FULL PAPER

Analysis of histamine and sisomicin in gentamicin: Search for the causative agents of adverse effects

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Abstract

In 1998, the aminoglycoside antibiotic gentamicin sulfate caused several cases of deaths in the United States, after the switch from twice- to once-daily application. Endotoxins were discussed as the cause for the adverse effects and sisomicin was identified as the lead impurity; batches containing sisomicin were contaminated with more impurities and were responsible for the fatalities. In 2016, anaphylactic reactions in horses, and later in humans with one fatality, were observed after application of gentamicin sulfate contaminated with histamine. To determine whether histamine was responsible for the 1990s death cases as well, histamine was quantified by means of liquid chromatography–tandem mass spectrometry (LC-MS/MS) in 30 samples of gentamicin sulfate analyzed in previous studies. Furthermore, a relative quantification of sisomicin was performed to check for a correlation between histamine and the lead impurity. A maximum amount of 11.52 ppm histamine was detected, which is below the limit for anaphylactic reactions of 16 ppm, and no correlation of the two impurities was observed. However, the European Medicines Agency recommends a stricter limit with regard to the maximum single dose of gentamicin sulfate to reach a greater gap between the maximum histamine exposition of 4.3 μg and the quantity known to cause hypotension of 7 μg. The low amounts of histamine and the fact that there is no connection with the contamination with sisomicin showed that histamine was not the cause for the death cases in the United States in 1998, and endotoxins remain the most probable explanation.

KEYWORDS
drug impurities, gentamicin sulfate, histamine, LC-MS/MS, sisomicin

1 | INTRODUCTION

The antibiotic gentamicin was first described in 1963 as a mixture of closely related aminoglycosides produced by *Micromonospora purpurea*.[1] The gentamicins C_1, C_{1a}, C_2, C_{2a}, and C_{2b} (Figure 1) are the main components and show similar antibacterial activities.[2,3] The sulfate

salt of the broad-spectrum antibiotic is used in the treatment of severe infections with various Gram-negative and Gram-positive microorganisms like *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Acinetobacter pittii*.[4] The oral bioavailability of gentamicin is low because of its hydrophilic (log P = −3.1) and cationic character, with five basic nitrogen atoms in the pK_a range of 5.7–9.9.[5,6] Hence, an intravenous or

FIGURE 1 Main components of gentamicin and their limitations[2]
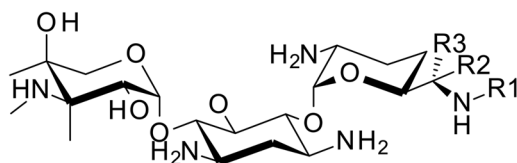
Component	R1	R2	R3	PhEur Limits
C₁	CH₃	CH₃	H	25 to 45%
C₁ₐ	H	H	H	10 to 30%
C₂	H	CH₃	H	35 to 55%
C₂ₐ	H	H	CH₃	
C₂ᵦ	CH₃	H	H	

FIGURE 2 Sisomicin

intramuscular application is necessary for systemic antibiosis.[7] Moreover, the topical application of gentamicin via (eye) ointments and eye drops is common in the therapy of local infections, often in combination with glucocorticoids.[8,9] Several resistance mechanisms like enzymatic drug modification (e.g., acetylation and phosphorylation), target modification (16S rRNA methylation), and efflux-mediated resistance have been described for aminoglycosides.[10]

Like for other aminoglycosides, the most relevant adverse effects of gentamicin are ototoxicity and nephrotoxicity.[11] During the first years after its introduction, the market authorization holders stated twice- or thrice-daily dosing (every 8–12 h).[12] In the 1990s, a lower nephrotoxicity was discussed for single daily dosing when compared to a multiple daily dosing regimen.[13–15] However, a distinct increase in deaths following severe endotoxin-like reactions was reported after a once-daily application of gentamicin sulfate in the United States,[16] even though the endotoxin concentrations of the affected batches were within the limits proposed by the US Food and Drug Administration. Perhaps, these limits were inappropriate as they considered multiple daily dosing. It was argued that higher peak concentrations of endotoxins were reached after application of a single, but higher dose of gentamicin sulfate when compared to multiple lower doses. As a result, the immunogenicity of the endotoxins exceeded tolerable limits and led to severe reactions.[17] Interestingly, an endotoxin contamination was never proven unequivocally as the root cause for the reported fatalities.

Following up these events, the Holzgrabe lab at the University of Würzburg developed several impurity profiling methods for gentamicin sulfate using capillary electrophoresis, micellar electrokinetic chromatography (MEKC), nuclear magnetic resonance spectroscopy, and multivariate analysis.[18–23] Batches containing the aminoglycoside sisomicin (4,5-dehydrogentamicin C1a; Figure 2) could be related to the ones that had caused the deaths. Hence, sisomicin was recognized as a lead impurity: Batches containing sisomicin were contaminated with more impurities of a higher quantity. The assessed batches of gentamicin sulfate were divided into two major groups: a sisomicin-containing group responsible for the deaths and a sisomicin-free group without linkage to the adverse effects.

In 2016, anaphylactic reactions including tachypnea, tachycardia, sweating, and shivering were reported owing to the application of
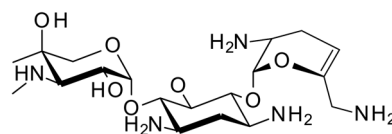
gentamicin sulfate to horses. Later, humans were also affected, with one fatality reported. The reactions were caused by elevated levels of histamine in the drug substance, which occurred after the manufacturer had changed his supplier of fish peptone, a raw material required for the fermentative production of gentamicin. The levels of histamine produced with the new supplier's fish peptone were distinctly higher than those in the batches produced before, that is, about 100 ppm versus max. 12 ppm, because the new supplier had not stored the fish under suitable conditions.[24] Hence, microorganisms like *M. morganii* or *K. pneumoniae*, which grow during spoilage of fish, decarboxylated free histidine to histamine.[25] Moreover, *M. purpurea*, which is used for the production of gentamicin, can produce histamine from histidine by its enzyme aromatic L-amino acid decarboxylase as well.[26]

As a consequence, the manufacturer changed the supplier and the European Medicines Agency defined limits for histamine in both fish peptone and gentamicin sulfate, that is, 16 ppm, as no adverse reactions had been observed with batches complying with this limit.[27] After this, the General Monograph "Products of Fermentation" in the European Pharmacopoeia (PhEur) was revised. In earlier versions, the raw materials were required to be "of suitable quality for the intended purpose."[28] Since the implementation of PhEur 9.6, the levels of free histidine in fish peptones must be considered to prevent the formation of histamine during fermentation processes.[29] Another revision, published in PhEur 10.4 and effective since 04/2021, states the following: "It must be demonstrated that the process or processes chosen reduce to a minimum or remove [...] histamine and other biogenic amines from fish and fishery products used in raw materials."[30]

In this study, histamine was quantified using liquid chromatography (LC) and mass spectrometric (MS) detection in 30 gentamicin batches that had been analyzed earlier in the context of the deaths in the United States. In addition, the lead impurity sisomicin was quantified by means of normalization to assess whether the contamination with sisomicin and its accompanying impurities, respectively, is linked to elevated contents of histamine. The aim of the work was to determine whether the deaths in the 1990s were caused by histamine instead of the hypothesized endotoxins.

2 | RESULTS AND DISCUSSION

2.1 | Quantification of histamine in gentamicin sulfate

The quantification of histamine was performed according to a method provided by Sandoz Canada Inc.[31] The 30 batches were
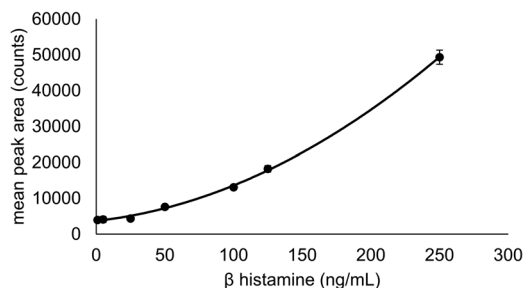
FIGURE 3 External calibration for histamine by quadratic regression ($n = 3$, ±1 SD), $y = 0.5591x^2 + 43.1342x + 3675.9810$; $R^2 = .9993$

analyzed using a hydrophilic interaction liquid chromatography (HILIC) column of unbound silica with a mixture of ammonium formate and acetonitrile (ACN) as the mobile phase and MS detection in multiple reaction monitoring (MRM) mode. Quantification was performed by external calibration (Figure 3) in the range of 1–250 ng/ml, equivalent to 0.2–50 ppm histamine. As described in the original method, a quadratic calibration curve was obtained, which is in line with previous reports on histamine quantification.[32] System suitability according to the original method requires a relative standard deviation of below 15% at the calibration level of 100 ng/ml (20 ppm) and a recovery of 70–130% at the calibration level of 125 ng/ml (25 ppm).[31] The relative standard deviation at 20 ppm was found to be 4.78% and the recovery at 25 ppm was 101.9% (±3.8). Moreover, the recovery at the calibrator below the 16 ppm limit, that is, 10 ppm, was determined to be 107.8% (±6.3%).

The content of histamine could be quantified in 6 of the 30 tested samples and ranged from 3.4 to 11.5 ppm (Table 1). All other batches showed contamination with histamine, but at a level below the quantification limit of 0.2 ppm. Exemplary chromatograms of G22 (11.5 ppm) and G24 (≤0.2 ppm) are shown in Figure 4. The neutral loss of ammonia, represented by the transition of m/z 112 → 95, was the most favored fragmentation reaction of histamine and yielded higher peak areas than the formation of the imidazolyl radical (m/z 112 → 68; Figure 5). The detection limit of the more sensitive transition (m/z 112 → 95) is lower than 0.25 ng/ml, equivalent to 0.05 ppm (signal-to-noise ratio, 12.9 ± 2.1).

2.2 | Contamination with the lead impurity sisomicin

To check the correlation of the contamination with histamine and sisomicin, a relative quantification of sisomicin was performed. A HILIC (zwitterionic) method for the chromatographic separation of aminoglycosides suitable for MS detection was applied with slight modifications.[33] A quantification by means of normalization is appropriate in this case as the analytes' structures (cf. Figures 1 and 2) are closely related and thus are conjectured to show very similar ionization efficiencies.[34] Samples G02, G05, G11, G22, and M5 were

**TABLE 1** Content of histamine and assignment to groups defined in previous studies of gentamicin sulfate

Sample	Histamine (ppm)	Sisomicin
G02	≤0.2	-
G04	≤0.2	-
G05	≤0.2	X
G06	≤0.2	-
G07	≤0.2	-
G08	≤0.2	-
G09	≤0.2	-
G10	≤0.2	-
G11	3.4	-
G12	≤0.2	X
G13	≤0.2	-
G14	≤0.2	X
G15	≤0.2	-
G16	≤0.2	X
G18	≤0.2	X
G20	≤0.2	X
G21	≤0.2	X
G22	11.5	X
G23	≤0.2	-
G24	≤0.2	-
G25	≤0.2	X
G26	≤0.2	X
G27	8.9	-
M1	3.9	n/a
M2	≤0.2	X
M3	≤0.2	-
M4	≤0.2	-
M5	8.7	X
M6	7.8	X
M7	≤0.2	-

Abbreviations: n/a, assignment to documentation ambiguous; X, sisomicin-containing group, -, sisomicin-free group.

selected for the measurements considering their histamine content and presumed sisomicin contamination according to the documentation (cf. Table 1).

As reported in previous studies, the analyzed gentamicin samples could be divided into two groups: one contaminated with sisomicin and one showing significantly lower amounts of the lead impurity (Figure 6). As expected, elevated levels of the lead impurity occurred in the batches reported to contain sisomicin and many other
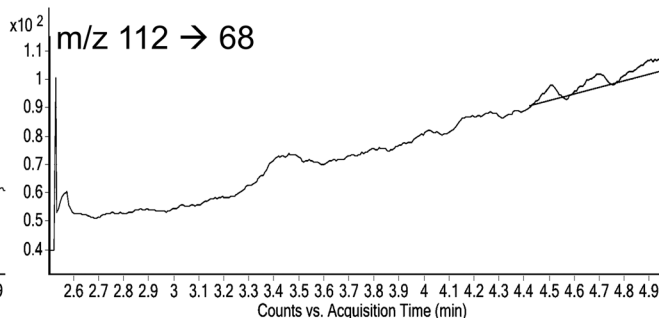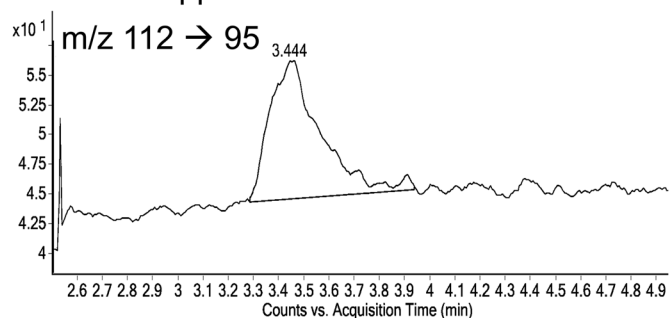
G22: 11.5 ppm
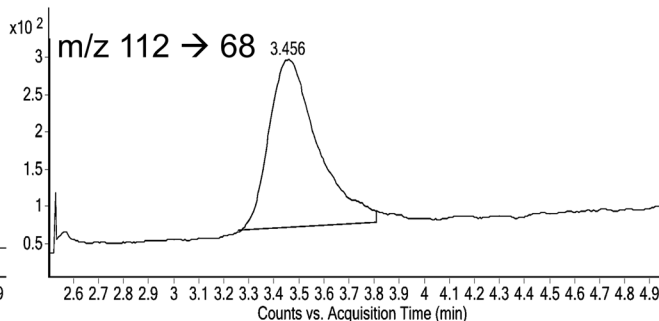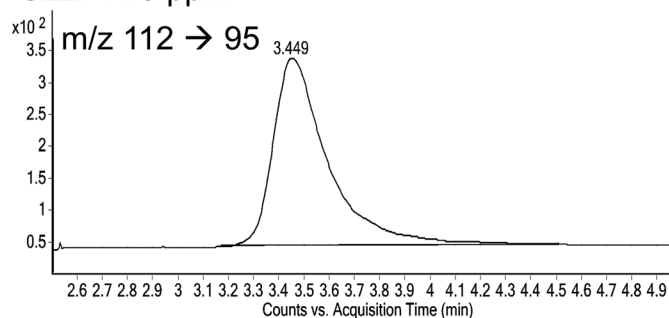
G24: < 0.2 ppm

FIGURE 4 Characteristic extracted ion chromatograms for m/z 112 → 95 and m/z 112 → 68 of two gentamicin samples: G22, which is contaminated with 11.5 ppm histamine, and G24, which shows a peak below the quantification limit (like all samples with histamine contents below 0.2 ppm)
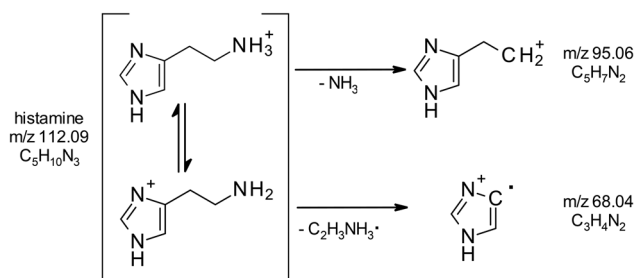

FIGURE 5 Fragmentation reactions of histamine monitored for quantification
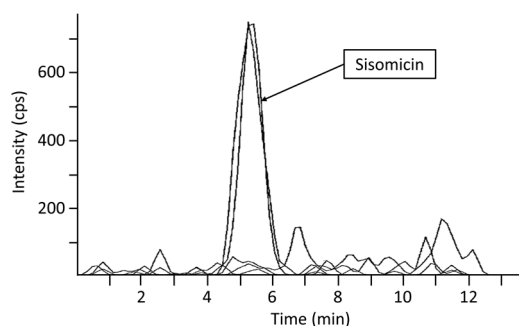

FIGURE 6 Overlay of exemplary extracted ion chromatograms of the sisomicin-containing group and the sisomicin-free group

impurities, in concordance with previous studies (MEKC).[21] G05 contained sisomicin, but no histamine, and G11 vice versa. G22 and M5 contained both contaminants. Taken together, there is no link between the occurrence of histamine and sisomicin.

3 | CONCLUSION

Two main conclusions can be drawn from our studies with regard to the "old" batches: Since the concentrations of maximal 11.5 ppm of histamine detected are below the limit of 16 ppm, the occurrence of anaphylactic reactions upon application of these batches is unlikely. However, the limit of 16 ppm, which refers to the maximum single dose of 160 mg of gentamicin, results in a maximum intake of 4.3 µg of histamine and is regarded "not sufficiently below the quantity of histamine which is known to cause hypotension (7 µg)."[24] Thus, a stricter limit is recommended to ensure the absence of anaphylactic reactions. The fact that the content of sisomicin in the batches is not related to the histamine contamination strengthens the conclusion that histamine was not the causative agent of the deaths in the United States in 1998.

The occurrence of histamine in gentamicin sulfate illustrates the relevance of raw material quality in the production of drug substances. The change in the General Monograph "Products of Fermentation" of the PhEur was implemented to prevent the emergence of histamine in the drugs affected by the monograph by controlling the contamination of the raw material with free histidine and is now

even more rigid, requiring suitable purification processes regarding biogenic amines from fishery products.[29,30] The fish peptone already contained histamine instead of its amino acid precursor,[27] which shows that testing for histidine alone could be insufficient. Instead, control of both free histidine and histamine is necessary to consequently ensure appropriate quality of fish peptones. Moreover, similar events with other biogenic amines like serotonin and noradrenaline might be possible if the respective amino acids tryptophan and tyrosine were present in a fermentation broth together with bacteria capable of amino acid decarboxylation and hydroxylation. Especially with intravenous application, serious adverse events like the serotonin syndrome, elevated blood pressure, and tachycardia could result.[35,36] Thus, raw materials and bacterial strains must be selected considering possible degradation products of biomolecules.

4 | EXPERIMENTAL

4.1 | Materials and instrumentation

The quantification of histamine was performed using a modular Agilent 1200 LC system, equipped with an online degasser, a binary pump, and a column oven (Agilent Technologies). A Kinetex HILIC 50 × 2.1 mm, 2.6 μm, 100 Å (Phenomenex) column was used. The system was coupled to an Agilent 6460 TripleQuad LC/MS using electrospray ionization (ESI).

For the relative quantification of sisomicin, a modular Agilent 1100 LC system, equipped with an online degasser, a binary pump, and a diode array detector, was used with a VDSpher PUR 100 HILIC-Z, 150 × 2.1 mm, 5 μm (VDS optilab) column. The system was coupled to an Agilent LC/MSD Trap SL equipped with an ESI source.

Thirty samples (Table 1) of gentamicin sulfate provided by the Federal Institute of Drugs and Medical Devices for previous works were reused for this study. Histamine dihydrochloride as well as MS-grade ACN and water were purchased from Sigma-Aldrich. Ultrapure water was produced by a water purification system from Merck Millipore.

4.2 | Quantification of histamine

Chromatographic separation was performed using a HILIC column of unbound silica (Kinetex HILIC). Mobile phase A was aqueous 25 mM ammonium formate and mobile phase B was a mixture of mobile phase A and ACN (30 + 70). The LC gradient and flow were adapted to the dimensions of the HILIC column used.[37] The gradient started with 100% B, which was decreased to 25% within 6 min. The system was cleaned and re-equilibrated by flushing the column with 100% B for 9 min. The flow rate was set to 0.2 ml/min and was directed to the mass spectrometer between 2.5 and 5 min (retention time of histamine: 3.5 min). The injection volume was set to 1 μl. The ESI parameters were applied according to the original method (gas temperature, 350°C; gas flow, 10 l/min; nebulizer, 40 psi; sheath gas

temperature, 400°C; sheath gas flow, 11 l/min; voltage, 5000 V; fragmentor, 135; collision energy, 25 V). Tandem MS (MS/MS) data were acquired in MRM mode. The neutral losses of ammonia (m/z 112 → 95) and an aminoethyl radical (m/z 112 → 68) were monitored (Figure 5). The sum of the peak areas in the extracted ion chromatograms (XIC) of both transitions was used for the quantification by means of a quadratic regression model.

10.35 mg of histamine dihydrochloride was weighed and dissolved in 25 ml of 0.01 M HCl (β = 414 μg/ml equivalent to 250 μg/ml of the free base). Seven standard solutions in the range of 1–250 ng/ml were prepared by dilution of the stock solution for the external calibration. The solutions were injected in triplicate in the order of increasing concentration.

For sample preparation, 25.0 mg of each sample was weighed and dissolved in 5.0 ml of 0.01 M HCl to reach a concentration of 5 mg/ml gentamicin sulfate. The solutions were transferred to chromatographic vials and analyzed using the method stated above.

4.3 | Analysis of the lead impurity sisomicin

A published method suitable for the chromatographic separation of aminoglycosides using HILIC with a zwitterionic stationary phase (VDSpher PUR 100 HILIC-Z) was applied with slight modifications.[33] Mobile phase A consisted of 5 mM ammonium acetate + 0.2% formic acid in a mixture of 5% water and 95% ACN. Mobile phase B contained the same buffer salts in 95% water and 5% ACN. After an isocratic step of 2.7 min at 100% B, mobile phase B was decreased to 10% within 2.2 min and held for 6.1 min to clean the column thoroughly. The system was re-equilibrated by flushing the column for 3 min with the start conditions. The injection volume was set to 5 μl, and the flow rate was 0.8 ml/min. The ESI and MS/MS parameters were set considering the LC flow rate: dry temperature, 350°C; nebulizer, 70 psi; dry gas, 12 l/min; skimmer, 40 V; and fragmentation amplitude, 0.6. The [M+H]$^+$ species for the main components of gentamicin (C_1 m/z 478, C_{1a} m/z 450, C_2/C_{2a}/C_{2b} m/z 464) and sisomicin (m/z 448) were isolated and fragmented. The evaluation was performed based on the XICs for the most abundant fragment ion of m/z 322 for all aminoglycosides, which emerges upon cleavage of a glycosidic bond (neutral loss of the amino sugar purpurosamine bearing R1–R3, displayed on the right side in Figure 1).

Five gentamicin samples were selected considering their histamine contamination (see Section 2.1) and their characteristics based on the documentation of previous works of the Holzgrabe lab.[18–22] Solutions with a concentration of 100 μg/ml of the gentamicin sulfate samples were created by dissolving 10 mg in 100.0 ml of a mixture of mobile phases A and B (3 + 8) and injected in triplicate.

CONFLICTS OF INTERESTS

The authors declare that there are no conflicts of interests.

AUTHOR CONTRIBUTIONS

Jonas Wohlfart: Methodology, formal analysis; investigation; writing—original draft. **Ulrike Holzgrabe:** Conceptualization; writing—review and editing; supervision; project administration.

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