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Ampicillin sodium: Isolation, identification and synthesis of the last unknown impurity after 60 years of clinical use

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AMPICILLIN SODIUM: IDENTIFICATION OF THE LAST UNKNOWN IMPURITY AFTER 60 YEARS OF CLINICAL USE

*Alessandra Tolomelli,¹ Antonio Ricci,^{*2} Angelo Viola,² Michele Bassan,² Luca Ferrari,² Lucia Ferrazzano,¹ Giulia Martelli,¹ Alexia Mattellone,¹ and Walter Cabri^{*1}*

Abstract

Ampicillin, discovered in 1958, was the first broad spectrum semisynthetic penicillin introduced into the market. Despite its wide use not all the impurities have been identified to date. Herein, the last unknown impurity present in commercially available medicines was isolated and identified. This impurity that accounts up to 0.8 in area % by HPLC (EP 10.0) in the Reference Listed Drugs (RLD) was characterized and identified to be the 16-keto penicillin G. The structure was confirmed by comparison with a chemically synthesized sample. The determination of the Relative Response Factor (RRF) of the impurity respect to the parent drug allowed to recalculate the real amount that is consistently below the reporting threshold.

Keywords: ampicillin; unknown impurity; degradation; penicillins; relative response factor

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1. Introduction

Ampicillin sodium **1** is the first semisynthetic broad-spectrum antibiotic that after the discovery in 1958 by Doyle, Nayler and Smith (Doyle et al. 1958; Doyle et al. 1960) reached the market in 1961 (Acred et al. 1962), during the golden age of the pharmaceutical industry (Daemmerich and Bowden, 2005). This drug is currently used for the treatment of several bacterial infections, comprising both Gram-positive and Gram-negative bacteria, and displays excellent resistance under acid conditions providing the further advantage of an efficacious oral administration (Saga and Yamaguchi 2009).

Ampicillin sodium **1** is marketed worldwide as generic drug by several companies. Interestingly, a big pharma like Pfizer is still on the market with this legacy product, with the brand names Amplital® and Penbritin-S® for the European and American markets respectively. More than two decades after ampicillin sodium **1** launch, the product was revitalized in 1986 by Pfizer in combination with the β -lactamase inhibitor sulbactam with the name of Unasyn® (Campoli-Richards and Brogden 1987). In USA, Penbritin® and Unasyn® are the Reference Listed Drugs (RLD).

As expected, the wide-spread clinical use of this antibiotic induced the occurrence of pathogens with acquired resistance, thus leading to the preferential prescription of other classes of antimicrobials. Recently, a remarkable reversal in the resistance pattern was observed as a result of its decreased use, and ampicillin **1** has been again re-emerging as an excellent therapeutic tool for its excellent safety profile, low cost and availability in developing countries (Kaushik et al. 2014).

In 2019, considering only the parenteral administration, 66 Million Units (MU) of ampicillin sodium **1** and 94 MU of ampicillin/sulbactam sodium sterile have been sold worldwide accounting for a turnover of 65 M\$ and 235 M\$ respectively (IQVIA 2020).

The identification and, when necessary, the qualification of impurities present in the active pharmaceutical ingredients (APIs) as well as in the corresponding drug products, at the beginning and end of the shelf life, is critical to avoid any concern by regulatory authorities and to guarantee patients safety. This screening must consider impurities deriving from the production process and from stressed stability conditions.

Concerning **1**, in addition to the process related impurities coming from variants of the Dane salt route (Dane and Dockner 1964), there are several stability related impurities coming from the reactivity of the β -lactam ring (Figure 1). Typical penicillin impurities are penicilloic, penilloic acids, and oligomers, resulting from the nucleophilic attack of the amino function to the β -lactam ring of a second molecule. Furthermore, the free amino group of **1** can react intramolecularly with other functionalities of the backbone, leading to rearranged products as the diketopiperazine derivative. The comprehensive list of impurities reported in the European Pharmacopeia 10.0 (EP 10.0, 2019) is

summarized in Table 1. Ampicillin sulfoxides (RRT 0.52) are generated only during stress tests and are never present in the final product; for this reason, they are not listed in the EP.

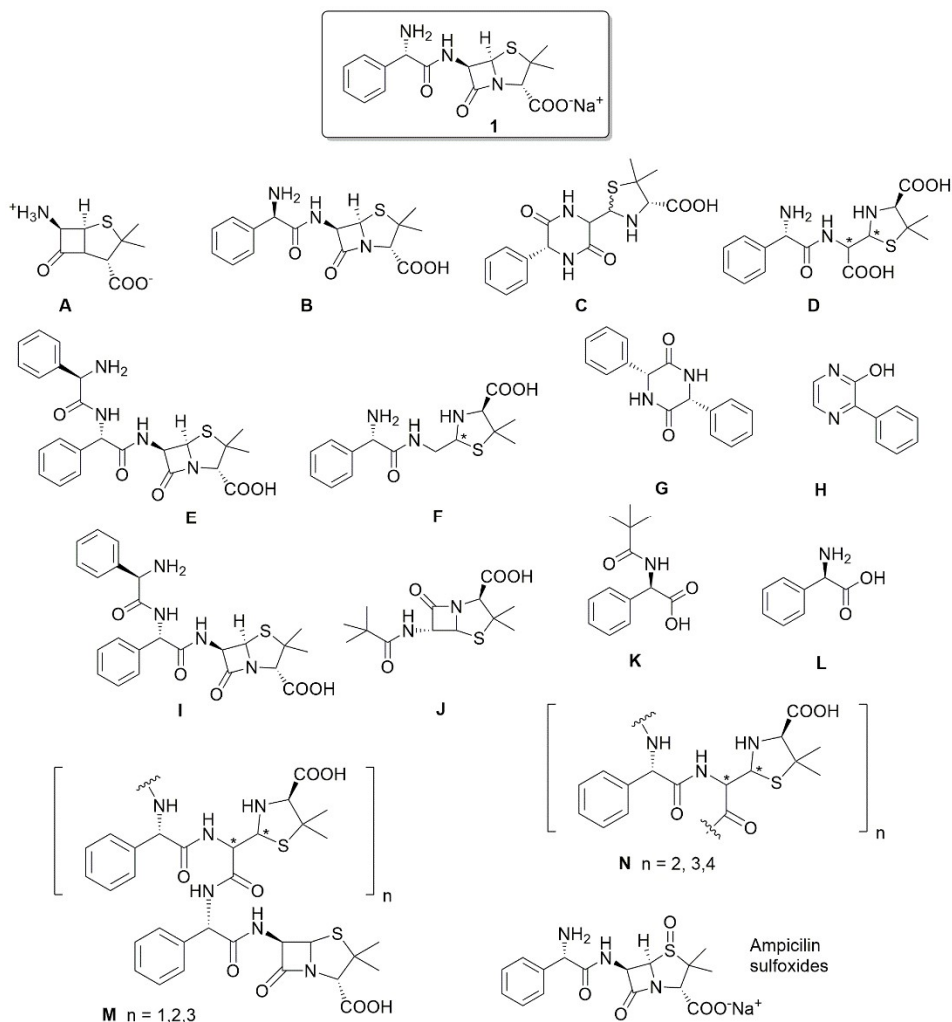


Figure 1: Molecular structure of ampicillin Sodium **1** and related substances reported in European Pharmacopoeia 10.0 method. Sulfoxides were also reported.

A representative HPLC chromatogram displaying these impurities is reported in Figure 2. Interestingly, although the list of impurities of ampicillin sodium **1** sterile and ampicillin/sulbactam sodium sterile comprehends a large number of compounds, a specified unknown impurity (**SUI**) is always present in the commercial samples, with an RRT of about 4.23 using the EP HPLC method (see Figure 2, peak underlined in red). The amount in area % ranges between 0.44 and 0.76 in different samples of the corresponding RLDs, namely Unasyn® and Amplital® (Table 2).

Table 1. Ampicillin **1** related substances reported in the European Pharmacopoeia 10.0.

NAME		Origin	RRT ^a
L	D-phenylglycine	Process	0.37
A	6-APA	Process	0.42
B	L-ampicillin	Process	0.54
D	Penicilloic acid	Degradation	0.57
ampicillin			1.00
F	Penilloic acids	Degradation	1.10/1.70
C	Diketopiperazine of ampicillin	Degradation	2.45
I	D-Phenyl-glycil-ampicillin	Process	2.60
H	3-phenylpiperazin-2-olo	Degradation	2.95
G	(3R,6R)-3,6-diphenylpiperazine-2,5-dione	Degradation	3.31
K	Pivaloyl phenylglycine	Process	3.35
N_(N=2)	Dimer of penicilloic acids of ampicillin	Degradation	3.64
J	Pivaloyl 6-APA	Process	3.71
M	ampicillin dimer	Degradation	3.87
E	ampicillin-D-phenylglycine	Degradation	4.20
SUI	Unknown impurity	Process/ degradation	4.23
N_(N=3)	Trimer of penicilloic acids of ampicillin	Degradation	4.42
M_(N=2)	Trimer of Ampicillin and penicilloic acids of ampicillin	Degradation	4.90
N_(N=4)	Tetramer of penicilloic acids of ampicillin	Degradation	5.04
M_(M=3)	Tetramer of ampicillin and penicilloic acids of ampicillin	Degradation	5.16

a. Impurities are sorted by relative retention time (RRT) respect to ampicillin **1** applying the official EP 10.0 analytical method for related substances.

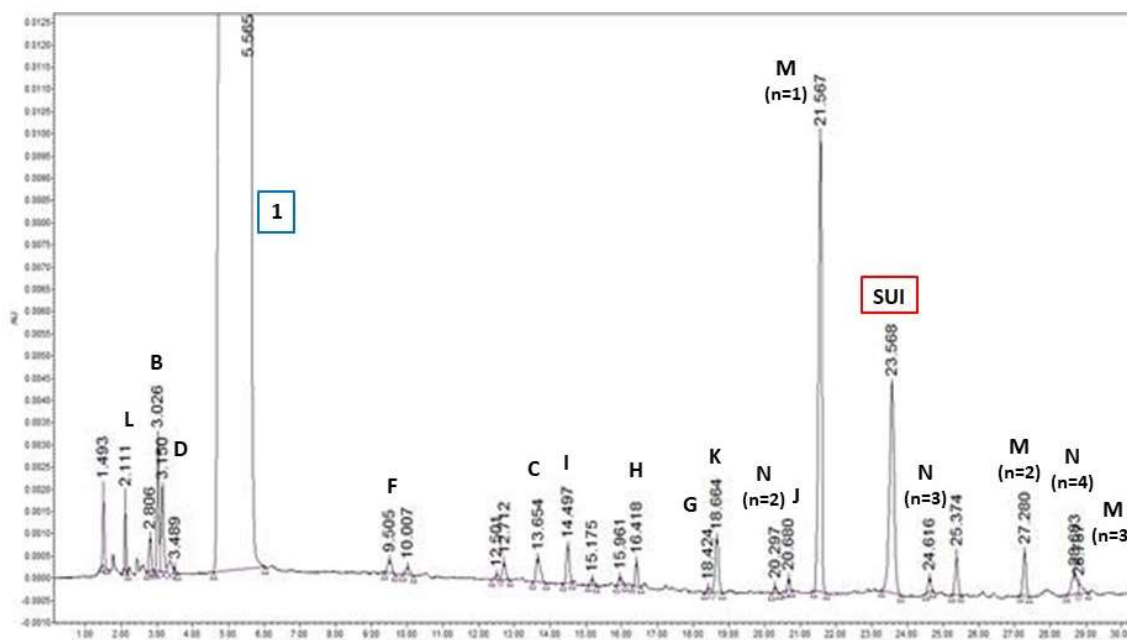
**Figure 2.** HPLC Chromatogram of ampicillin sodium **1** according to EP 10.0.

Table 2. Content of SUI in commercial RLDs (Amplital® and Unasyn®) using EP 10.0 HPLC method.

Product	batch	Age (months)	SUI (area %)
Amplital®	23922800	1	0.50
Amplital®	23922800	24	0.76
Amplital®	23917401	1	0.60
Unasyn®	12507103	3	0.44

This unknown impurity is considered qualified for generic version of the drug, being present in the RLD. In fact, the reported values (Table 2) exceed by far the specification for unknown impurities (<0.15%) for small molecules with a daily dosage >2g, according to the International Conference on Harmonization guideline Q3b (ICH quality guidelines).

However, the quantification of this impurity and therefore the potential overall impact on ampicillin and ampicillin/Sulbactam assay has not been really evaluated till date.

This issue prompted us in studying the chemical structure of SUI RRT 4.23 and its RRF.

2. Materials and methods

2.1 General information

The commercial samples of Unasyn® and Amplital® have been purchased from a pharmacy. Ampicillin sodium **1** for degradation studies was given by Fresenius Kabi. All chemicals were purchased from commercial suppliers and used without further purification. NMR spectra were recorded with an INOVA 400 MHz spectrometer. Chemical shifts were reported as δ values (ppm) relative to the solvent peak of CDCl₃ set at δ = 7.27 ppm (¹H-NMR) or δ = 77.0 ppm (¹³C-NMR), CD₃OD set at δ = 3.31 ppm (¹H-NMR) or δ = 49.0 ppm (¹³C-NMR), D₂O set at δ = 4.79 ppm, DMSO-d₆ set at δ = 2.50 ppm (¹H-NMR). Coupling constants are given in Hertz.

2.2 LC analytical conditions for commercial RLD studies according to EP 10.0

Analysis of the samples content (1 mg/mL) was performed on Agilent HPLC 1260 instrument coupled with DAD and ESI-MS detectors. Column: ACE C18 (3 μ particle size, 150 \times 4.6 mm), flow: 1 mL/min, temperature: 25°C; injection volume: 50 μ L; solvent A: 1.36 g KH₂PO₄ in 500 mL of MilliQ water with 0.5 mL of acetic acid (2M), 50 mL of CH₃CN diluted in 1L of MilliQ water; solvent B: 1.36 g KH₂PO₄ in 500 mL of MilliQ water with 0.5 mL of acetic acid (2M), 400 mL of CH₃CN diluted in 1L of MilliQ water; elution program starting with isocratic 85/15 A/B (6 minutes)

followed by a gradient from A/B = 85/15 to A/B = 0/100 in 36 min; UV absorption was monitored at 254 nm.

2.3 LC-MS analytical conditions reproducing the EP 10.0 profile

A program of elution starting with isocratic 85/15 H₂O/CH₃CN containing 0.2% of formic acid (18 minutes) followed by a gradient to 50/50 H₂O/CH₃CN containing 0.2% of formic acid in 12 minutes was applied. ESI-MS nebulizer was set at 50 psi with dry gas 12 L/min at 325 °C. Mass range of acquisition was from 100 to 1200 m/z.

2.4 Formation of the unknown impurity in the stressed ampicillin sodium 1 sample

A sample of ampicillin sodium **1** (5 g), containing an initial amount of **SUI** of 0.2%, was placed in an oven at 90°C to follow the degradation. After 3 days, the degraded sample was dissolved in 20 mL of water (conc 250 g/L), analyzed, and purified through HPLC chromatography.

2.5 Isolation of the unknown impurity by preparative HPLC

The stressed sample (5g) was dissolved in 20 mL of water (conc 250 g/L) and subjected to preparative HPLC treatment. Chromatographic runs were performed on an Agilent 1260 Preparative HPLC with binary pump, using column: Phenomenex Luna AXIA® C18 (250 x 30 mm), 10 µm particle size, flow: 20 mL/min, temperature: 25°C; injection volume: 5 mL; solvent A: 1.36 g ammonium acetate dissolved in 1L of MilliQ water adjusted to pH 4.9 with glacial acetic acid, solvent B: CH₃CN; elution program starting with isocratic 80/20 A/B (10 minutes) followed by a gradient from A/B = 80/20 to A/B = 60/40 in 40 min. The injection was repeated five times.

Fractions were monitored with analytical HPLC-MS before collection. The fractions showing HPLC purity > 95% were collected, acetonitrile was removed under vacuum and the remaining aqueous solution was treated with an equivalent volume of DCM, cooled to 5°C and the pH adjusted to 2.0 with HCl 4%. The layers were separated and the organic one collected and washed with an equivalent volume of water (2 times). Keeping the temperature at 5°C, the pH of the mixture was adjusted to 5.5 with a sodium bicarbonate saturated solution and a separation of the two phases was performed. The aqueous layers were collected, washed with 10 mL of DCM, stripped under vacuum until complete removal of the volatile organic residue and lyophilized to obtain 1.5 mg of **SUI** as a yellow solid with an HPLC purity of 94.1% (see LC method reported above in section 2.2). Complete characterization of **SUI** is reported in the supporting material.

2.6 Synthesis of 16-Keto-Penicillin G 2 (SUI)

To a solution of phenylglycolic acid (2 g, 13.33 mmol) in DCM (5 mL) and DIMAC (1.5 mL) at -40°C, 1.84 mL of TEA was added. At the same temperature, pivaloyl chloride (1.43 mL) and pyridine (53 μ L) were added dropwise at the same temperature and mixed for 1h. In a separate flask, 6-APA (2.4 g, 11.11 mmol) was dissolved in 20 mL of DCM at room temperature. This mixture was cooled at -10°C and TEA (3 mL) and water (200 μ L) were added. After complete dissolution, the solution of 6-APA was added to the solution of phenylglycolic acid at -35°C in 30 seconds. The mixture was stirred at -15°C for 1h. After this time the solution was warmed to 0-5°C and 20 mL of water were added. The pH was adjusted to 4.5 with HCl 4%, a separation of the two phases was performed and the aqueous layer was collected, washed with 10 mL of DCM and purified by preparative HPLC chromatography following the protocol above described. The work-up afforded 2.5 g of 16-Keto-Penicillin G **2** as a yellow solid (65% yield) with an HPLC purity of 98.0%. Complete characterization of **2** is reported in the supporting material.

2.7 RRF calculation

Compound **2** was used for RRF determination immediately after isolation due to its low stability. Three samples (about 10 mg) containing different ratios of **1/2** were dissolved in DMSO- d_6 (1 mL) and TFA (30 μ L) was added to the solutions. A portion (50 μ L) of these solutions was further diluted with H₂O/CH₃CN/TFA (80/20/0.08) for HPLC analysis, that was performed on an Agilent 1260 Infinity II system instrument coupled with DAD detector, Column: Phenomenex C18 Luna (3 μ Phenyl-Hexyl, 250 \times 4.6 mm), flow: 1 mL/min, temperature: 25 °C; injection volume: 20 μ L; solvent: H₂O+0.08%TFA/CH₃CN+0.08%TFA. UV absorption was monitored at 230 and 254 nm. The following gradient elution program was applied: H₂O+0.08%TFA/CH₃CN+0.08%TFA from 80:20 to 60:40 in 15 minutes, 60:40 from 15 to 20 minutes, from 60:40 to 80:20 from 20 to 50 minutes. The remaining part of the solutions was transferred into 5 mm NMR tubes to perform qNMR experiments with an INOVA 400 instrument, using a relaxation delay = 60s, an acquisition time = 4s and a number of scans = 12. The RRF values were determined at two wavelengths: 254 nm (EP 10.0) and 230 nm (USP 43-NF38), as average of the three experimental values.

3. Results and discussion

3.1 Isolation and determination of SUI structure

Previous studies in our laboratory showed that the formation of **SUI** can be expedited by thermal stress. Thus, a sample of ampicillin sodium salt **1**, containing an initial amount of **SUI** of 0.2%, was placed in an oven at 90°C for 3 days and treated as reported above.

The HPLC profile, recorded according to European Pharmacopoeia 10.0 method, where the peaks are reported in area %, showed an increased amount of **SUI** at RRT 4.23 (0.59%). The unmodified ampicillin sodium salt **1** remained the main component (96.88%).

The HPLC-MS analysis, performed both in positive and in negative mode, provided in both cases an exact mass of 348 uma, one mass unit less respect to ampicillin **1** (exact mass 349 uma; spectra reported in SI). In addition, the UV profile of **SUI**, having a maximum at 260 nm, is completely different compared to the one of the parent drugs **1**. The molecular weight suggested to focus the attention on a structure close to ampicillin **1**, excluding any possibility of a multimer; on the other hand, the UV spectra showed the presence of a conjugated system. The impurity was then isolated via preparative HPLC to perform NMR experiments.

The NMR analysis in *d*6-DMSO+3%TFA (Figure 3) showed some distinctive chemical shifts of the penicillin structure, as those at 1.5-2.0 ppm, associated to the protons of the two methyl groups bound to the thiazolidine ring, and those at 5.5 ppm related to both protons bound to the β -lactam ring (Branch et al. 1987). In particular, when ring opening occurs, the proton in position 6 usually displays a chemical shift at higher fields, while it resonates at 5.5 ppm, close to the proton in position 5, when the cyclic lactam structure is maintained.

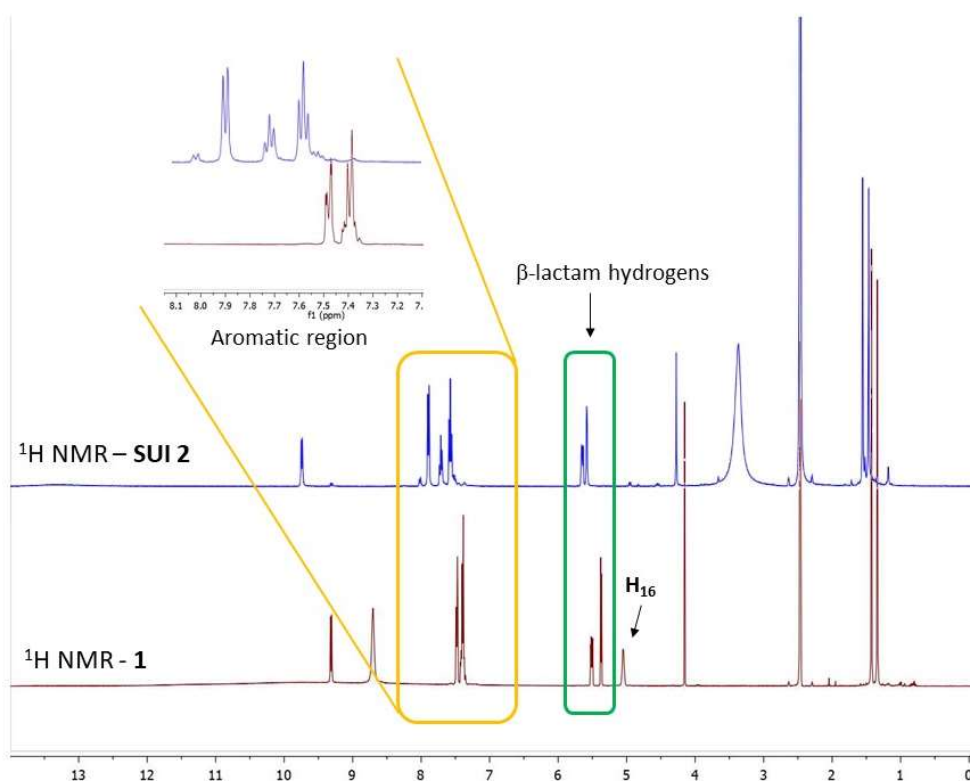


Figure 3: ¹H NMR spectra of the isolated **SUI 2** and ampicillin **1**.

The chemical shift of these protons in **SUI** confirms that the β -lactam ring is intact. On the contrary, the proton on C16 is completely missing and another relevant difference is found in the chemical shifts of the aromatic region. The ordered pattern of the **SUI** spectrum from 7.9 to 7.5 ppm, constituted by a doublet and two triplets, completely differs from the multiplets displayed by ampicillin **1** (Figure 3).

The lack of the hydrogen on C16, the molecular weight that is one unit less than ampicillin **1** and the high level of conjugation of the aromatic fragment support the presence of a carbonyl group in place of the amine on C16 (Compound **2**, Figure 4).

In the proposed structure **2** the side chain in position 6 contains the phenyl glyoxyl amide function that is very similar to the phenyl glyoxylic acid, which has an UV maximum of absorbance of 258 nm (Spectrabase 2020). Moreover, the comparison between the aromatic region in the $^1\text{H-NMR}$ experiments of phenyl glyoxylic acid and the isolated impurity further supported the attribution, displaying the distinctive pattern composed by two triplets and one doublet.

Concerning the mass analysis, the fragmentation pattern of **SUI 2** observed in the LC-MS closely resembles the one observed for ampicillin **1** (Franski et al. 2014), with the difference of one mass unit in each fragment (Figure 4, mass spectra reported in SI).

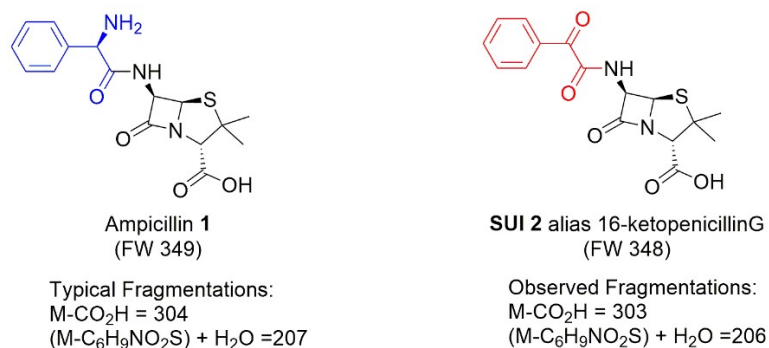


Figure 4: MS fragmentation pattern comparison of ampicillin **1** and **SUI 2**.

To finally confirm the hypothesized backbone, the molecule was chemically synthesized from 6-APA and phenylglyoxylic acid. The synthetic procedure is summarized in Figure 5.

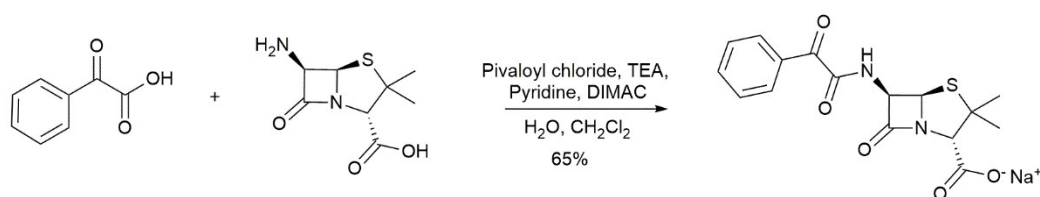


Figure 5: Synthetic scheme for the impurity 16-keto-penicillin G **2**

The product was isolated by preparative HPLC and fully characterized, showing identical data to those recorded for the product obtained by the impurity isolation study. All together, these evidences confirmed the identification of SUI with RRT 4.23 as compound **2**.

3.2 Calculation of relative response factor (RRF) by combination of ¹H NMR/HPLC

In order to understand the impact of this impurity on ampicillin **1** quality, we decided to determine the relative response factor (RRF) at two wavelengths, 254 nm (EP 10.0) and 230 nm (USP 39). To overcome the issue of **2** instability, we decided to apply the HPLC/NMR combination. This method is very rapid and allows to calculate the RRF by comparison of the two analysis, performed on the same mixture of impurity and parent drug, independently from the weighted amounts and purity of the components (Webster et al. 2009; Maggio et al. 2014). The analysis was performed on three samples and the RRF was calculated as the average value. At 254 nm, the RRF was 27, as a consequence of the increased molar absorptivity due to conjugation in structure **2**. Applying this conversion factor, the amount of this impurity in the above reported samples of Amplital® and Unasyn® ranges from 0.016% to 0.028%. On the other hand, the value of RRF at 230 nm is 1.3, as also confirmed by the fact that this impurity is not even detected in USP.

*3.3 Mechanism of formation of 16-keto-penicillin G **2***

To explain the formation of the investigated impurity, the simple transamination reaction, widely known to directly exchange an amino function with a ketone, cannot be taken into consideration. In fact, no partner α -ketoacids are present in the reaction mixture in any of the steps of the manufacturing process of ampicillin **1**. The generation of **2** may instead occur through a mild metal catalyzed oxidation of the amine. Several works report that oxidation is particularly efficient for benzylic amines, as the one in phenylglycine side chain of ampicillin **1**. Generally, in medicinal formulations, oxidation processes are favored by the presence of oxygen and metals with low oxidation potential, like iron (Zhang et al. 2013). Iron (III) salts are safe common impurities in pharmaceutical drugs, since they may be released in traces by the stainless-steel used to build industrial plants. Therefore, the presence of metals, as iron, even in ppb concentrations, may be responsible for the oxidation of the amine to afford 16-keto-penicillin G **2**. To verify this hypothesis, an intimate mixture of ampicillin **1** and iron salts was prepared and submitted to the above reported stress conditions. The results are reported in Table 3.

Table 3. Ampicillin sodium **1** stress studies at solid state (90° for 3 days).

Entry	additive	2 (area %)	2 (recalculated with RRF)
1 ^a	/	0.20	0.007
2	/	0.59	0.022
3	FeCl ₂ (10 ppm)	0.59	0.022
4	FeCl ₃ (10 ppm)	0.74	0.027

^a Starting content before stress experiment.

The strongest effect seems to be related to thermal stress (entry 2) but a slight increase in the formation of **2** could be detected also in the experiment performed with FeCl₃ (entry 4), while FeCl₂ did not show any influence (entry 3). However, taking into consideration the negligible amount of **2** after recalculation with RRF, these results are anyway not conclusive. Ampicillin sodium **1** commercial samples were submitted to ICP-MS analysis and iron content was always less than 0.5 ppm.

4. Conclusions

The recurrent impurity at RRT 4.23 (EP 10.0 method) detected in commercial ampicillin **1** sodium sterile and ampicillin/sulbactam sodium sterile combination was identified as the oxidized 16-keto penicillin G **2**. The structure was both isolated from a stressed sample of **1** with an enriched content of the investigated impurity and synthesized from 6-APA. Mass spectrometry as well as NMR analysis are in agreement with the ketoamide structure. The calculation of the RRF, performed using the NMR/HPLC combination method, pointed out that there is an overestimation of impurity **2** in the European pharmacopeia HPLC analysis, which is carried out at 254 nm, a wavelength close to the absorbance maximum of **2**. On the contrary, the USP method, being recorded at 230 nm, do not overestimate **2** since the RRF is 1.3 under these conditions. The amount of impurity **2** hence results below the reporting threshold of 0.05% and largely below the 0.15% qualification threshold. The formation of impurity **2** is most probably due to a metal catalyzed oxidation of the phenylglycine side chain in the presence of oxygen, occurring on the surface of the crystalline powder inside the vials. The negligible amount of impurity **2** in ampicillin **1** sodium sterile and ampicillin/sulbactam sodium sterile does not affect by any means the quality, efficacy and safety of the commercial antibiotics. Based on the RRF calculation this impurity is below the reporting limit or in other words “Much ado about nothing” (Shakespeare 1623).

5. Acknowledgments

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Declaration of authors' contribution

AR, WC and AT are responsible for conceptualization, methodology planning and writing of the original draft. AV, MB and LF² performed all the experiments. GM, AM and LF¹ synthesized compound 2 and analyzed the data, AV validated the results. The final version of the text was reviewed by all the authors.

Declaration of interest: The authors declare no conflict of interest.

This work has been developed in the context of a collaborative project between Fresenius Kabi Ipsum, pharmaceutical company involved in the market of generic antibiotics, and the Alma Mater Studiorum -University of Bologna. AV, AR LF² and MB are company's employees.

6. References

- Acred et al. 1962 P. Acred, D. M. Brown, D. H. Turner, M. J. Wilson, Pharmacology And Chemotherapy Of Ampicillin—A New Broad-Spectrum Penicillin, Brit. J. Pharmacol. Chemother. 18 (2), pp. 356–69. <https://doi.org/10.1111/j.1476-5381.1962.tb01416.x>.
- Branch et al. 1987 S. K. Branch, A. F. Casy, E.M.A. Ominde, Application of ¹H nuclear magnetic resonance spectroscopy to the analysis of β -lactam antibiotics and their common degradation products, J. Pharm. Biomed. Anal. 5, pp 73-103, [https://doi.org/10.1016/0731-7085\(87\)80011-0](https://doi.org/10.1016/0731-7085(87)80011-0).
- Campoli-Richards and Brogden 1987 D. M. Campoli-Richards, R. N. Brogden, R.N. Sulbactam/ampicillin. A Review of Its Antibacterial Activity, Pharmacokinetic Properties, and Therapeutic Use, Drugs 33, pp.577–609, doi: 10.2165/00003495-198733060-00003.
- Daemmerich and Bowden 2005 A. Daemmerich, M. E. Bowden, Cover Stories: A Rising Drug Industry The Pharmaceutical Golden Era: 1930 – 60, Chem. Eng. News , 83 (25).
- Dane and Dockner 1964 E. Dane, T. Dockner, Synthesis of 6-[(D-a-amino-a-phenylacetyl)amino]-penicillanic acid using β -dicarbonyl compounds as amino protecting groups, Angew. Chem. Int. Ed., 3, p. 439. <https://doi.org/10.1002/anie.196404391>.

Doyle et al. 1958 Beecham Research Laboratories Limited (UK) Patents, F. P. Doyle, J. H. Nayler, H. Smith, Improvements in or relating to penicillin derivatives, GB873049.

Doyle et al. 1960 Beecham Research Laboratories Limited (UK) Patents F. P. Doyle, J. H. Nayler, H. Smith, H. Penicillins, GB902703.

EP10.0, 2019, edqm.eu/en/european-pharmacopoeia-ph-eur-10th-edition.

Franski et al. 2014 R. Franski, J. Czerniel, M. Kowalska, M. Franska, Electrospray ionization collision-induced dissociation tandem mass spectrometry of amoxicillin and ampicillin and their degradation products. *Rapid Commun. Mass Spectrom.*, 28, pp 713-722, doi: 10.1002/rcm.6834.

ICH quality guidelines, <https://www.ich.org/page/quality-guidelines>.

IQUVIA 2020 healthcare market data: www.iqvia.com.

Kaushik et al. 2014 D. Kaushik, M. Mohan, D. M. Borade, O. C. Swami, Ampicillin: rise, fall and resurgence, *J. Clinic. Diagn. Res.* 8, pp. ME01-ME03, doi: 10.7860/JCDR/2014/8777.4356.

Maggio et al. 2014 R. M. Maggio, N. L. Calvo, S. E. Vignaduzzo, T. S. Kaufman, Pharmaceutical Impurities and Degradation Products: Uses and Applications of NMR Techniques, *J. Pharm. Biomed. Anal.* 101, pp. 102–122; doi: 10.1016/j.jpba.2014.04.016.

Saga and Yamaguchi 2009 T. Saga K. Yamaguchi History of antimicrobial agents and resistant bacteria. *Japan Med. Assoc. J.* 52, pp. 103-108.

Shakespeare, W. 1623 *Much Adoe about Nothing* play included in the First Folio collection.

Spectrabase 2020 Biorad free database 2020, <https://spectrabase.com/spectrum/1zi0wvWPHaH>.

Webster et al. 2009 G. K. Webster, I. Marsden, C. A. Pommerening, C. M. Tyrakowski, B. Tobias, Determination of Relative Response Factors for Chromatographic Investigations Using NMR Spectrometry *J. Pharm. Biomed. Anal.* 49, pp 1261–1265, doi: 10.1016/j.jpba.2009.02.027.

Zhang et al. 2013 E. Zhang, H. Tian, S. Xu, X. Yu, Q. Xu, Iron-Catalyzed Direct Synthesis of Imines from Amines or Alcohols and Amines via Aerobic Oxidative Reactions under Air, *Org. Lett.*, 15(11), pp. 2704-2707, <https://doi.org/10.1021/ol4010118>).