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A simply and sensitive fluorometric method for determination of gentamicin in liposomal suspensions

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Abstract

A new method for measuring gentamicin in liposomes fluorometrically is described. The assay is based on the reaction between the amino groups in the gentamicin molecule and *o*-phthaldialdehyde (OPA), under basic pH conditions; the product's fluorescence can be read directly on a simple fluorimeter. The effects of several factors (time of reaction, volume of the OPA reagent, and product stability) were investigated. The standard curve was linear in the concentration range of 0.5–4.0 μg, showing an excellent determination coefficient of $r^2 = 0.99$. Additionally, the influence of different liposomal lipids on gentamicin determination was tested. Liposomal lipids containing no free amino groups (PC, Chol, DOTAP) have no influence on the reaction when present in the reaction mixture. In contrast, amino groups containing lipid (SA) showed intense method interference. Therefore, a method of lipid extraction was adapted to remove undesired lipids. The described method was successfully utilised during 2 years of liposomal gentamicin experiments.

Keywords: Liposomes; Gentamicin determination

1. Introduction

Gentamicin is an aminoglycoside antibiotic produced by the fermentation of *Micromonospora purpurea* or *M. echinospora* (Weinstein et al., 1963a). However, gentamicin is not a single molecule, but a complex of three major and several minor components. Gentamicin C_1, C_{1a}, and C_2 are the three molecules of the gentamicin mixture and they differ in their degree of methylation on the purpurosamine ring (Weinstein et al., 1963b; Cooper et al., 1971). Some animal data also indicated differences in toxicity of the gentamicin components (Kohlhepp et al., 1984). Gentamicin, as other aminoglycosides, binds to the surface of bacteria and is transported through the cell wall. Once inside the cell it inhibits protein synthesis by the microorganism, resulting in a rapid, concentration-dependent bactericidal action. Severe side effects limit the medical application of gentamicin, the most important of which are its nephrotoxicity and ototoxicity (Karlowsky and Zhanel, 1992; Prins et al., 1993). The use of the drug is thus limited and concentrated mainly on the treatment of serious sepsis. Therefore, numerous reports involving liposomes as gentamicin antibiotic carriers have been published (Swenson et al., 1990; Schiffelerrs et al., 2000, 2001a,b). Several methods of drug incorporation in liposomes were applied and *in vitro* tests on clinical strains were performed. The use of liposomes in *in vivo* models showed reduced side effects and increased therapeutic index of the antibiotic through increased concentration of the drug at the site of infection.

One of the major problems related to the use of gentamicin, and all aminoglycoside antibiotics, in the laboratory is the limited means of quantifying the drug. Since gentamicin absorbs ultraviolet and visible light poorly and the molecule lacks fluorophores, no direct spectrophotometric or fluorometric method can be applied. Therefore, its detection often requires derivatisation (Tawa et al., 1998). Several methods of measuring gentamicin have been established. It may be quantified using the polarisation fluorescence-immunoassay (PFIA) (Gurtler et al., 1995), enzyme immunosorbent assay (ELISA) (Ara et al., 1995), enzyme-immunoassay (EMIT) (Miglioli et al., 1993),

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fluorescence-immunoassay (DTX) (Miglioli et al., 1993), colorimetric methods (Swenson et al., 1990; Frutos et al., 2000), microbiological methods (Reamer et al., 1998), HPLC (Stead and Richards, 1997; Tawa et al., 1998; Isoherranen and Soback, 1999, 2000), some fluorometric assay (Wang et al., 2000), and liposome immunoassay (LIA) (Kim and Park, 1994). Most of the above methods suffer from a lack of sensitivity and reproducibility (colorimetric and microbiologic methods), are uneconomical (enzyme-immunoassays), require specialised equipment (HPLC with a fluorescence detector, EMIT assay), or are just time consuming. Additionally, some of these methods do not have similar response to free and liposome-encapsulated drug (Lovering et al., 1999).

When we started experiments with gentamicin encapsulated in cationic liposomes and its evaluation on *in vitro* clinically resistant Gram-negative bacterial strains, we needed a reliable method for routine gentamicin quantification. Most of the above methods were inconvenient and some of the others required equipment not present in the laboratory. Therefore, we established a simple and sensitive fluorimetric method based on a derivative of gentamicin with a commonly used OPA reagent (Al-Amoud et al., 2002). The method requires one step of sample preparation and access to a spectrofluorimeter. The method is simple, reproducible, and sensitive, and may be used for both drug quantification within liposomes as well in other samples containing gentamicin. The limitation of the method is the necessity of avoiding or removing other molecules containing primary amino groups prior to gentamicin quantification.

2. Materials and methods

2.1. Chemicals

Gentamicin sulphate (606 U/mg) was obtained from ICN Biomedicals Inc. (Aurora, OH, USA). 1,2-Dioleoyloxy-3-trimethylammonium-propane (DOTAP), phosphatidylcholine (PC), and cholesterol (Chol) were purchased from Northern Lipids Inc. (Vancouver, BC, Canada). HPLC solvents (chloroform, dichloromethane, methanol) were supplied by J.T. Baker (Deventer, The Netherlands). Phthaldialdehyde (OPA) was purchased from Fluka Chemie GmbH (Buchs, Germany). Boric acid, potassium hydroxide, sodium phosphates, sterylamine (SA), and 2-mercaptoethanol were obtained from Sigma–Aldrich Chemie GmbH (Steinheim, Germany).

2.2. Equipment

A Kontron SMF 25 fluorophotometer (Kontron Instruments, Italy) was used and operated at 380 V.

2.3. Preparation of liposomes

The lipid compositions of the various liposomes used in the experiments were: PC/Chol (7:3), PC/Chol/DOTAP (5:3:2), and PC/Chol/SA (5:3:2). Appropriate amounts of lipids dissolved in chloroform (20 mg/ml) were mixed in a 100 ml round-bottom flask. By evaporating the organic solvent at 40 °C, a thin film of dry lipid was deposited on the inner wall of the flask. Residual solvent was removed under high vacuum applied for at least 1 h. The dry lipid films (with 10 mg of total lipid) were hydrated in PBS (pH 7.4). Hydration was performed at a room temperature and facilitated by adding two 5 mm glass beads and vortexing the liposomal suspension. Unilamellar liposomes were prepared by extrusion through two-stacked polycarbonate filters of 100 nm pore size (Nucleopore, Whatman) at 20 °C on a Thermobarrel Extruder (Lipex Biomembranes, Vancouver, BC, Canada). The mean vesicle size was between 110 and 130 nm. Lipid concentration was determined colorimetrically with ammonium ferrothiocyanate (Stewart, 1980). For experimental purposes, every liposomal formulation was diluted in water to achieve a total lipid stock suspension of 500 μg/ml.

2.4. Gentamicin standard solution

An accurately weighed 0.05 g standard sample of gentamicin sulphate was dissolved in HPLC grade water, transferred to a 50 ml standard flask, diluted to the mark with water, and mixed well. This solution, containing 1 mg/ml gentamicin sulphate, was diluted 10 times in water (10–100 ml) and used for the preparation of standard curves and all other experiments.

2.5. Boric acid buffer solution preparation

Boric acid (0.4 M, 24.7 g) was dissolved in 900 ml of HPLC grade water, the pH was adjusted to 10.4 with a 50% (w/v) potassium hydroxide solution, and then sufficient water was added to produce 1000 ml.

2.6. Preparation of the OPA reagent

OPA reagent was prepared according to following procedure: 0.2 g of *o*-phthaldialdehyde was dissolved in 1 ml of methanol and the solution was mixed with 19 ml of a 0.4 M boric buffer (pH 10.4). Then, 0.4 ml of 2-mercaptoethanole was added and the pH was adjusted to 10.4 with the potassium hydroxide solution. This reagent was kept in the dark at 4 °C and was stable for 2–3 days.

2.7. Effect of OPA reagent concentration on the OPA–gentamicin reaction

To optimise the volume of OPA reagent necessary for efficient reaction with gentamicin in its higher concentration limit, several OPA reagent volumes were mixed with samples containing 4 μg of gentamicin according to the following procedure.

One milliliter of water containing 4 μg of gentamicin was placed into six glass tubes and 0.6 ml of methanol was added. Then, 0.4, 0.2, 0.1, 0.075, 0.05, and 0.025 ml of the OPA reagent was added, stirred vigorously, and a sufficient volume of methanol was added to produce 2.5 ml of total sample volume. Blank samples for every OPA reagent concentration were prepared according to this procedure, with water replacing the

aliquots of gentamicin solution. After 10 min of incubation, the relative fluorescence of the blank and test samples were measured on the Kontron SMF 25 fluorophotometer at an excitation wavelength of 340 nm and emission at 455 nm. As a result of these measurements, a 100 μl OPA reagent sample was selected for further tests.

2.8. Effect of incubation time on gentamicin–OPA fluorescent complex formation

Four micrograms of gentamicin, as gentamicin sulphate solution in water (100 μg/ml), was placed in a glass tube and sufficient water was added to produce 1 ml samples. Then, 0.6 ml of methanol was added and the samples were vigorously vortexed, after which a 0.9 ml mixture of 0.1 ml of OPA reagent and 0.8 ml of methanol was added. Blank samples were also prepared. The samples were then vortexed repetitively and the relative fluorescence of the test samples was measured on the Kontron SMF 25 fluorophotometer with an excitation wavelength of 340 nm and emission at 455 nm at 1, 3, 5, 10, 15, 20, 30, 60, 90, and 120 min after fluorescent complex formation.

2.9. Method linearity and preparation of the standard curve

The linearity of the method was estimated on the base of several series of gentamicin sulphate standard curves prepared using 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, and 4 μg of drug, this range covering the applicable spectrofluorimeter reading range (0–200 relative units). Every concentration as well as blank samples were prepared in triplicate. For the measurements, 100 μl of OPA reagent in the samples was chosen. All measurements were performed in glass tubes. Standard samples containing from 50 to 400 μl of gentamicin sulphate solution were placed in dry glass tubes and then appropriate volumes of water were added to produce 1 ml samples. Then, 0.6 ml of methanol was added to the samples and mixed, followed by 0.9 ml of the reagent solution (0.1 ml of OPA reagent and 0.8 ml of methanol). The relative fluorescence of the standard samples was then measured after 10 min of incubation.

2.10. Gentamicin determination in the presence of the liposomal lipids

To test the effect of liposomal lipids on the method under study, known amounts of gentamicin sulphate (2 μg) were measured in the presence of liposome formulations containing various lipids. Gentamicin was also tested in the presence of different amounts of lipid (10–100 μg total lipid (TL)). Briefly, 200 μl of gentamicin sulphate solution (2 μg) was mixed with different amounts of liposomes suspended in water (0, 10, 20, 30, 40, 60, 80, and 100 μg of TL) and then water was supplemented to produce 1 ml aliquots, followed by 0.6 ml of methanol, and mixed well. The reaction was started by adding 0.9 ml of the reagent solution (0.1 ml of OPA reagent and 0.8 ml of methanol). The amount of gentamicin was then read from the simultaneously prepared standard curve.

2.11. Liposomal lipid extraction procedure

Two hundred microliters of the liposomal suspension was mixed with 250 μl of dichloromethane followed by 500 μl of methanol. The mixture was then vortexed until a clear solution was obtained, to which 250 μl of a 0.2 M NaOH solution and 250 μl dichloromethane were introduced and then vortexed vigorously. The sample, containing two phases, was then centrifuged for 5 min (10,000 rpm) in mini glass tubes on a bench centrifuge. The liposomal lipids were located in the lower phase and gentamicin in the upper, water–methanol phase. The volume of the phase containing gentamicin was 960 μl. As a rule, 480 μl of the samples (50% of the volume) were used for drug determination.

3. Results

In this study, different gentamicin concentrations were measured using a fluorometric method involving the reaction between *o*-phthaldialdehyde and primary amino groups present in the antibiotic molecule. The OPA reagent was prepared according to HPLC gentamicin-analysis procedures and adapted for an easy, tube fluorometric test (Frutos et al., 2000). The amount of the drug which can be detected was in the range of 0.5–4 μg when the spectrofluorimeter was operated near its optimal sensitivity field (380 V).

First the effect of OPA reagent volume on the gentamicin–OPA reaction was measured. Different volumes of reagent (6.25–400 μl) were mixed with a water/methanol solution containing the maximal amount of drug (4 μg) and the reaction mixture was incubated for 10 min and then read. Fig. 1 presents the results expressed as a function of relative fluorescence intensity and OPA reagent volume. The reaction is linear until the OPA reagent concentration is reduced to 12.5 μl. Then, the relative fluorescence intensity decreases. Therefore, higher volumes of the OPA reagent (100 μl) have to be applied for all the studies to assure reaction linearity for even higher gentamicin concentrations than those measured. Surprisingly, the volume of OPA reagent used in this test had only a negligible effect on the background fluorescence of the blank samples (data not shown).

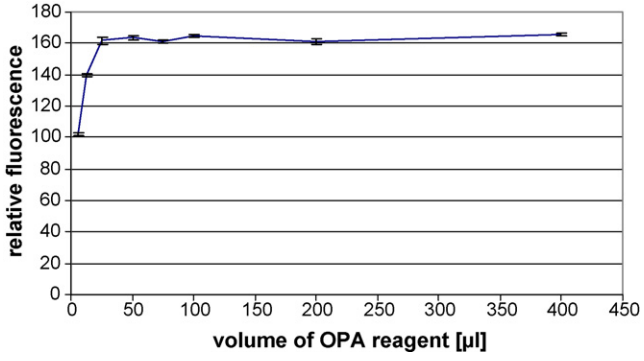

Fig. 1. The influence of the OPA reagent volume on the OPA–gentamicin sulphate reaction.

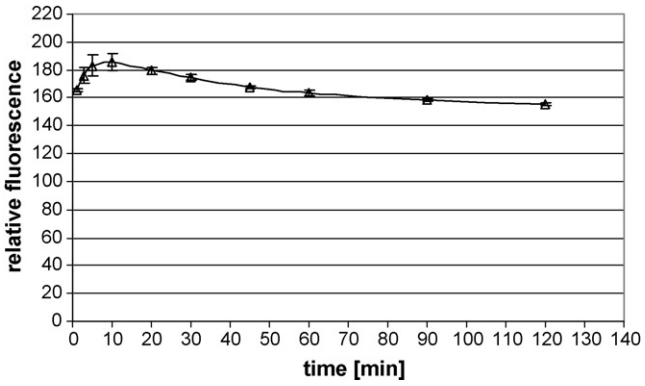

Fig. 2. The kinetics of the complex formation between gentamicin sulphate (4 μg) and the OPA reagent (100 μl) and its quenching in time.

The kinetics of fluorescent complex formation was then examined (Fig. 2). In the first 4 min, the sample's fluorescence increased, reached its maximum at about 10 min after the start of the reaction, and then slowly decreased. Therefore, 10 min of incubation was chosen for the other readings. When the basic parameters of the reaction were set, the linearity of the gentamicin standard curve was examined. The linearity of the method was evaluated by analysing a series of gentamicin sulphate standards. In this study, eight concentrations were chosen which covered the whole usable spectrofluorimeter reading range (0–200). As shown in Fig. 3, the relative fluorescence is linear in the range of all the amounts applied (0.5–4 μg), with a determination coefficient of $r^2 = 0.9996$.

As this method was devised to determine gentamicin sulphate in liposomes, three different liposomal formulations were examined in terms of liposomal lipid interference with the OPA reagent. Because *o*-phthaldialdehyde reacts with primary amino groups, it should not react with other groups present in phospholipid/cholesterol molecules. Only lipids containing primary amino groups (phosphatidyletanoloanine, phospatidylserine, or sterylamine) can influence the reaction. To check this assumption, the unilamellar liposome suspensions PC/Chol (7:3 m/m), PC/Chol/DOTAP (5:3:2), and PC/Chol/SA (5:3:2), representing total lipid concentrations from 10 to 100 μg, were dissolved in 0.6 ml of methanol, then mixed with a gentamicin solution containing 2 μg of the drug and assayed for drug concentration. In the event the liposomal lipids do not influence the method, the

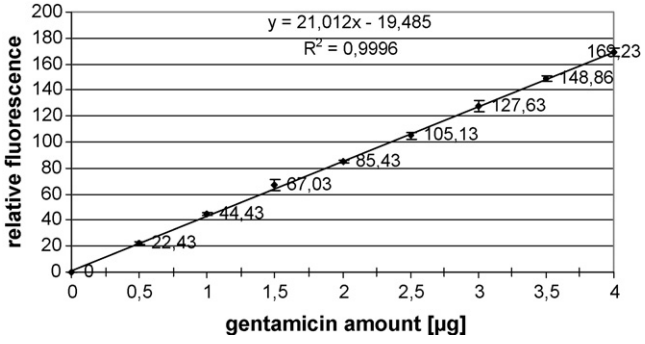

Fig. 3. The calibration curve for fluorescence intensity vs. concentration of gentamicin sulphate.

Table 1
Determination of gentamicin in the presence of increasing amounts of liposomal lipids (PC/Chol 7:3)

Amount of liposomal lipids mixed with 2 μg of gentamicin (μg)	Measured gentamicin amount (μg)	S.D.	Percent of recovery
0	1.92	0.08	96.0
10	1.94	0.08	97.0
20	1.87	0.07	93.5
30	2.05	0.16	102.5
40	1.95	0.09	97.5
60	1.96	0.09	98.0
80	2.07	0.16	103.5
100	1.91	0.08	95.5

Table 2
Determination of gentamicin in the presence of increasing amounts of liposomal lipids (PC/Chol/DOTAP 5:3:2)

Amount of liposomal lipids mixed with 2 μg of gentamicin (μg)	Measured gentamicin amount (μg)	S.D.	Percent of recovery
0	1.99	0.07	99.3
10	1.96	0.03	97.9
20	1.92	0.02	96.0
30	2.11	0.11	105.5
40	1.99	0.01	99.4
60	2.00	0.07	100.0
80	2.05	0.11	102.4
100	1.99	0.02	99.9

results should be close to the amount of drug applied; otherwise the measured amount can be lower or higher depending on the way of the interaction. As shown in Tables 1 and 2, when non-primary amino groups containing lipids were present in the reaction mixture, the drug concentration measured was close to applied. Unfortunately, in the case of liposomes with sterylamine, the lipid amino groups strongly increased the fluorescence of the assayed samples; thus the gentamicin concentration was adulterated and far above of its real value (Table 3).

To assure reliable drug readings, lipids possessing primary amino groups should first be extracted. Fortunately, the physicochemical proprieties of gentamicin and lipid molecules are different. Gentamicin, as a water-soluble substance, does not dissolve in organic solvents such as chloroform. Lipids, in turn, do not dissolve in water. One possible way is the Bligh–Dyer extraction method (Bligh and Dyer, 1959), which can be adapted for this procedure. The method applies chloroform and methanol

Table 3
The interference of liposomal lipids (PC/Chol/SA 5:3:2) present in the reaction mixture on the OPA–gentamicin sulphate reaction

Amount of liposomal lipids mixed with 2 μg of gentamicin (μg)	Measured gentamicin amount (μg)	S.D.	Percent of recovery
0	1.99	0.04	99.4
10	3.22	0.11	161.0
20	4.00	0.16	200.0
30	–	–	–

Table 4
The recovery of gentamicin sulphate after extraction of different amounts liposomal lipids (PC/Chol/SA 5:3:2) from 2 μg gentamicin samples and its influence on the OPA–gentamicin sulphate reaction

Amount of liposomal lipids mixed with 2 μg of gentamicin and extracted (μg)	Measured gentamicin amount (μg)	S.D.	Percent of recovery
0	2.00	0.05	100.0
10	1.95	0.13	97.5
20	1.98	0.03	99.0
30	2.03	0.12	102.5
40	1.91	0.07	95.5
60	2.03	0.02	101.5
80	1.98	0.14	99.0
100	2.10	0.07	105.0

for lipid extraction and has already been proposed for separating ciprofloxacin from liposomal lipids (Maurer et al., 1998). We therefore tested this method and found it to cause a substantial decrease in sample fluorescence. After a short investigation we found that traces of chloroform present in the measured samples interfered with the OPA reagent, leading to a decrease in method sensitivity (data not shown). The chloroform was then replaced by dichloromethane, which showed only minimal influence on the method's sensitivity.

Table 4 shows the results of gentamicin determination (2 μg) in samples with increasing liposomal lipid content (PC/Chol/SA, 0–100 μg) subjected to the lipid extraction method prior to drug quantification. The calibration curve was simultaneously prepared by extracting known amounts of gentamicin (2–8 μg) by the same method to ensure the same experimental conditions. The measured amounts of drug were not altered by the presence of liposomal sterylamine, and the feasibility of the applied extraction method was demonstrated.

4. Discussion

The method described here is based on the well-known reaction between *o*-phthaldialdehyde (OPA) and primary amino groups. The gentamicin molecule, as other aminoglycosides, possesses several groups which are able to form fluorescent complexes suitable for drug quantification. Since the molecule lacks a sufficient chromophore necessary for its determination, there are several different pre- and post-gentamicin derivatisation methods involving OPA used in HPLC procedures. According to our knowledge, no method has been established so far involving OPA for a simple and sensitive fluorometric assay requiring only commonly used spectrofluorimeters, in contrast to expensive HPLC instruments equipped with fluorescence detectors rarely present in microbiological laboratories The procedure for preparing the OPA reagent was adapted from one of the HPLC post-derivatisation methods (Frutos et al., 2000) and requires the use of a boric buffer of pH 10.4 and 2-mercaptoethanol for high reaction efficiency and fluorescent product stability. The volume of OPA reagent was fixed at 100 μl and exceeded the amount necessary to assure the required excess usually utilised in all colorimetric and fluorimetric methods. The initial fluorescence of the blank samples was almost equal regardless of the volume of OPA reagent used in the experiments (6.25–400 μl), suggesting that this fluorescence was produced mainly by the reaction between the OPA reagent and residual solvent contaminants present in the water and methanol used in the experiments.

Fig. 2 shows the kinetics of the fluorescent complex formation. The fluorescent product is formed almost immediately after mixing all the reaction reagents, but the fluorescence increases slightly over about 10 min, when it reaches its maximum level and then slowly decreases. Therefore, we decided to use 10 min of incubation prior to the fluorescence readings, but in fact all times between 10 and 120 min can be used and the results are reliable (data not shown). The sensitivity of the method is satisfactory in the concentration range of 0.5–4 μg when normal spectrofluorimeter sensitivity is applied. This concentration range, of course, depends of the type of spectrofluorimeter and the high voltage applied to the photomultiplier, and may be modified to some extent, depending on the instrument type used. The above method was established particularly for the measurement of gentamicin sulphate in liposomes used in antibacterial tests. Since gentamicin is encapsulated in spherical phospholipid vesicles, the drug must be released prior to measurement. Methanol is a water-miscible solvent able to dissolve liposomal lipids and liberate encapsulated gentamicin when the methanol concentration markedly exceeds the water concentration (e.g. 9:1 v/v). For this purpose, in this procedure 1 ml of water is mixed with 0.6 ml of methanol, which can be utilised as a solvent for liposomes. The drug concentration in liposomes is usually relatively high to compare with method sensitivity, so the liposomal suspension should be first diluted with water to obtain a relatively low drug concentration in the liposomal suspension (e.g. ∼50 μg/ml), and then 20- to 40-μl liposomal aliquots can be mixed with 0.6 ml of methanol followed by 960–980 μl of water to produce the total 1.6 ml gentamicin sample. At this stage, vigorous mixing is required. Next the gentamicin sample is mixed and vortexed with 0.9 ml of methanol containing 0.1 ml of the OPA reagent, thereby starting the reaction.

One of the disadvantages of the method is certainly its sensitivity to every molecule containing free primary amino groups. Therefore, the use of amino acids containing buffers (glycine, histidine) or amino group molecules of Tris, Hepes, or similar is prohibited. Fortunately, PBS buffer or saline is usually used in most liposome-based antibacterial experiments (Desai et al., 2002; Rukholm et al., 2006). If lipids having a free amino group such as sterylamine, phosphatidyl ethanolamine, or phosphatidyl serine are used in the liposome preparation, the lipids must first be extracted before drug quantification. The Bligh–Dyer extraction method was our first choice because of its versatility and simplicity. Although the presence of traces of chloroform in the extracted gentamicin samples influenced the method sensitivity, the fluorescence of the measured samples was almost three times lower than the initial values. This problem was not related to incomplete gentamicin recovery, since blank samples subjected to the extraction procedure and then enriched with known amounts of the drugs also showed decreased fluorescence. One of the possible explanations of

this phenomenon is the reaction of phosgene, usually present in chloroform in small quantities, with gentamicin amino groups before reaction with the OPA reagent. Chloroform was therefore replaced by dichloromethane, which produced only a negligible drop in method sensitivity. It is thus proposed to perform lipid extraction simultaneously with the preparation of a standard curve using the same procedure. The described method was successfully used during 2 years of liposomal gentamicin experiments in our laboratory, delivering coherent and reliable data (Drulis-Kawa et al., 2006). The method, with some modification, can be easily adapted for the determination of other commonly used aminoglycosides and, possibly, other molecules containing amino groups.

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