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Determination of gentamicin in pharmaceutical formulations using peroxyoxalate chemiluminescent detection in flow-injection analysis

José M. Fernández-Ramos, Ana M. García-Campaña, Fermín Alés-Barrero, Juan M. Bosque-Sendra *

Department of Analytical Chemistry, Faculty of Sciences, University of Granada, E-18071 Granada, Spain

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Abstract

A simple and sensitive procedure for the determination of gentamicin is presented, based on the use of the peroxyoxalate chemiluminescent (PO-CL) system in presence of imidazole as a catalyst. The gentamicin has to be previously derivatized with *o*-phthaladehyde (OPA) in order to obtain a fluorophore, which participates in the PO reaction, producing a CL emission proportional to the gentamicin concentration. The method is developed by using a particular flow-injection analysis (FIA) manifold, employing sodium dodecyl sulfate (SDS) micellar medium as a carrier in order to avoid the degradation of PO in water. The optimization of the instrumental and chemical variables affecting the CL reaction was rigorously carried out by using experimental design methodology. The method has been successfully applied to pharmaceutical formulations.

Keywords: Gentamicin; Peroxyoxalate; FIA; Chemiluminescence; Pharmaceutical analysis

1. Introduction

Gentamicin is an aminoglycoside antibiotic produced from *Micromonospora purpura,* which is effective against a wide variety of a susceptible gram-positive and gram-negative bacteria [1]. It is a mixture of five major components designated as C_1, C_2, C_{1a}, C_{2a} and C_{2b}, these forms differ each other in their degree of methylation on the purpurosamine ring [2]. Gentamicin is a highly polar polycationic molecule, which is relatively lipid-insoluble; therefore, it is poorly absorbed on the gastrointestinal tract, being the oral bioavailability around 0.2% [3]. This antibiotic is eliminated by the renal route via glomerular filtration almost entirely in the active form. In the management of systematic infections it is administered by injection and for topical infections it is administered directly to the site.

Numerous methods for the determination of gentamicin have been developed. These are based on a microbiological assay [4], enzyme immunoassay [5], polarization fluoroimmunoassay [6] gas–liquid chromatography [7], capillary electrophoresis [8] and high-performance liquid chromatography (HPLC). The microbiological assay is inexpensive and simple, but the reproducibility is limited and it has a long incubation period. The immunoassay is more specific and accurate, but it depends on the purity of enzyme. Both methods may be subject to possible interference from other antibiotics. Mass spectrometry could permits very sensitive and specific measurements, but it is unavailable to many facilities because of its relatively high-cost. Several modes of detection including UV [9], fluorescence [10], electrochemical [11] and mass spectrophotometry [12], have been reported.

Chemiluminescence (CL) is a very sensitive analytical technique that has been used for the analysis of pharmaceutical compounds [13], mainly in combination of flow-injection analysis (FIA) manifold [14] due to the advantages of this coupling, such as small analysis time, automation, high-versatility and satisfactory precision. Some CL methods have been proposed for the analysis of gentamicin and other aminoglicosides. The luminol reaction has been proposed as CL system for the indirect detection of the aminoglycoside amikacin, which is based on the strong inhibition of the CL emission from the oxidation of the luminol in alkaline medium in presence of Cu(II) as a catalyst [15]. A flow-injection analyzer with a thin-layer electrochemical cell modified for electrogenerated chemiluminescence is proposed to determine streptomycin and gentamicin using

* Corresponding author. Tel.: +34 958 248435; fax: +34 958 243328.
E-mail address: jbosque@ugr.es (J.M. Bosque-Sendra).

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immobilized tris(4,7-diphenyl-1,10-phenanthrolinedisulfonic acid) ruthenium(II) on an electrode surface [16]. A flow-injection system is used for the chemiluminescence determination of gentamicin based on the CL reaction of gentamicin and Co(III), which is electrogenerated on-line [17]. CL emitted during the oxidation of tobramycin, micronomicin, amikacin and gentamicin with *N*-bromosuccinimide in alkaline medium in the presence of fluorescein is uses to propose a flow-injection chemiluminescence method for the determination of these aminoglicosides [18]. Gentamicin and other nine antibiotics have been determined in milk using a multianalyte immunoassays combined with the detection of the CL generated from the luminol system in the presence of horseradish peroxidase [19].

Peroxyoxalate chemiluminescent reaction (PO-CL) [20,21] is a kind of sensitized CL, very efficient for the determination of hydrogen peroxide and some energy-acceptors such as fluorescent molecules and compounds that are not natively fluorescent, which can be derivatized with fluorescent labels to be quantified by this technique. Gentamicin is not natively fluorescent, but it could be derivatized in order to participate in the PO-CL detection. Based on its chemical structure, derivatization of gentamicin has been carried out with *o*-phthaladehyde (OPA) [22], dansyl chloride [23], fluorescamine [24], 9-fluorenylmethyl chloroformate, (FMOC-CL) [25] and 1-fluoro-2,4-dinitrobenzene (FDNB) [26].

In this paper, we propose for the first time the application of the PO-CL system for the determination of an aminoglycoside antibiotic. The proposed CL detection is coupled to a special FIA manifold [27,28], which allows the application of the PO reaction in micellar medium, avoiding the use of organic solvents to prevent the decomposition of the POs. In relation to the above mentioned methods for the determination of gentamicin based on CL detection present suitable linear ranges and good limits of detection, but the proposed method is easier to implement for routine analysis in pharmaceutical control using a cheaper and simple equipment. This straightforward method has shown it is usefulness in the analysis of gentamicin in pharmaceutical formulations.

2. Experimental

2.1. Chemicals and solutions

All the reagents or solvents were of analytical reagent or HPLC grade. Ultrapure water (Milli-Q Plus 185, Millipore Corporation) was used for the experimental work.

A stock solution of OPA (Sigma-Aldrich) was prepared daily by dissolving 0.05 g of OPA in 1 mL of methanol (Panreac). A stock solution of *N*-acetylcysteine (NAC, Fluka) was prepared daily by dissolving 0.075 g in 10 ml of deionized water. Working derivatization solutions were prepared daily by adding the adequate volumes of the stock solutions of OPA and NAC in 100 mL of 2.5 mM sodium borate (Sigma-Aldrich) buffer, pH 9.2. A 10 mM sodium dodecyl sulphate (SDS, Panreac) solution was prepared in 0.1 M sodium dihydrogenphosphate (Panreac) buffer (pH 5.0) and used as carrier and solvent. A 1 M stock solution of imidazole (IMZ, Sigma-Aldrich) was prepared weekly by dissolving 6.8 g of IMZ in water. Proper working solutions were prepared daily in SDS solution and used as a catalyst. A working solution of the appropriate concentration of hydrogen peroxide (from 30% w/v solution, Panreac) was prepared daily by dilution in SDS and used as oxidant. A working solution of bis(2,4,6-trichlorophenyl)oxalate (TCPO, Sigma-Aldrich) was prepared daily by dissolving 0.044 g in acetonitrile (Panreac). A 100 mg l^{-1} stock solution of Gentamicin sulfate (665 mg/gr potency) (Sigma-Aldrich) was prepared daily by dissolving 7.5 mg in 50 ml of ultrapure water. The standard solutions were freshly prepared by dilution of the stock solution with ultrapure water in propylene volumetric labware.

2.2. Apparatus and software

A Campecs CL-1 luminometer (Campecs, United Kingdom), equipped with a quartz flow-cell with a volume of 120 μl, data control and acquisition programme (CSW 32) was used for the CL measurements and data processing.

The FIA manifold used (Fig. 1) includes a Gilson Minipuls 3 peristaltic pump to deliver the carrier and to regulate the flow, a Rheodyne 5020 manual injection valve to inject working and sample solutions into the carrier stream and PTFE connecting tubing (0.5 mm i.d.).

For statistical treatment of data ALAMIN software [29] and STATGRAPHICS [30] package were used.

2.3. Derivatization procedure

1 ml of the standard or sample solution containing gentamicin was added to 1 ml of OPA reaction solution (23 mM for OPA concentration and 0.36 mM for NAC concentration). They were mixed and placed in an ultrasound bath during a minute at room temperature.

2.4. General procedure

The FIA manifold consisted in a three-channel configuration where the standard or sample and TCPO solutions are incorporated to the carrier (SDS solution, 3 mL/min) with the aid of two rotary valves, respectively (Fig. 1). Hydrogen peroxide and imidazole in SDS solution are pumped at 1.63 mL/min and 2.20 mL/min, respectively. A volume of 500 μL of TCPO solution is injected manually in valve 1 and 300 μL of the standard

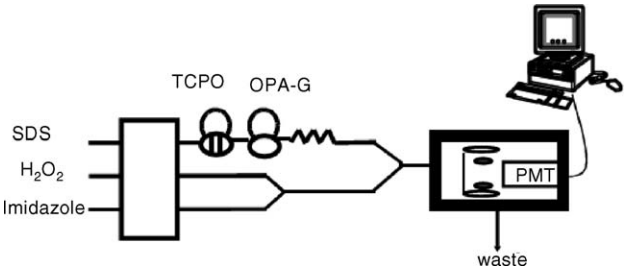

Fig. 1. Proposed manifold: PMT: photomultiplier tube; TCPO: bis(2,4,6-trichlorophenyl)oxalate; SDS: sodium dodecyl sulphate; OPA: *o*-phthalaldeyde; G: gentamicin.

or sample solutions containing the gentamicin derivatized with OPA (G-OPA) is injected using valve 2. Both valves are turned with a delay of 2 s between injections. In the carrier, the TCPO and sample solutions are mixed in a reaction coil (50 cm. length, 0.5 mm i.d.) and then, merged with the imidazole and hydrogen peroxide streams, allowing the production of the CL signal in the flow-cell placed just in front of the detector. Derivatization solutions produce a residual signal-blank due to the fluorescent compound produced in the reaction between OPA and NAC. Blank solutions (derivatization solution-water, 50:50 v/v) are measured in the same way. The peak of the blank (5–10% of the sample peak) is subtracted from the high-peak of the sample.

2.5. Sample preparation

Two types of treatments were used depending on the characteristics of the pharmaceutical formulations. The first one is applicable to the injection ampoules (Genta-Gobens) and eyes drop (Coliscursi gentamicina 1) formulations. An appropriate volume of these solutions is diluted up to the adequate concentration of gentamicin, between the ranges of the calibration curve (10 mg/L), with ultrapure water. Specifically, 50 μl of the injection ampoules were diluted up to 100 ml and 42 μl of the eyes drop formulation were diluted up to 25 ml.

The second treatment is useful for the eye cream (Oftalmolosa). 1 g of eye cream was added to 80 ml of water and the suspension obtained was heated to 70 °C during 10 min and then it was cooled at room temperature. The samples were filtered through a filter paper and the vaseline and colesterine precipitated were eliminated. The filtrate was diluted with ultrapure water in a 100 ml standard flask and finally 8.3 ml of this solution was diluted to 25 ml.

3. Results and discussion

3.1. Optimization of experimental conditions

The PO-CL reaction for the determination of gentamicin is based on the previous formation of a fluorescent derivative with OPA and NAC in alkaline medium (Fig. 2). OPA reacts with the free primary amino groups of gentamicin. TCPO is oxidized by hydrogen peroxide in the presence of IMZ as a catalyst, leading to the formation of a energy-rich intermediate, 1,2-dioxetane-3,4-dione [31], capable of exciting the fluorophore through the CIEEL (chemically initiated electron exchange) mechanism [32], by which the intermediate forms a charge transfer complex with the fluorophore, donating one electron to the intermediate, which is transferred back to the fluorophore raising it to an excited state and liberating an emission typical for the nature of this fluorescent derivative.

A multivariate optimization [33] was used to obtain the optimum values for the chemical and instrumental variables involved in this CL-based method. The optimization process implied four steps: (i) optimization of chemical variables implied in the CL process, such as pH, hydrogen peroxide and imidazole concentrations; (ii) optimization of instrumental variables related to the FIA system, such as H_2O_2 and imidazole flow-rates; (iii) optimization of the labelling reaction (OPA and *N*-acetylcysteine concentrations); (iv) optimization of the injection volumes for TCPO and gentamicin-OPA solutions.

3.1.1. Optimization of the chemical variables affecting the CL intensity

A central composite design, 2^3+ star (faced center), was used to optimize the pH and concentrations of hydrogen peroxide and imidazole. The factorial design was evaluated using as analytical signal the difference between the peak height (signal) in the presence of gentamicin and the peak height of the blank (blank). The magnitude of the blank peaks is 4–10% of signal peaks (between 100 and 1200 depending of the conditions). The experimental conditions for log [H_2O_2] and log [imidazole] were studied in the range between −2 and 0 and −3 and −1, respectively and between 5 and 6.5 for pH. The values of instrumental variables and chemical variables affecting the derivatization reaction were maintained fixed (Table 1). The SDS concentration was selected above the CMC in order to avoid the decomposition of TCPO [34]. Seventeen experiments are required by

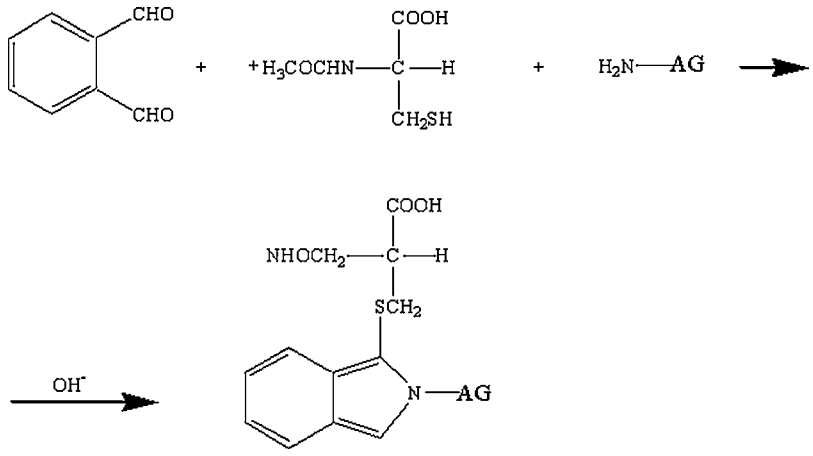

Fig. 2. Chemical reaction performed for the derivatization of gentamicin using OPA in presence of NAC. AG represents the aminoglicoside gentamicin showing a primary amine group to be labelled.

Table 1
Conditions of the labelling reaction and instrumental variables

Variable	Value
[TCPO]	1 mM
[NaH_2PO_4] (buffer)	100 mM
SDS	10 mM
Flow-rate imidazole	2 mL/min
Flow-rate H_2O_2	2 mL/min
Inject volume of TCPO	500 μL
Sample inject volume	60 μL
OPA (pH 9.2)[a]	2 mM
N-acetylcysteine (pH 9.2)[a]	2 mM

[a] Sodium borate buffer 2.5 mM.

using a 2^3 + star (Table 2). The significance of the effects was checked by application of the analysis of variance (ANOVA) and using *P*-values for the significance levels. The results have demonstrated that the second-order effect of the imidazole and two interaction effects (H_2O_2-pH and imidazole-pH) were non-significant. The function representing the relationship among pH, H_2O_2 and imidazole concentrations and the chosen response is:

$$\begin{aligned}(\text{Signal} - \text{blank}) = {} & 365.4 + 36.4 \times \log[H_2O_2] + 7.2 \\ & \times \log[\text{Imidazole}] - 63.2 \times \text{pH} - 115.3 \\ & \times \log [H_2O_2]^2 - 66.0 \\ & \times \log[H_2O_2] \times \log[\text{Imidazole}] - 190.8 \\ & \times \log [\text{Imidazole}]^2 + 30.0 \times \text{pH}^2\end{aligned}$$

This model, where the non-significant effects have been removed, fits the experimental data (*P*-value, lack-of-fit test 20%). The optimum value obtained for each variable was 0.15 M for H_2O_2, 10 mM for imidazole and a pH value of five.

Table 3
Final optimum values from the experimental designs

Carrier flow-rate (SDS, ml min^{-1})	3.00
Imidazole flow-rate (ml min^{-1})	2.20
Peroxide flow-rate (ml min^{-1})	1.63
TCPO injection volume (μl)	500
AG-OPA injection volume (μl)	300
Time of labeling reaction (min)	1
Turn time between valves (s)	2
OPA concentration in derivatization solution (mM)	23
NAC concentration in derivatization solution (mM)	0.36
pH	5
TCPO (mM)	1
Peroxide (mM)	150
Imidazole (mM)	10

3.1.2. Optimization of the imidazole and hydrogen peroxide flow-rates

As a previous step, the flow-rate of the carrier (SDS solution) was optimized separately. This variable is the only one affecting the interval of time between the turnings of both injection valves. Finally, a flow-rate of 3 mL/min, with a 2 s lapse between both injections, was finally selected because of the best sensitivity and precision of the signals were obtained at these conditions. A 3^2 factorial design was used for the flow-rate optimization of the other two channels. The flow-rates of imidazole and hydrogen peroxide were studied in the range between 0.75 mL/min and 3.5 mL/min and using a flow-rate of 2 mL/min as the center point. The function representing the relationship among flow-rates of imidazole and hydrogen peroxide and the chosen response is:

$$\begin{aligned}(\text{Signal} - \text{blank}) = {} & 328.5 - 45.5 \times \text{Flow-rate}\, H_2O_2 + 12.9 \\ & \times \text{Flow-rate imidazole} - 115.5 \\ & \times \text{Flow-rate}\, H_2O_2^2 - 31.0 \times \text{Flow-rate}\, H_2O_2 \\ & \times \text{Flow-rate imidazole} - 84.5 \\ & \times \text{Flow-rate imidazole}^2\end{aligned}$$

Table 2
Design matrix for the optimization of chemical factors

Experiment	H_2O_2	Imidazole	pH	Signal-blank		
				Experimental	Expected	Residual
1	0	0	0	409.7	365.4	44.3
2	−1	−1	−1	5.9	42.9	−36.9
3	1	−1	−1	248.5	247.6	0.9
4	−1	1	−1	265.1	189.3	75.8
5	1	1	−1	125.7	130.1	−4.4
6	−1	−1	1	21.2	−83.5	104.8
7	1	−1	1	122.0	121.1	0.9
8	−1	1	1	56.8	62.9	−6.1
9	0	0	0	516.7	365.4	151.3
10	1	1	1	11.9	3.7	8.2
11	−1	0	0	76.1	213.7	−137.6
12	1	0	0	280.9	286.4	−5.6
13	0	−1	0	97.7	167.4	−69.7
14	0	1	0	108.4	181.9	−73.5
15	0	0	−1	423.2	458.6	−35.4
16	0	0	1	224.4	332.1	−107.7
17	0	0	0	456.2	365.4	90.8

Table 4
Determination of gentamicin in pharmaceutical formulations

Formulation	Concentration ($n=5$) (mg/ml)	R.S.D (%)	Nominal value (mg/ml)	t calculated	$t_{(0.05,4)}$ tabulated	P-value (%)
Genta-gobens®	21 ± 1	11.63	20	0.629	2.776	56.46
Oftalmolosa cusi gentamicina®	3.03 ± 0.06[a]	4.70	3[a]	0.447	2.776	67.79
Colircusí gentamicina 1®	20.5 ± 0.6	6.28	20	0.889	2.776	42.41

Composition: genta-gobens®: sodium metabisulfite, methylparaben, propylparaben and purified water; Oftalmolosa cusi gentamicina®: gentamicin sulfate, cholesterine and vaseline; Colircusí gentamicina 1®: gentamicin sulfate, benzalkonium chloride, monosodium phosphate, disodium phosphate, sodium chloride and purified water.

[a] Values expressed in mg/g.

This model, where non-significant effects have been removed, fits the experimental data (P-value, lack-of-fit test 70% and $R^2 = 97.98\%$). The optimum values for the hydrogen peroxide and imidazole flow-rates were 1.63 and 2.20 mL/min, respectively.

3.1.3. Optimization of the derivatization reaction

The derivatization reaction (Fig. 2) is carried out off-line, using OPA to obtain the fluorescent derivative, which will acts as an energy-accepting fluorophore in the PO-CL reaction. NAC was used as the provider of the thiol group in the labeling reaction [35], instead of 2-mercaptoethanol, avoiding its high-toxicity and the instability of the isoindole derivates obtained with this compound.

Some experiments were performed in order to establish the most appropriate conditions for the production of the fluorescent derivative from the gentamicin and OPA reaction solution (G-OPA). A 3^2 factorial design was used to optimize the OPA and NAC concentrations. The pH was fixed at 9.2 due to it is the best pH for the labelling reaction [35]. The concentration of both reagents, OPA and NAC, was studied in the range between 10^{-4} and 10^{-2} M.

The second-order function obtained fits the experimental data (P-value, lack-of-fit test 10.0% and $R^2 = 94.89\%$). The optimum values obtained were 23 mM for OPA concentration and 0.36 mM for NAC concentration.

3.1.4. Optimization of G-OPA and TCPO injection volumes

A 3^2 factorial design was selected to optimize these variables. The levels of the experimental domain selected were 20–500 μL for inject volume of TCPO and 100–500 μL for inject volume of G-OPA. A response surface described by a second-order equation, which represents the relationship among the analytical signal and the injection volumes, was obtained and employed for establishing the optimum values of the both variables. They were 500 μL for TCPO injection volume and 300 μL for G-OPA injection volume.

The optimum values found for all the significant variables involved in the general procedure are shown in Table 3.

3.2. Calibration curves and performance characteristics

Under the optimum conditions, a calibration curve was estimated by measuring the peak heights of the CL emission (signal-blank) of gentamicin standard solutions and using three replicates for each concentration level. It was established by applying univariate linear regression in the range of 3.93–30 μg/mL. The linear equation obtained was CL emission = 171.26 × [Gentamicin] − 25.91 with a determination coefficient (R^2) of 99.4% and a residual standard deviation of 69.8 CL unit × ml × mg^{-1}. The on-line linearity (97.8%) and analytical resolution (1.01 CL unit × ml × μg^{-1}) were estimated using the calibration dataset [29]. The detection and quantification limits according to IUPAC recommendations [36] were 1.18 and 3.93 μg ml^{-1}.

3.3. Applications

To check the trueness of the proposed method, the analytical procedure was applied to pharmaceutical formulations, such as Genta-Gobens® (Normon, Spain), Oftalmolosa Cusi Gentamicina® (Alcon Cusi, Spain) and Colircusí Gentamicina 1® (Alcon Cusi, Spain). A t-test was applied to compare if the nominal values (claimed on the formulation label) were similar to the obtained average from the application of the proposed method. The results are shown in Table 4. As can be seen, no significant differences were found between the compared values, being the method applicable to these pharmaceutical formulations.

4. Conclusions

The application of the PO-CL system is proposed for the first time for the detection of an aminoglycoside antibiotic. The use of a SDS micellar medium and the special FIA configuration allow the use of the TCPO in absence of organic solvent in the flowing stream. The method implies a previous formation of a fluorescent derivative gentamicin-OPA in presence of *N*-acetylcysteine and the subsequent oxidation of TCPO by hydrogen peroxide using imidazole as catalyst in alkaline medium. The CL emission generated will be proportional to the gentamicin concentration due to the participation of the fluorophore into the energy-transfer reaction. This method has shown its usefulness in the control of gentamicin content in different pharmaceutical products.

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