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Chemiluminescence determination of amikacin based on the inhibition of the luminol reaction catalyzed by copper

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Abstract

A simple and sensitive method has been proposed for the amikacin sulphate determination. It is based on the inhibition of the chemiluminescence (CL) emission generated from the oxidation of luminol in alkaline medium by H_2O_2 catalyzed by Cu(II), due to the interaction caused by amikacin, which forms a robust complex with the catalyst. The optimization of the experimental and instrumental variables affecting this CL inhibition effect has been carried out using statistical models, based on the application of two-level full factorial and Box–Behnken designs. The performance characteristics of the proposed method have been established, showing that the method is efficient to determine amikacin sulphate in the linear range of 9.89−20 mg/L with a detection limit of 2.97 mg/L. It has been successfully applied to the amikacin sulphate determination in pharmaceutical formulations.

Keywords: Chemiluminescent inhibition; Luminol; Copper; Amikacin sulphate; Hydrogen peroxide; FIA

1. Introduction

The growing necessity to establish methods able to detect smaller quantities in the analysis of biomedical products has given rise to the search of more sensitive and versatile techniques, which must cover the necessities in this field. Such a research is very important in clinical practise in relation to drug quality control. The coupling of a flow injection analysis (FIA) system with CL detection in the determination of pharmaceutical products is an interesting alternative, because it offers a serial of advantages such as high sensitivity, easy implementation, and low cost [1]. Thus, this technique could be a useful tool in the quality control of pharmaceutical products and drugs [2].

The oxidation of luminol (3-aminophthalhydrazide) in alkaline medium is one of the most efficient CL reactions. The produced CL emission is strongly enhanced when different metal ions (Co(II), Cu(II), Cr(III), etc.) are used as a catalyst, being applied with an analytical purpose for the sensitive detection of these metal ions [3–5]. Taking into account this catalytic effect, it is possible to achieve the suppression of the metal ion-enhanced luminol CL by the use of chelating agents such as citrate and EDTA [6]. Considering the fact that aminoglycoside antibiotics have been proposed as chelating agents of metal ions [7–9], our interest in this paper has been to study this effect on the emission generated from the metal ion enhanced luminol CL reaction, so as to check the possibility of a simple detection of these compounds.

Among the aminoglycoside antibiotics, amikacin (*O*-3-amino-3-desoxy-α-D-glucopyranosyl-(1-6)-*O*-[6-amino-6-desoxy-α-D-glucopyranosyl-(1-4)]-N1-(4-amino-2-hydroxy-1-oxobutyl)-2-desoxy-D-streptamine, $C_{22}H_{43}N_5O_{13}$) sulphate [10] is one of the most widely used in medicine and veterinary, due to its effect on Gram-positive and Gram-negative bacteria [11]. However, inadequate doses in patients can also produce problems in the renal system and severe dysfunction. This is the reason why its therapeutic monitoring is mandatory in pharmacology with the aim to control the adequate ingest in patients [12].

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Amikacin sulphate has been analysed by using fluorimetric [13,14], amperometric [15] or UV–vis detection [16,17]. However, no detection methods based on CL detection have been reported. In this paper, we propose the first CL method for the determination of this antibiotic based on the fact that amikacin sulphate inhibits strongly the CL emission from the oxidation of luminol in alkaline medium by hydrogen peroxide in the presence of Cu(II) as a catalyst. This is due to the fact that amikacin forms a strong complex with Cu(II) [7], decreasing the free concentration of this metal ion and avoiding its function as a catalyst. The net inhibition of the CL intensity of the luminol–H_2O_2–Cu(II) is proportional to the amikacin concentration and has permitted the establishment of a flow injection method with inhibited CL detection for the determination of the aminoglycoside. This simple and sensitive method can be used as an alternative method for quality control of pharmaceutical products. The proposed method has been satisfactorily applied to the determination of amikacin sulphate in different pharmaceutical formulations.

2. Experimental

2.1. Chemicals and solutions

All chemicals used were of analytical-reagents grade with no further purifications; reverse osmosis water (18.2 MΩ cm^{-1}) from a Millipore Milli-Q Plus water system (Milford, MA, USA) was used for diluting reagents and samples and all experiments were carried out at room temperature.

A 100 mg/L aqueous solutions of amikacin sulphate was prepared daily by dissolving 50 mg (Sigma, St. Louis, MO, USA) of the product in 50 mL of water, this solution was stable for 2 days at 2–8 °C, followed by appropriate dilution. A 4.24×10^{-4} M luminol solution was prepared daily by dissolving 0.0188 g of the substance (Sigma, St. Louis, MO, USA) in a 8×10^{-5} M NaOH solution.

A 1 M hydrogen peroxide solution was prepared daily by diluting 10.12 mL of the product (30% (w/v), Panreac Barcelona, Spain) in 100 mL of water. A 1 M stock solution of sodium hydroxide was prepared daily by dissolving 50 mg of the substance (Panreac Barcelona, Spain) in 100 mL of water. Working solutions were prepared by appropriate dilution in water. A 10^{-2} M Cu(II) stock solution was prepared by dissolving 0.253 g of $CuSO_4 \cdot 5/2H_2O$ (Riedel-de-Haën, Germany) in 10^{-3} M H_2SO_4 solution. Working solutions were prepared daily by dilution. Solutions of drug excipients (sodium metabisulfite and sodium citrate) were prepared by dissolving 2.5 g of these substances (Sigma, St. Louis, MO, USA) in 50 mL of water.

Dowex-11X2 (100–200 mesh) anion-exchange resin from Sigma (USA) was used in the chloride form. It was properly conditioned before use.

2.2. Apparatus

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

The FIA system used includes a Gilson Minipuls 3 peristaltic pump to deliver the carrier and to regulate the flow, and PTFE connecting tubing (0.5 mm i.d.). A Rheodyme 5020 manual injection valve was used to inject working and sample solutions into the carrier stream. The flow diagram system is shown schematically in Fig. 1.

2.3. Statistical software

Data analysis was carried out by using the ALAMIN software [18] to estimate the performance characteristics of the proposed method. STATGRAPHICS [19] was used for the estimation of regression models and for the application of experimental designs.

2.4. Procedure

The selected FIA manifold consisted of in a three-channel configuration where either the standard or sample solutions where incorporated to the carrier with the aid of a rotary valve (Fig. 1). The carrier (H_2O) and a 10^{-2} M H_2O_2 solution were pumped continuously at 2.3 mL/min and the resulting stream was then mixed with the 8×10^{-6} M luminol solution in a 8×10^{-5} M NaOH solution, pumped at 1 mL/min, into a flow cell by the peristaltic pump. Standard solutions of the complex amikacin–Cu(II) were prepared by transferring increasing aliquots of the amikacin standard solution into a 10 mL calibrated flask and 3.45 μL of a 10^{-2} M Cu(II) solution and then diluting to the mark with water. A volume of 70 μL was incorporated into the carried through the injection valve. The same procedure was carried out to measure the blank signal, considering it as the signal corresponding to the injection of a similar volume of a 3.45×10^{-5} M Cu(II) solution into the carrier stream. The decrease in the CL intensity produced when a solution containing the amikacin–Cu(II) complex is incorporated into the carrier stream, in relation to the original CL emission corresponding to a blank containing the same Cu(II) concentration, is proportional to the amikacin sulphate concentration and has been used as analytical signal. This signal is measured as peak height.

For the analysis of the selected pharmaceutical formulations, appropriately treated solutions were diluted with water and a suitable volume of Cu(II) solution was added to obtain a final concentration of 3.45×10^{-5} M. This final solution can be directly injected into the carrier steam in the FIA manifold.

2.5. Sample preparation

The level of sodium citrate used as additive in some pharmaceutical formulations could produce some interference in

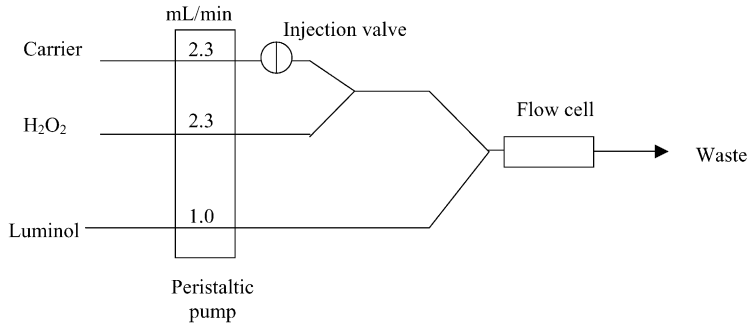

Fig. 1. Schematic diagram of the flow injection system used in the CL determination of amikacin.

the quantification of these samples. For this reason, in the case of Biclin® (Brystol Myers, USA) and Amikacina Normon® (Normon, Spain) formulations, diluted solutions were prepared as follows: 200 μL of the sample was diluted with water up to 100 mL, then 2 mL of this solution was diluted up to 100 mL with water and finally, 5 mL of this solution was passed through a 3 g of Dowex-1 anion-exchange resin to remove the high concentration in sodium citrate. A total volume of 20 mL was used to wash the resin, avoiding a possible retention of the analyte. The obtained eluate was collected and a suitable volume of Cu(II) solution was added to obtain a final concentration of 3.45×10^{-5} M, diluting to 50 mL with water. The Amukin® formulation (Brystol Myers, Belgium) was not pretreated with the resin because sodium citrate is not used as excipient. In this case, 50 mg of Amukin was dissolved with water and the volume was made up to 50 mL. Then, 1 mL of this solution was mixed with a suitable volume of Cu(II) solution to obtain a final concentration of 3.45×10^{-5} M, and diluted up to 10 mL with water.

3. Results and discussion

3.1. Optimization of experimental conditions

A multivariate optimization [20] was used for obtaining the optimum values for the chemical and instrumental variables involved in this CL-based method. The optimization process implied three steps: (i) use of a screening design in order to select the significant factors in the CL inhibition; (ii) application of a second-order design to estimate the real functional relationship between the CL response and the significant chemical variables; (iii) application of a multivariate optimization of flow rates and an univariate optimization of the injection volume in the FIA system.

3.1.1. Screening design

The chosen factors to be study were: Cu(II), luminol and H_2O_2 concentrations and the flow rates, considering for the three streams an unique value. It is well-known that the CL reaction of luminol with H_2O_2 catalyzed by Cu(II) ion is carried out in a strong alkaline medium [21]. For this reason, the NaOH concentration to dissolve the luminol was kept constant (Table 1) to assure an alkaline pH. A two-level full factorial 2^4 design was used for screening purposes to identify the significant variables affecting the response. Minimum and maximum levels for each factor are indicated in Table 1. The factorial design was evaluated using the analytical signal (peak height). 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 demonstrated that the luminol concentration is the most significant factor, together with H_2O_2 and Cu(II) concentrations. Besides, the interactions (luminol concentration × Cu(II) concentration) and (luminol concentration × H_2O_2 concentration) are also statistically significant. In addition, the flow rate factor had not a significant effect on the CL inhibition, finally, a 2.3 mL/min flow rate for the three channels was selected.

3.1.2. Optimization of chemical variables

From the previous experience, luminol, H_2O_2 and Cu(II) concentrations are the significant factors that can be optimized. Seventeen experiments are required by using a Box–Behnken experimental design for this purpose (Table 2). Luminol, H_2O_2 and Cu(II) concentrations were modified in the ranges: 8×10^{-6} to 10^{-6}, 10^{-2} to 10^{-4}, and 5×10^{-5} to 10^{-5} M, respectively. For this optimization, a mathematical function was selected as the response:

$$R = \frac{H_A}{H_B} + \frac{H_A}{1000}$$

In which R is the response, H_A the peak height corresponding to the amikacin–Cu(II) solutions, H_B the peak height of the blank. This response was chosen in order to achieve a high inhibition of the CL intensity proportional to the amikacin

Table 1
Factors and levels used in the factorial design

Variable	Low (−)	High (+)
Luminol (M)	10^{-6}	5×10^{-6}
Cu^{2+} (M)	5×10^{-6}	5×10^{-5}
[H_2O_2] (M)	10^{-2}	10^{-4}
Flow rates (mL/min)	1	2.3
[NaOH] (M)	8×10^{-5}	8×10^{-5}

Table 2
Box–Benhken matrix for the optimization of chemical factors

Experiment	Luminol	Cu(II)	H_2O_2	*R*		
				Experimental	Expected	Residual
1	0	0	0	0.496	0.537	−0.041
2	−1	−1	0	0.202	0.077	0.12
3	1	−1	0	0.320	0.408	−0.087
4	−1	1	0	0.149	0.117	0.032
5	0	0	0	0.430	0.537	−0.107
6	1	1	0	0.467	0.448	0.019
7	−1	0	−1	0.048	0.204	−0.155
8	1	0	−1	0.237	0.280	−0.042
9	0	0	0	0.481	0.537	−0.056
10	−1	0	1	0.251	0.253	−0.0016
11	1	0	1	0.949	0.838	0.111
12	0	−1	−1	0.375	0.317	0.058
13	0	0	0	0.731	0.537	0.193
14	0	1	−1	0.242	0.191	0.051
15	0	−1	1	0.361	0.456	−0.095
16	0	1	1	0.557	0.660	−0.102
17	0	0	0	0.636	0.537	0.099

R: analytical response.

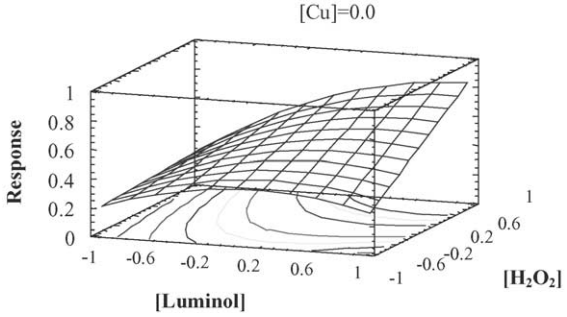

Fig. 2. Estimated response surface for [Cu(II)] = 0.

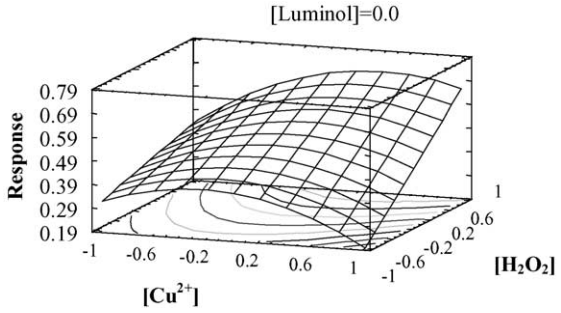

Fig. 3. Estimated response surface for [luminol] = 0.

concentration and also to obtain a better control of the peak height of the blank because an excessively high CL intensity for the blank signal produces saturation of the detector.

The function representing the relationship among luminol, H_2O_2 and Cu(II) concentrations and the chosen response is

$$R = 0.53731 + 0.165494 \times [\text{luminol}] + 0.0195797 \times [\text{Cu(II)}] + 0.151876 \times [H_2O_2] - 0.143258 \times [\text{luminol}]^2 + 0.127381 \times [\text{luminol}] \times [H_2O_2] - 0.131123 \times [\text{Cu(II)}]^2 + 0.082499 \times [\text{Cu(II)}] \times [H_2O_2]$$

This model, in which non-significant effects have been removed, fits the experimental data (*P*-value, lack-of-fit test 45.45%). The corresponding surface responses (Figs. 2–4) show that the optimum values for luminol and H_2O_2 concentrations are out of the experimental region while the optimum value for Cu(II) concentration is into the experimental region and corresponds to a 3.45×10^{-5} M. However, concentrations of 8×10^{-6} M of luminol and 10^{-2} M of H_2O_2 were chosen as suitable for the purposes of the study, because higher concentrations of luminol and H_2O_2 could produce the saturation of the detector due to the high CL intensity of the blank signal obtained.

3.1.3. Selection of flow rates and injected volume in the FIA system

The same value of the flow rate, for the three streams, was considered in the previously described screening design.

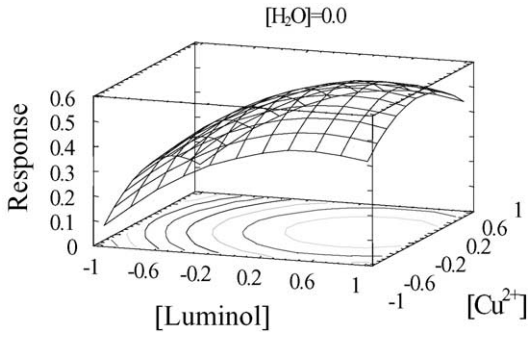

Fig. 4. Estimated response surface for [H_2O_2] = 0.

Table 3
Statistics and performance characteristics of the analytical method

Statistic	
Intercept	3.572
Slope	−0.041
Residual standard deviation	0.041
R^2 (%)	97.96
Performance	
On-line linearity (%)	102
Resolution (mg/L)	1.01
Detection limit (mg/L)	2.97
Quantification limit (mg/L)	9.89
Precision[a]	14.0 (3), 7.2 (5), 4.3 (10), 3.3 (15), 2.6 (20)

The parameters were calculated as indicated in Ref. [18].
[a] R.S.D.% (mg/L).

However, the experimental data suggested that the CL intensity could be improved if different flow rates were used for each stream. Therefore, a study of these flow rates was carried out, to check the significant factors and their possible interactions, by using a 2^3 full factorial design. The flow rates of luminol, carrier and H_2O_2 streams were varied from 2.3 to 1 mL/min. ANOVA shows that the flow rate for both the luminol and the carrier streams has a significant effect on the CL response, but the flow rate for the H_2O_2 stream was not a significant factor. Taking into account the repeatability and symmetry of the peak at different levels, values of 2.3 mL/min for H_2O_2 and carrier streams, and 1 mL/min for the luminol stream were chosen.

Finally, a univariate optimization was used to obtain the optimum value of the volume of sample injected. The injection volumes were changed from 10 to 120 μL. An injection volume of 70 μL was chosen as suitable because the intensity of the peaks is high enough to produce good sensitivity with adequate precision.

3.2. Linear calibration range

Calibration graph (log (peak height) versus [amikacin]) was established by applying univariate linear regression in the range of 2.97−20 mg/L. Six injections were used for each concentration level. The statistics and performance characteristics are summarized in Table 3.

3.3. Precision

The repeatability of the proposed method, expressed as the relative standard deviations (R.S.D.) of the peak height, for four series of three independent solutions containing 5, 10, 15 and 20 mg/L of amikacin sulphate, and included into the linear range were 6.71%, 6.49%, 7.41% and 4.35%, respectively. Table 3 presents the R.S.D. estimated for the different concentrations over this working range. The reproducibility (inter-day), expressed as R.S.D., for 10 mg/L of amikacin sulphate ($n = 15$) is 6.58%. Thus, the precision of the proposed method is verified.

3.4. Robustness

The consistency [22] of an analytical method, robustness and ruggedness, is defined by its capacity to produce constant and unbiased results when it is applied under different operating conditions. When the intrinsic operating conditions (instrumentals and/or experimental) of a method are slightly modified in an intralaboratory study, the robustness can be determined. These small changes are any variation in the nominal value of experimental variables.

The robustness of the proposed analytical method in relation to slight modifications of the optimized values of the experimental variables (luminol, H_2O_2 and carrier flow rates and luminol, H_2O_2 and Cu(II) concentrations) was studied using a 2^{7-4} saturated factorial fractional design using an amikacin sulphate concentration of 10 mg/L. A dummy variable was confounded with the most probable interaction. The choice of this most probable interaction was based on our knowledge of the analytical system; it was necessary to order the experimental variables according to a confounding pattern obtained from the generator of the design. The results obtained are summarized in Table 4. The significance of effects was estimated according with the variance in reproducibility for the selected concentration of amikacin. As can be seen, for a variation of ±10%, only Cu(II), luminol, and H_2O_2 concentrations were significant. Another study of robustness at ±5% was carried out for these three significant variables (luminol, H_2O_2 and Cu(II) concentrations), using a 2^{7-4} saturated factorial fractional design and adding four dummies variables. The results show that none of the effects is significant (Table 4).

Therefore, the analytical procedure is robust for variations of ±10% of the luminol, H_2O_2 and carrier flow rates and for variations of ±5% of luminol, H_2O_2 and Cu(II) concentrations. Thus, a complete knowledge of the system has been achieved as well as the possibility of controlling the factors that contribute to the experimental error.

Table 4
Study of the robustness of the method proposed

Variables	Nominal values	Significance (*P*-value (%))	
		10%	5%
[Luminol] (M)	8×10^{-6}	S (5.13)	NS (9.05)
[H_2O_2](M)	10^{-2}	S (0.04)	NS (81.13)
[Cu(II)](M)	3.45×10^{-6}	S (0)	NS (48.98)
Luminol flow rate (mL/min)	1	NS (14.30)	–
H_2O_2 flow rate (mL/min)	2.3	NS (22.70)	–
Carrier flow rate (mL/min)	2.3	NS (39.21)	–
Dummy	–	NS (96.67)	–

S: significant effect; NS: non-significant effect.

Table 5
Determination of amikacin in pharmaceutical formulations

Formulation	Concentration ($n = 6$) (mg/mL)	R.S.D. (%)	Nominal value (mg/mL)	t Calculated	$t_{(0.05,5)}$ Tabulated	P-value (%)
Biclin®	249 ± 7	2.76	250	0.492	2.571	64.34
Amikacin Normon®	254 ± 9	3.59	250	1.176	2.571	29.26
Amukin®	249 ± 12[a]	4.75	250[a]	0.297	2.571	77.83

Composition: Biclin® and Amikacin Normon®, amikacin, sodium metabisulfite and sodium citrate; Amukin®, Amikacin lyophilised.
[a] Values are expressed in mg.

3.5. Trueness

To check the trueness of the proposed method, the analytical procedure was applied to pharmaceutical formulations, such as Amikacin Normon® (Normon, Spain), Biclin® (Brystol Myers, USA) and Amukin® (Brystol Myers, Belgium) due to its effect on Gram-positive and Gram-negative bacteria. A *t*-test was applied to compare if nominal values (claimed on the formulation label) were similar to the obtained average. The results are shown in Table 5. As can be seen, no significant differences were found between the compared values, being the method applicable to these pharmaceutical formulations.

4. Conclusion

Amikacin sulphate inhibits strongly the CL intensity of the luminol–H_2O_2–Cu(II) system, due to the formation of a complex between amikacin and the Cu(II) removes free concentration of Cu(II), decreasing its activity as a catalyst in the CL reaction. Based on this fact, a simple and sensitive FIA method for the determination of amikacin sulphate is established, the decrease in the CL signal being proportional to the amikacin content. This procedure can be used in pharmaceutical quality control due to the low cost of instrumentation and reagents, simplicity and speed of analysis, satisfactory precision and high sensitivity.

Acknowledgements

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