PAPER

LC with electrochemical and UV detection for analysis of a formulation containing gentamicin and parabens

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This work describes two separate liquid chromatographic (LC) methods which were developed to control gentamicin sulphate and its preservatives methylparaben and propylparaben in an injectable formulation for veterinary purposes. Owing to the absence of a UV absorbing chromophore in the gentamicin molecule, LC combined with pulsed electrochemical detection (LC-PED) was found to be suitable for the determination of this drug in the formulation. The LC-PED methods from edition 7.0 (valid till 06/2012) and 7.5 (supplement valid from 07/2012) of the European Pharmacopoeia (Ph. Eur.) were compared. The currently recommended LC-PED method allowed good separation between the main gentamicin components and their impurities without interference from additives. Because of its better performance the actual method was used to investigate the degradation profile of the gentamicin sulphate injection. Results obtained from the forced stability study showed that the formulation was stable to oxidative and thermal stress. For the control of the preservatives, a LC-UV method was applied. Both methods were found to be specific, linear and precise. Hence, these two LC methods can be used for routine analysis of the gentamicin sulphate injection.

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1 Introduction

Gentamicin sulphate is a potent broad spectrum aminoglycoside antibiotic, which is used as an active pharmaceutical ingredient (API) for the treatment of severe infections caused by Gram-negative bacteria. It is a widely used aminoglycoside, mainly in veterinary medicine, for the treatment of septicemia, infections of the lungs, bones, gastrointestinal and urogenital tracts, skins and soft tissues. Gentamicin sulphate is available in various formulations including injections, creams, eyedrops, ophthalmic solutions and eardrops. Gentamicin was originally obtained from fermentation of a strain of *Micromonospora purpurea*¹ and consists of two amino sugars glycosidically linked to positions 4 and 6 of 2-deoxystreptamine. It occurs as a complex mixture of four major components: C₁, C_{1a}, C₂, C_{2a} and the minor component C_{2b} (Fig. 1).

Since gentamicin is a fermentation product, it can contain several structurally related substances like gentamicin B₁, 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

Gentamicin	Mol. Formula	R1	R2	R3
C1	C ₂₁ H ₄₃ N ₅ O ₇	CH ₃	CH ₃	H
C1a	C ₁₉ H ₃₉ N ₅ O ₇	H	H	H
C2	C ₂₀ H ₄₁ N ₅ O ₇	H	CH ₃	H
C2a	C ₂₀ H ₄₁ N ₅ O ₇	H	H	CH ₃
C2b	C ₂₀ H ₄₁ N ₅ O ₇	CH ₃	H	H

sisomicin

2-deoxystreptamine

Fig. 1 Chemical structure of the main gentamicin sulphate components.

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commercial samples. Impurities are assumed to be responsible for the severe side effects often encountered after administration of gentamicin sulphate.² The impurities may arise due to

faulty manufacture or degradation upon storage. To ensure optimal therapeutic efficacy with less side effects, close control of this drug is mandatory.

Routine analysis of gentamicin is not so easy. This is due to its polar basic nature and the absence of a UV absorbing chromophore. Several efforts have been made in order to analyze gentamicin in bulk samples, biological samples and formulations. These techniques involve mainly ion exchange procedures and include LC,^{3–22} paper chromatography,²³ thin layer chromatography,²⁴ Craig distribution²⁵ and capillary electrophoresis^{26–28} with either direct or indirect detection mechanisms. Indirect methods involved pre-column derivatization with *o*-phthaldehyde (OPA),^{6–10} 2,4,6-trinitrobenzenesulphonic acid¹¹ or post-column derivatization with OPA.^{4,12,13} Nevertheless, derivatization techniques are not preferred for routine analysis because they are tedious, time consuming and can give problems with quantitation due to reaction incompleteness and instability of the derivatized products. For proper quantitative work, direct detection is preferred. In gentamicin analysis, refractive index (RI),¹² evaporative light scattering (ELSD),^{14,15} charged aerosol detector (CAD),¹⁶ pulsed electrochemical detection (PED)^{17–20} and mass spectrometry (MS)²¹ have been employed. RI detection shows poor sensitivity, ELSD and CAD show non-linear response, and MS is an expensive technique for routine analysis. Also, ELSD, CAD and MS require only volatile mobile phases. So, LC-PED was found to be the method of choice for the analysis of aminoglycosides.²² This is because of its high sensitivity, selectivity and relatively low operation cost for routine use.

Some organic acids and their esters are used as single preservatives, but a combination of them is more common in cosmetics, food and pharmaceutical products.²⁹ They are the important constituents to prevent alteration and degradation of the formulation, especially for multi-dose products. In pharmaceutical products, the benzoic acid esters methylparaben (MP) and propylparaben (PP) (Fig. 2) are the most commonly used, usually combined together for a maximum synergistic effect in a molar ratio between 7.5 : 1 and 9 : 1. The doses are relatively low, varying from 0.002% to 0.2%.^{30,31} These parabens have a broad antimicrobial activity in the pH range of 4–8. Outside this range, they will be hydrolyzed.³¹ Also, adsorption of the parabens may occur in some plastic containers and so lower their effectiveness. Hence, determination of these preservatives in formulations is particularly important both for quality assurance and consumer safety.

Owing to the presence of a UV absorbing chromophore in the preservative's molecules, direct analysis by LC-UV is possible. In the literature, several LC methods have been described for the

analysis of these esters in pharmaceuticals, food and cosmetics.^{32–45} Simultaneous determination of MP and PP was preferred, as they are usually used in combination. Hence, the LC-UV method previously developed in our laboratory by Aluoch-Orwa *et al.*⁴⁵ was used as the starting point.

This work describes two LC methods which were developed for the control of an injection for veterinary purposes. The formulation contains gentamicin sulphate (5 mg ml⁻¹ as base) as the API and MP (0.18% w/v), PP (0.02% w/v), sodium metabisulphite (SMB) (0.3% w/v) and sodium edetate (EDTA) (0.01%) as additives. When this study was started, the official method in the 7.0 edition (edn) of the European Pharmacopoeia (Ph. Eur.), valid till 06/2012,¹⁸ for the determination of related substances and composition of gentamicin bulk samples was LC-PED using a poly(styrene-divinylbenzene)copolymer (PSDVB) column. During the time that this method was introduced in the Ph. Eur., this reversed phase polymer packing showed a better stability compared to silica based columns, but its efficiency was rather low. In the mean time, new types of silica based columns became available and column properties improved a lot. Recently, an improved LC-PED method that utilizes a silica-based reversed phase column has been incorporated in supplement 7.5 of the Ph. Eur.²⁰ and is based on the method of Manyanga *et al.*¹⁹

This work investigates the efficiency of these two methods, focusing on the separation of the main components and their related impurities. The influence of the additives was also investigated. Based on the results of the experiments, the more performant method was selected for further quantitative work. This method was also used to conduct a stability study by forced degradation in order to generate formulation product related impurities. For the control of the paraben preservatives, the suitability of the LC-UV method developed by Aluoch-Orwa *et al.*⁴⁵ was examined. Here also quantitative parameters like sensitivity, linearity, repeatability, accuracy and specificity were checked according to the Veterinary International Conference on Harmonization (VICH) guidelines.⁴⁶

2 Experimental

2.1 Reagents and samples

All the reagents used were of HPLC grade. Tetrahydrofuran (THF), stabilized with 2,6-di-*tert*-butyl-4-methylphenol, 30% hydrogen peroxide (H₂O₂) and extra pure anhydrous sodium sulphate (Na₂SO₄) were obtained from Merck (Darmstadt, Germany). Potassium dihydrogen phosphate, dipotassium hydrogen phosphate, methanol, 99% trifluoroacetic acid (TFA), 97% pentafluoropropionic acid (PFPA) and sodium octanesulphonate (SOS) were purchased from Acros Organics (Geel, Belgium). Acetonitrile (ACN) was from Fisher Scientific UK Limited (Loughborough, Leicestershire, UK). A 50% sodium hydroxide solution was from J.T. Baker (Deventer, The Netherlands) and 85% phosphoric acid was from Sigma Aldrich (Steinheim, Germany). Water was produced in-house using a Milli-Q water purification system (Millipore, Bedford, MA). Helium gas was obtained from Air Liquide (Liège, Belgium). All mobile phases were degassed by sparging helium gas.

Fig. 2 Chemical structure of methylparaben and propylparaben.

The samples used to conduct this study were obtained from a company: gentamicin injection (commercial veterinary formulation containing gentamicin sulphate (5 mg ml⁻¹ as base), MP (0.18% w/v), PP (0.02% w/v), sodium metabisulphite (SMB) (0.3% w/v) and sodium edetate (EDTA) (0.01%)), blank with and without preservatives, gentamicin sulphate bulk samples and reference samples for MP, PP, SMB and disodium EDTA. Gentamicin sulphate chemical reference substance (CRS) and sisomicin sulphate CRS were obtained from European Directorate for the Quality of Medicines (EDQM) (Strasbourg, France). All solutions were prepared using a mobile phase before injection.

2.2 LC instrumentation and chromatographic conditions

2.2.1 LC-PED. The LC apparatus consisted of an L-6200 Intelligent Pump (Merck Hitachi, Darmstadt, Germany), an autosampler AS 100 Spectra Series with a fixed 20 µl loop (San Jose, CA, USA), a Decade II electrochemical detector (Antec, Leyden, the Netherlands) and Chromeleon 6.70 software (Dionex Corporation, Sunnyvale, CA, USA) for data acquisition. The detector cell consisted of a gold working electrode, a HyRef reference electrode and a carbon filled polytetrafluoroethylene (PTFE) counter electrode. HyREF is an alternative for a Ag/AgCl reference electrode. It is principally maintenance free and does not contain a salt bridge. The detector cell was kept at 35 °C in a hot air oven. The pulse settings were as follows: t_{det} 400 ms with $E_{\text{det}} + 0.05$ V, t_{oxd} 200 ms with $E_{\text{oxd}} + 0.75$ V and t_{red} 400 ms with $E_{\text{red}} - 0.15$ V. Integration of the signal occurred between 200 ms and 400 ms.

During this study, the following columns were used: poly(styrene-divinylbenzene) copolymer, 250 × 4.6 mm ID, 100 nm, 8 µm (Polymer Laboratories, Shropshire, UK) for the Ph. Eur. 7.0 method and Zorbax SB C18, 250 × 4.6 mm ID, 5 µm (Agilent Technologies, Inc., CA, USA) for the new improved LC-PED. The columns were kept at a constant temperature by using a water bath with a heating immersion circulator (Julabo, Seelbach, Germany). In the final conditions, the mobile phase was composed of 35 ml l⁻¹ of ACN and 965 ml l⁻¹ of an aqueous solution containing TFA (7 ml l⁻¹), PFA (250 µl l⁻¹) and 50% NaOH (4 ml l⁻¹). The aqueous phase was previously adjusted to pH 2.6 with 0.5 M NaOH. The flow rate was 1.0 ml min⁻¹.

For good detection of aminoglycosides with PED, at least pH 12 is necessary. Since the mobile phase has a lower pH, 0.5 M NaOH was pulselessly added post-column using a helium-pressurized reservoir. The column effluent was mixed with the base in a packed reaction coil from Dionex (1.2 m, 500 µl). The 0.5 M NaOH solution was prepared starting from a 50% (m/m) aqueous solution which was pipetted in helium degassed water. This solution was degassed for about 10 minutes in order to avoid the formation of carbonates that foul the working electrode.

2.2.2 LC-UV. The LC apparatus consisted of an L-6200 intelligent pump (Merck Hitachi), an L-2200 Elite LaChrom autosampler (Merck Hitachi), an L-2400 Elite LaChrom UV detector (Merck-Hitachi) set at 254 nm and Chromeleon 6.60 software (Dionex) for data acquisition. This method utilizes a

PSDVB column kept at a temperature of 60 °C. The mobile phase consisted of ACN–0.2 M phosphate buffer pH 7.0–water A (35 : 5 : 60, v/v/v) at a flow rate of 1 ml min⁻¹. The injection volume was 100 µl. UV detection was performed at 254 nm.

3 Results and discussions

3.1 Gentamicin injection analysis

3.1.1 Comparison between the new and old method of the Ph. Eur. First, a comparison between the LC-PED methods described for the analysis of gentamicin in the Ph. Eur. 7.0 and 7.5 supplement was performed.^{18,20} The two methods were compared on the basis of their specificity, selectivity and sensitivity.

During this initial stage, formulation blank, gentamicin sulphate CRS for identification, gentamicin bulk sample which was used for the preparation of the formulation and the gentamicin formulation itself were injected in both systems. Typical chromatograms obtained under the above mentioned experimental conditions are shown in Fig. 3 and 4 for the Ph. Eur. method and the improved LC-PED respectively. As can be seen from these chromatograms, the Ph. Eur. 7.0 method is less selective since fewer impurities can be separated from the main gentamicin components. This is because the efficiency of PSDVB as a stationary phase is lower and as a consequence, the selectivity and sensitivity are rather poor. On the other hand, polymer columns show a good stability and reproducibility. The new improved method utilizes a C18 reversed phase column for the determination of gentamicin sulphate. With this method, many impurities are well separated from the main components and from each other with no interference from preservatives or blank solutions. Because of its better selectivity, this method was further used for quantitative work and for the stability study. In general better peak shapes and baseline separations were observed for the LC-PED method from the 7.5 supplement. Another noteworthy observation here was the difference in elution order of the C_{2b} component in the two methods which utilize different stationary phases.

Fig. 3 Typical chromatograms obtained after analyzing formulation blank (1), gentamicin sulphate CRS (2), gentamicin sulphate powder used for the formulation (3) and gentamicin formulation (4) using the Ph. Eur. 7.0 method.

Fig. 4 Typical chromatograms obtained after analyzing formulation blank (1), gentamicin sulphate CRS (2), gentamicin sulphate powder used for the formulation (3) and gentamicin formulation (4) using the new LC-PED method for gentamicin described in the 7.5 supplement of the Ph. Eur.

3.1.2 Validation of the improved LC-PED method. For the quantitative work, two sets of solutions were prepared. Set one was prepared with a concentration of 1.0 mg ml^{-1} ($20 \mu\text{g}$ injected, corresponding to 100%) gentamicin sulphate and was used for the determination of related substances. Set two was prepared with a concentration of 0.2 mg ml^{-1} gentamicin sulphate and was used for the determination of composition of the main gentamicin C components. Sisomicin sulphate CRS was used at a concentration of 0.01 mg ml^{-1} (1%) as an external standard for impurity content determination. The use of gentamicin as a standard for impurity content determination is less reliable since it consists of several compounds, which proportions vary from sample to sample.

The sensitivity, linearity and repeatability of the method were evaluated. For the determination of impurities, the limit of detection (LOD) (corresponding to a signal-to-noise ratio of 3) and limit of quantitation (LOQ) (corresponding to a signal-to-noise ratio of 10) were assessed by using sisomicin sulphate CRS. An LOD of 3 ng corresponding to 0.015% was found and an LOQ value of 10 ng, corresponding to 0.05%. The linearity was examined from LOQ to 5%, since above this concentration overloading of the detector was observed. The following linearity was obtained $y = 577989x + 114$, where y = peak area and x = concentration in mg ml^{-1} , with coefficient of determination (R^2) = 0.999. For determination of the composition of the main gentamicin components, the linearity was checked according to the limits prescribed in the Ph. Eur.¹⁸ The ranges were examined and their results are summarized in Table 1. As can be seen good results were obtained. Repeatability for the main components was examined at a concentration of 0.2 mg ml^{-1} . The gentamicin formulation was analysed six times ($n = 6$). The relative standard deviation (RSD) values in the areas of the main gentamicin components are 0.4% for C_{1a} , 0.5% for C_2 , 1.2% for C_{2b} , 0.4% for C_{2a} and 0.3% for C_1 . These values demonstrate the good precision of the method. The accuracy of the method was determined by checking the recovery of sisomicin sulphate dissolved in the blank. Accuracy data are shown in Table 2.

Table 1 Linearity data for the main gentamicin components^a

Gentamicin	Concentration ranges (mg ml^{-1})	Regression equation	R^2	S_{yx}	n_i	n_c
C_{1a}	0.0025–0.0752	$y = 726447x + 2533$	0.9873	3628	3	5
C_2	0.0042–0.0851	$y = 717267x + 1881$	0.9952	2857	3	4
C_{2b}	0.0003–0.0079	$y = 545515x + 43$	0.9962	120	3	4
C_{2a}	0.0030–0.0616	$y = 654659x + 541$	0.9990	828	3	4
C_1	0.0047–0.0951	$y = 653713x + 987$	0.9981	1735	3	4

^a Where R^2 = coefficient of determination, n_c = number of experimental concentrations studied, n_i = number of injections for each concentration, y = peak area, x = concentration in mg ml^{-1} , and S_{yx} = standard error of estimate.

Table 2 Recovery of sisomicin

% Concentration	Concentration (mg ml^{-1})	% Recovery of sisomicin sulphate
0.5	0.005	98.0
1.0	0.01	97.9
1.5	0.015	97.2
3.0	0.03	99.7

3.2 Control of preservatives

3.2.1 Preliminary investigations. Preliminary investigations showed that MP and PP present in the formulation were well separated under these chromatographic conditions without any interference from the blank. Typical chromatograms are shown in Fig. 5. Although not the primary aim, SMB could not be detected. Decreasing the wavelength to 210 nm allowed detection of SMB at around 2.4 minutes, but only with a very poor sensitivity.

3.2.2 Method validation. Linearity was examined for MP and PP simultaneously at five different concentrations from 50% to 120% (100% corresponds to $18 \mu\text{g ml}^{-1}$ for MP and $2 \mu\text{g ml}^{-1}$ for PP). The following results were obtained; $y = 100.7x + 0.9$, where y = peak area and x = concentration, with $R^2 = 1$ for MP and $y = 92.4x - 0.3$ with $R^2 = 1$ for PP.

Fig. 5 Typical chromatograms obtained after analyzing methylparaben and propylparaben in the formulation (1) and formulation blank (2) using the chosen LC-UV method.

Table 3 Recovery of methylparaben and propylparaben

Sample concentration (%)	Recovery (%)	
	Methylparaben	Propylparaben
80	99.7	97.8
100	101.9	99.8
120	100.0	98.7

Repeatability for MP and PP was examined at 100% concentration. The formulation was analysed six times ($n = 6$). The RSD values were found to be 0.7% for MP and 0.2% for PP which indicates good precision of this method.

The accuracy of the method was determined by checking the recovery of MP and PP dissolved in the blank. The data obtained for recovery, shown in Table 3, demonstrate good method performance.

3.3 Stability studies

This study was done with the objective of determining the stability of the API in the formulation. Since gentamicin sulphate is quite stable under ambient conditions, the stability study for the formulation was carried out under accelerated heat and oxidative conditions.

3.3.1 Stability of the formulation under oxidative and thermal conditions

3.3.1.1 Degradation using hydrogen peroxide. One drop of 15% H_2O_2 solution was added per ml of the formulation. The samples were withdrawn after 0, 2, 8 and 24 hours and diluted to 1 mg ml^{-1} using a mobile phase. Reference solution was prepared by taking 1.0 ml of the formulation and adding to it one of drop of water instead of H_2O_2 . The reference solution was then also diluted to 1.0 mg ml^{-1} using a mobile phase. Under these experimental conditions, no degradation of the sample was observed. Furthermore, the concentration of H_2O_2 was increased to 25%. Here also no degradation was observed.

3.3.1.2 Degradation using hydrogen peroxide and heating. Since no significant degradation was obtained by using H_2O_2 at room temperature, it was decided to combine it with heating. This was performed by adding 5 drops of 25% H_2O_2 solution per ml of sample. The resultant solution was kept in an oven at a temperature of 60 °C. The samples were withdrawn after 0, 2, 8 and 24 hours and diluted to 1.0 mg ml^{-1} using a mobile phase. The reference solution was prepared in a similar way by adding 5 drops of water instead of H_2O_2 . The sample was then analysed at different time points. The results obtained showed that no degradation of the formulation occurred.

3.3.2 Stability of the solutions to be injected in the LC system. The stability of the solutions of the formulation that are used for injecting in the LC system was checked by heating the formulation at a concentration of 1.0 mg ml^{-1} at 60 °C and 100 °C at 0, 2, 8 and 24 hours. Blank solution was prepared by diluting the blank of the formulation the same way as for formulation using a mobile phase. The experiments showed that there was no degradation of the sample solutions at 60 °C and 100 °C.

4 Conclusions

Compared to the Ph. Eur. 7.0 method for the analysis of gentamicin sulphate, the improved LC-PED method allowed better separation between the main gentamicin components and their impurities in the gentamicin formulation studied. Moreover, the method proved to be sensitive, selective, specific, accurate, linear and repeatable. The method was also applied for forced stability studies of the gentamicin injection. The results of these experiments showed that the formulation was stable to oxidative and thermal stress conditions. For the control of preservatives, the LC-UV was successfully applied for their simultaneous analysis in the pharmaceutical dosage form. This method was also found to be specific, accurate, linear and precise. Hence, the two LC methods can be used for routine quality control of the gentamicin injection.

Abbreviations

ACN	Acetonitrile;
API	Active pharmaceutical ingredient;
CAD	Charged aerosol detector;
CRS	Chemical reference substance;
EDTA	Sodium edetate;
EDQM	European Directorate for the Quality of Medicines;
ELSD	Evaporative light scattering;
H_2O_2	Hydrogen peroxide;
PED	Pulsed electrochemical detection;
PFFA	Pentafluoropropionic acid;
LC	Liquid chromatographic;
LC-PED	LC combined with pulsed electrochemical detection;
MP	Methylparaben;
MS	Mass spectrometry;
NaOH	Sodium hydroxide;
Na_2SO_4	Sodium sulphate;
OPA	<i>o</i> -Phthaldehyde;
Ph. Eur.	European Pharmacopoeia;
PP	Propylparaben;
PSDVB	Poly(styrene-divinylbenzene) copolymer;
PTFE	Polytetrafluoroethylene;
LOD	Limit of detection;
LOQ	Limit of quantitation;
RI	Refractive index;
RSD	Relative standard deviation;
SMB	Sodium metabisulphite;
SOS	Sodium octanesulphonate;
TFA	Trifluoroacetic acid;
THF	Tetrahydrofuran;
VICH	Veterinary International Conference on Harmonization Guidelines.

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