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Original Research

Applying Liquid Chromatography with Fluorescence Detection to Determine Gentamicin

Dorota Kowalczuk*, Rafał Pietraś, Beata Paw, Agnieszka Czerkies

Chair and Department of Medicinal Chemistry, Medical University of Lublin,
Jaczewskiego 4, 20-090 Lublin, Poland

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Abstract

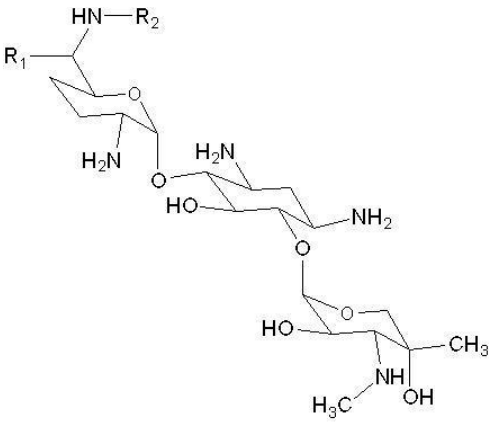
A simple and sensitive procedure for the determination of gentamicin is presented, based on reaction with o-phthaldialdehyde (OPA) in combination with N-acetylcysteine (NAC) in a basic medium. The use of N-acetylcysteine instead of liquid mercaptans for the formation of isoindole derivatives is a good solution for avoiding the unpleasant odour of thiol compounds. Determination of the resulting fluorescent derivatives was carried out by reversed-phase chromatography at λex=328 nm and λem=423. Stability of OPA/NAC/gentamicin-conjugate was improved by the addition of hydroxypropyl-β-cyclodextrin. The calibration graph was linear (r = 0.9997) over the range 0.4-12.8 μg mL^{-1}. The precision (RSD%) of the method varied from 1.5 to 8.5. The method was satisfactorily applied to the determination of gentamicin in pharmaceutical preparations with a mean recovery of 100.02% for injections and 88.94% for drops.

Keywords: gentamicin, HPLC with pre-column derivatization, fluorescence detection

Introduction

Gentamicin (a mixture of three components, gentamicin C1a, C2, and C1 that have different patterns of methylation at the 6 positions of ring I, Fig. 1) is a representative of the class of aminoglycoside antibiotics that interfere with essential steps of protein synthesis. This bactericidal antibiotic acts by binding to the ribosomal A site, misreading the genetic code, and inhibiting translocation. The clinically important gentamicin is useful in many types of bacterial infections, particularly aerobic gram-negative infections.

The HPLC methods have been often used to determine gentamicin in biological matrices and in pharmaceutical preparations [1-9]. In the HPLC analysis, the application of o-phthaldialdehyde in the presence of a thiol component (OPA-method) for the quantitative determination of free amino acids, peptides and aminoglicosides is well known.



Gentamicin C_1 $R_1 = R_2 = CH_3$
Gentamicin C_2 $R_1 = CH_3$, $R_2 = H$
Gentamicin C_{1a} $R_1 = R_2 = H$

Fig. 1. Chemical structures of the analyzed gentamicin components.

*e-mail: dorota.kowalczuk@am.lublin.pl

The most frequently used thiol compound is mercaptoethanol.

The present paper describes a modified RP-HPLC method with fluorescence detection for analysis of gentamicin in which mercaptoethanol was replaced by N-acetylcysteine (NAC). We recommend the use of hydroxypropyl-β-cyclodextrin to improve the stability of OPA/NAC gentamicin derivatives. The proposed method under the selected conditions was applied to determine gentamicin in the pure substance and pharmaceutical preparations.

Experimental

Chromatographic Conditions

The HPLC system (Waters, USA) comprised a 2475 fluorescent Detector, Model 515 isocratic pump, a Rheodyne valve with a 20-μL loop. Millenium software was used for collected data. Chromatographic analysis was performed on a Nova-Pak® RP-C18 (150 mm x 3.9 mm, 5 μm particle size) column from Waters. A mixture of methanol, glacial acetic acid, and aqueous solution of sodium hexanesulphonate at a concentration of 0.02 mol L^{-1} (65:3:32, v/v) was applied as mobile phase at a flow-rate of 1.0 mL min^{-1}. Measurements were made at λex=328 nm and λem=423 nm.

Chemical and Reagents

Gentamicin sulphate, $C_{21}H_{43}N_5O_7 \cdot 2.5\ H_2SO_4$ (Genta) pure substance and *o*-phthaldialdehyde (OPA) were products acquired from Fluka (Steinheim, Germany). N-acetylcysteine (NAC) and hydroxypropyl-β-cyclodextrin (HP-β-CD) were purchased from Sigma (St. Louis, USA). Sodium hexanesulphonate and methanol (both for chromatography) were purchased from Merck (Darmstadt, Germany). All other chemicals (boric acid, sodium hydroxide, glacial acetic acid, ethanol 95º) were obtained from POCh (Gliwice, Poland). The gentamicin injection contained 10 mg gentamicin base per 1 mL and gentamicin ophthalmic drops contained 3 mg gentamicin base per 1 mL. *Phthalaldehyde reagent* was freshly prepared by dissolving 20 mg of OPA in 1.0 mL of methanol, adding 1.0 mL of 10% solution of NAC, and diluting to 10 mL with 0.2 mol L^{-1} solution of borate buffer, pH 10. *Borate buffer* was obtained by adjusting the pH of 0.2 M solution of boric acid to 10.0 with 40% sodium hydroxide. *10% solution of NAC* was prepared by dissolving 10 g of NAC substance in 100 mL of redistilled water. *2.5 mM solution of HP-β-CD* was prepared by dissolving 0.345 g of HP-β-CD substance in 100 mL of ethanol 95º.

Preparation of Calibration Solutions

The stock solution of genta at a concentration of 1.0 mg mL^{-1} was prepared by dissolving 5 mg of the substance to be examined (calculated with reference to gentamicin base) in 5.0 mL of water. A 2.0 mL aliquot of this solution was transferred into a 10 mL volumetric flask and diluted to the mark with the same solvent (0.2 mg mL^{-1}). Calibration solutions at concentrations of 2, 4, 8, 16, 32, and 64 μg mL^{-1} were prepared by appropriately diluting the diluted stock solution of genta to 10 mL with water and subjected to derivatization procedure.

Preparation of Samples

A 0.1 mL aliquot of gentamicin injection equivalent to 1.0 mg of genta was introduced into a 50 mL volumetric flask and diluted to the mark with water (20 μg mL^{-1}). A 0.1 mL aliquot of gentamicin ophthalmic drops equivalent to 0.3 mg of genta was transferred into a 10 mL volumetric flask and complied to the mark with water (30 μg mL^{-1}).

Derivatization procedure: The calibration solutions (0.1 mL, corresponding to 0.2, 0.4, 0.8, 1.6, 3.2, and 6.4 μg of genta) and injection samples (0.1 mL, corresponding to 2.0 μg of genta) or ophthalmic drops samples (0.1 mL, corresponding to 3.0 μg of genta) were transferred separately into series of the glass test-tube. To each test tube a 0.1 aliquot of phthalaldehyde reagent and 0.3 mL aliquot of 2.5 mM solution of HP-β-CD were added. Resulting mixtures were heated in a water-bath at 50ºC for 20 min. After cooling they were subjected to HPLC analysis at λex=328 nm and λem=423 nm.

Results and Discussion

Gentamicin, an aminoglycoside antibiotic, does not absorb UV light in its native form (it is a weak chromophore). Therefore, gentamicin was chemically modified (pre-column derivatization) and the obtained fluorescent product was detected at much higher sensitivity by using the high performance liquid chromatographic method with fluorescence detector. Gentamicin reacts with essentially nonfluorescent o-phthaldialdehyde (OPA) in the presence of an excess of N-acetylcysteine as a thiol compound (nucleophile) to yield a fluorescent isoindole. The use of N-acetylcysteine is a satisfactory alternative to other mercaptans characterized by an unpleasant odour.

In order to choose the analytical conditions, different concentration of derivatization reagents were investigated. On the basis of the results, the borate buffer pH 10 at concentration of 0.2 M (Fig. 2A), N-acetylcysteine at concentration of 10% (Fig. 2B), and the mole ratio of genta/OPA = 1:64 (Fig. 2C) were selected. The obtained data proved that a significant excess of OPA reagent is necessary for performing the derivatization process. Further tests permitted us to evaluate the most profitable conditions for obtaining the stable fluorescent product. On the basis of the results, heating in the water at 50ºC for 20 min was selected (Figs. 3A and 3B). The addition of HP-β-CD resulted in improved stability of the OPA/NAC derivatives of genta. The concentrations of HP-β-CD in the considered range from 0.25 to 40 mM do not influence significantly on the fluorescence response. In order to evaluate the stability of genta derivatives, the UV and fluorescence responses of the

genta sample at the genta concentration of 10 μg mL^{-1} in a function of the time were measured. The genta derivatives stored at room temperature were stable for 12 h. Under applied conditions, gentamicin was eluated as three big baseline separated peaks at the retention times (±RSD) of 6.81 min ± 1.53%, 11.68 min ± 1.80%, and 14.05 min ± 2.04%, which were labeled as G1, G2, G3 (Fig. 4). The intensity of the fluorescent products was directly proportional to the concentration of gentamicin at the excitation wavelength of λ = 328 nm and the emission wavelength of λ = 423 nm.

To evaluate specificity, a chromatogram of the blank sample was used. No interferences in the range of the retention times of gentamicin components were observed.

A linearity study was made by preparing six calibration samples covering the concentration range of 0.4-12.8 μg mL^{-1}. An assay was repeated five times and each sample was injected in duplicate. Calibration graphs were constructed by plotting the peak area versus the sample concentration. The used Mandel fitting test indicated the linearity of the method.

The limit of detection (LD) was evaluated by analysis of a series of samples containing decreasing concentrations of the drug below the lowest limit of the calibration range. LD values were found to be 1.0 ng mL^{-1} (G1) and 2.5 ng mL^{-1} (G2, G3). The limits of quantification calculated as three times the corresponding LD were 3.0 ng mL^{-1} (G1) and 7.5 ng mL^{-1} (G2, G3).

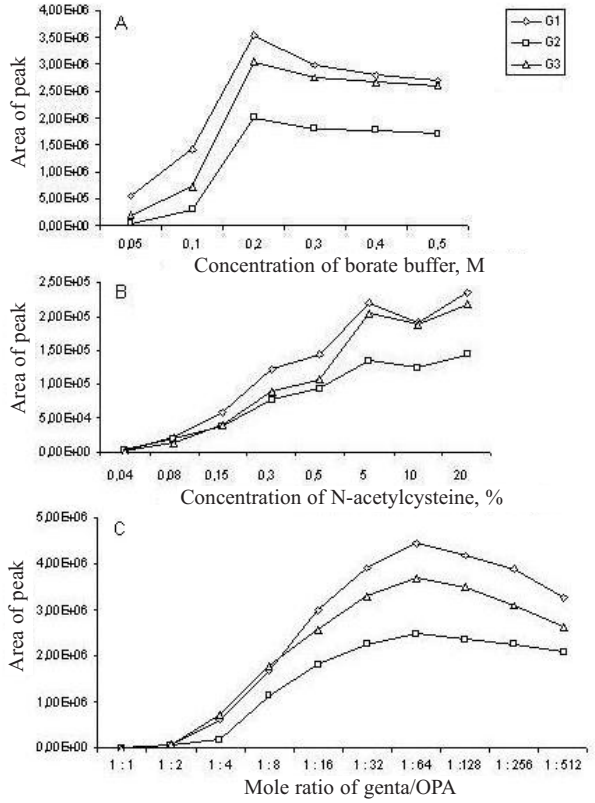


Fig. 2. Effect of the borate buffer concentration at pH 10 (A), N-acetylcysteine concentration (B), and the mole ratio of gentamicin (Genta) to *o*-phthaldialdehyde (OPA) (C) on the derivatization reaction.

Precision of the method was studied by the repeated analysis of three standard solutions at three concentration levels of 1.2, 4.0, and 8.0 μg/ml using precisely the same equipment and analytical procedure. The RSD values varied in the range 7.2-8.5%, 1.5-5.0%, and 2.1-4.5% at the low, medium and high concentration levels, respectively.

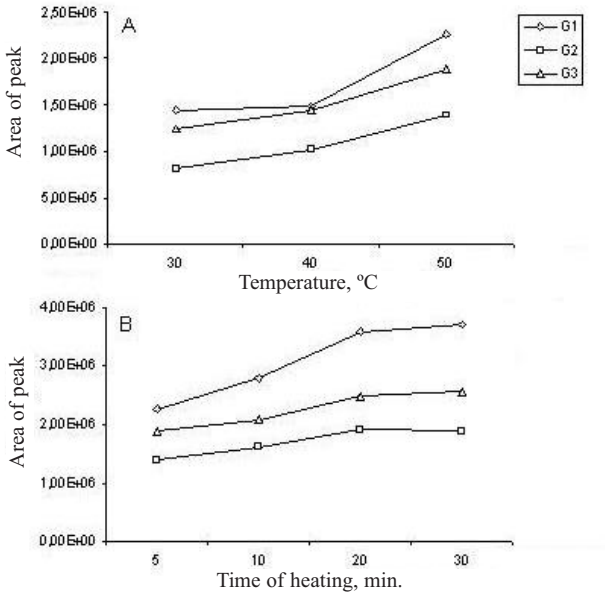


Fig. 3. Effect of temperature (A) and heating time (B) on the derivatization reaction.

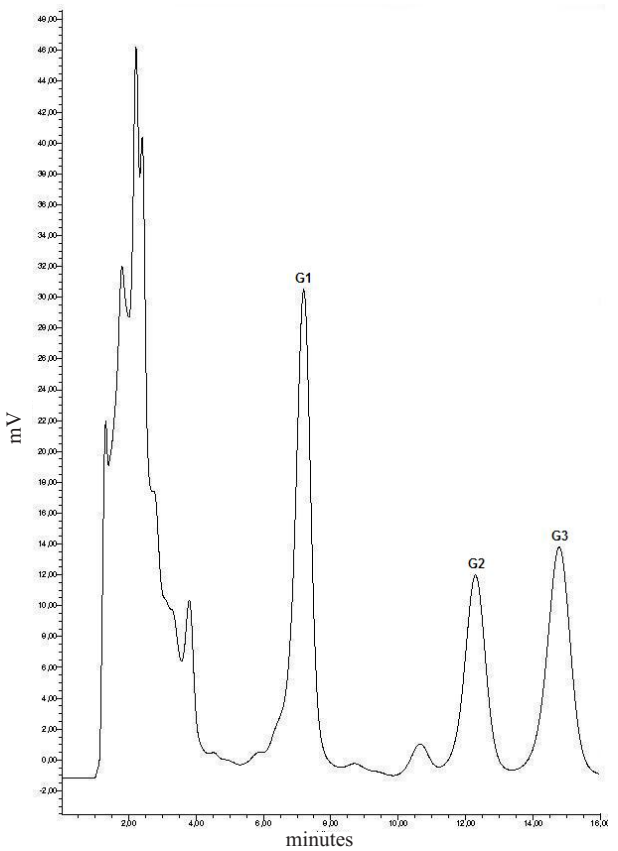


Fig. 4. Typical chromatogram of the analyzed gentamicin components labeled as G1, G2, G3.

Table 1. Results obtained from the determination of gentamicin components G1, G2, G3 and their sum (ΣG1,G2,G3) in calibration solutions (evaluation of linearity) and in pharmaceutical preparations using the HPLC method.

	G1	G2	G3	ΣG1,G2,G3
Calibration solutions				
Regression equation	y = 149475.5x -33417.1	y=89567.3x -11676.7	y=125982.2x -24126.5	y=365025.0x -69220.2
Correlation coefficient	0.9997	0.9997	0.9996	0.9997
Mandel fitting test ($n = 18$, $F_{99\%\,(1,15)} = 8.68$)	$TV = 1.87$	$TV = 1.09$	$TV = 1.61$	$TV = 1.66$
Gentamicin injections (10 mg/ml, n = 8)				
Amount found, mg/ml	9.6062	9.3792	10.9152	10.0023
Standard deviation	0.5500	0.5117	0.5767	0.4937
Variance	0.3025	0.2619	0.3326	0.2437
Relative standard deviation (%)	5.73	5.46	5.28	4.94
95% confidence interval	0.4599	0.4279	0.4822	0.4128
Relative error (%)	-3.94	-6.21	9.15	0.02
Gentamicin drops (3 mg/ml, n = 6),				
Amount found, mg/ml	2.4853	2.6288	2.9134	2.6682
Standard deviation	0.2440	0.3464	0.3388	0.2970
Variance	0.0595	0.1200	0.1148	0.0882
Relative standard deviation (%)	9.82	13.18	11.63	11.13
95% confidence interval	0.2560	0.3635	0.3555	0.3117
Relative error (%)	-17.16	-12.37	-2.89	-11.06

TV – the tested value.

The elaborated HPLC method was applied for the determination of gentamicin in the pharmaceutical preparations such as gentamicin injections and gentamicin drops. The drug content in 1 ml of preparation was found to be 10.0023 mg ± 4.94% (*RSD*) for gentamicin injections, and 2.6682 mg ± 11.13% (*RSD*) for gentamicin drops.

The accuracy of this method was confirmed after application of Student's *t*-test. There was not significant difference between the mean recovery of the total content of gentamicin (injections: 100.02%; drops: 88.94%) and 100% (injections: $TV = 0.0044 < t_{95\%} = 2.37$, $n = 8$; drops: $TV = 1.03 < t_{95\%} = 2.57$, $n = 6$). The obtained results for the calibration method and for analysis of gentamicin in the pharmaceutical formulations are summarized in Table 1.

Conclusion

The developed HPLC-pre column derivatization method with fluorescence detection is simple in performance, selective, accurate, precise, and can be recommended for the quality control of gentamicin commercial formulations. This method can be also be extended to the determination of other aminoglycoside antibiotics.

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