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A Q-TOF LC/MS method for identification and quantitation of Histamine in the antibiotic Gentamicin at ppm level: Validation and uncertainty evaluation

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ABSTRACT

Adverse reactions have been reported for antibiotics produced via fermentation with fish peptone due to Histamine contamination. Just few micrograms of Histamine can result in adverse reactions when administered intravenously. Thus in this paper a new method for identification and quantitation of Histamine at ppm levels in the antibiotic Gentamicin is described. The method is based on separation of Histamine from Gentamicin and other excipients present in the drug matrix, by hydrophilic interaction liquid chromatography (HILIC) coupled to a Q-TOF/MS detector; quantitation is based on the standard addition approach.

The method was validated for repeatability, inter-day precision, specificity, accuracy (relative and absolute bias) linearity, limit of detection and quantitation. Uncertainty was estimated and evaluated by comparison with values expected according to the Horwitz theory. The method showed satisfactory performances and good sensitivity, reaching a limit of quantitation of approximately 1 ppm.

The method proposed can be a starting point for the development of Histamine quantitation methods in other antibiotics or even in other medicinal products which active ingredient is produced via fermentation in presence of fish peptone.

1. Introduction

Since the end of 2015 many adverse drug reactions (ADR) related to Gentamicin intravenous administration were reported. Initially they concerned only horses, but later also human patients were involved. The ADR reported in EU were anaphylactic reactions (e.g. flushing, itching, urticaria and shortness of breath), hypotensive reactions, increased heart rate and even death.

It was supposed that such ADR could be caused by Histamine, present at levels higher than expected in some batches of the Gentamicin sulphate active pharmaceutical ingredient (API). This contamination was considered linked to the use of fish peptone in the Gentamicin production process.

Histamine is an endogenous substance derived from the decarboxylation of the amino acid Histidine by L-Histidine decarboxylase. It is mainly involved in immune response and inflammatory reactions, but it is also a neurotransmitter in brain. It is also present in some food: for example, it is produced by bacterial fermentation of fish and at high concentrations it is the main cause of fish poisoning [Taylor 1986]: Histamine is actually called Scombrotoxin.

The amount of Histamine and other biogenic amines in spoiled fish depends on the kind of fish (mostly tuna and mackerel) and on time and temperature abuse.

Man can assume up to 180 mg of Histamine from the diet without incurring in ADR, but when administered intravenously, just 7 μg of Histamine may induce vasodilation and increase in hearth rate [1]. It was hypothesized that there is a different sensitivity to Histamine in human population due to a particular genetic polymorphism, but also to some gastro-enteral conditions, the assumption of certain medications, age and even lifestyle [2].

Just 13 ppm of Histamine in Gentamicin are sufficient to reach the danger threshold of 7 μg when Gentamicin is administered intravenously at its maximum dosage (the maximum permitted single daily dose of Gentamicin is 520 mg [3]).

After a life threatening ADR occurred at the beginning of 2017 (a woman died after intravenous administration of an 80 mg/2 ml solution of Gentamicin from a batch produced by an Italian manufacturer, which in turn used an active ingredient imported from China) the Italian Official Medicines Control Laboratory was asked

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to ascertain the quantity of Histamine present in two batches of injectable Gentamicin medicinal product (40 mg/mL as free base) and two batches of Gentamicin sulphate API.

Although various methods for detection and quantitation of Histamine exist (i.e. ELISA, enzymatic and colorimetric tests as well as spectrofluorometric and also some HPLC methods [4–7]) they are specifically designed for food matrices (fish products) and not for pharmaceutical ones and moreover often require a time consuming derivatization. Presently, no method has been published for the quantitation of Histamine in products containing Gentamicin: Thus in this paper we report the development, validation and uncertainty determination of a new method for Histamine identification and quantitation at ppm level in Gentamicin API and medicinal products.

Considering the sensitivity and specificity required, this new method was developed using LC with Q-TOF MS detector.

2. Experimental

2.1. Materials and methods

A *Fast* LC Mod.1290 Infinity, equipped with a diode array detector (DAD) and a Dual ESI source Q-TOF mass spectrometer detector Mod. G6520B (Agilent Technologies, Santa Clara, CA, USA) was used. Data were processed with MassHunter® workstation software. Solvents and reagents employed were of LC–MS grade; Histamine di-hydrochloride reference standard (≥99% purity) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.1.1. LC method development

Initially a simple gradient separation on a C18 column was tried, hoping to exploit the high selectivity provided by Mass Spectrometry. Unfortunately, both Gentamicin and Histamine are extremely hydrophilic and could not be separated chromatographically; even operating in targeted MS/MS mode no clean fragmentation spectrum of Histamine could be obtained, due to the very much higher concentration of Gentamicin (10^5–10^6 times Histamine's).

Eventually chromatographic separation of Histamine from Gentamicin and other excipients (methyl paraben, propyl paraben e sodium metabisulphite) was achieved using a hydrophilic interaction liquid chromatography (HILIC) column (Zorbax RRHDD, 2.1 × 50 mm, 1.8 μm) and the following elution pattern:

1 isocratic step with acetonitrile-ammonium formate (pH = 3.5; 20 mM) 80/20 v/v for 5 minutes
2 gradient step from initial conditions to acetonitrile-ammonium formate (pH = 3.5; 20 mM) 35/65 v/v in 8 minutes
3 restore of initial isocratic conditions in 1 minute
4 reconditioning of the column for 6 min

Flow rate was 0.5 mL/min and the injection volume was 1 μL. Column temperature was set to 30 °C. The sample was thermostated at 15 °C.

Histamine was eluted in the first step, while Gentamicin in the second.

2.1.2. Mass spectrometry parameters

The mass spectrometer instrumental parameters were: nitrogen temperature 300 °C, drying gas 10 L/min, Nebulizer 40 psig and Fragmentor 100 V.

Because of the low Histamine concentration, its peak could not be distinguished in the TIC (Total Ion Chromatogram); it was necessary to recur to the EIC (Extracted Ion Chromatogram) at ± 50 ppm of the calculated monoisotopic mass of the Histamine molecular ion (i.e. m/z = 112.0869 as provided by the Isotope Distribution Calculator application of the Agilent MassHunter® software).

2.1.3. Histamine identification

Histamine identification was accomplished first by confirming that the mass peak in the extracted chromatogram (together with its isotopic pattern) was in fact the one proposed by MassHunter® for Histamine molecular formula; second by confirming that the fragmentation spectrum of a Histamine reference standard (operating in MS/MS with a collision offset voltage of 30 V) matched the one obtained at the same retention time in Gentamicin samples.

The absence of compounds (or fragments) that have a mass similar to that of Histamine, which could contribute to the intensity of the EIC Histamine peak (thus determining an overestimation of Histamine concentration) was confirmed by the mass spectrum of the Histamine peak, verifying that around the Histamine molecular ion peak there were no other significant mass signals (up to ±7 m/z).

2.1.4. Histamine quantitation

For the quantitation of Histamine in Gentamicin, the standard additions method was used.

The following solutions were prepared.

2.1.4.1. Solution A (Gentamicin API). Gentamicin API was dissolved in water at the same concentration of the medicinal product (i.e. 63.5 mg/mL as Gentamicin sulphate, 40 mg/mL as Gentamicin free base).

2.1.4.2. Solution B (Histamine standard). 25 mg of Histamine dihydrochloride were dissolved in 100 mL of water and then diluted 1:100 with water and then 1:10 with acetonitrile/water 1:1 + 0.1% formic acid (diluent), reaching a concentration of $2.5 \cdot 10^{-4}$ μg/μL.

2.1.4.3. Spiked solutions. Four spiked solutions were prepared adding 1, 2, 3 and 4 mL of Solution B to 5 mL of Solution A and each one was then made up to 10 mL with the diluent.

The spiked solutions obtained were analysed together with the unspiked one (prepared adding 5 mL of diluent to 5 mL of Solution A). Thus 5 solutions in total were analysed for each Gentamicin sample.

For the medicinal products the same process was followed, the only difference being that in this case samples were already in form of solution: the content from 15 vials (each of 2 mL in volume) was mixed; from this mixture the 5 solutions to be analysed were then prepared as for Gentamicin API: 5 mL of the mixture were spiked with 0, 1, 2, 3 and 4 mL of solution B and made up to10 mL with the diluent.

A straight line was then obtained plotting the 5 areas of the Histamine chromatographic peaks in EIC (m/z = 112.0869) vs the concentrations of the added Histamine.

The absolute value of the intersection of this line with the negative X-axis of the plot, provided the calculated Histamine concentration in each Gentamicin sample.

2.2. Method validation

Validation was performed in order to verify specificity, linearity, repeatability, intermediate precision, accuracy, limit of detection, limit of quantitation and uncertainty of measurement. The validation was conducted following the general scheme proposed by [8] and [9].

Validation was conducted entirely on one of the two available batches of Gentamicin API. One of the two batches of Gentamicin medicinal product was used to ascertain method specificity. The second batch of API and both batches of medicinal product were then analysed with the same method in order to confirm method