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Improved liquid chromatographic method with pulsed electrochemical detection for the analysis of gentamicin

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Abstract

The official method for the determination of the composition and related substances of gentamicin prescribed by the European Pharmacopoeia (Ph. Eur.) is liquid chromatography combined with pulsed electrochemical detection (LC-PED). However, this method utilizes a polymer stationary phase which shows rather low efficiency towards the separation of the main gentamicin components. Moreover, the mobile phase contains a lot of non volatile salts: sodium sulphate and sodium octanesulphonate. Following a comparative study, the most performant LC–PED method has been evaluated and validated using a reversed phase C18 column (C18, 250 × 4.6 mm ID, 110 Å, 5 μm) kept at 35 °C with a mobile phase containing volatile ion pairing agents: trifluoroacetic acetic acid (TFA) and pentafluoropropionic acid (PFPA). In addition to the selectivity of the main gentamicin components and its related substances, the method is repeatable, linear and proves to be robust. It is also applicable to a wider number of C18 columns.

Keywords: Evaluation; Validation; Composition; Related substances; Gentamicin sulphate; Aminoglycoside antibiotics

1. Introduction

Gentamicin is an important member of the class of aminoglycoside antibiotics that has a broad spectrum towards severe infections caused by Gram-negative bacteria both in human and veterinary medicine. It was originally obtained from fermentation of a strain of *Micromonospora purpurea* [1]. Gentamicin consists of two amino sugars glycosidically linked to positions 4 and 6 of 2-deoxystreptamine**.** Gentamicin is a complex mixture of four major components: C_1, C_{1a}, C_2, C_{2a} and the minor component C_{2b} (Fig. 1). Since it is a fermentation product, it can contain several structurally related substances like gentamicin B_1, sisomicin, dihydroxygentamicin C_{1a}, JI-20B, degradation products like garamine, 2-deoxystreptamine and several other, unknown compounds, formed in small amounts. The differences in antimicrobial potency and sometimes toxicity necessitate to limit and control carefully the amount of impurities in commercial samples. In 2002, Hildebrand [2] reported 66 deaths in addition to hundreds of patients suffering from severe side effects after administration of gentamicin sulphate. It was assumed that these cases were caused by impurities due to faulty manufacture. Therefore there is a need of a powerful separation technique combined with a sensitive detector in order to separate and detect impurities, even if they are present at lower concentrations.

Gentamicin is highly polar, non-volatile and lacks a UV absorbing chromophore. These properties, for many years, pose a great challenge in the analysis of this drug. In 1963, Rosselet and coworkers [3] first reported on the isolation of the gentamicin complex using ion exchange chromatography. From that time, ion exchange procedures continued to be used extensively for the separation and purification of gentamicin. Several separation methods have been applied for the determination of the main gentamicin C component ratio in commercial samples and include paper [4] and thin layer chromatography [5], Craig distribution [6], cation and anion exchange LC [7–9], reversed phase LC [10–26] and capillary electrophoresis (CE) [27–28]. Determination of related substances has been reported only in a few papers [23–26]. To solve the detection problems, direct and indirect methods have been applied. Pre and post-column derivatization by *o*-phthaldialdehyde, dansylchloride and mercaptoacetic acid with either UV or fluorescence detection have

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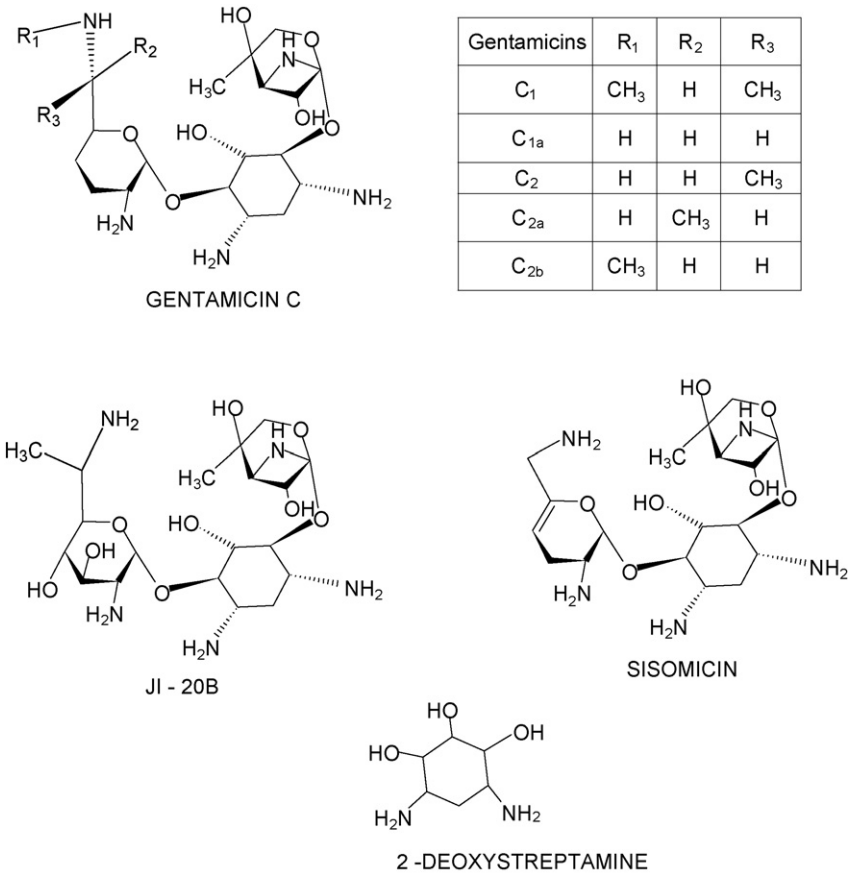

Gentamicins	R_1	R_2	R_3
C_1	CH_3	H	CH_3
C_{1a}	H	H	H
C_2	H	H	CH_3
C_{2a}	H	CH_3	H
C_{2b}	CH_3	H	H

Fig. 1. Chemical structures of the main gentamicin compounds and some of its impurities.

been reported [8,10–13,15,16,19,25,27,28]. These derivatization methods have proved to be tedious, time consuming and not free of toxic side effects. They also may lead to reaction incompleteness and to formation of reaction by-products which will lead to difficulties in quantitation. For the sake of simplicity and correct quantitation, direct detection methods are preferred. Direct detection with potential gradient [28], refractive index (RI) [14], evaporative light scattering (ELSD) [22,23], mass spectrometry (MS) [26] and electrochemical detection (ED) [24] have been investigated. Potential gradient detection has been used to determine the composition of the main components. Even though RI, ELSD and MS are described as universal detectors, their usefulness for routine analysis is limited. RI shows poor sensitivity, ELSD shows a non-linear response to the amount of sample injected and is not very sensitive either. ELSD like MS, requires volatile mobile phases, which unfortunately showed less selectivity. These methods are not always very robust so that they are less suitable for routine analysis. In addition, MS is an expensive technique. So, ED seems to be the method of choice since it shows the highest sensitivity, selectivity, robustness and relatively low operation costs for routine use. ED for gentamicin started its history back in 1983 when Getek et al. [17] used a glassy carbon electrode to detect gentamicin. Later on, Kaine and Wolnik [9] used pulsed electrochemical detection (PED) on a gold electrode. More recently, Adams et al. [24] developed an ion pair reversed phase LC method with PED using a gold working electrode. This method was slightly adapted and is the current official method in the monograph of gentamicin in the European Pharmacopoeia (Ph. Eur.) [29].

PED of aminoglycosides is based on the oxidation of the analyte following applied potentials on the surface of a gold electrode [30]. In practice, a three electrode system is used, which allows the precise control of the applied potentials. PED may suffer from some signal stability, but with some experience it is possible to obtain nice and reproducible results. Proper selection and handling of the reagents to avoid fouling of the electrodes are of vital importance. Using a polymer column, the official method for the analysis of gentamicin proved to be not very selective, especially towards the separation between gentamicins C_{2b}–C_2 and C_2–C_{2a}. The method cannot be transferred as such to silica-based columns because the mobile phase contains a lot of non-volatile salts, which would seriously limit the life cycle of this type of stationary phase.

The aim of this study was to make available to the Ph. Eur a more selective method, which should be robust, linear, repeatable and easy to use in routine analysis. After comparison of several LC methods, the most performant LC-PED was selected [31]. This method, which utilizes a silica based Gemini C18 column, was further evaluated and validated in this study. The mobile phase contains acetonitrile as organic modifier and an aqueous solution of volatile ion pairing agents (trifluoroacetic acid and pentafluoropropionic acid) adjusted to pH 2.6 with