Structure Elucidation of Degradation Products of the Antibiotic Amoxicillin with Ion Trap MS^n and Accurate Mass Determination by ESI TOF

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Today, it is necessary to identify relevant compounds appearing in discovery and development of new drug substances in the pharmaceutical industry. For that purpose, the measurement of accurate molecular mass and empirical formula calculation is very important for structure elucidation in addition to other available analytical methods. In this work, the identification and confirmation of degradation products in a finished dosage form of the antibiotic drug amoxicillin obtained under stress conditions will be demonstrated. Structure elucidation is performed utilizing liquid chromatography (LC) ion trap MS/MS and MS^3 together with accurate mass measurement of the molecular ions and of the collision induced dissociation (CID) fragments by liquid chromatography electro spray ionization time-of-flight mass spectrometry (LC/ESI-TOF). (J Am Soc Mass Spectrom 2005, 16, 1670–1676) © 2005 American Society for Mass Spectrometry

In modern pharmaceutical drug discovery and development, it is of crucial importance to identify unknown compounds with the highest possible confidence because of their potential pharmacologic effects on humans. Such compounds could be, for instance, the pharmaceutically active substances themselves, minor byproducts emerging during the production process, secondary substances in drugs isolated from a natural source, metabolites created in the human body, or degradation products of the pharmaceutical agent arising during storage. In addition to the repertoire of analytical methods for structure elucidation, the mass spectrometric measurement of accurate molecular mass and, consequently, the determination of the empirical formula is a common strategy for the identification of unknown compounds. Until a few years ago, the only instruments available to perform these measurements with highest mass accuracy were the magnetic sector mass spectrometers. Nowadays, ESI orthogonal acceleration TOF (oaTOF) instruments are also capable of handling this task sufficiently for compound confirmation. This is demonstrated by a comparison study of different types of mass spectrometer instruments for the determination of accurate masses of small molecules [1]. The improved molecular mass determination capability of oaTOF instruments was made possible by several technical innovations in TOF technology introduced during the past few years. One of the main technical improvements is the development of orthogonal acceleration (oa) TOF technology, which decouples ion beam velocity spread from the TOF axis and therefore provides better resolution [2]. In such an environment, today's routine of coupling continuous ionization sources like the electrospray ionization (ESI) source with oaTOF mass analyzers is of special importance for LC/ESI-TOF applications. High mass accuracy is only achieved when a reference compound, e.g., a reference mass solution, is simultaneously introduced into the mass spectrometer. However, mixing the LC column effluent with a stream of reference material can result in ion suppression, discrimination, or adduct formation. To preclude the need for mixing the analyte and the reference compound before spray ionization, a dual sprayer interface is used for ESI [3, 4]. Such an instrument is capable of achieving resolving powers of better than 15,000 and mass accuracies in the low single digit ppm range for small molecules [1]. Recently, the implementation of oaTOF instruments for the measurement of accurate molecular mass, the calculation of the empirical formula, and consequently, the identity confirmation of an unknown compound, was impressively demonstrated for a large number of published applications [5–10]. For instance, LC/ESI oaTOF was used for

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the characterization of in vitro drug metabolites by accurate mass measurement of the molecular ion and the CID fragments [5, 6]. The instrument found its place for the quantification and accurate mass measurement of pharmaceutical drugs in plasma [7] as well as for the characterization of trace level impurities in a drug substance [8]. In conjunction with an ion trap instrument for MS/MS and MS^3 experiments to gain structural information, the ESI oaTOF was used for the identification of a photooxygenation product of a broad spectrum antibiotic used for live stock [9] and for the identification of highly complex polyene macrolides isolated from *Streptomyces noursei* by means of an ion trap monitored purification process [10].

The identification of degradation products from antibiotics like amoxicillin is a known analytical method by LC/UV and LC/single quadrupole mass spectrometry [11]. In this work, the identification of degradation products from the antibiotic drug amoxicillin obtained under stability test conditions will be demonstrated by means of an instrument combination not utilized before. This task will be solved by a combination of structure elucidation using an LC ion trap with MS/MS and MS^3 and accurate mass measurement using ESI oaTOF. Both sets of data are combined and used to construct a degradation pathway of amoxicillin under the applied stress conditions. Besides antibiotics, the mass spectrometric approach described in this article can also be applied for many drugs belonging to other chemical classes than penicillin to identify degradation products or metabolites to construct degradation or metabolism pathways.

Experimental

Equipment

The ESI ion trap and the ESI oaTOF MS analyses were performed with the Agilent 1100 series LC/MSD XCT+ ion trap and with the Agilent LC/MSD TOF (Agilent Technologies, Santa Clara, CA), which was equipped with a dual sprayer source for the simultaneous but separate infusion of the reference mass solution with LC eluent. The LC system used was an Agilent 1100 series capillary LC system (Agilent Technologies, Waldbronn, Germany) consisting of a capillary pump with a micro vacuum degasser, a temperature controlled micro well-plate autosampler 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 Agilent ChemStation A10.02 (Agilent Technologies, Wilmington, DE), ion trap software 5.2, and TOF software A01. Data analysis was performed using ion trap data analysis software and Analyst QS for ESI TOF data.

Methods

The Agilent 1100 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 autosampler was used to make injections of 1 μL and the samples were maintained at 4 °C. The sample loop was switched to bypass after 1 min to reduce delay volume.

The mass spectrometers were operated under the following conditions:

(1) Ion trap MS: source: ESI in positive mode; drying gas: 5.0 L/min; gas temperature: 300 °C; nebulizer: 15 psi; ion current control: 150,000; maximum accumulation time: 50 ms; scan: 200–600. For the acquisition of MS/MS and MS^3 the automated data dependent acquisition functionality was used.

(2) ESI oaTOF MS: source: ESI in positive mode with dual sprayer; drying gas: 7.0 L/min; gas temperature: 300 °C; nebulizer: 15 psi; scan: 50–1000; fragmentor: 150 or 300 V for CID; skimmer: 60 V; capillary: 5000 V.

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 h 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 acidic conditions, as described in the experimental section. Aliquots of this solution were collected at various time points and subjected to capillary chromatography to separate the accumulated degradation products. The base peak chromatogram (BPC) clearly shows the degradation of amoxicillin into various products (Figure 1). The degradation products were identified by structure elucidation of the molecular ions and the CID fragments using ion trap MS/MS and MS^3 experiments. Identity confirmation was provided by accurate mass determination using ESI oaTOF MS to deduce molecular formulae. The extracted ion chromatogram (EIC) for

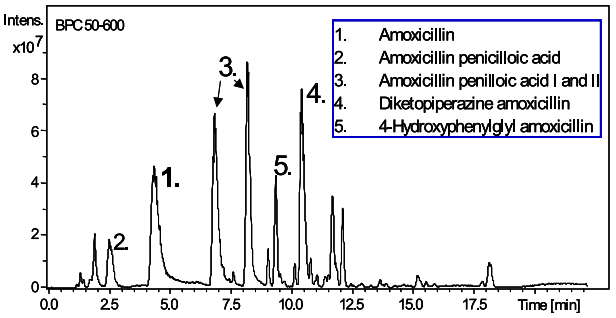

Figure 1. BPC of amoxicillin **(1)** and its degradation products after acid exposure.

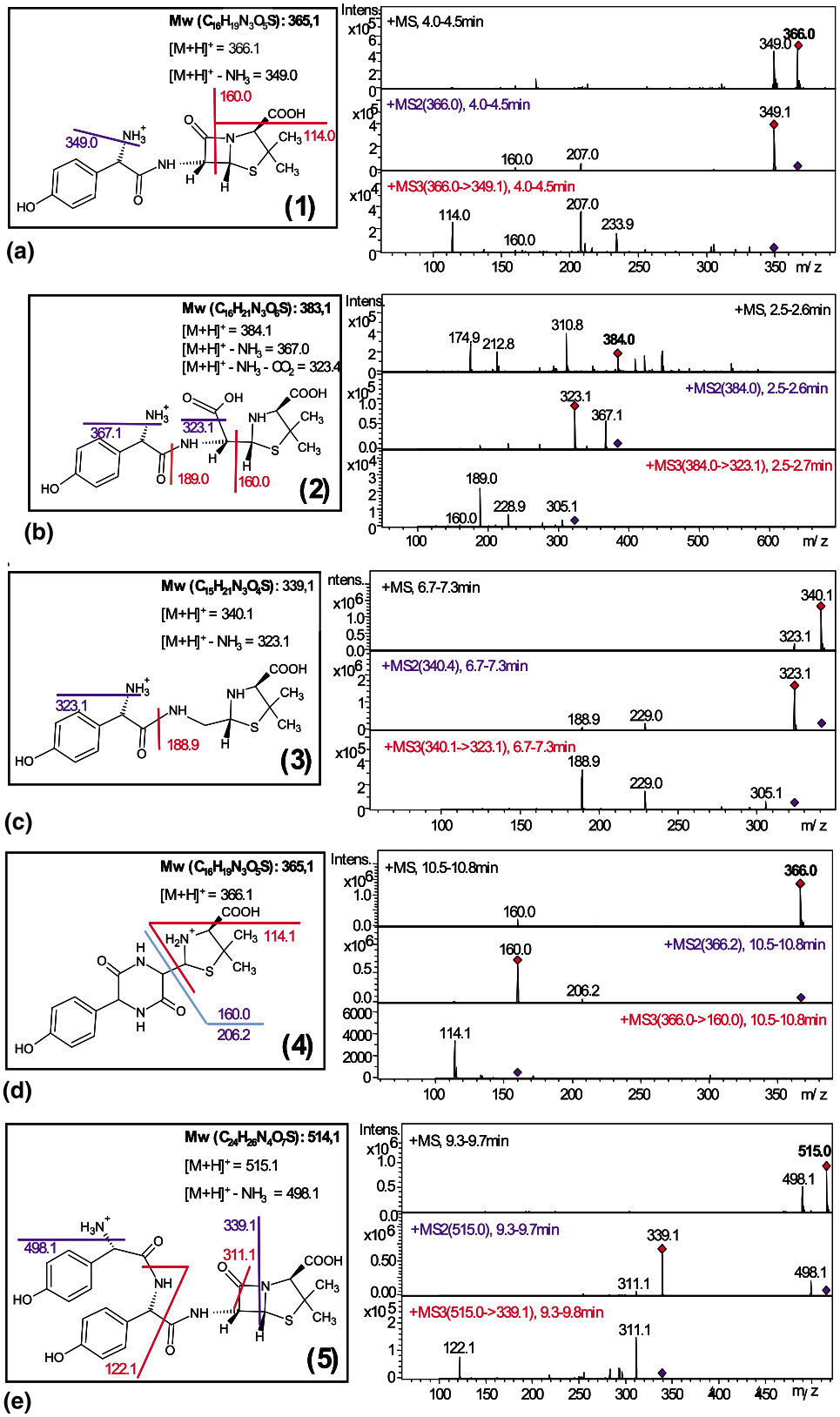

Figure 2. Ion Trap MS/MS and MS^3 analysis of amoxicillin **(1)** and its degradation products after acidic exposure. (**a**) Amoxicillin **(1)** ($C_{16}H_{19}N_3O_5S$), $[M + H]^+ = m/z$ 366.1. (**b**) Amoxicillin penicilloic acid **(2)** ($C_{16}H_{21}N_3O_6S$), $[M + H]^+ = m/z$ 384.1 (**c**) Amoxicillin penilloic acid I and II **(3)** ($C_{15}H_{21}N_3O_4S$), $[M + H]^+ = m/z$ 340.1 (**d**) Diketopiperazine amoxicillin **(4)** ($C_{16}H_{19}N_3O_5S$), $[M + H]^+ = m/z$ 366.1. (**e**) 4-Hydroxyphenylglycyl amoxicillin **(5)** ($C_{24}H_{26}N_4O_7S$), $[M + H]^+ = m/z$ 515.1.

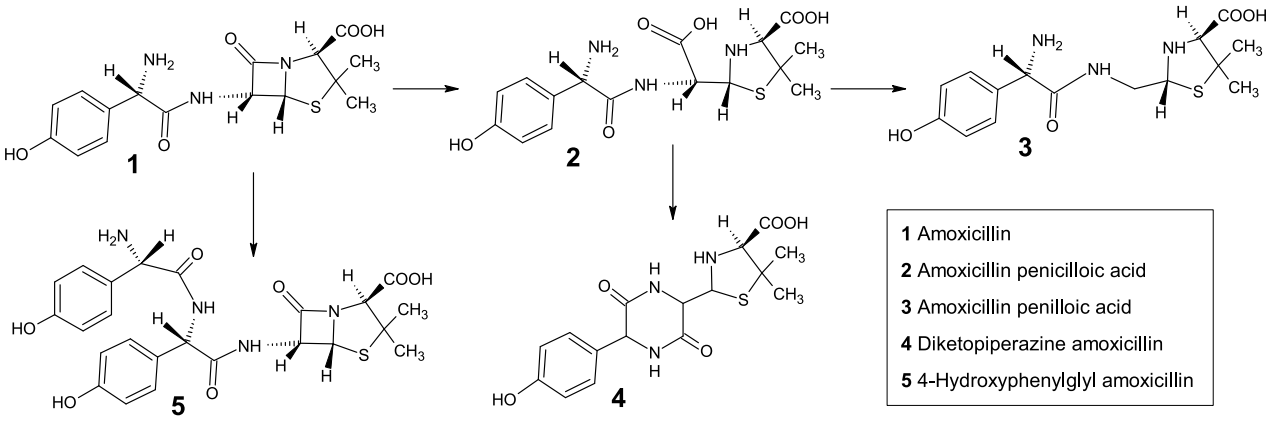

Figure 3. Degradation pathway of amoxicillin.

protonated amoxicillin **(1)** at *m/z* 366.1 shows a retention time of 4.0 min for amoxicillin **(1)** and 10.5 min for the isomeric compound **(4)**. This mass spectrum showed a potassium adduct at *m/z* 404.0 and a product generated by the loss of ammonium at *m/z* 349.0. The product ion from ammonia loss is also generated under CID conditions in the ion trap as the major product in the first stage of MS/MS (Figure 2a). In MS^3, isolation and fragmentation of the ion at *m/z* 349.0 results in two ions at *m/z* 114.0 and at 160.0, which could be easily assigned to the corresponding structural components (Figures 2a). According to a fragmentation which could not be assigned the ions at *m/z* 207 and 234 occur in this spectrum. The degradation of amoxicillin **(1)** in an acidic medium starts with the opening of the four-membered β-lactam ring and yields the product amoxicillin penicilloic acid **(2)**, which contains a free carboxylic acid group and gives a higher polarity to this molecule. This leads to a shift towards an earlier retention time in the reverse phase liquid chromatography (RP LC) separation, which was seen in the EIC of protonated amoxicillin penicilloic acid **(2)** at *m/z* 384.0 with a retention time at 2.6 min. The mass spectrum shows the protonated molecular ion of **(2)** and also its potassium adduct at *m/z* 422.0. The fragmentation of the molecular ion **(2)** results in two product ions, one from a loss of ammonia at *m/z* 367.1 and the other from subsequent decarboxylation at *m/z* 323.1. This ion (*m/z* 323.1) is the precursor for the MS^3 fragmentation, which yields fragment ions at *m/z* 189.0 (fragmentation of the amide bond) and at *m/z* 160.0, the thiazolidine ring (Figure 2b).

Starting with Compound **(2)**, there are two possible pathways for further degradation. The first one is based on the decarboxylation of the free carboxylic acid and leads to the stereoisomeric compounds amoxicillin penilloic acid I and II **(3)**. The second possible degradation reaction of intermediate **(2)** is the formation of a new, stable, six-membered ring giving diketopiperazine amoxicillin **(4)**. Both reaction products were identified in the amoxicillin solution stored under acidic conditions. The protonated stereo isomeric amoxicillin penilloic acids I and II **(3)** both at *m/z* 340.2 were extracted from the BPC at 6.7–7.3 and 8.5–9.0 min. They were detected as the protonated species and as the potassium adducted species at *m/z* 378.0. From the MS/MS analysis of the protonated molecular ion, the product of a deamination reaction is assigned. From the MS^3 fragmentation of the deamidation ion at *m/z* 323.1 a product is detected at *m/z* 188.9 (Figure 2c). The MS, MS/MS and MS^3 spectra of the stereoisomer occurring at 8.5 min are identical to those obtained for the isomer eluting at 6.7 min.

The second reaction product derived from compound **(2)** the protonated diketopiperazine amoxicillin **(4)** with a protonated molecular ion at *m/z* 366.0 was extracted from the BPC and the corresponding peak was seen in the EIC at a retention time of 10.4 min. Additionally, the protonated form of the compound is also accompanied by a potassium adduct giving a positively charged ion at *m/z* 404.0. In the MS/MS experiment, the molecule undergoes fragmentation by cleavage of the bond between the six-membered diketopiperazine ring and the five-membered thiazolidine ring yielding fragments at *m/z* 206.2 and 160.0, respectively. Isolation and MS^3 fragmentation of the ion at *m/z* 160.0 yields a product ion at *m/z* 114.1 obtained from the cleavage of a carboxylic acid group from the thiazolidine ring moiety (Figure 2d).

In another reaction pathway, amoxicillin **(1)** undergoes a nucleophilic attack on itself, where the benzylic carbonyl group is attacked by the free amino group to form 4-hydroxyphenylglycyl amoxicillin **(5)**. The protonated molecular ion at *m/z* 515.0 was seen in the extracted ion chromatogram at 9.3 min. The MS spectrum showed the protonated molecular ion and the potassium adduct at *m/z* 552.9. The MS/MS spectrum of *m/z* 515.0 reveals a product at *m/z* 498.0 from a loss of ammonia and also a product at *m/z* 339.1 from fragmentation of the five-membered thiazolidine ring. The MS^3 fragmentation on the ion at *m/z* 339.1 gave major ions at *m/z* 311.1 (loss of a carbonyl group) and 122.1 (a benzylic amino fragment) (Figure 2e). The complete degradation pathway of amoxicillin **(1)** with the proposed degradation products is summarized in Figure 3.

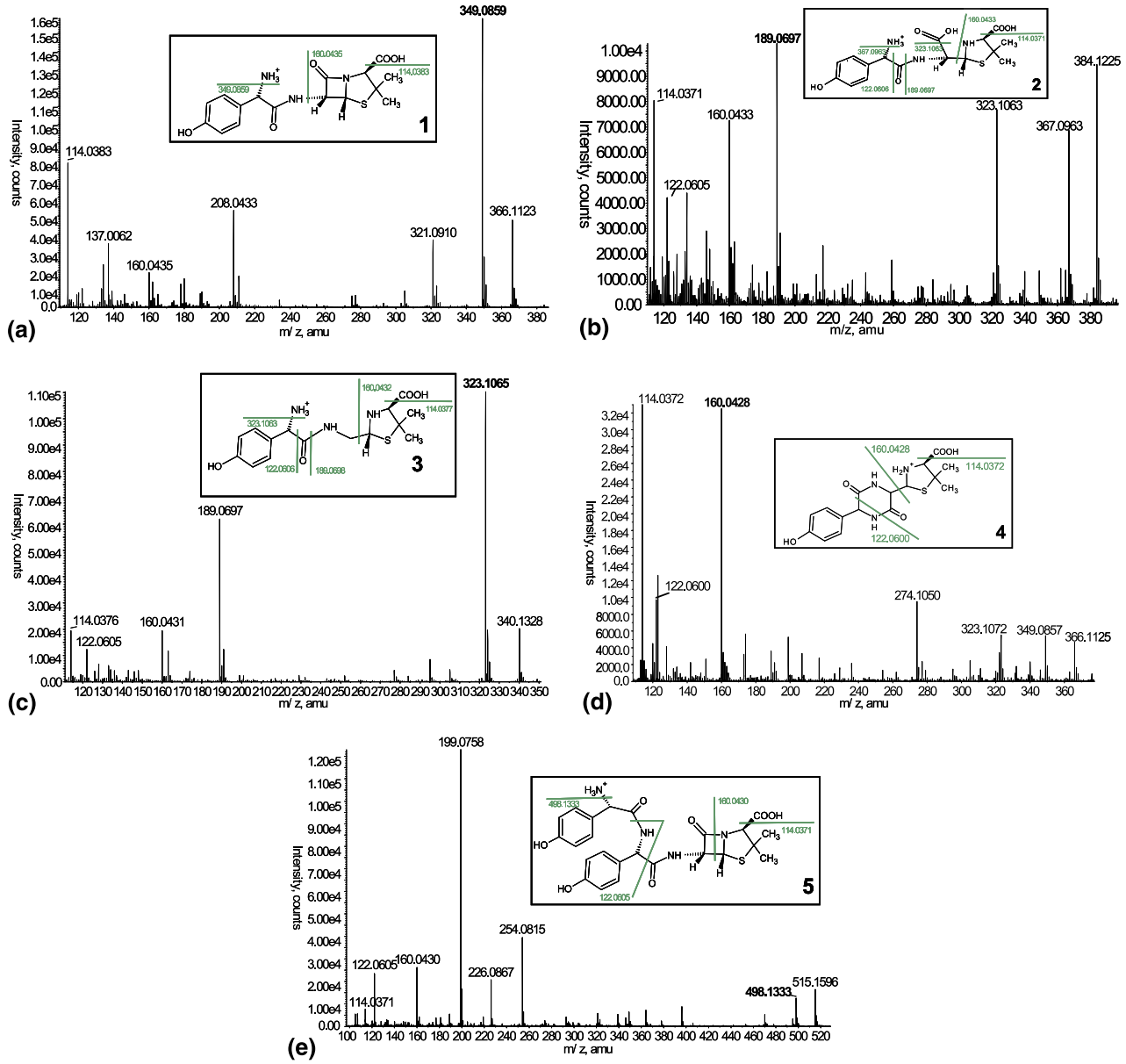

Figure 4. ESI oaTOF analysis of amoxicillin **(1)** and its degradation products after acidic exposure for accurate mass measurement and compound confirmation. (**a**) Amoxicillin **(1)** ($C_{16}H_{19}N_3O_5S$), $[M + H]^+ = m/z$ 366.1124. (**b**) Amoxicillin penicilloic acid **(2)** ($C_{16}H_{21}N_3O_6S$), $[M + H]^+ = m/z$ 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 amoxicillin **(5)** ($C_{24}H_{26}N_4O_7S$), $[M + H]^+ = m/z$ 515.1600.

For the identity confirmation of the proposed intermediates°(Figure°3)°involved°in°the°degradation°pathway of amoxicillin **(1)**, accurate mass measurement were 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 and 300 V in the oaTOF MS. At a fragmentor voltage of 150 V there is no CID observed whereas good fragmentation of the molecular ion from each of the separated degradation products is observed at a voltage of 300 V (only these data are discussed below). The measured mass (m/z) for protonated amoxicillin **(1)** is 366.1123, which has a deviation of 0.1 mDa or 0.27 ppm from the calculated° molecular° monoisotopic° mass° (Figure° 4a). For structural confirmation, fragmentation of amoxicillin **(1)** is obtained by the application of an increased fragmentor°voltage°up°to°300°V°(Figure°4a).°The°main fragment shown in the mass spectrum is the loss of ammonia at m/z 349.0859, which has a deviation from the calculated mass of 0.1 mDa or 0.28 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 molecular ion is calculated with a

mass accuracy of 0.3 mDa or 1.87 ppm. The complete fragmentation is shown in Figure 4a. The mass accuracy in mDa and the ppm value of the obtained molecular ions and fragments calculated from the mean measured mass of five experiments is shown in Table 1a. For more detailed information about the masses measured in the five experiments as well as the means and standard deviations for the masses; mass accuracies in mDa and ppm of each measured molecule and their corresponding mean and standard deviation, see the Supplementary Material in the online version of the article. The higher, but still acceptable, values for the mean and the standard deviation of the fragment ion at *m/z* 114.0377 could be explained by the fact that this value is below the lowest measured point of the calibration curve of the instrument.

The first degradation product of amoxicillin **(1)** obtained after breaking the four-membered β-lactam ring is amoxicillin penicilloic acid **(2)** and was confirmed by accurate mass measurement at *m/z* 384.1225, with 1.04 ppm mass accuracy and formula calculation with the ESI oaTOF at a fragmentor voltage of 300 V. The structure of this degradation product was confirmed by the appearance of the characteristic fragment at *m/z* 323.1063 with 0.93 ppm mass accuracy, which is the product of a decarboxylation reaction. The complete fragmentation pattern of amoxicillin penicilloic acid **(2)** also reveals fragments at *m/z* 122.0606 and 189.0697, which are important for the structure confirmation (Figure 4b). The complete information obtained from the CID fragments of amoxicillin penicilloic acid **(2)** is summarized in Table 1b in the Supplementary Material with their molecular formula and the measured mass accuracies based on the mean mass measured in five experiments.

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* 340.1328 ($C_{15}H_{22}N_3O_4S$) with a deviation of 0.30 mDa or 0.89 ppm from the theoretical mass. The main fragment ion obtained in the CID spectrum is the loss of ammonia at *m/z* 323.1065 (Figure 4c). The same fragment ion was also observed in the CID spectrum of amoxicillin penicilloic acid **(2)** (Figure 4b) and gives further evidence for the identity of the degradation products amoxicillin penilloic acid I and II **(3)**. The identified fragmentation reactions and fragment ions are outlined in Figure 4c and summarized in Table 1c. The detailed table is again summarized in the Supplementary Material online.

Beginning with amoxicillin penicilloic acid **(2)**, the degradation pathway also leads to diketopiperazine amoxicillin **(4)** by the formation of a six-membered ring structure (Figure 3). The molecular formula $C_{16}H_{19}N_3O_5S$ was confirmed by accurate mass measurement with the ESI oaTOF at *m/z* 366.1125 with 0.27 ppm accuracy. The main CID fragment belongs to a cleavage of the molecule at the bond between the two rings with the charge remaining with the five-membered thiazolidine ring at *m/z* 160.0428 with a mass accuracy of 2.65 ppm (Figure 4d). The confirmed fragmentation is shown in Figure 4d and the mean measured masses of the fragments and their calculated accuracies are summarized in Table 1d in the Supplementary Material.

Finally, the identity of the product obtained from a self-condensation reaction of amoxicillin **(1)** to 4-hydroxyphenylglycyl amoxicillin **(5)** was confirmed by using the ESI oaTOF instrument. The protonated molecular ion at *m/z* 516.1596 with the calculated formula $C_{24}H_{27}N_4O_7S$ was confirmed with a mass accuracy of 0.77 ppm. The CID fragments useful for the identification come from loss of ammonia (*m/z* 498.1333), a loss of the five-membered thiazolidine ring from the parent molecule and from a residual benzyl imminium ion (*m/z* 122.0605) (Figure 4e), which show accuracies of 0.40 and 0.82 ppm, respectively. The mean masses of the fragment ions and the molecular ion are determined with sufficient accuracy for unambiguous identification of the fragments and consequently the molecule (Table 1e, see Supplementary Material).

Conclusions

The presented work describes the elucidation of the degradation pathway of the pharmaceutical drug substance amoxicillin, a commonly used antibiotic drug. The degradation of amoxicillin was induced by acidic stress. The degradation products created from amoxicillin were separated by capillary LC and analyzed in the first step by an ion trap in the MS, MS/MS, and MS^3 modes. The fragments obtained were used for the structure elucidation of the degradation products. The identities of the proposed products were confirmed by accurate mass measurement with ESI oaTOF and empirical formula calculation for the molecular ions of the degradation products. Additionally, a CID experiment was carried out with the ESI oaTOF 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 by accurate mass. The same fragments were generated in a controlled manner in the ion trap experiment and therefore support the conclusions. With low single digit ppm mass accuracy on both the molecular ions and the CID fragments, the elemental compositions of the amoxicillin degradation products, and thus the pathway, were confirmed.

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