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Validation of gentamicin congeners using HPLC with electrochemical detection: comparison with fluorimetric detection

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Abstract

OPA is often used as an off-line derivatisation agent for fluorimetric detection in gentamicin analysis. As, in general, electrochemical detection is more sensitive than fluorimetric detection, the analysis of gentamicin as its OPA derivatives by high-performance liquid chromatography and electrochemical detection is proposed. The derivatisation procedure is that described by the European pharmacopoeia. The method allows not only the detection of all major gentamicin congeners C₁, C_{1A}, C_{2A} and C₂, but also of other minor unidentified components. A full validation of the method is presented and comparison is made with fluorimetric detection. A good linearity is found for the four congeners within the dynamic ranges 1–50 µg ml⁻¹ for electrochemical detection and 4–80 µg ml⁻¹ for fluorimetric detection. Both methods presented good accuracy and fidelity, although electrochemical detection is more sensitive than fluorimetric detection. The electrochemical detection method presented in this work appears to be a good alternative to the fluorimetric and pulsed electrochemical detection methods. **To cite this article:** I.T. Somé *et al.*, *C. R. Chimie* 7 (2004).

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Résumé

Validation du dosage des congénères de la gentamicine par chromatographie liquide haute performance suivi d'une détection électrochimique : comparaison avec la détection fluorimétrique. L'orthophthaldialdéhyde (OPA) est un dérivé utilisé comme agent de dérivation dans la détection fluorimétrique de la gentamicine. La détection électrochimique étant en général plus sensible que la détection fluorimétrique, une méthode de chromatographie liquide haute performance suivie d'une détection électrochimique utilisant les dérivés OPA de la gentamicine est proposée dans ce travail. La méthode de dérivation est celle décrite dans la pharmacopée européenne. Cette méthode permet non seulement la détection des congénères majeurs C₁, C_{1A}, C_{2A} et C₂ de la gentamicine, mais également des congénères mineurs non identifiés. Une validation complète de la méthode est présentée et une comparaison est faite avec la détection fluorimétrique. On trouve une bonne linéarité pour les quatre congénères dans la gamme de concentration de 1 à 50 µg ml⁻¹ pour la détection électrochimique et de 4 à 80 µg ml⁻¹ pour la

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détection fluorimétrique. Les deux méthodes de détection présentent de bonnes exactitude et précision, la détection électrochimique ayant effectivement une sensibilité meilleure. Cette détection électrochimique semble une bonne alternative à la détection fluorimétrique. **Pour citer cet article : I.T. Somé et al., C. R. Chimie 7 (2004).**

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Keywords: Gentamicin congeners; Validation; Electrochemical detection; Fluorimetric detection

Mots clés: Congénères de la gentamicine; Validation; Détection électrochimique; Détection fluorimétrique

1. Introduction

Gentamicin is a complex of more than four aminoglycoside congeners obtained by fermentation from *Micromonospora purpurea*. Gentamicin has a broad antibiotic activity and is used in diverse infectious diseases such as leishmaniosis, osteomyelitis and against other gram-negative bacteria. It is the main drug used in the treatment of chronic osteomyelitis [1,2]. Gentamicin is also used in food production to promote growth and for prophylactic reasons [3].

- There are a number of methods dealing with the separation and/or the quantification of gentamicin either as a bulk or as congeners in different media. Among bulk quantification methods, the microbiological assays are those recommended by different pharmacopoeias [4,5] and many publications deal with this method. Immunological and ultraviolet measurement methods are also used for bulk gentamicin analysis [7,8]. Gas chromatography, liquid chromatography and thin-layer chromatography have been introduced for congeners' separation. The advantage of the chromatographic methods over bulk methods is that they can perform individual component separation and quantification. Congeners can be detected either by UV detection [9–11], MS detection [12–14], fluorimetric detection [15–18] or electrochemical detection [2,19,20].
- The detection of gentamicin after chromatographic separation was an analytical problem due to the lack of chemical detectable characteristics. So pre-derivatization or post-derivatization processes are used to allow detection. Gentamicin can then be detected as FMOC-Cl derivatives, dansylchloride derivatives or OPA derivatives [17], the more commonly encountered in the literature being the OPA-mercaptoacetic acid derivatives [3,9]. The advantage of these OPA derivatives is that they are used

either for UV or fluorescence detection [10,17]. Getek et al. [19] described a chromatographic separation of gentamicin congeners followed by electrochemical detection with a glassy carbon electrode at a 1200-mV potential and pulsed electrochemical detections with a gold electrode have also been used [1,2]. The electrochemical detections encountered in literature are pulsed electrochemical detections [1,2]. This detection technique is difficult to manage for many laboratories and many others cannot afford it.

Because, in general, electrochemical detection is more sensitive than fluorimetric detection, an analysis by high-performance liquid chromatography that uses the electrochemical properties of gentamicin-OPA derivatives is proposed. In this work, gentamicin-OPA derivatives are simultaneously determined by electrochemical and fluorimetric detections after chromatographic separations. The comparison of electrochemical detection with fluorimetric detection in gentamicin congeners analysis has been investigated.

2. Experimental

2.1. Reagents

Gentamicin sulphate was a kind gift from Schering Plough (Belgium). Orthophthaldialdehyde (OPA) was purchased from Sigma (Austria). 1-Heptanesulfonic acid, thioglycolic acid, potassium hydroxide 45% (w/v) and acetic acid were obtained from Acros Organics (Belgium). Methanol was HPLC grade from Acros Organics (Belgium). Water was double distilled and purified with a Millipore Milli-Q (Millipore, USA). The borate buffer was a 4-mM boric acid solution whose pH was adjusted to 10.4 with potassium hydroxide 45% (w/v).

2.2. OPA–thioglycolic acid solution

The OPA–thioglycolic acid solution was prepared as followed: 400 mg of OPA was dissolved in 2 ml of methanol and 37 ml borate buffer. Thioglycolic acid (0.8 ml) was added and the pH was then adjusted to 10.4 with potassium hydroxide (45% w/v). It is important to proceed in this way to avoid precipitation.

2.3. Derivatisation procedure

10 ml of gentamicin solution were introduced into a 25-ml volumetric flask and 800 μl of the OPA solution (approximately 10 mg ml^{-1} at pH 10.4) are added. The volume was brought to the mark with methanol. The flask was then placed at 60 °C for 15 min in a water bath. After the reaction had taken place, the solution was cooled and delivered into vials. A volume of 20 μl of the reaction mixture was injected.

2.4. HPLC system

The analysis was performed with a computer-assisted Agilent 1100 HPLC series comprising a binary pump equipped with an autosampler. For the fluorimetric detection, we used an Agilent 1100 HPLC series fluorimetric detector. The excitation wavelength was set at 340 nm and the emission was measured at 430 nm. Data were handled with Chemstation software. For the electrochemical detection, an electrochemical detector with glassy-carbon working and auxiliary electrodes (BAS CC-5E, *petit ampère*, Bioanalytical Systems Inc, West Lafayette, USA) was used; the electrochemical cell potential was set at 700 mV. This detector is connected in series to the fluorimetric detector. The data from the electrochemical detector were processed via Borwin software (GMBS, France). The chromatographic column was an Alltech Hypurity C18 octadecylsilane (100 \times 4.6 mm). The mobile phase consisted of water, methanol and acetic acid (28/67/5; v/v/v) containing 25 mmol of sodium heptanesulfonate as counter ion. A linear flow rate of 1 ml min^{-1} was used.

2.5. Linearity, accuracy and precision evaluation

A three-day validation procedure was implemented, as indicated by Caporal-Gauthier et al. [21], with mi-

nor modifications. Each day, a quantity of about 25 mg of gentamicin was weighed and dissolved in water (25 ml). Independent dilutions were then undertaken to cover our analytical dynamic range. For each concentration triplicate, analysis was carried out. For each congener, the exactitude was calculated, referring to the given linear equation. For precision assessment, the recovery values at each concentration throughout the three-day validation are compared and then the assessment of the inter-day precision is made.

3. Results and discussion

The objective of this study was to validate an isocratic HPLC assay for gentamicin analysis with electrochemical detection that allows low gentamicin concentration analysis as an alternative to fluorimetric detection. OPA is often used as an off-line derivatisation agent for fluorimetric detection in gentamicin analysis. Attempts were made to evaluate the possibility of using the same OPA derivatives for electrochemical detection, as electrochemical detection is generally more sensitive than fluorimetric detection. Preliminary investigations show that OPA derivatives have also electrochemical properties. The bulk gentamicin received from Schering Plough consists of four major components. The separation profiles of gentamicin congeners are shown in Figs. 1 and 2 for electrochemical and fluorimetric detections, respectively. The order of elution is congener C₁, congener C_{1A}, congener C_{2A} and congener C₂. This elution behaviour was in accordance with that established by Schering Plough and brought to us through the certificate of analysis. Chromatograms also show unidentified congeners that are better detected by the electrochemical detector as opposed to the fluorimetric technique. These unidentified congeners have been reported by other publications [2,20]. The validation process was then undertaken during three days to estimate the performance of this electrochemical detection for routine analysis.

During the time course of the validation process, the stability of the OPA derivatives was investigated, as the chromatographic system was equipped with an autosampler and analysis took place all day long. It is established that these OPA derivatives are stable up to 48 h; thus the automatic injection procedure was used

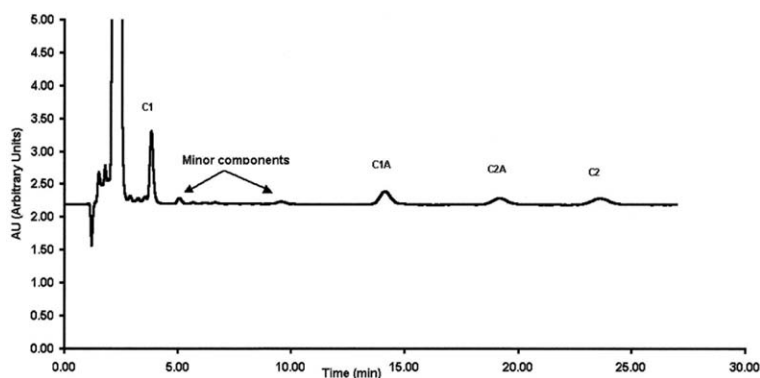


Fig. 1. LC-electrochemical detection of gentamicin congeners (C1, C1a, C2a and C2) at $10 \mu\text{g ml}^{-1}$.

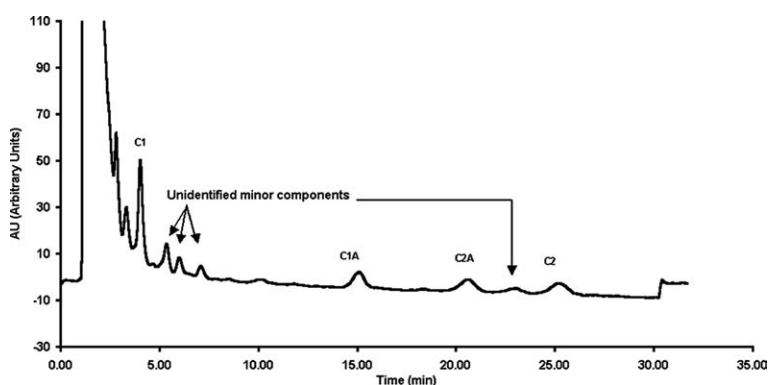


Fig. 2. LC-fluorimetric detection of gentamicin congeners (C1, C1a, C2a and C2) at $10 \mu\text{g ml}^{-1}$.

without further problems. It is also well known that glassy electrodes have a tendency to encounter stability problems during long-time analysis. No specific problem regarding the stability of the glassy electrodes was encountered during this analysis; long-time automation analysis was consequently possible.

3.1. Linearity

For each day, at least six concentrations were used for the linearity profile. Each concentration was injected as a triplicate. Regression parameters were calculated every day. The regression parameters calculated from the data of electrochemical and fluorimetric detections are given in Tables 1 and 2, respectively, for each day. For each congener, the regression determination coefficient (r^2) approached unity, and comparison of the intercepts with zero in both detection methods indicates that they are not different from the zero value

($p < 0.05$). Comparisons of regression parameters between days do not show any statistical difference as well.

For the electrochemical detection, although a good linearity was found from 0.2 to $50 \mu\text{g ml}^{-1}$ for congeners C_1 and congener C_{1A} , bad recovery rates were noticed at 0.2 and $0.4 \mu\text{g ml}^{-1}$. So, depending on the congener, the analytical dynamic range was set from 1 to $50 \mu\text{g ml}^{-1}$ for congeners C_1 and C_{1A} , and from 2 to $50 \mu\text{g ml}^{-1}$ for congeners C_{2A} and C_2 , for which good recovery rates and precisions were found. The dynamic range for the fluorimetric detection goes from 8 to $80 \mu\text{g ml}^{-1}$ for the four congeners, taking into account accuracy and fidelity parameters.

3.2. Accuracy

The results concerning accuracy were calculated for each concentration by using the regression equation and data generated during independent concentration

Table 1
Regression parameters for the linearity test of gentamicin congeners using electrochemical detection

Linearity parameters		Gentamicin congeners			
		C ₁	C _{1A}	C _{2A}	C ₂
Day 1	Slope	138.77 (10.62) ^a	215.57 (3.36)	114.68 (1.882)	143.46 (3.73)
	Intercept	-12.80 (24.27)	-208.82 (76.81)	-104.61 (43.013)	-1.93 (85.27)
	r ²	0.999	0.999	0.999	0.997
Day 2	Slope	134.59 (32.54)	220.95 (4.46)	117.67 (2.97)	146.70 (3.712)
	Intercept	-32.63 (74.37)	-273.88 (102.01)	-158.88 (67.96)	-1.11 (84.82)
	r ²	0.997	0.998	0.997	0.997
Day 3	Slope	140.22 (2.35)	231.84 (4.41)	122.67 (2.56)	144.5 (2.78)
	Intercept	-16.33 (53.61)	-239.30 (100.71)	-160.97 (58.717)	-2.15 (63.95)
	r ²	0.998	0.998	0.998	0.999

^a Standard errors of the estimated parameters as given by least-square regression analysis. The concentrations are expressed in $\mu\text{g ml}^{-1}$.

Table 2
Regression parameters for the linearity test of gentamicin congeners using fluorescence detection

Linearity parameters		Gentamicin congeners			
		C _{1A}	C ₁	C _{2A}	C ₂
Day 1	Slope	0.126 (0.003) ^a	0.088 (0.002)	0.0507 (0.0007)	0.065 (0.001)
	Intercept	-0.7 (0.3)	-0.4 (0.2)	-0.05 (0.08)	-0.3 (0.2)
	r ²	0.996	0.996	0.998	0.995
Day 2	Slope	0.140 (0.003)	0.095 (0.002)	0.054 (0.001)	0.066 (0.001)
	Intercept	-1.1 (0.4)	-0.6 (0.2)	-0.2 (0.1)	-0.2 (0.2)
	r ²	0.995	0.997	0.996	0.989
Day 3	Slope	0.139 (0.003)	0.095 (0.003)	0.05 (0.05)	0.064 (0.002)
	Intercept	-1.0 (0.4)	-0.5 (0.4)	-0.1 (0.1)	-0.3 (0.3)
	r ²	0.996	0.989	0.996	0.989

^a Standard errors of the estimated parameters as given by least-square regression analysis. The concentrations are expressed in $\mu\text{g ml}^{-1}$.

measurements. The results are shown in Tables 3 and 4. With electrochemical detection, a good accuracy was noticed for each congener and the recovery rates for each concentration, except for congener C₂ within the dynamic range, are between 90 and 110% of the nominal value, indicating a good stability of the OPA derivatives during analysis. This congener is less sensitive to electrochemical detection of the gentamicin congener as an OPA derivative. No significant difference was noticed between the other congeners in terms

of recovery within their respective dynamic ranges. With fluorimetric detection, the recovery rate for each concentration within the dynamic range is between 90 and 115% of the nominal value, indicating a good accuracy. Moreover, no significant difference was noticed between the four congeners.

3.3. Fidelity

The fidelity of both detection methods was assessed by analysing the between-day precision through the

Table 3
Accuracy of electrochemical detection expressed in terms of % recovery and between-day precision

Concentration ($\mu\text{g ml}^{-1}$)	Congeners mean recovery			
	C_1	C_{1A}	C_{2A}	C_2
1	108.41 (12.41) ^a	101.14 (0.56)		
2	107.10 (4.12)	98.84 (2.93)	104.5 (10.84)	137.67 (9.67)
5	81.20 (10.74)	93.58 (4.40)	93.67 (8.68)	99.52 (8.78)
10	98.57 (1.79)	102.82 (7.60)	91.48 (6.3)	99.2 (0.89)
20	97.96 (3.22)	103.11 (0.90)	105.35 (8.23)	89.4 (7.47)
25	103.25 (3.17)	109.18 (11.11)	98.36 (2.46)	97.39 (4.57)
50	99.7 (1.12)	108.09 (2.38)	99.93 (1.47)	102.29 (0.26)

^a Coefficients of variation that are considered as inter-day precisions ($n = 9$).

Table 4
Accuracy of fluorimetric detection expressed in terms of % recovery and between-day precision

Concentration ($\mu\text{g ml}^{-1}$)	Congener's mean recovery			
	C_1	C_{1A}	C_{2A}	C_2
8	108.62 (2.99) ^a	112.68 (6.95)	108.46 (1.51)	114.2 (0.63)
10	102.89 (2)	103.99 (7.5)	104.86 (7.38)	107.72 (8.99)
20	92.57 (7.68)	94.23 (9.86)	97.27 (11.44)	96.81 (14.81)
30	97.22 (2.28)	95.95 (9.25)	94.3 (7.38)	92.74 (7.1)
40	97.2 (0.61)	96.78 (4.03)	96.54 (3.14)	96.71 (3.13)
50	97.31 (4.34)	97.03 (6)	100.13 (2.83)	97.56 (7.93)
60	100.92 (3.38)	99.96 (1.55)	100.45 (1.56)	100.01 (1.16)
70	100.23 (1.36)	100.43 (3.02)	100.41 (1.5)	100.38 (2.9)
80	101.93 (2.32)	102.5 (3.88)	101.19 (1.16)	102.53 (3.74)

^a Coefficients of variation that are considered as inter-day precisions ($n = 9$).

coefficient of variation (Tables 3 and 4). They range from 1.12% to 12.41% for congener C_1 , and from 0.56 to 11.11% for congener C_{1A} . The fidelity values for congener C_{2A} range from 1.47 to 10.84% and from 0.63 to 14.81% for congener C_2 with electrochemical detection. Fluorimetric detection gives values from 0.61% to 7.68% for congener C_1 , from 1.55% to 9.86% for congener C_{1A} , from 1.16% to 11.44% for

congener C_{2A} , and from 0.63% to 14.81% for congener C_2 . Both detection methods present similar fidelity characteristics.

3.4. Detection and quantification limits

The limit of quantification was determined, taking into account the linearity parameters by using data of

gentamicin concentrations as low as possible and the accuracy and fidelity parameters concerning these concentrations. For the electrochemical detection, the quantification limit was $1 \mu\text{g ml}^{-1}$ for congeners C_1 and C_{1A} , with the accuracy of 90%, which was not the case of congener C_2 . The detection limit in electrochemical detection was calculated by analysing subsequent dilution up to the lowest level of detection. The concentration is $0.05 \mu\text{g ml}^{-1}$ for congeners C_1 and C_{1A} and $0.1 \mu\text{g ml}^{-1}$ for congeners C_{2A} and C_2 . For fluorimetric detection, the limit of quantification was found to be $8 \mu\text{g ml}^{-1}$ for each congener, whereas the detection limit was $1 \mu\text{g ml}^{-1}$, C_2 congener being the more sensitive.

4. Conclusion

We report in this work results of gentamicin congeners analysis as OPA derivatives using electrochemical detection with full validation data. The method allows detection not only of all major gentamicin congeners C_1 , C_{1A} , C_{2A} and C_2 , but also of other minor unidentified components. The results of the validation show that a good linearity is found for the four congeners within the dynamic range $1\text{--}50 \mu\text{g ml}^{-1}$ for electrochemical detection and from $8\text{--}80 \mu\text{g ml}^{-1}$ for fluorimetric detection. These OPA derivatives are stable up to 48 h, whereas the electrochemical electrodes were also very stable during the whole validation experiment and no specific problem was encountered within the work time. For analysis with electrochemical detection, it is better to choose congeners C_1 , and C_{1A} , as they are more sensitive to this electrochemical method, whereas congeners C_{2A} and C_2 have better responses to fluorimetric detection.

The comparison of the results shows that electrochemical detection is more sensitive than the fluorescence detection, although both methods can be used with good accuracy and precision within their respective dynamic ranges. The stability of the OPA derivatives, in addition to the stability of the glassy carbon and working electrodes during the whole analytical time, made the electrochemical detection method a

good alternative to the fluorimetric and pulsed electrochemical detection methods.

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