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Selective kinetic determination of amikacin in serum using long-wavelength fluorimetry

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Abstract

A simple and rapid method for the determination of the antibiotic amikacin, involving the use of a long-wavelength fluorophor, namely indocyanine green, (ICG) is presented. The dye is oxidised by cerium(IV) in acidic medium, resulting in a sharp decrease of the fluorescence, but this fluorescence quenching is inhibited in the presence of amikacin, which can be ascribed to the formation of an ion pair between the fluorophor and the analyte. The initial rate of the system is monitored at λ_{ex}: 765 nm and λ_{em}: 812 nm as excitation and emission wavelengths, respectively, using the stopped-flow mixing technique, which makes the method applicable to automatic routine analysis. Each measurement is obtained in only 2–3 s. The method presents a detection limit of 0.02 $\mu g\,m1^{-1}$ in standard solutions, which corresponds to 2.5 $\mu g\,ml^{-1}$ in serum samples. The precision is in the range 4.8–6%. The good selectivity of the method allows amikacin to be determined in the presence of other antibiotics, including other aminoglycoside antibiotics, in serum. The recoveries obtained from the analysis of different samples were in the range 89.4–104.7%.

Keywords: Amikacin; Indocyanine green; Stopped-flow mixing technique; Serum samples

1. Introduction

The potential nephrotoxicity and ototoxicity during long-term therapy with aminoglycoside antibiotics and their relatively narrow safety levels pose a great interest in the control of these levels in serum, being their use limited by this toxicity. However, despite of the introduction of newer, less toxic antimicrobials, they continue to serve a useful role as therapeutic agents for serious infections because of the increasing resistance of pathogenic organisms to the new ones [1,2]. Amikacin is a semisynthetic derivative of kanamycin [3], that has a bactericidal action and antimicrobial spectrum similar to those of gentamicin. This antibiotic is currently the first choice aminoglycoside, although amikacin is more active against mycobacteria. Also, it is less affected by enzymatic degradation than other antibiotics from this group what contributes to resolve problems of drug resistance to gentamicin, kanamycin and tobramycin. Amikacin does not appear to be metabolised and is excreted unchanged, as are aminoglycoside antibiotics in general.

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Microbiological assays have been traditionally used to determine amikacin and other aminoglycosides in serum, but immunoassay methods have replaced them and several commercial kits are now available [1,4–6], although in some instances, these methods feature cross-reactivity problems due to the similar structures of this type of antibiotics. The absence of chromogenic or fluorogenic groups in the amikacin molecule makes necessary the use of different reactions to provide an adequate detection by photometry and fluorimetry. A non-derivatisation approach based on ligand displacement reaction after ion-exchange liquid chromatography has been described [7]. However, derivatisation reactions have been more used although they are general for all aminoglycosides. Nitrophenylation reagents [8–10], 1-naphtylisothiocyanate [11], 1-fluoro-2,4-dinitrobenzene [12] have been used to develop HPLC methods with UV detection for amikacin determination in plasma. The use of *o*-phthalaldehyde (OPA) has been reported in some chromatographic methods involving post-column derivatisation [13,14] in which the analyte is separated from plasma matrix through the formation of ion pairs using alkylsulphonate compounds as reagents at pH 3.0. The formation of ion pairs has been also used for the development of liquid chromatography methods incorporating electrochemical detection [15,16] and they have been mostly applied to the analysis of bulk pharmaceutical samples. The use of OPA as reagent has been also reported for the kinetic fluorimetric determination of amikacin, tobramycin and kanamycin in pharmaceutical samples [17]. A method involving micellar electrokinetic chromatography with fluorimetric detection has been described for the determination of amikacin alone in plasma [18]. The quantitation limit was $5\,\mu g\,ml^{-1}$ and the retention time was 16.7 min. Similar retention times were obtained for amikacin in other capillary electrophoresis methods with electrochemical detection [19,20].

The method presented here reports the selective kinetic determination of amikacin using indocyanine green (ICG) and cerium(IV) as reagents. ICG is an anionic cyanine dye with maximum emission in the 800 nm region. Luminescence measurements in this region provide a good spectral selectivity because the interference of potential fluorescent signals from sample matrix can be minimised as they usually appear at shorter wavelengths. The basis of the method is the oxidation of ICG by cerium(IV) producing a quenching of its fluorescence, which is hindered in the presence of amikacin. This decrease in the fluorescence inhibition can be due to the formation of an ion pair through the two sulphonate groups of the ICG molecule, phenomenon that disturbs the oxidation of the dye. Kinetics of these reactions are very fast requiring the use of stopped-flow mixing technique for the adequate monitoring of initial rate, which is used as analytical parameter. The expeditiousness of this technique makes it suitable for the performance of the reaction. Also, it allows the partial automation of the method since the handling of reactants is considerably reduced, as they are run into the mixing chamber directly from the drive syringes. The proposed method can be applied to the selective determination of amikacin in the presence of other antibiotics at their therapeutic levels in serum. Also, an additional advantage of the use of kinetic methodology is that background static signals can be minimised.

2. Experimental

2.1. Instrumentation

An SLM Aminco Bowman (Urbana, IL, USA) Model 8100 photon-counting spectrofluorimeter, equipped with a 450 xenon arc source and a R928 photomultiplier tube, was used. The instrument was furnished with an SLM Aminco Milliflow stopped-flow module, which was fitted with an observation cell of 0.2 cm path-length and controlled by the associated electronics, the computer and a pneumatic syringe drive system. The temperature of the solutions in the stopped-flow module and the cell compartment was kept constant at $20 \pm 0.1\,^{\circ}C$ by circulating water from a thermostated tank.

2.2. Reagents

All chemicals used were of analytical grade. A $250\,\mu g\,ml^{-1}$ stock solution of amikacin (Sigma, Poole, Dorset, UK) was prepared in distilled water. ICG (5.1×10^{-4} M) solution was prepared in dimethylsulfoxide (DMSO) and stored at room temperature. Cerium(IV) solution (5×10^{-3} M) was daily prepared from cerium(IV) sulfate tetrahydrate

(Merck, Darmstadt, Germany) in 0.05 M sulfuric acid. A monochloroacetic/monochloroacetate solution (0.2 M, pH 2.9) was prepared in distilled water to adjust the pH.

2.3. Procedure

2.3.1. Determination of amikacin

An aqueous solution containing ICG (3.9×10^{-5} M), monochloroacetate buffer (7×10^{-3} M), amikacin standard or sample (0.06–3.0 μg ml^{-1}) and DMSO (20% (v/v)) was used to fill one of the 2 ml drive syringes of the stopped-flow module. The other syringe was filled with a premixed aqueous solution containing cerium(IV) (10^{-4} M) and monochloroacetate buffer (7×10^{-3} M). In each run, 0.04 ml of each syringe were mixed in the mixing chamber and the variation of fluorescence intensity with time throughout the reaction was monitored at λ_{ex}: 765 nm and λ_{em}: 812 nm for 5 s. Data were processed by the computer furnished with a linear regression program for application of the initial rate method. The initial rate was measured in ca. 2–3 s, being the integration time used 0.3 s. All measurements were carried out at 20 ± 0.1 °C. Each standard or sample was assayed in triplicate. A linear calibration graph was obtained by plotting the reaction rate difference obtained in the presence and in the absence of amikacin versus the amikacin concentration.

2.3.2. Determination of amikacin in serum samples

A volume of 500 μl of serum was mixed to 1 ml of acetonitrile and centrifuged at 2000 rpm for 10 min. Then, 120 μl of this solution were analysed following the above mentioned procedure, using a final volume of 5 ml.

3. Results and discussion

3.1. Study of the chemical system

As mentioned above, measurements at long wavelengths allow potential spectral interferences from sample matrix to be minimised as their emission usually occurs at shorter wavelengths. Also, the short lifetime of these fluorophors diminishes the probability of non-radiative quenching processes. It has been reported that aminoglycosides are polybasic cations at low pH and they can form ion pairs with reagents containing sulphonate groups. This approach has been used in several ion-pair HPLC methods [15,16]. ICG has two sulphonate groups in its molecule which can form the corresponding ion pair which amikacin at low pH. However, no change in the fluorescent properties of the dye due to the direct interaction with amikacin was observed.

A limitation of long-wavelength dyes such as oxazines and cyanines is the lack of sufficient reactive groups for the tagging of analytes. However, the reactivity of these compounds can be improved by electrostatic interactions [21–23] or redox reactions [24,25]. Several redox agents such as sodium metaperiodate, hydrogen peroxide and cerium(IV) were assayed with the aim of modifying the fluorescent features of ICG allowing its use as reagent for the selective determination of amikacin. It was found that sodium metaperiodate, in the range 2.5×10^{-5} to 2×10^{-3} M, and H_2O_2, at concentrations ranged between 2.5×10^{-5} and 0.05 M, did not affect to the fluorescence of the dye. However, the presence of cerium(IV) originated the quenching of fluorescence of the ICG, which could be partly ascribed to the elevated oxidative ability of cerium(IV), and also to a process which probably involves transfer of electron density from the fluorophor to the metal ion to form a charge-transfer complex [26]. This quenching effect was partially inhibited in the presence of amikacin, what could be ascribed to a different redox behaviour of ICG due to the ion pair formation. Fig. 1 shows the kinetic curves obtained in the absence and in the presence of amikacin at different concentrations. It was found that the changes in the decrease of the fluorescence intensity of the dye can be correlated with amikacin concentration.

The study of the distribution of the reagents in the stopped-flow module syringes showed that it plays an important role on the selectivity of the system, concerning to the potential interference from other aminoglycoside antibiotics. It is necessary to place ICG in one syringe and cerium(IV) in the other. The placement of amikacin in the ICG syringe or in the cerium(IV) syringe led to similar kinetic results but the analytical signal was modified when other aminoglycoside was also placed together with cerium(IV). This effect could be ascribed to possible interactions of aminoglycoside antibiotics with cerium(IV), as it

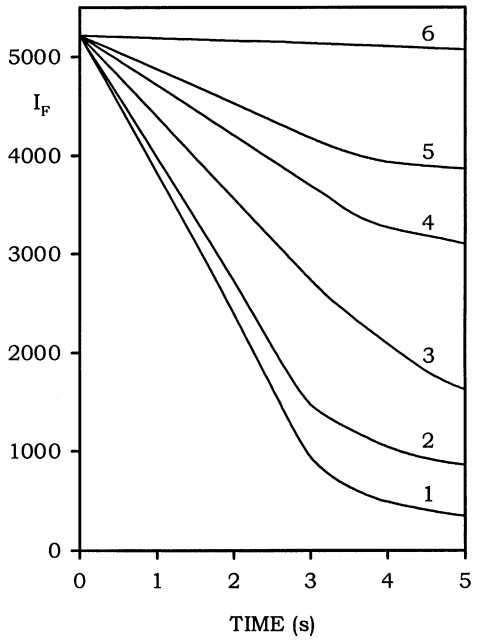

Fig. 1. Kinetic curves obtained under optimum conditions: [ICG] = 3.9×10^{-5} M; [cerium(IV)] = 10^{-4} M, pH 3.2; [chloroacetate] = 7×10^{-3} M. [Amikacin] values were: (1) 0; (2) 0.1; (3) 0.5; (4) 1.5; (5) 2; (6) 3 μg ml^{-1}.

has been described for other metal ions, such as Cu(II) [27]. However, these interactions were overcome when aminoglycosides were in the same syringe than ICG. In this case, the oxidation of the dye was the fastest step, together with the higher ability of the amino groups of amikacin molecule to form the ion pair with ICG. Thus, the use of stopped-flow mixing technique proves to be a useful tool for improving the selectivity of this system.

3.2. Influence of variables

The variables affecting the system were optimised by the univariate method. All reported concentrations are initial concentrations in the syringes (twice the actual concentrations in the reaction mixture at time zero after mixing). Each kinetic result was the average of three measurements. Those values yielding the minimum possible standard deviation for the initial rate, under conditions where the reaction order with respect to the species concerned was zero or near zero, were taken as optimal.

DMSO has been chosen to stabilise the ICG solution, as the fluorescence of this dye is efficiently quenched in aqueous solution [23]. The influence of this solvent in the kinetic behaviour of the system was studied in the range 10–30% obtaining that the optimum concentration in the ICG syringe was in the 19–22% interval. Higher amounts of DMSO decreased the initial rate of the system, probably due to an increase of the apparent pH which could favour cerium precipitation as its hydroxide, although the analyte deprotonation could also contribute to this negative effect. The study of the distribution of DMSO in both syringes showed that the initial rate of the system is lower in this instance than when it is only placed in the ICG syringe. The optimum apparent pH of the mixture, which was investigated in the waste, was in the range 3.1–3.3. Potasium hydrogenphthalate, citrate and monochloroacetate buffer solutions were assayed to adjust the apparent pH. The presence of phthalate or citrate solutions decreased the initial rate of the system if compared to those obtained in its absence. Monochloroacetate buffer only increased slightly the initial rate in the optimum range (6×10^{-3} to 1.1×10^{-2} M) and it was prepared in aqueous solution at pH 2.9. This value allowed the apparent pH of 3.2 to be obtained in the reaction mixture.

Cerium(IV) concentration is a key variable of this system as it is shown in Fig. 2A). The initial rate showed its maximum value in the range 8×10^{-5} to 1.2×10^{-4} M. A higher cerium(IV) concentration increased the oxidation rate of ICG, decreasing the differences between blank and analyte signals. The study of ICG concentration showed that the system was independent of this variable in the range 3.1×10^{-5} to 4.1×10^{-5} M (Fig. 2B). The influence of the temperature on kinetics was also studied and the best results were obtained at 20–22 °C. The initial rate of the chemical system in the presence or in the absence of amikacin increases at increasing temperatures, but the effect of this variable is higher in the absence of the analyte, so that the reaction rate difference, which is the analytical parameter, as indicated above, decreases when the temperature increases.

3.3. Analytical features of the proposed method

Kinetic data were obtained from the fluorescence intensity-time curves monitored at λ_{ex} 765 and λ_{em}

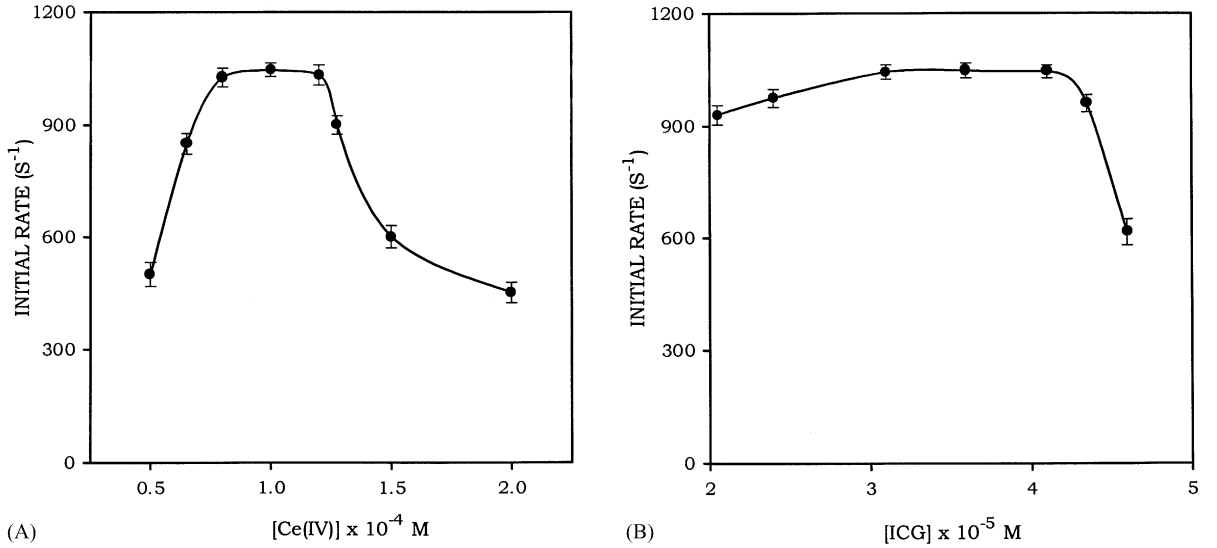

Fig. 2. Effect of cerium(IV) (A) and indocyanine green (B) concentration on the initial rate of amikacin system. Experimental conditions: [amikacin] = 2 μg ml^{-1}; pH 3.2; [chloroacetate] = 7×10^{-3} M. In (A) 4×10^{-5} M [ICG] was used and in (B) 10^{-4} M [cerium(IV)] was used.

812 nm under the optimum conditions. The initial rate was measured in ca. 2–3 s. The method presents two linear ranges, 0.06–0.4 and 0.4–3 μg ml^{-1}, respectively. The regression equations are $v = (26 \pm 9) + (1290 \pm 20)$[amikacin] and $v = (410 \pm 20) + (310 \pm 10)$[amikacin], respectively, for each range, being amikacin concentration expressed in μg ml^{-1}. The correlation coefficients, which are 0.998 and 0.996, respectively, suggest good calibration linearity. The detection limit, calculated according IUPAC recommendations [28] was 0.02 μg ml^{-1}, which corresponds to 2.5 μg ml^{-1} in the original serum sample. This value is under the lower limit of its therapeutic range [3], which oscillates between 20 and 30 μg ml^{-1}. Toxic effects are more likely to occur at concentrations above 35 μg ml^{-1}. The precision was determined by calculating the relative standard deviation at two analyte concentrations, 0.1 and 1 μg ml^{-1}, giving 6 and 4.8% values, respectively.

The selectivity of amikacin determination was studied by assaying other antibiotics as potential interferents. A substance was considered not to interfere at a given concentration if the initial rate in the presence of this substance was within one standard deviation of the value obtained with the analyte alone. Table 1 summarizes the maximum tolerated interferent/analyte ratios where it can be seen that the highest ratios are obtained for non-structurally related antibiotics such as tetracyclines, glycopeptides or fluoroquinolones. None of the other aminoglycoside antibiotics assayed interfered at the same concentration level than that of amikacin. Also, some of these antibiotics, which are frequently used for the treatment of several infectious diseases in humans, such as streptomycin, gentamycin, neomycin, tobramycin and kanamycin, were tolerated at higher concentrations than those corresponding

Table 1
Influence of foreign substances over the determination of 0.3 μg ml^{-1} of amikacin

Compound	Maximum tolerated interferent/analyte ratio
Tetracycline	20
Vancomycin	16.5
Norfloxacin	10
Spectinomycin	10
Amoxycillin	8
Streptomycin	6.5
Tobramycin	6.5
Hygromycin B	6.5
Paromomycin sulphate	6.5
Gentamicin sulphate	5
Kanamycin monosulphate	5
Erythromycin	5
Neomycin sulphate	3

to their therapeutic concentrations in serum [3]. This fact enables the selective determination of amikacin at its therapeutic levels in presence of other aminoglycosides.

The different kinetic behaviour observed between amikacin and the other aminoglycosides in the cerium(IV)–ICG system could be ascribed to the presence of an amino group out of the aminocyclitol ring of amikacin, which is not present in the structure of the other aminoglycosides. As it has been mentioned above, the interaction of these compounds with ICG can be justified through the formation of an ion pair with the protonated aminoglycoside, so that the different ability of the cited amino group in terms of protonation could be responsible of the different reactivity. Also, it has been described elsewhere [18] that not necessarily all amino groups are involved in the derivatisation reaction. This can explain the similar reactivity of streptomycin, tobramycin, paromomycin and hygromycin B in spite of the differences in the number and nature of amino groups (Table 1). Also, the presence of sulfate ions in some of these aminoglycosides can originate a positive interference, owing to their interaction with cerium(IV), which can modify the kinetics of the oxidative action of cerium(IV) [29]. In this instance, higher differences in the initial rate of the blank and analyte systems are obtained. This can contribute to explain the behaviour of neomycin sulphate in the system as there are three sulphate ions per each antibiotic molecule.

3.4. Applications

The method was applied to the analysis of three different serum samples. The sample pretreatment consisted of a simple deproteinisation step as it is known that ICG can interact with proteins [23]. Acetonitrile was assayed to remove proteins from the sample and it was found that the initial rate was not affected by the presence of up to 5% of acetonitrile, being subsequently chosen. The calibration graph was constructed in the presence of 0.8% (v/v) of serum matrix because as blank as analyte signals were slightly increased. Results were calculated by interpolating in the calibration graph. None of the analysed samples contained amikacin. The recovery study was carried out adding three different amounts of analyte to the serum samples so that the final concentration was within the therapeutic range, and subtracting the results from similarly prepared unspiked samples. Table 2 lists the recoveries obtained which were in the range 89.4–104.7%.

Table 2
Recoveries of the amikacin added to serum samples

Sample	Added ($\mu g\,ml^{-1}$)	Found[a] ($\mu g\,ml^{-1}$)	Recovery (%)
Serum 1	12.5	11.8 ± 0.3	94.6
	18.75	16.8 ± 0.7	89.4
	31.25	32 ± 1	101.6
Serum 2	12.5	13.1 ± 0.5	104.7
	18.75	18.3 ± 0.3	97.8
	31.25	31 ± 1	98.7
Serum 3	12.5	11.4 ± 0.7	91.1
	18.75	19 ± 1	102.5
	31.25	29 ± 2	92.8

[a] Mean ± S.D.

4. Conclusions

The results obtained with the proposed method show the suitability of redox modification of the dye, which does not react directly in its oxidized form but its oxidation reaction allows the interaction between the dye and amikacin to be monitored. The stopped-flow mixing technique automates the mixing of all the reactants involved and enables the measurement of the initial rate of the reaction. The combined use of a long-wavelength fluorophor together with dynamic measurements contributes to obtain the spectral and temporal discrimination of the analytical signal, attaining the required selectivity for the application of the method to the analysis of serum samples. Although the dilution factor of the sample is relatively high, no sample preconcentration methods are used, because the sensitivity achieved with the method is adequate for amikacin levels in serum and only a simple deproteinisation step, common to some of the previously reported systems [1,13] is needed.

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