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Rapid determination of aminoglycosides in pharmaceutical preparations by electrospray ionization mass spectrometry

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Abstract

Aminoglycosides are broad-spectrum antibiotics often employed to combat Gram-negative bacterial infections. A technique based on electrospray-ionization mass spectrometry (ESI-MS) was developed for rapid determination of aminoglycosides. This method, which does not require prior chromatographic separation, or derivatization and extensive sample preparation steps, was deployed to estimate gentamicin, tobramycin, and amikacin in pharmaceutical formulations. Upon gas-phase collisional activation, protonated gentamicin, tobramycin, and amikacin undergo a facile loss of their respective "C" ring moiety to produce characteristic ions of *m/z* 322, 324, and 425, respectively. The mass spectral peak intensities for these specific product ions were monitored either by a flow-injection analysis selected-ion monitoring (FIA-SIM) time-intensity method or by a mass spectrometric internal-standard method. The linear dynamic ranges of detection for both methods were evaluated to be 10–1000 ng/mL for gentamicin, 25–2500 ng/mL for tobramycin, and 10–1000 ng/mL for amikacin. The internal-standard mass spectrometric method afforded lower intra-day and inter-day variations (2.3–3.0% RSD) compared to those from FIA-SIM method (4.5–5.0% RSD). This method was applied as a potential alternative procedure to determine gentamicin in commercial pharmaceutical samples and to monitor the release of gentamicin from "self-defensive" tannic acid-based layer-by-layer films into phosphate buffer solutions at different pHs.

Keywords: Aminoglycosides, ESI-MS, Quantitative determination, FIA-SIM, Pharmaceutical sample

Introduction

Aminoglycosides are a group of highly potent, broad-spectrum antibiotics that are often used against aerobic Gram-negative bacillary infections (Bailey 1981; Gilbert 2009; Davies 2006; Hermann 2007; Vakulenko and Mobashery 2003). These glycosides constitute a large and diverse class of antibiotics that bear two or more aminosugars linked to an aminocyclitol core (Stead 2000; Rhinehart and Shield 1980). Due to their high-polarity, non-volatility, and the absence of a strongly absorbing chromophore, quantitative analyses of aminoglycosides in simple matrices, such as bulk pharmaceutical formulations, require derivatization, chromatographic separation, specialized detectors, and/or the use of fluorinated ion-pairing agents (Rhinehart and Shield 1980; Joshi 2002; Soltés 1999; Niessen 1998; Marzo 1998; Tawa et al. 1998). Several derivatization-free analytical methodologies have been described in literature for the determination of aminoglycosides in simple matrices, mostly involving prior separation by reversed-phase high-performance liquid chromatography (RP-HPLC), hydrophilic interaction chromatography (HILIC), or capillary electrophoresis (CE) and subsequent detection using either refractive index (RI), evaporative light scattering detector (ELSD), charged aerosol detector (CAD), pulsed electrochemical detector (PED), or mass spectrometry (MS) (Kumar et al. 2012; Kahsay et al. 2014; Kaufmann et al. 2012; Almeling et al. 2012; Pérez-Fernández et al. 2012; Castro-Puyana et al. 2010; Castro-Puyana et al. 2008). In all reported cases involving liquid chromatography-mass spectrometry (LC-MS), the methods call for the use of fluorinated ion-pairing agents, such as trifluoroacetic acid (TFA), pentafluoropropanoic acid (PFPA), or heptafluorobutyric acid (HFBA) (Rhinehart and Shield 1980; Niessen 1998;

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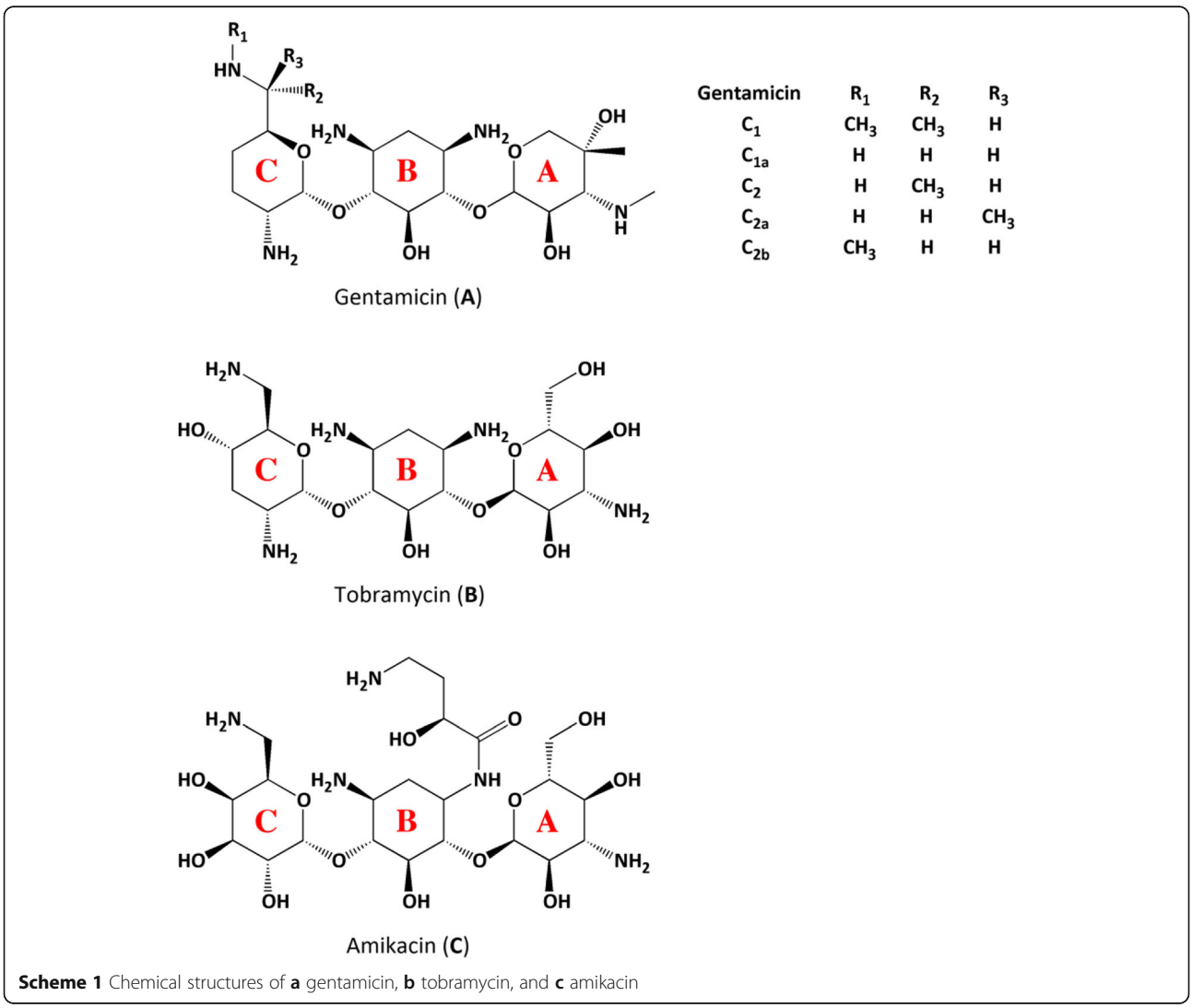
Kaufmann et al. 2012; Almeling et al. 2012). Therefore, a rapid method that does not require derivatization, chromatographic separation, or the use of fluorinated ion-pairing agents was deemed useful. Herein, we report a fast and simple electrospray-ionization mass spectrometric (ESI-MS) procedure which can be used for quantification of aminoglycosides, specifically gentamicin (A), tobramycin (B), and amikacin (C), in simple matrix solutions, such as pharmaceutical formulations. The method was also applied towards the determination of gentamicin, released from "self-defensive" multi-layered films, into phosphate-buffered solutions (Zhuk et al. 2014). These films can be prepared by layer-by-layer technique, which enables incorporation of desired amounts of antibiotics to solid matrices. The "smart," self-defensive films made in this way release the incorporated antibiotics only as a response to a bacteria-caused pH drop (Albright et al. 2017).

Methods

Materials

Gentamicin (10 mg/mL solution in deionized water), tobramycin, amikacin disulfate, tannic acid, sodium chloride, dibasic sodium phosphate, and branched poly(ethylenimine) (BPEI; M_w 65,000, 50% aqueous solution) were purchased from Sigma-Aldrich Co. (Milwaukee, WI, USA). Acetonitrile (HPLC grade), hydrochloric acid (37%, ACS grade), sulfuric acid (95–98%, ACS grade), and formic acid (88%, ACS grade) were purchased from Pharmco-AAPER (Brookefield, CT, USA). Gentamicin sulfate (40 mg/mL injection USP) and tobramycin (40 mg/mL injection USP) pharmaceutical samples were obtained from Hospira (Lake Forest, IL, USA). House nitrogen was generated by a Parker-Balston Model 75-A74 Nitrogen Generation System (Haverhill, MA, USA).



Scheme 1 Chemical structures of **a** gentamicin, **b** tobramycin, and **c** amikacin

Mass spectrometry

All experiments were performed on an Applied Biosystems API 3000 (Concord, ON, Canada) tandem quadrupole mass spectrometer equipped with a TurboIonSpray® source. A Shimadzu LC-10 AD (Columbia, MD, USA) HPLC system fitted with a Rheodyne 7725i (Rohnert Park, CA, USA) sample injection valve with a 20-μL loop was used for solvent delivery to the ion source. An isocratic solvent flow of acetonitrile-water-formic acid (10:90:0.01, v/v/v) was maintained at 1.0 mL/min, and the mass spectrometric source temperature was held at 550 °C. Both the nebulizer gas (N_2) and curtain gas (N_2) flow rate settings were set at 15 (arbitrary units). The drying gas (N_2) was maintained at a flow rate of 7 L/min at 60 psi. The ionspray voltage was held at 5500 V, and a declustering potential of 50 V was used. For the FIA-SIM method, the instrument was operated in SIM mode monitoring the intensities of the peaks at *m/z* 322, 324, and 425 for gentamicin, tobramycin, and amikacin, respectively. For the internal-standard method, the instrument was operated in MS^1 mode and spectra were acquired from *m/z* 320 to 326 at a rate of 0.1 s/scan. Calibration standards and diluted pharmaceutical sample solutions were directly injected into the solvent stream, and mass spectra were acquired in a continuous manner. For the internal-standard method, an average mass spectrum of 120 scans was reconstructed. For each sample/standard, the mass spectral peak areas (for *m/z* 322 for gentamicin, and *m/z* 324 for tobramycin) were integrated using Analyst 1.4.2 software.

Preparation of calibration standards and pharmaceutical samples

Stock solutions (1 mg/mL) of gentamicin, tobramycin, and amikacin were prepared in deionized water. Standard solutions (1–100,000 ng/mL) of gentamicin, tobramycin, and amikacin for the FIA-SIM method were prepared by serial dilution using deionized water as diluent. For the internal-standard method, gentamicin standards (10–10,000 ng/mL) each containing 1000 ng/mL of tobramycin were used to develop the calibration curve. For tobramycin (10–10,000 ng/mL), 1000 ng/mL of gentamicin was used as the internal standard. For each concentration level, five replicate calibration standards were prepared and analyzed to develop the calibration curves. Commercial injectable pharmaceutical sample solutions (40 mg/mL gentamicin and tobramycin) were withdrawn from the crimp-top vials using a syringe (3 mL) attached with a septum-piercing needle (22 gauge, 1 inch). The solutions were diluted to a final concentration of approximately 400 ng/mL using deionized water as diluent.

Experiments with layer-by-layer films

To remove organic impurities, silicon wafers were UV irradiated for 2 h, treated with concentrated sulfuric acid for 20 min, rinsed with deionized water, and dried under a flow of nitrogen. Spin-assisted layer-by-layer depositions were made on the wafer by a Laurel WS-650-23NNP/UD3/UD3B spin coater at a rotational

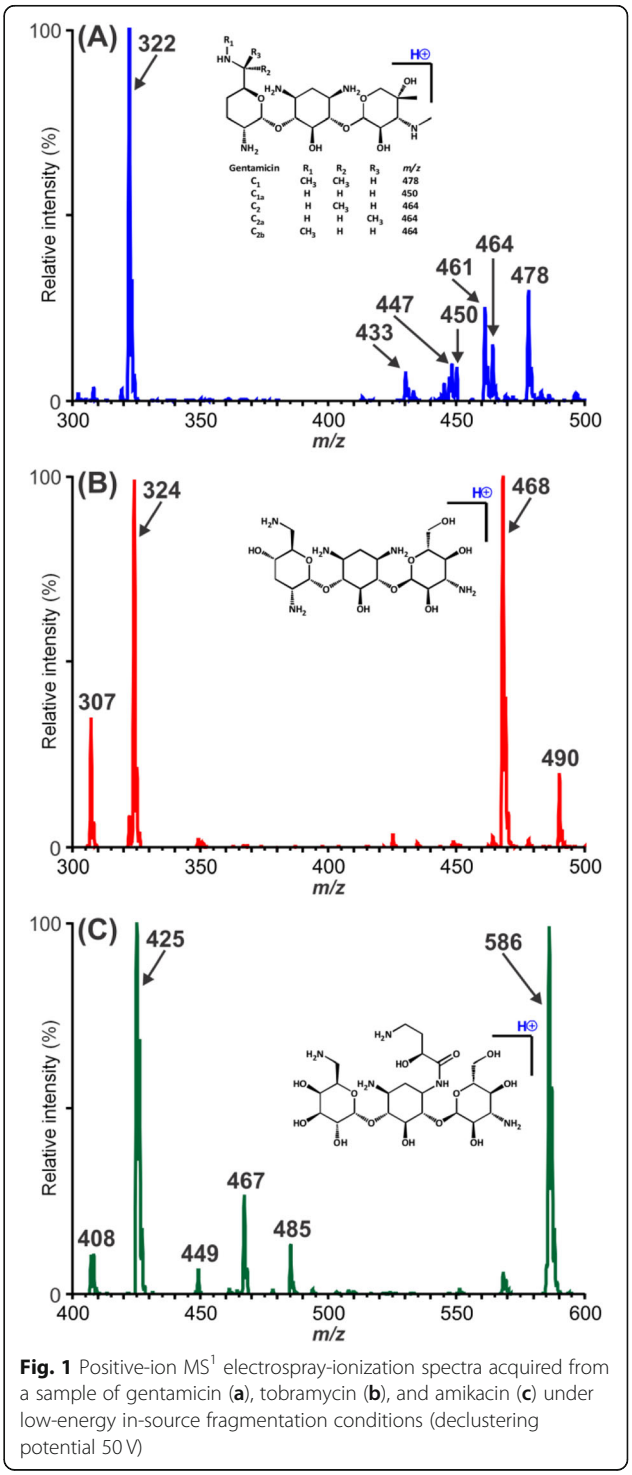


Fig. 1 Positive-ion MS^1 electrospray-ionization spectra acquired from a sample of gentamicin (**a**), tobramycin (**b**), and amikacin (**c**) under low-energy in-source fragmentation conditions (declustering potential 50 V)

speed of 3000 rpm. To enhance adhesion of multilayer films to the wafer surface, a solution of BPEI (0.5 mg/mL in 0.01 M phosphate buffer of pH 5.5) was initially deposited for 15 min, using a 40-s deposition cycle. A solution of tannic acid (0.5 mg/mL in 0.01 M phosphate buffer of pH 7.5), followed by a solution of gentamicin (0.1 mg/mL in 0.01 M phosphate buffer of pH 7.5), was deposited on the BPEI layer. After each deposition step, the substrates were thoroughly washed with pH 7.5 phosphate buffer (0.01 M) and dried. The speed and spinning for the rinsing step were identical to those used for film deposition.

To quantify the amount of gentamicin released from layer-by-layer films, a calibration curve was generated for the four calibration standards (5, 10, 20, 30 μg/mL gentamicin solutions, each containing 50 μg/mL tobramycin) using tobramycin as the internal standard. The integrated area of *m/z* 322 and 324 mass spectral peaks,

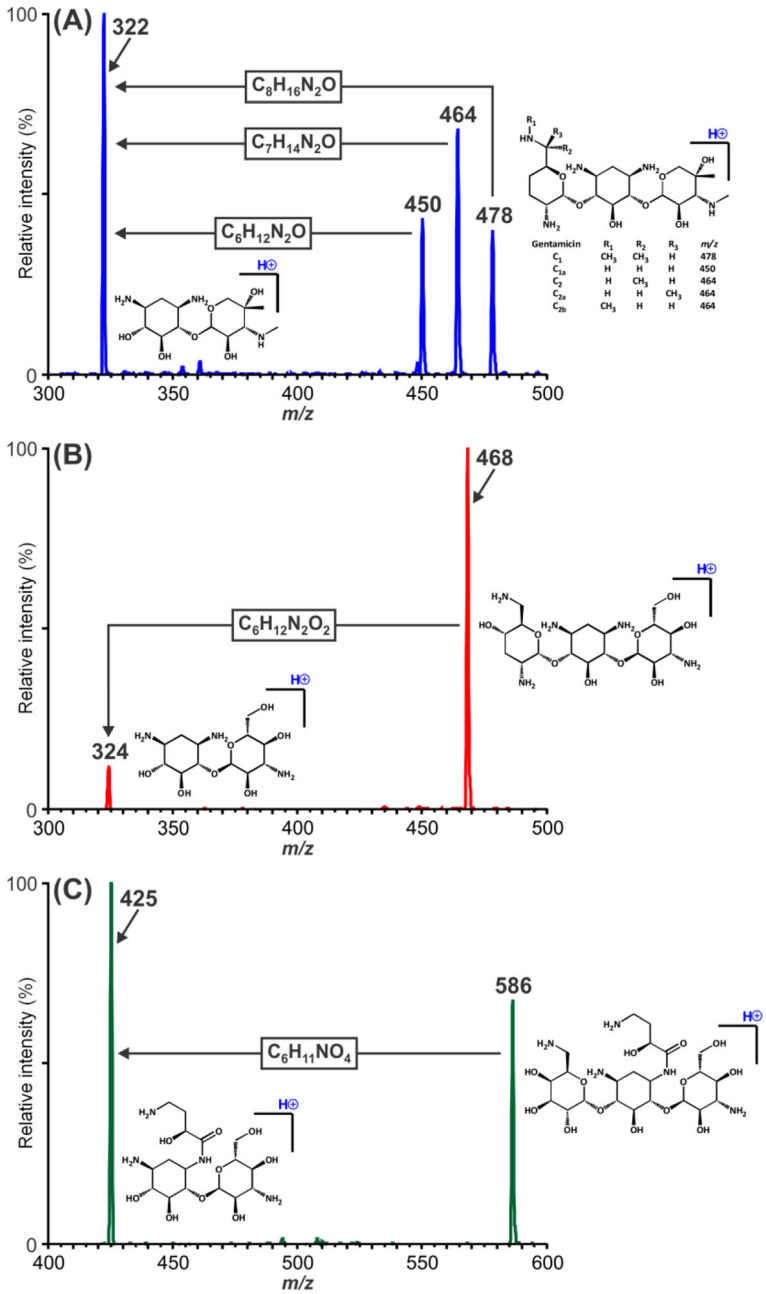


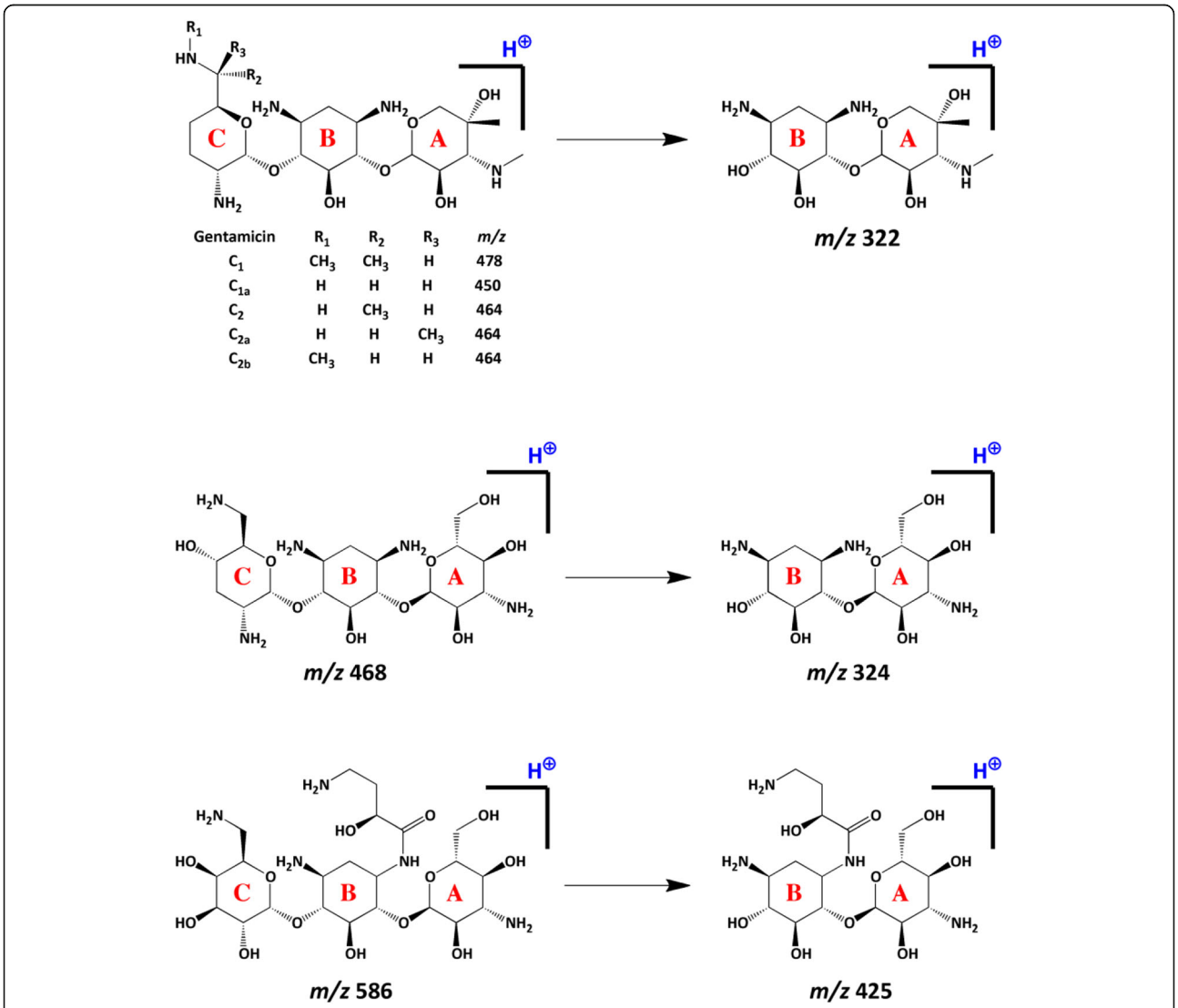
Fig. 2 Precursor-ion spectrum acquired for *m/z* 322 from a solution of gentamicin (**a**), and those for *m/z* 324 and 425 from solutions of tobramycin (**b**) and amikacin (**c**), respectively

which are characteristic for gentamicin and tobramycin (internal standard), respectively, were used for quantitation calculations. Silicon wafers, coated with 300 tannic acid/gentamicin layer-by-layer films, were exposed for 48 h to 0.01 M phosphate buffer solutions of pH values ranging from 7.5 to 5.5, and aliquots of the buffer were subjected to analysis. Calibration standards and gentamicin-containing solutions (spiked with 50 μg/mL of tobramycin) were directly injected into an isocratic solvent flow of acetonitrile-water-formic acid (10:90:0.01, v/v/v) at 1.0 mL/min, and mass spectra were acquired in a continuous manner. An average mass spectrum generated from 120 scans was used for each sample/standard, and areas of mass spectral peak profiles for *m/z* 322 and 324 were integrated using Analyst 1.4.2 software.

Results and discussion

Mass spectrometry of aminoglycosides

Commercially available gentamicin is a mixture of five components [C_1 (M.Wt. 477.60 g/mol), C_{1a} (M.Wt. 449.54 g/mol), C_2 (M.Wt. 463.57 g/mol), C_{2a} (M.Wt. 463.57 g/mol), and C_{2b} (M.Wt. 463.57 g/mol); Scheme 1]. A full-scan ESI mass spectrum recorded from a sample of gentamicin showed mass spectral peaks that represented the protonated species of all aforementioned components (*m/z* 478, 450, and 464). In addition, the base peak of the spectrum was observed at *m/z* 322 (Fig. 1a). Analogously, major fragment-ion peaks were observed at *m/z* 324 and 425 in full-scan ESI spectra recorded from tobramycin (M.Wt. 467.51 g/mol; Fig. 1b) and amikacin (M.Wt. 585.60 g/mol; Fig. 1c), respectively, in addition to peaks at *m/z* 468 and 586 for the



Scheme 2 Proposed fragmentation pathways for protonated components of gentamicin, tobramycin, and amikacin

protonated precursor molecules (Fig. 1b, c). A precursor-ion mass spectrum recorded for the *m/z* 322 ion from a solution of gentamicin showered three peaks at *m/z* 450, 464, and 478. Analogously, *m/z* 468 was determined to be the precursor of the *m/z* 324 ion from tobramycin (Fig. 2b). A similar precursor-ion experiment demonstrated *m/z* 586 to be the precursor of *m/z* 425 ion of amikacin (Fig. 2c). Taken together, these results demonstrated that the *m/z* 322, 324, and 425 ions originate directly from their corresponding protonated molecules. Moreover, the fragmentation pathway follows a common dissociation channel, which eliminates a neutral molecule bearing the "C" ring moiety, after an initial charge-remote hydrogen-transfer (Fig. 2; Scheme 2). The fragmentation pathways of protonated aminoglycosides have been previously reported; however, the origin of

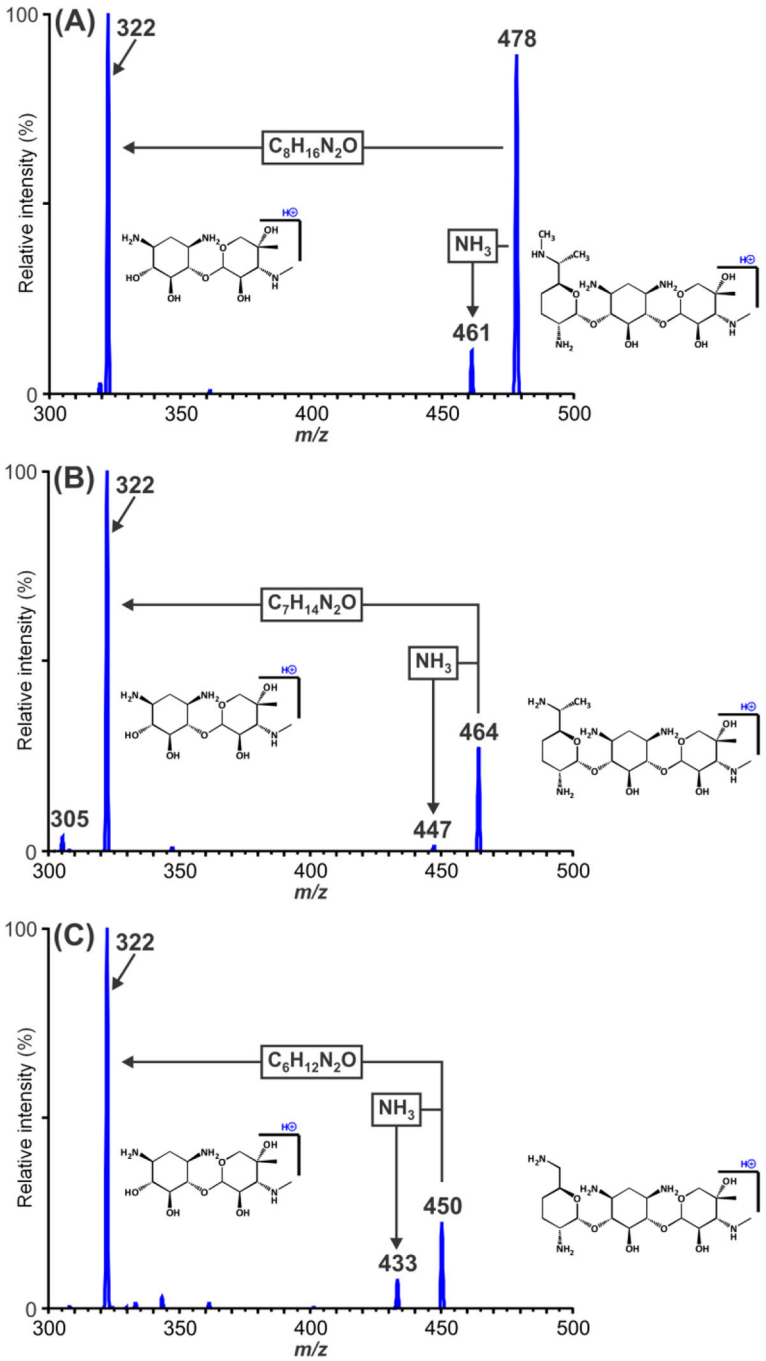


Fig. 3 Product-ion spectra of *m/z* 478 (**a**), *m/z* 464 (**b**), and *m/z* 450 (**c**) acquired from ions generated from a solution of gentamicin

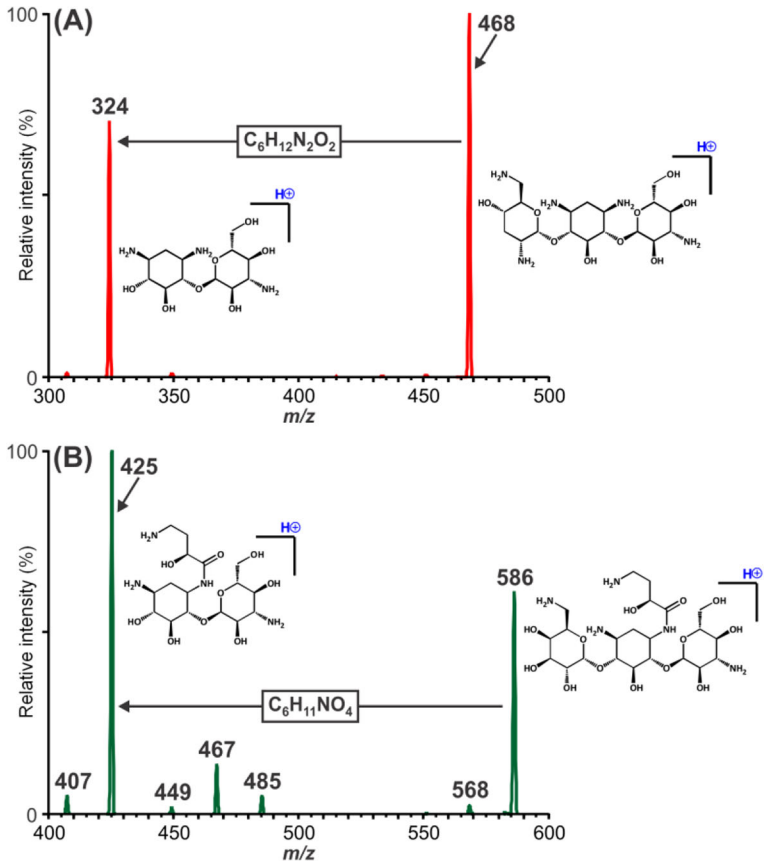


Fig. 4 Product-ion spectra acquired from *m/z* 468 (**a**) and *m/z* 586 (**b**) ions from tobramycin and amikacin, respectively

the transferred hydrogen atom remains elusive (Kotretsou and Constantinou-Kokotou 1998; Hu et al. 2000; Kotretsou 2004; Kaale et al. 2005; Grahek and Zupancic-Kralj 2009; Li et al. 2011). Product-ion spectra of protonated gentamicin components (*m/z* 478, 464, and 450), tobramycin (*m/z* 468), and amikacin (*m/z* 586) further confirmed that the elimination of the "C" ring is the most favored fragmentation pathway of protonated aminoglycosides under low-energy CID conditions (Figs. 3 and 4). The fragmentation was so facile that even under the mildest of in-source fragmentation conditions, the elimination of the "C" ring could not be completely inhibited.

Since the elimination of the "C" ring was a strongly favored general phenomenon, we opted to use the fragment ions *m/z* 322, 324, and 425 as respective diagnostic marker ions for gentamicin, tobramycin, and amikacin. In fact, it is fortuitous that all protonated gentamicin components generated *m/z* 322 fragment upon activation. As a result, the *m/z* 322 ion could be utilized for selected-ion

Table 1 Summary of flow-injection analysis single-ion monitoring (FIA-SIM) method versus the internal standard method

	FIA-SIM method			Internal standard method	
	Gentamicin	Tobramycin	Amikacin	Gentamicin	Tobramycin
Selected *m/z*	322	324	425	322	324
Internal standard (*m/z*)	–	–	–	Tobramycin (324)	Gentamicin (322)
Limit of detection (ng/mL)	1	5	1	1	5
Linear dynamic range (ng/mL)	10–1000	25–2500	10–1000	10–1000	25–2500
RSD intra-day, $n = 5$ (%)	6.1	5.2	11.7	2.2	2.9
RSD Inter-day, $n = 15$ (%)	8.0	7.8	–	3.5	3.8
Concentration in commercial sample (mg/mL)	41.3 ± 2.2	42.2 ± 1.9	–	40.5 ± 1.2	41.0 ± 0.9

monitoring experiments to determine all gentamicin components simultaneously. Consequently, all the methods reported herein were optimized for in-source fragmentation conditions to produce the aforementioned fragment ions.

Comparison of quantitative methods

Two separate analytical methods were designed and evaluated: a flow-injection analysis single-ion monitoring (FIA-SIM) method and an internal standard method (see Table 1 for a summary of conditions and results). For the FIA-SIM method, samples were injected via a sample loop directly into the isocratic solvent stream whilst the mass spectrometer was set to record the intensity of a specific diagnostic ion. A plot of chronographic peak area against the concentration of each injected sample showed a dynamic increase of the peak intensities as the concentrations were increased (Additional file 1: Figure S1). The linear dynamic ranges were determined to be from 10 to 1000, 25 to 2500, and 10 to 1000 ng/mL for gentamicin, tobramycin, and amikacin, respectively (Fig. 5). Of course, this is not a validated protocol for accurately estimating gentamicin, tobramycin, or amikacin levels because errors can be introduced due to isobaric impurity ions in the samples. On the other hand, the intercepts of Fig. 5 are very low, which indicated the interferences from isobaric impurities in the background are not very significant. The current method could be considered as a first-pass protocol for high-throughput sample analysis. The specificity can be improved when necessary by adding liquid-chromatographic separation step prior to MS analysis.

For the internal-standard method, samples were injected into the solvent stream whilst acquiring full-scan mass spectra from *m/z* 320 to 326. For data analysis, the ratio of the mass spectral peak areas (Additional file 2: Figure S2) was plotted against the ratio

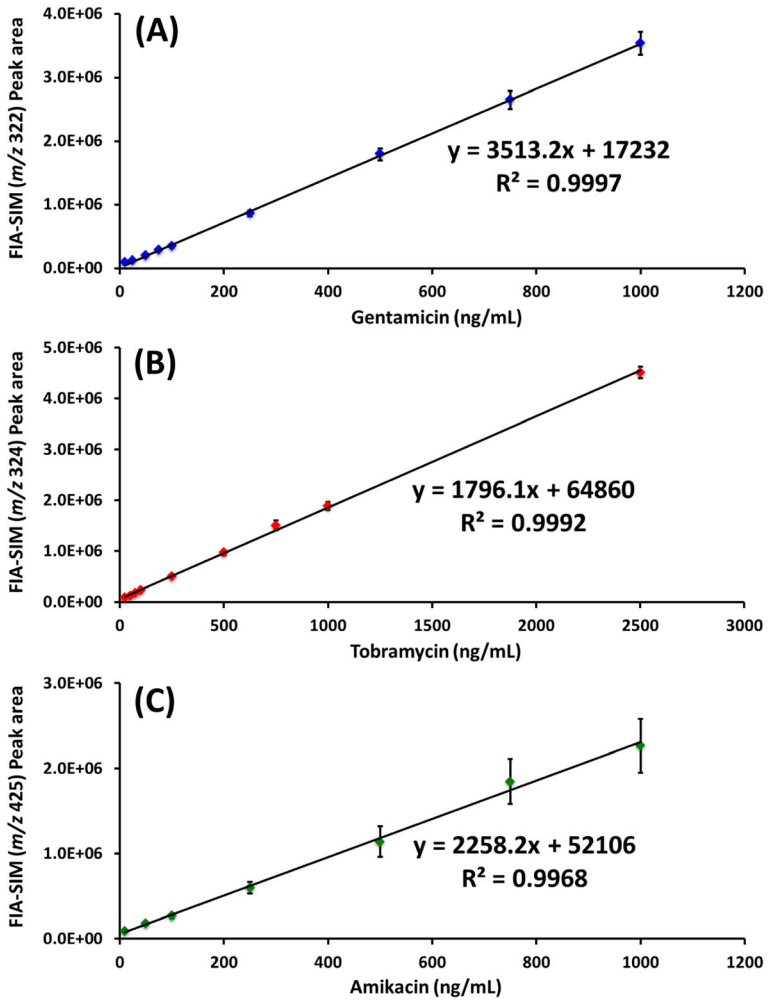


Fig. 5 Linear regression curves obtained for gentamicin (**a**), tobramycin (**b**), and amikacin (**c**) by the FIA-SIM method (error bars smaller than the width of the data-point bullets are not shown)

of the concentrations of the solutions (Additional file 3: Figure S3). For example, for gentamicin quantification, using tobramycin as the internal standard, the intensity ratio of *m/z* 322 and *m/z* 324 peaks was plotted against the concentration ratio of the two components (the tobramycin concentration was kept constant as the internal standard). Although the linear dynamic range appeared narrow, the regression curves showed the promise of the method as a rapid non-chromatographic procedure for rapid first-pass analysis (Fig. 6). Although both flow-injection and internal-standard methods gave similar quantitative results, the latter gave significantly better inter-day and intra-day precision (Table 1). Results obtained for gentamicin and tobramycin in commercial samples are presented in Table 1.

For convenience and economic reasons, tobramycin was used as the internal standard for current study. However, some errors due to the small contributions from the "M+2" isotopologues of gentamicin can be expected. For accurate determinations, an isotopically labeled tobramycin is recommended as the internal standard. Furthermore, the use of a high-resolution mass analyzer and determination under accurate-mass conditions should improve specificity.

Quantitation of gentamicin released from layer-by-layer films

We reported previously on the controlled release of antibacterial agents from layer-by-layer constructs (Zhuk et al. 2014). These films exhibit a distinct "self-defensive" behavior triggered by acidification of the immediate environment by pathogenic bacteria. We applied the internal standard method to monitor the release of gentamicin from tannic acid/gentamicin multi-layer films into phosphate buffer solutions at various pHs. A set of 1 cm × 1 cm silicon wafers coated at pH 7.5 with 300-bilayer tannic acid/gentamicin films were exposed, for 48 h to ensure complete release of antibiotic agent, to a small volume (0.5 mL) of 10^{-3} M phosphate buffer of pH ranging from 7.5 to 5.5 containing 0.2 M NaCl. Each extract solution was spiked with tobramycin as the internal standard, and the concentration of gentamicin in each solution after 48 h was quantified by the present method. As expected from the pH responsive multi-layer films, the amount of gentamicin

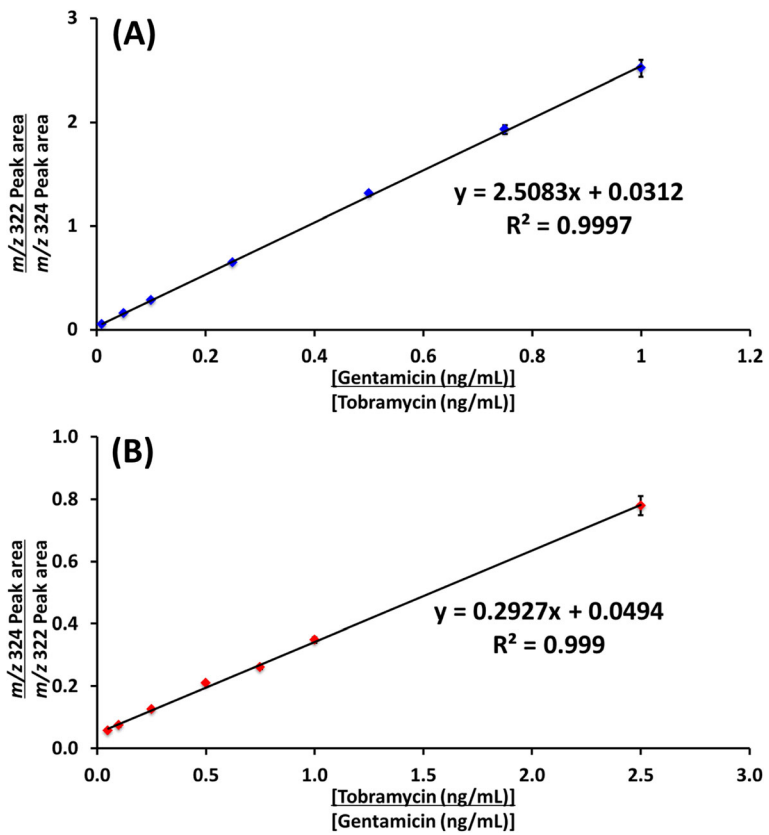


Fig. 6 Representative linear regression curves generated using the internal standard method for quantification of gentamicin with tobramycin as internal standard (**a**) and tobramycin with gentamicin as internal standard (**b**). Error bars smaller than the width of the data-point bullets are not shown

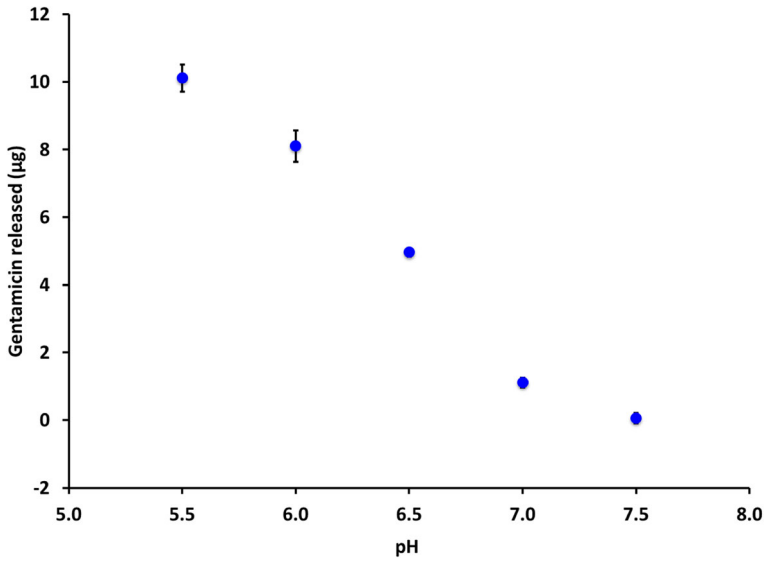


Fig. 7 Amount of gentamicin released from a tannic acid/gentamicin multi-layer construct, into phosphate buffer solutions of various pHs. Error bars smaller than the data-point bullets are not shown

released into solution increased as the pH value decreased (Fig. 7).

Conclusions

A rapid, simple, and sensitive ESI-MS method for quantitation of certain aminoglycosides (gentamicin, tobramycin, and amikacin) in simple matrices, such as pharmaceutical samples, was demonstrated. Unlike previously published methods that either requires a derivatization or a separation step, the current method can be used to analyze samples directly on any ESI-MS instrument. In general, both flow-injection and internal-standard methods gave somewhat similar quantitative results. The latter however gave significantly better inter-day and intra-day precision. Although reproducibility was significantly improved by the use of an internal standard, the FIA-SIM method could still be used as a rapid method for determination of aminoglycosides.

The new methods were deployed to determine the gentamicin or tobramycin content of commercial injectable drug products, as well as gentamicin release from "self-protective" multi-layer films. Quantification by mass spectrometry is usually accomplished by incorporating a prior chromatographic separation and then integrating chromatographic peak areas, and not by integrating peaks areas in a mass spectrum. Our results support the concept that compounds that fragment under mass spectrometric conditions in a similar manner can be quantified directly by mass spectrometric data without the need of a chromatographic step. Although the specificity of the current method is not high, and interferences could arise from isobaric matrix ions, the method has the potential to be developed as a more reliable method by employing a high-resolution mass analyzer and monitoring ions under high mass-accuracy conditions.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s40543-019-0202-4.

Additional file 1: Figure S1. Concentration vs. integrated FIA-SIM peak area plots for gentamicin (**A**), tobramycin (**B**), and amikacin (**C**) by monitoring *m/z* 322, 324, and 425 ions, respectively (error bars smaller than the width of the data-point bullets are not shown). A 20-μL aliquot of each sample solution was injected to a 1.0 mL/min solvent flow of acetonitrile-water-formic acid.

Additional file 2: Figure S2. Representative electrospray ionization mass spectrum (*m/z* 320–326) acquired from a solution of gentamicin (500 ng/mL) with tobramycin (1000 ng/mL) as internal standard. For the internal standard method, the relative areas of *m/z* 322 (gentamicin) and 324 (tobramycin) peaks were used to plot the calibration curves.

Additional file 3: Figure S3. Relative concentration versus relative mass spectrometric peak area plots generated using the internal standard method for gentamicin with tobramycin as internal standard (**A**), and tobramycin with gentamicin as internal standard (**B**). Error bars smaller than the width of the data-point bullets are not shown.

Abbreviations

CAD: Charged aerosol detector; CE: Capillary electrophoresis; ELSD: Evaporative light scattering detector; ESI-MS: Electrospray ionization mass spectrometry; FIA-SIM: Flow-injection analysis single-ion monitoring; HFB: Heptafluorobutyric acid; HILIC: Hydrophilic interaction chromatography; LC-MS: Liquid chromatrography-mass spectrometry; MS: Mass spectrometry; M.Wt.: Molecular weight; PED: Pulsed electrochemical detector; PFPA: Pentafluoropropanoic acid; RI: Refractive index; RP-HPLC: Reversed-phase high-performance liquid chromatography; TFA: Trifluoroacetic acid

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Authors' contributions
FBJ, JAH, and IZ carried out the experiments and collected, analyzed, and interpreted the experimental results. SAS and ABA supervised the work and interpreted the experimental results. All authors contributed to the manuscript drafts and read and approved the final manuscript.

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Availability of data and materials
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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