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Development and validation of liquid chromatography tandem mass spectrometry methods for the determination of gentamicin, lincomycin, and spectinomycin in the presence of their impurities in pharmaceutical formulations

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ABSTRACT

Liquid chromatography with tandem mass spectrometry (LC/MS/MS) methods for the determination of gentamicin, lincomycin and spectinomycin in the presence of their impurities were developed and tested. Chromatographic separations were achieved using gradient elution on a C18 column. All components were ionized by positive-ion electrospray and detected by multi reaction monitoring (MRM) with an LC-tandem mass spectrometer. Calibration curves were linear with correlation coefficients better than 0.99. The developed method for the determination of gentamicin provides complete base line separation of components C1, C1a, C2, C2a and C2b mentioned in the European and British Pharmacopoeias. The second developed method makes possible a simultaneous analysis of the active compounds of both lincomycin and spectinomycin. Additionally, all impurities defined in the pharmacopoeias for all three active components were determined and their identities confirmed. The methods were tested in routine quality control analysis.

1. Introduction

Aminoglycosides represent broad spectrum antibiotics which exert bactericidal activity against some Gram-positive as well as Gram-negative bacteria. Gentamicin, spectinomycin and lincomycin are among the most commonly used aminoglycosides effective in both human and veterinary applications [1,2]. Pharmaceutical formulations containing these compounds as active substances are repeatedly tested for composition as well as for the presence of impurities (Figs. 1–3). Pharmacopoeias are defining a number of HPLC, GC and microbiological methods for their determination. Gentamicin is an aminoglycoside complex mainly consisting of gentamicin C1, C1a, C2, C2a and the minor component C2b. Routinely, in the pharmaceutical industry, due to the multi component nature of gentamicin, only the relative percentage of its major constituents is measured. For the analysis of gentamicin and spectinomycin composition, Ph. Eur. defines a reversed phase LC method with electrochemical detection after postcolumn derivatization. According to the same source, lincomycin is tested for the presence of lincomycin B as the main impurity. Up to now, microbiological assays [British and European Pharmacopoeias], immunoassays, and ELISA methods neither provided the means of quantifying individual components of gentamicin, nor of impurities present in the pharmaceutical dosage forms.

Analysis of aminoglycosides is challenging due to a lack of any significant chromophore or fluorophore in these molecules. Numerous analytical methods have been used to quantify aminoglycosides, such as TLC [3], LC with spectroscopic and fluorescence detection [4–8], electrochemical detection [9–13], with evaporative light scattering detection [14–16] and also capillary electrophoresis (CE) [17,18]. Earlier LC methods and CE methods necessitated a precolumn or postcolumn derivatization (e.g. *o*-phthalaldehyde (OPA)/mercaptoacetic acid (MAA) or dansyl chloride) step to enable either UV or fluorescence detection. Although these modes of detection are quite sensitive, the obligatory derivatization step is a time-consuming process and needs well-controlled experimental conditions to produce repeatable results. If we take all this into account, mass spectrometry seems to be the technique of choice for aminoglycosides' detection in respect to very high sensitivity and positive identification, and with no derivatization steps required [18–31]. In this study, to meet this demand, reliable LC/MS/MS methods for the determination of active compounds of the antibiotics (gentamicin, lincomycin and spectinomycin) and the inherent impurities were developed and

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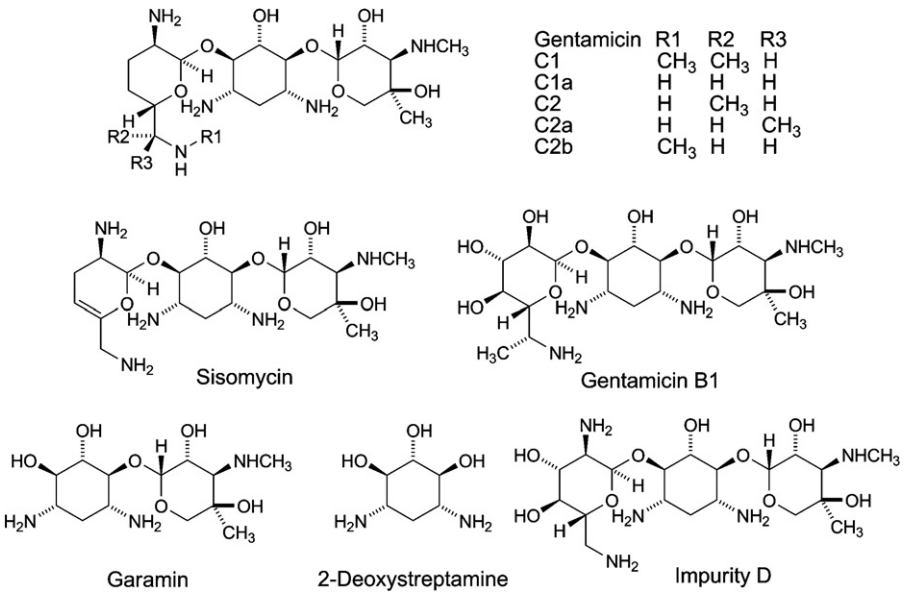

Fig. 1. Structures of gentamicin active compounds and its impurities.

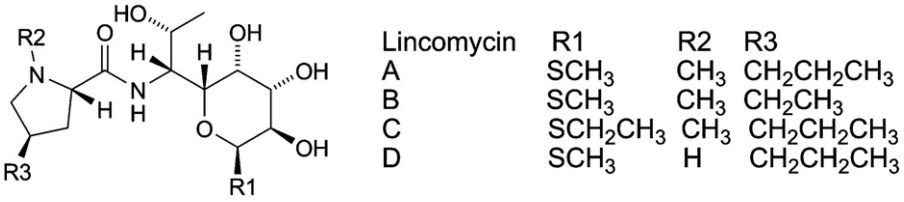

Fig. 2. Structures of lincomycin and its impurities.

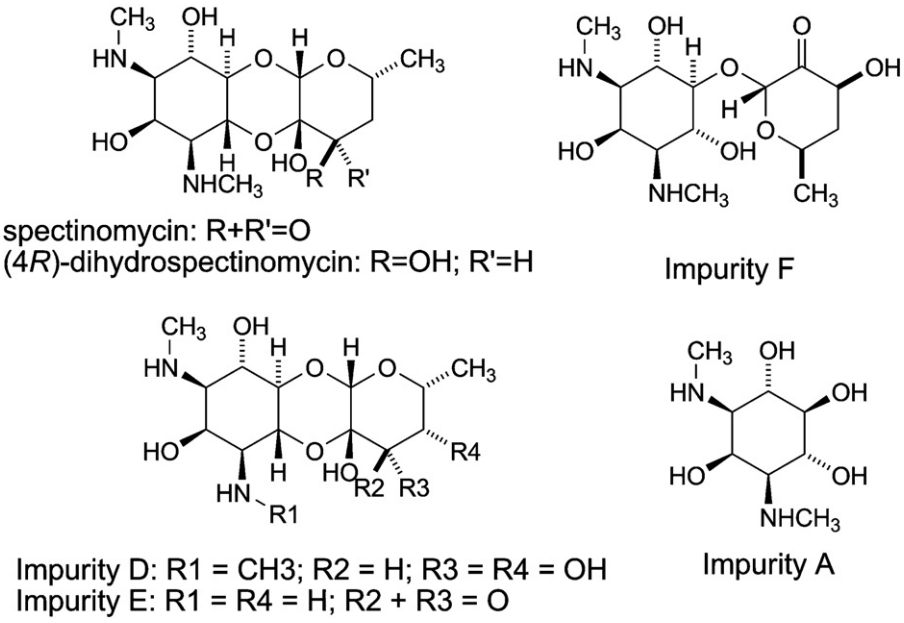

Fig. 3. Structures of spectinomycin and its impurities.

tested. The developed (two) methods were validated for assay of the active compounds and determination of their impurities with quantification expressed as percentage of the active compound. The methods were tested through the analysis of commercially available pharmaceutical dosage forms that contained either one or two antibiotics.

2. Experimental

2.1. Instrumentation

LC was performed using an Agilent Technologies HPLC system 1200 series (Waldbronn, Germany) equipped with a quaternary

Table 1
Gentamicin gradient elution conditions.

Time/min	A[a]	B[b]	C[c]
0	99%	1%	0%
14	94.5%	1%	4.5%
15	59%	1%	40%
18	59%	1%	40%
19	99%	1%	0%
30	99%	1%	0%

[a] 0.1% (v/v) aqueous trifluoroacetic acid (with the addition of ammonia up to pH 2.5).
[b] 0.1% (v/v) aqueous trifluoroacetic acid (plus triethylamine up to pH 2.5).
[c] Acetonitrile.

pump, vacuum degasser, thermostated autosampler, thermostated column compartment and a diode array detector. Chromatographic separation was carried out using Eclipse Plus C18 column (50 mm × 4.6 mm, particle size 1.8 μm; Agilent Technologies, Waldbronn, Germany) at 32 °C in case of gentamicin and at 25 °C for lincomycin and spectinomycin. In the case of gentamicin, the mobile phase consisted of varying solutions A – 0.1% (v/v) aqueous trifluoroacetic acid (with the addition of ammonia up to pH 2.5), B – 0.1% (v/v) aqueous trifluoroacetic acid (plus triethylamine up to pH 2.5) and C – acetonitrile. Optimal gradient elution was achieved as presented in Table 1. The constant flow rate was adjusted to 0.25 mL/min.

Analysis of lincomycin and spectinomycin were done using an isocratic method with a mobile phase consisting of aqueous trifluoroacetic acid (0.05%, v/v, pH 3.0, adjusted with NH_3(aq)) and acetonitrile (90%:10%, v/v) with a total flow of 0.5 mL/min.

HPLC system was connected to an Agilent Technologies 6410 Series Triple Quadrupole Tandem Mass Spectrometer (Santa Clara, USA) equipped with a multimode source. For this application, ESI positive mode was used with gas temperature of 325 °C and vaporizer temperature of 200 °C. Nitrogen was used as the drying gas at a flow rate of 5 L/min and also as the nebulizer gas at 60 psi. Capillary voltage was 2000 V, multiple reaction monitoring (MRM) dwell time was 200 ms, fragmentation voltage was 70 V for gentamicin and lincomycin and 80 V for spectinomycin and collision energy of 10 V for gentamicin and lincomycin and 24 V for spectinomycin was applied.

2.2. Chemicals and materials

Gentamicin sulphate, lincomycin hydrochloride and spectinomycin dihydrochloride were purchased from the European Directorate for the Quality of Medicines and Health Care (EDQM). Acetonitrile (HPLC grade), 25% aqueous ammonia, triethylamine (TEA) and trifluoroacetic acid (TFA) were purchased from JTBaker (Breda, The Netherlands). Ultrapure water was obtained in a Milli-Q system from Millipore (Bedford, MA, USA). Commercial samples containing gentamicin sulphate, spectinomycin sulphate and lincomycin hydrochloride were produced and acquired from FMPharm (Subotica, Serbia). Methods were tested on the following commercially available formulations: Neogent® injections containing 80 mg of gentamicin sulphate in 1 mL sample, Neolincogent® powder containing 360 mg of lincomycin hydrochloride and 10 mg of gentamicin sulphate in 1 g of powder, Neoli-spec® injections containing 50 mg of lincomycin hydrochloride and 100 mg of spectinomycin dihydrochloride in 1 mL of solution and Neoli-spec P-44® powder with 22 mg of lincomycin hydrochloride and 22 mg of spectinomycin dihydrochloride in 1 g of the powder.

The quantity of detected impurities was expressed as percentage (%) of the active compounds and obtained by comparing the impurity peak areas with the area of active compounds.

2.3. Preparation of standards

A stock solution of gentamicin sulphate was prepared by dissolving the aforementioned standard in water in order to obtain a staring concentration of 500 μg/mL. Solutions (25–500 μg/mL) for the test calibration plot were obtained by diluting the stock solution with water.

Standard solutions of lincomycin hydrochloride and spectinomycin dihydrochloride, both in the concentration range from 10 to 100 μg/mL, were prepared by diluting stock solutions containing 500 μg/mL of lincomycin hydrochloride or spectinomycin dihydrochloride with water.

2.4. Solutions for precision testing

Three series containing 25, 100 and 500 μg/mL of gentamicin sulphate, with six standard solutions in each, were prepared and injected in order to test the precision of the method. Also, for the precision testing of lincomycin hydrochloride and spectinomycin dihydrochloride analysis method, three series containing 10, 50 and 100 μg/mL of lincomycin hydrochloride or spectinomycin dihydrochloride, with six standard solutions in each, were prepared from the stock standard solutions (500 μg/mL).

2.5. Solutions for accuracy testing

Laboratory (model) mixtures containing placebo components and active substances (gentamicin sulphate, lincomycin hydrochloride and spectinomycin dihydrochloride) were prepared in water in adequate concentrations corresponding to the investigated formulations. The model mixtures were treated in the same manner as the tested formulations used for the preparation of sample solutions. For the quantitative analysis of the model mixtures, three series of dilutions with six solutions in each, calculated as 80, 100 and 120% of the concentrations corresponding to those in the tested formulations, were prepared.

2.6. Preparation of sample solutions

Sample solutions of Neogent® – injections containing 80 mg/mL of gentamicin sulphate, were prepared by diluting the sample with water to a working concentration of 100 μg/mL of total gentamicin. Sample solution of Neolincogent®, a formulation that contains 10 mg of gentamicin sulphate and 360 mg of lincomycin hydrochloride in 1 g of the powder, was prepared by dissolving the sample in water in order to obtain solutions with a final concentration of 100 μg/mL of gentamicin sulphate and 50 μg/mL of lincomycin hydrochloride. Neoli-spec® injections, containing 50 mg of lincomycin hydrochloride and 100 mg of spectinomycin dihydrochloride in 1 mL, were prepared by diluting the samples to get separate solutions having the concentration of 50 μg/mL per active component. Samples for Neoli-spec P-44® powder, which contains 22 mg of lincomycin hydrochloride and 22 mg of spectinomycin dihydrochloride in 1 g of the powder, were prepared by dissolving the sample in such a quantity of water to obtain the solution with both 50 μg/mL of lincomycin hydrochloride and spectinomycin dihydrochloride.

3. Results and discussion

3.1. Methods development

The main challenge in the development of an LC/MS/MS method for gentamicin sulphate determination was achieving the chromatographic baseline separation of its C2 components. The investigated C2 components – C2a, C2 and C2b are positional and/or

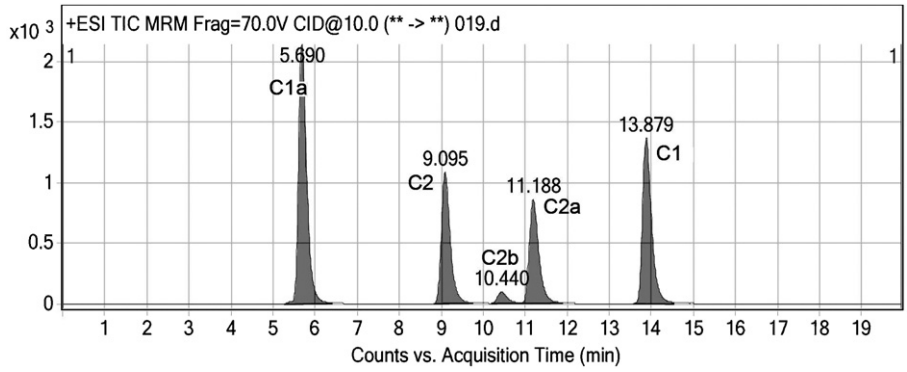

Fig. 4. Chromatogram (MRM) showing the separation of gentamicin sulphate active compounds.

stereoisomers and therefore have the same molecular weights and give ions with the same *m*/*z* values. The optimization of chromatographic conditions included the usage of different buffers of different pH values and different gradient conditions as well as the utilization of different stationary phases. The presence of triethylamine (TEA) in the buffers resulted in better peak shape and shorter retention times; however, the C2b component, under such conditions, could not be separated from other active compounds. Also, the responses of the targeted MS/MS transitions were very low as TEA influenced ionization. Therefore, as a compromise between peak shape and sensitivity on one side, and resolution on the other, a buffer with 0.1% of TFA with ammonia was used with only 1% of the buffer with 0.1% of TFA with TEA to improve peak shape and resolution. Also, C18 non-end capped and double end-capped stationary phases were tested and double end-capped phase provided better separation. The influence of pH was also tested. Significant changes of the chromatographic behavior to pH variation were noticed. The main influence was observed on the peak shape and therefore on the resolution of the C2 components of gentamicin. Even a difference of 0.2 units in the pH value resulted in significant peak tailing and therefore lowered peak resolution. This parameter was the critical one as the C2b compound was present in low percentage and the resolution was crucial in order to quantify this component separately. A typically achieved chromatographic separation of gentamicin components is depicted in Fig. 4.

The development of methods for lincomycin hydrochloride and spectinomycin dihydrochloride was also centered on attaining the required separation through the use of an adequate mobile phase. Hence, different mobile phase compositions were trialed and the best separations were achieved with a mobile phase containing TFA. However, the usage of TFA influenced the MS response and lowered the sensitivity of the methods (TFA is a well known ionization suppressor). But as these methods are intended to be used in quality control testing of pharmaceutical formulations, sensitivity was not a crucial parameter but the complete separation of compounds.

The obtained typical chromatograms for lincomycin hydrochloride and spectinomycin dihydrochloride determination are presented in Figs. 5 and 6, respectively.

The method developed for the analysis of gentamicin allowed the identification and quantitation of all impurities and active compounds (Fig. 1) defined by the Pharmacopoeias except for 2-deoxystreptamine (not detected), alongside with some additional impurities also detected in other recent studies on this subject [13,23].

Up to now, MS detection was used on two instances (ion trap [13] and triple quadrupole mass spectrometers [23]) for the analysis of gentamicin and related compounds, but for identification/confirmation purposes only, while the quantification was done through the use of a pulsed electrochemical detector [13] and with a varying analysis time of either 85 or 30 min, respectively. The latter study [23] also provided for the first time an identity confirmation of most of the gentamicin impurities and pinpointed to some new previously unknown impurities. On the other hand the main focus of the present work, besides being able to identify and confirm the presence of the analytes, was the quantification of these components by the application of such a high selectivity (mass spectrometric) detector. Additionally, we validated the method for a routine quality control application, while cutting down on the analysis time to 20 min which could significantly improve the throughput and reduce costs of the analysis.

The developed method for lincomycin hydrochloride determination revealed the presence of one additional component (impurity) of commercial lincomycin that eluted at the retention time of 5.52 min and that was unreported up-to-now. The structure of this compound remained unelucidated, although we can speculate on its possible origin – degradation of lincomycin (a difference of 2 amu in the molecular weights suggests oxidation of an alcoholic function in lincomycin A). Also, worth noting is the fact that for lincomycin and its impurities it was difficult to determine reliable qualifiers as their ratio with the quantifier was not stable. Thus, the presented values for lincomycin hydrochloride qualifiers should be further tested.

Previous studies concerning the investigation of lincomycin were usually only centered on the active compound and in most studies, biological samples were analyzed. Our method was validated for a routine use in quality control process of dosage forms with analysis times maximally up to 10 min, and, thus, provides a reliable and fast analysis with an increase of the sample throughput.

The analysis of spectinomycin was carried out mostly using amperometric detectors or evaporative light scattering detectors and focused only on the active component again. One study that included the analysis of related substances [31] used an evaporative light scattering detector for the quantification and an ion trap mass spectrometer for the confirmation of the structures of the tested compounds. The method developed herein for analysis of spectinomycin includes both the identification and quantification by a triple quadrupole mass spectrometer and this highly selective detector provides a major advantage to most of the previous works. Moreover, as the method parameters are the same for the analysis of spectinomycin and lincomycin; the same method can be used for a complete analysis of the samples that contain a mixture of these two active compounds (and we showed this to be successful by the analysis of one of the commercial samples).

The methods were tested in the analysis of active components, as well as for the impurity testing, in pharmaceutical formulations that contained gentamicin, lincomycin and spectinomycin. The used pharmaceutical preparations were both in the form of injections or powder, thus, providing a different means of assaying the influence of the matrix.

3.2. Methods validation

3.2.1. Specificity

The specificity of the methods was guaranteed by MS/MS transitions of the analyzed compounds. Precursor and product ions of compounds considered are presented in Table 2.

3.2.2. Linearity

The methods were tested for linearity for all active principles at eight different concentrations for gentamicin and six different concentrations for lincomycin and spectinomycin. The obtained calibration curves for the working concentration ranges had correlation coefficients higher than 0.99 for each compound presented in Table 3, except for gentamicin C2b with correlation coefficient better than 0.98.

3.2.3. Precision and accuracy

Precision of the methods was defined through the repeatability testing as the standard deviation of a series of 6 injections for the lowest, medium and highest concentrations from the calibration set. The obtained results are presented in Table 4. Accuracy was calculated from a series of 6 sample injections with the known added amount of the standard. Results are expressed as the percentage recovery and are given in Table 4 for the different concentration levels correlating to 80%, 100% and 120% of the working concentrations.

3.2.4. Robustness

Robustness of the method in the case of the analysis of gentamicin was tested by the variation of temperature (34 and 30 °C) and pH value of the mobile phase (2.4 and 2.6). The lincomycin and spectinomycin method was investigated in this sense under the conditions of varying temperature (23 and 27 °C) and percentage

Table 2
Precursor and product ions in the developed LC/MS/MS methods.

Compound	*m/z*	Quantifier	Qualifier
Gentamicin sulphate – active compounds			
Gentamicin C1	478.0	322.0	157.0
Gentamicin C1a	450.0	322.0	160.0
Gentamicin C2	464.0	322.0	160.0
Gentamicin C2a	464.0	322.0	160.0
Gentamicin C2b	464.0	322.0	160.0
Gentamicin sulphate – impurities			
Sisomycin	448.4	254.1	270.1
Garamine	322.3	160.0	112.0
Gentamicin B1	497.4	338.0	162.8
Impurity D	482.4	307.0	161.0
2-Deoxystreptamine[b]	163.0	Not detected	Not detected
Gentamin C1	319.3	157.0	138.9
Gentamicin B	483.4	163.0	205.0
JI-20A[a]	482.4	161.0	307.0
Gentamicin A	469.4	163.0	323.9
Lincomycin hydrochloride – active compounds			
Lincomycin A	407.3	126.3	359.3[c]
Lincomycin hydrochloride – impurities			
Lincomycin B	393.2	112.3	70.1[c]
Unknown	405.3	124.2	[d]
Spectinomycin dihydrochloride – active compounds			
Spectinomycin	333.3	140.0	98.2
Spectinomycin dihydrochloride – impurities			
Impurity A	207.2	73.0	43.5
Impurity D	351.3	207.0	98.0
Impurity E	319.2	73.4	43.0
Impurity F	333.2	139.9	97.7
Impurity 4R	335.2	116.0	98.0

[a] Designation taken from reference [20].
[b] 2-Deoxystreptamine was not detected in our samples.
[c] Given values correspond to the only potential qualifier ions (no others were detected), but due to their low relative intensities these should be considered as unreliable.
[d] The MS/MS contained only one production.

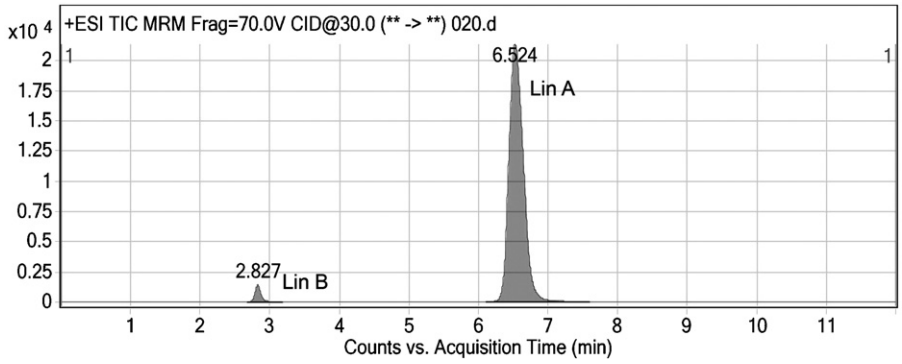

Fig. 5. Chromatogram (MRM) showing the separation of lincomycin and its impurity (lincomycin B) present in lincomycin hydrochloride.

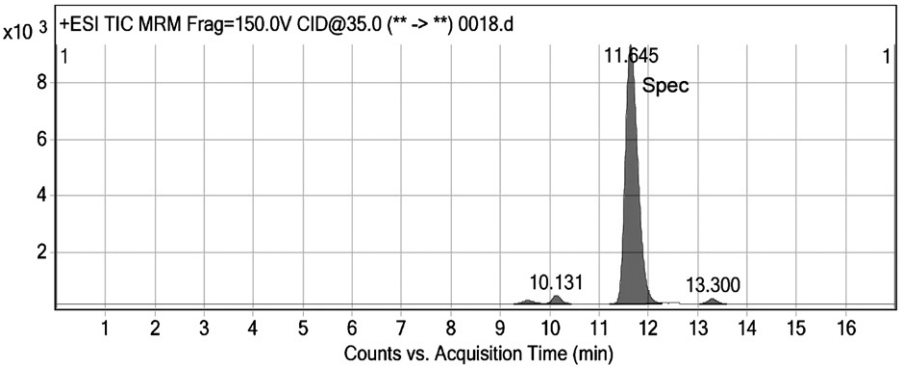

Fig. 6. Chromatogram (MRM) showing the separation of spectinomycin and its impurities present in spectinomycin dihydrochloride.

Table 3
Calibration curves of the developed methods.

Compound	Concentration range (μg/ml)	Equation	R^2
Gentamicin C1	16.39–163.9	$y = 441{,}361x - 21{,}933$	0.9977
Gentamicin C1a	21.74–217.4	$y = 139{,}074x - 3968$	0.9958
Gentamicin C2	7.62–76.2	$y = 416{,}649x - 10{,}724$	0.9958
Gentamicin C2a	4.20–42.0	$y = 406{,}126x - 13{,}688$	0.9919
Gentamicin C2b	0.49–4.9	$y = 220{,}363x - 464$	0.9875
Lincomycin A	10.00–100.0	$y = 48{,}897x + 2092$	0.9999
Spectinomycin	10.00–100.0	$y = 118198x - 5766$	0.9974

of TFA in the mobile phase (0.04 and 0.06%). In both methods, the parameters of the ion source were varied and tested under changed conditions (gas temperature 320 °C, vaporizer temperature 220 °C and nebulizer gas at 50 and 55 psi). All of these parameter variations showed no significant influence on the separation of the investigated compounds except that the pH value of mobile phase in the case of gentamicin was demonstrated to be a critical parameter (change of pH for 0.1 gave acceptable separation but changes in peak shape were noticed).

3.2.5. Sensitivity

Sensitivity of the methods was expressed through LOD and LOQ data. LOD was calculated from the peak information (signal to noise ratio of 3) and peaks with signal to noise ratio of 10 were used for LOQ. LOD for gentamicin C2b, the component present in the lowest concentration in gentamicin, was 9.85 ng/ml and LOQ was 32.85 ng/ml. For lincomycin and spectinomycin, LOD values were 4.21 and 12.36 ng/ml and LOQ were 14.03 and 41.2 ng/ml, respectively. These values are expected to be lower for MS/MS detection but as the mobile phases contained TFA and TEA, well known suppressors of ionization, this decrease of method sensitivity is easily interpreted in this way. As the proposed methods are intended to be used for routine analysis of pharmaceutical formulations, sensitivity of the methods is not a critical requirement but the individual determination of all components present in the investigated formulations, and this was achieved by chromatographic separation by applying the mentioned mobile phases with TFA and TEA.

The developed assays were applied in the determination of the active compounds in pharmaceutical dosage forms containing gentamicin sulphate, lincomycin hydrochloride or/and spectinomycin dihydrochloride in injections or in powder form. The obtained results are presented in Table 5. The influence of the matrix was more pronounced in the powder forms as expected due to the presence of added sugars in the dosage forms which further reduced the ionization efficiency.

3.3. Conclusions

This paper describes the development and validation of fast and reliable LC/MS/MS methods for the determination of gentamicin sulphate, lincomycin hydrochloride and spectinomycin dihydrochloride in the presence of their impurities. The separations in the case of gentamicin were achieved by employing a gradient mobile phase of simple composition and by this the quantification of individual components C1, C1a, C2, C2a and C2b was enabled.

Table 4
Precision and accuracy of the developed methods.

Compound	Precision			Accuracy		
	Concentration (μg/mL)	RSD [%]	Taken (μg/ml)	Found (μg/ml)	RSD [%]	Recovery
Gentamicin C1	5.77	1.95	18.47	17.89 ± 0.28	1.59	96.86%
	23.09	0.70	23.09	22.53 ± 0.31	1.35	97.59%
	115.45	0.91	27.71	28.32 ± 0.24	0.85	102.21%
Gentamicin C1a	8.81	1.97	28.18	27.38 ± 0.30	1.11	97.17%
	35.23	1.14	35.23	33.47 ± 0.41	1.16	95.02%
	176.15	1.11	42.28	41.37 ± 0.65	1.58	97.86%
Gentamicin C2	5.46	1.18	17.46	16.39 ± 0.23	1.42	93.90%
	21.83	0.74	21.83	20.73 ± 0.27	1.31	94.97%
	109.15	1.56	26.20	26.94 ± 0.37	1.39	102.84%
Gentamicin C2a	4.43	1.79	14.18	13.31 ± 0.23	1.77	93.91%
	17.72	1.17	17.72	16.67 ± 0.22	1.32	94.06%
	88.6	0.81	21.26	20.53 ± 0.14	0.71	96.57%
Gentamicin C2b	0.53	2.12	1.70	1.70 ± 0.05	3.21	99.83%
	2.13	1.22	2.13	2.08 ± 0.06	2.92	97.63%
	10.65	1.87	2.56	2.56 ± 0.08	3.19	100.01%
Lincomycin A	10.0	0.32	40.0	40.04 ± 0.17	0.42	100.12%
	50.0	0.40	50.0	50.05 ± 0.66	1.33	100.11%
	100.0	0.70	60.0	60.44 ± 0.45	0.75	100.73%
Spectinomycin	10.0	1.00	40.0	40.04 ± 0.38	0.38	100.09%
	50.0	0.71	50.0	51.20 ± 0.60	1.11	99.60%
	100.0	1.18	60.0	58.21 ± 0.46	0.79	97.12%

Table 5
The results obtained for commercially available samples using the developed methods.

Pharmaceutical	Found (mg)			RSD [%, $n = 6$]			Percentage of label claim		
	Gentamicin	Lincomycin	Spectinomycin	Gentamicin	Lincomycin	Spectinomycin	Gentamicin	Lincomycin	Spectinomycin
Neogent® injection	81.88	/	/	1.03	/	/	102.36	/	/
Neolincogent® powder	9.88	357.23	/	1.13	1.09	/	98.78	99.23	/
Neoli-spec® injections	/	50.90	100.16	/	0.45	1.06	/	101.8	100.16
Neoli-spec P-44® powder	/	21.8	21.7	/	0.69	0.59	/	99.09	99.86

/, not applicable.

MS/MS detection provided high selectivity of the investigated compounds and reduced the influence of the matrix and other present compounds in the samples. For all of the investigated compounds data about their MS/MS transitions are provided. Methods were validated according to ICH requirements and proved to be reliable in terms of specificity, linearity, precision, accuracy and robustness. We have shown the methods to be reliable and selective for the investigated compounds, with shorter analysis time compared to most of the available methods. The methods were proven to be appropriate for the determination of both the active compounds of the selected aminoglycoside antibiotics and their related impurities in complex newly developed pharmaceutical dosage forms for veterinary medicine. The dosage forms were shown to be of good quality, stability and within the requested specifications according to all of the pharmacopoeial requirements.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jpba.2011.07.031.

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