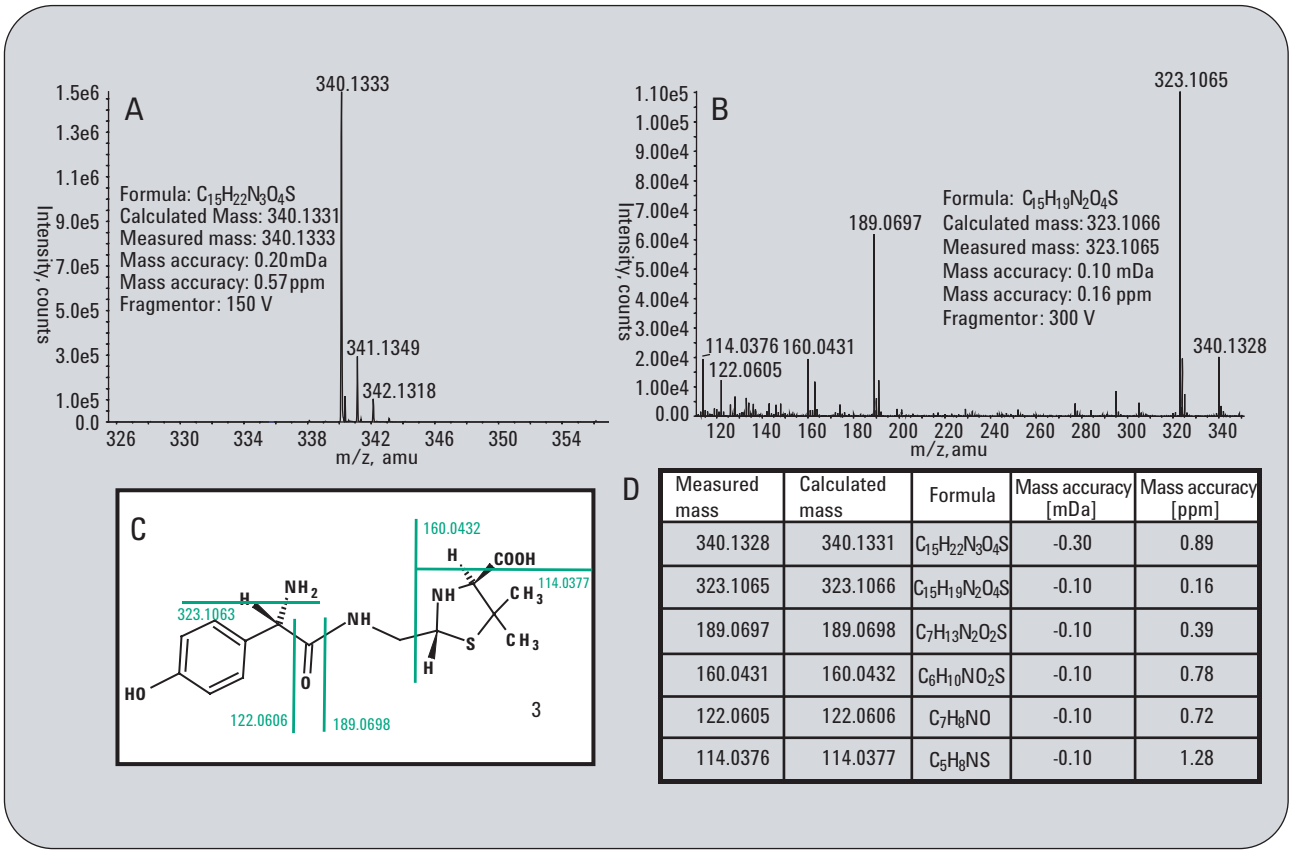


Measured mass	Calculated mass	Formula	Mass accuracy [mDa]	Mass accuracy [ppm]
384.1225	384.1229	$C_{16}H_{22}N_3O_6S$	-0.40	1.12
367.0963	367.0964	$C_{16}H_{19}N_2O_6S$	-0.10	0.22
323.1063	323.1066	$C_{15}H_{19}N_2O_4S$	-0.30	0.78
189.0697	189.0698	$C_7H_{13}N_2O_2S$	-0.10	0.39
160.0433	160.0432	$C_6H_{10}NO_2S$	0.10	-0.46
122.0605	122.0606	C_7H_8NO	-0.10	0.70
114.0371	114.0377	C_5H_8NS	-0.60	5.60

Figure 4
Amoxicillin penicilloic acid (2), ($C_{16}H_{21}N_3O_6S$), $[M+H]^+$ = 384.1229 *m/z*
A) Accurate mass measurement of the molecular ion of amoxicillin penicilloic acid.
B) Accurate mass measurement of the CID fragment ions of amoxicillin penicilloic acid.
C) CID fragmentation pattern of amoxicillin penicilloic acid.
D) Mass accuracy of all CID fragments of amoxicillin penicilloic acid.

m/z 122.0606 and at *m/z* 189.0697 with high mass accuracy, which are important for the structure confirmation (figures 4B and 4C). The complete information obtained from the CID fragments of amoxicillin penicilloic acid (2) is summarized in table 4D with their empirical formula and the measured mass accuracies.

The subsequent degradation products obtained from amoxicillin penicilloic acid (2) by a decarboxylation of the free carboxylic acid group are the stereoisomeric amoxicillin penilloic acids I and II (3). Their identity was confirmed by accurate mass measurement and formula confirmation at *m/z* 340.1333 with a deviation of 0.20 mDa, 0.57 ppm from the theoretical mass ($C_{15}H_{22}N_3O_4S$) (figure 5A). The main fragment ion obtained in the CID spectrum is the product from a loss of ammonium at *m/z* 323.1065 (figure 5B). The same fragment ion was obtained as a product after the loss of ammonia followed by a decarboxylation from amoxicillin penicilloic acid (2) (figure 4B) and therefore confirms the identity of the degradation products amoxicillin penilloic acid I and II (3). The identified fragmentations and fragment ions are outlined in fig-



Measured mass	Calculated mass	Formula	Mass accuracy [mDa]	Mass accuracy [ppm]
340.1328	340.1331	$C_{15}H_{22}N_3O_4S$	-0.30	0.89
323.1065	323.1066	$C_{15}H_{19}N_2O_4S$	-0.10	0.16
189.0697	189.0698	$C_7H_{13}N_2O_2S$	-0.10	0.39
160.0431	160.0432	$C_6H_{10}NO_2S$	-0.10	0.78
122.0605	122.0606	C_7H_8NO	-0.10	0.72
114.0376	114.0377	C_5H_8NS	-0.10	1.28

Figure 5

Amoxicillin penilloic acid I and II (3), ($C_{15}H_{21}N_3O_4S$), $[M+H]^+$ = 340.1331

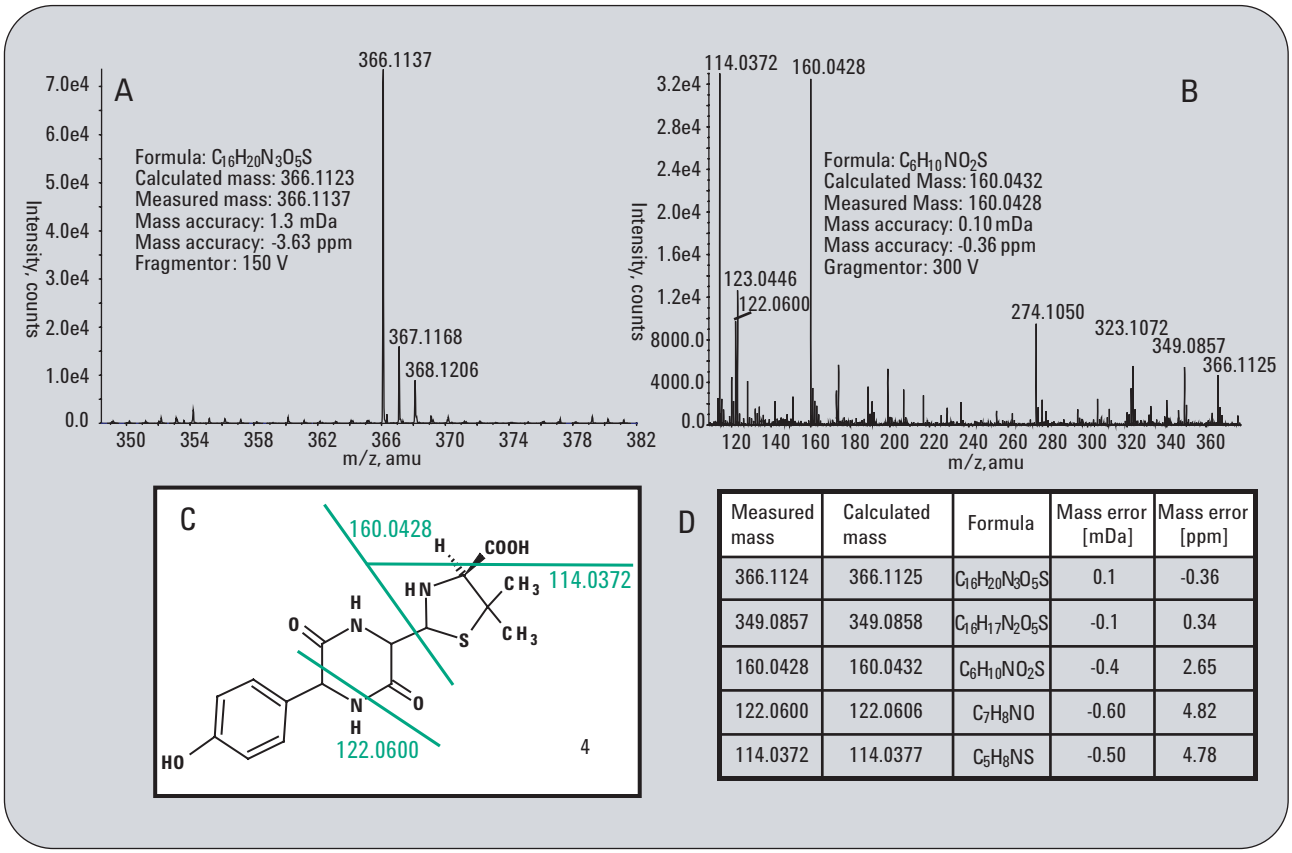
A) Accurate mass measurement of the molecular ion of amoxicillin penilloic acid I and II.

B) Accurate mass measurement of the CID fragment ions of amoxicillin penilloic acid I and II.

C) CID fragmentation pattern of amoxicillin penilloic acid I and II.

D) Mass accuracy of all CID fragments of amoxicillin penilloic acid I and II.

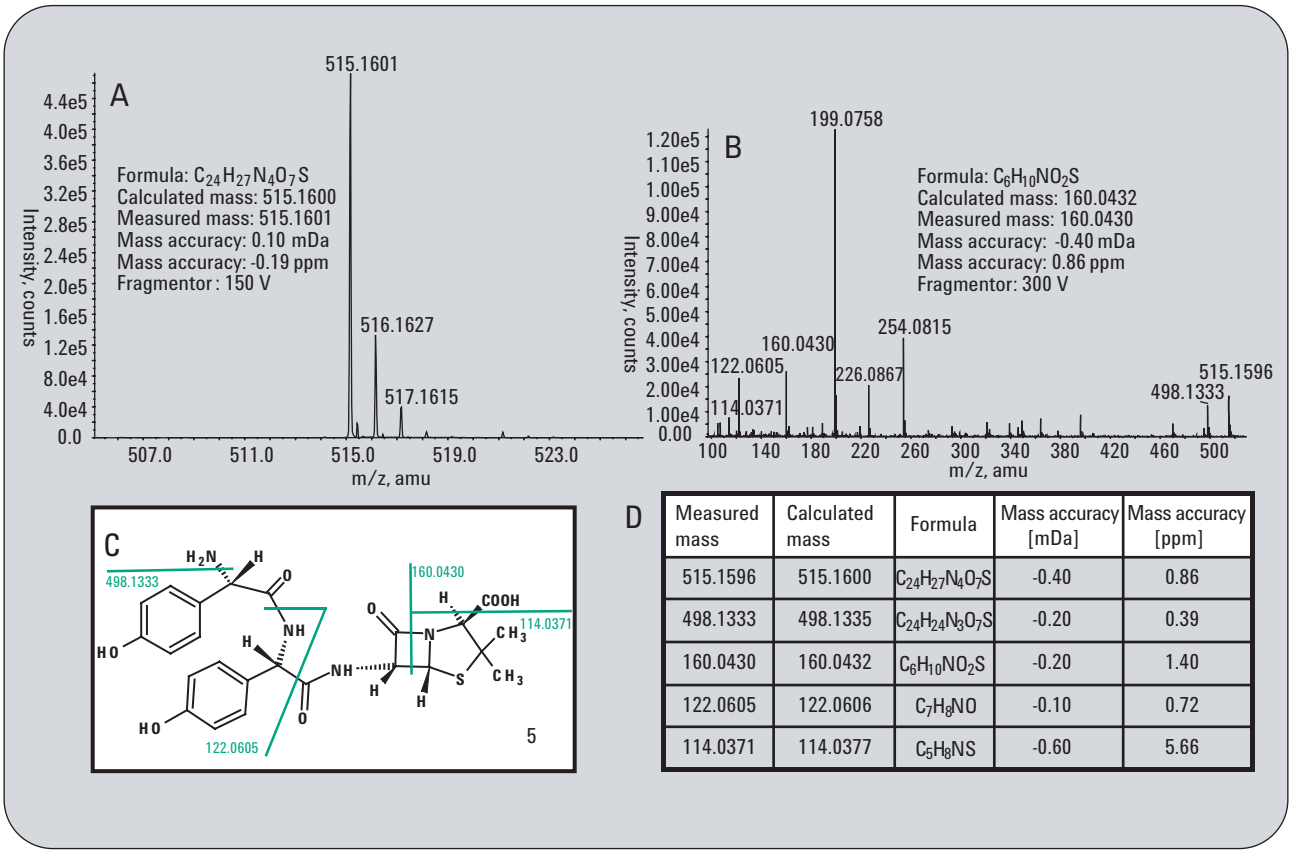
ure 5C and summarized in table 5D. The stereo isomeric forms of (3) resolved in the peaks at 6.7-7.3 min and 8.5-9.0 min gave the same mass accuracies and the same CID fragments. Beginning with amoxicillin penicilloic acid (2), the degradation pathway also leads to diketopiperazine amoxicillin (4) by a formation of a six membered ring structure (figure 6). The molecular identity of the protonated molecular ion given by the formula $C_{16}H_{19}N_3O_5S$ was confirmed by accurate mass measurement with the ESI TOF at *m/z* 366.1137 with 3.63 ppm accuracy (figure 6A). The main CID fragment accrues from a cleavage of the molecule at the bond between the six and the fife membered ring and belongs to the fife membered thiazolidine ring at *m/z* 160.0428 with a mass accuracy of 2.65 ppm (figure 6B). The confirmed fragmentation is shown in figure 6C and the fragments are summarized in table 6D.



Measured mass	Calculated mass	Formula	Mass error [mDa]	Mass error [ppm]
366.1124	366.1125	$C_{16}H_{20}N_3O_5S$	0.1	-0.36
349.0857	349.0858	$C_{16}H_{17}N_2O_5S$	-0.1	0.34
160.0428	160.0432	$C_6H_{10}NO_2S$	-0.4	2.65
122.0600	122.0606	C_7H_8NO	-0.60	4.82
114.0372	114.0377	C_5H_8NS	-0.50	4.78

Figure 6
Diketopiperazine amoxicillin (4) ($C_{16}H_{19}N_3O_5S$) $[M+H]^+$ = 366.1125 *m/z*
A) Accurate mass measurement of the molecular ion of diketopiperazine amoxicillin.
B) Accurate mass measurement of the CID fragment ions of diketopiperazine amoxicillin.
C) CID fragmentation pattern of diketopiperazine amoxicillin.
D) Mass accuracy of all CID fragments of diketopiperazine amoxicillin.

Finally, the chemical identity of the product obtained from a self-condensation reaction of Amoxicillin (1) to 4-Hydroxyphenylglyl amoxcillin (5) was confirmed by means of the ESI TOF instrument. The molecular ion at *m/z* 515.1601 with the calculated formula $C_{24}H_{27}N_4O_7S$ was confirmed with a mass accuracy of 0.19 ppm (figure 7A). The CID fragments useful for the identification come from a loss of ammonium, a loss of the fife membered thiazolidine ring from the parent molecule and from a residual benzyl imminium ion (figures 7B and 7C). The fragment ions are measured with sufficient accuracy for unambiguous identification of the fragments and consequently the molecule (table 7D).



Measured mass	Calculated mass	Formula	Mass accuracy [mDa]	Mass accuracy [ppm]
515.1596	515.1600	$C_{24}H_{27}N_4O_7S$	-0.40	0.86
498.1333	498.1335	$C_{24}H_{24}N_3O_7S$	-0.20	0.39
160.0430	160.0432	$C_6H_{10}NO_2S$	-0.20	1.40
122.0605	122.0606	C_7H_8NO	-0.10	0.72
114.0371	114.0377	C_5H_8NS	-0.60	5.66

Figure 7
4-hydroxyphenylglyl amoxcillin (5) ($C_{24}H_{26}N_4O_7S$), $[M+H]^+$= 515.1600
A) Accurate mass measurement of the molecular ion of 4-hydroxyphenylglyl amoxcillin.
B) Accurate mass measurement of the CID fragment ions of 4-hydroxyphenylglyl amoxcillin.
C) CID fragmentation pattern of 4-hydroxyphenylglyl amoxcillin.
D) Mass accuracy of all CID fragments of 4-hydroxyphenylglyl amoxcillin.

Conclusion

The presented work describes the confirmation of the degradation pathway of the pharmaceutical substance amoxicillin, a commonly used antibiotic drug, which was unraveled in Part I of this work[1]. The creation of the degradation products, which may possibly be formed under harsh storage conditions was induced artificially. The identity of the proposed degradation products were confirmed by accurate mass measurement with ESI TOF and empirical formula calculation for the molecular ions of the degradation products. Additionally, a CID experiment was carried out with the ESI TOF instrument by increasing the fragmentor voltage. In this experiment the molecular identity of the fragments, which are derived from the degradation products of amoxicillin, were confirmed. The same fragments were generated in a controlled manner in the ion trap experiments, which are described in detailed in Part I[1]. The molecular mass obtained from the molecular ions of the degradation products and the molecular masses of their CID fragments, which were measured with highest mass accuracy of less than 1 ppm by ESI TOF, confirm the proposed amoxicillin degradation pathway.

In Part III[12] the obtained knowledge obtained about the degradation of amoxicillin will be applied for the measurement of minor byproducts in a drug development formulation trial by accurate mass measurement with LC/ESI oaTOF and byproduct monitoring with ion trap MRM mode.

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