



# Rapid determination of multiple aminoglycoside antibiotics in veterinary formulations by ion-pair chromatography coupled with evaporative light scattering detection

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## ABSTRACT

A fast and simple method for simultaneous determination of eight aminoglycoside antibiotics using ion-pairing liquid chromatography (IPLC) coupled with evaporative light scattering detection (ELSD) has been developed and validated. Separation of amikacin, apramycin, gentamicin (as a sum of gentamicin C1, C2 and C1A), kanamycin A, neomycin B, paromomycin, streptomycin and tobramycin was achieved using C18 column with H<sub>2</sub>O and MeOH/acetone, both with addition of nonafluoropentanoic acid. Limits of detection ranged between 1.2 µg/mL and 2.8 µg/mL. The total analysis time was 9.5 min. The developed method showed the suitability for the routine quality control of veterinary drugs (drops, powders and paraffin-based suspensions).

## 1. Introduction

Aminoglycosides (AGs) are broad spectrum antibiotics which are used in cases of infections caused by Gram-negative and rarely Gram-positive bacteria [1]. AGs are successfully used in human and veterinary treatment. In the case of veterinary treatment, pharmaceuticals containing AGs are used to treat domestic as well as farm animals. The examples of therapeutic applications include: treatment of the infections caused by *Escherichia coli* and *Salmonella* bacteria, respiratory tract infections (e.g. caused by *Actinobacillus Pleuropneumoniae*, *Pasteurella multocida*), eye infections, ear infections, etc. [1,2]. Additionally they can be used in animal sperm diluters and as conservative agents for vaccines. Aminoglycosides are also used to treat reptiles, birds, bees, and other small species. AGs can be used in different formulation types, such as paraffin-based suspensions, injections, drops, tablets or powders (for feed supplementation). Exemplary uses in veterinary treatment are shown in Fig. S1.

According to European Medicines Agency [3], the total (registered) sale of AGs equals to 3.5% among all veterinary antibiotics.

Despite being valuable therapeutic agents, AGs can cause toxic effects as a consequence of inappropriate treatment and dosage. The above applies not only to animals being treated with AGs but also to the general population due to the fact that most AGs are excreted in the unchanged form and released to the environment (waters, soils) [4,5]. The

main toxic effects include ototoxicity and nephrotoxicity, as well as digestive and nervous system damage [1,3]. However, due to low cost of treatment and great antimicrobial properties, AGs are still the most often used medicines, especially in large-scale farms.

Another problem is the availability of low quality or inappropriately stored veterinary products. Quality control of such drugs may be more challenging than quality control of pharmaceuticals meant for human treatment. Very often veterinary formulations, in comparison to human medicines, are characterized by different compositions (e.g. different kinds of excipients) as well as the form of the drug (like granulates, suspensions etc.) which can cause problems during analysis. In order to provide the safe treatment for animals it is essential to develop robust, reliable, cheap and convenient procedures for the determination of AGs in veterinary formulations.

Considering the chemical structure, AGs are characterized by presence of aminosugar molecules (two or more), e.g. D-glucosamine, D-kanosamine, which are connected with cyclitol by glycosidic bond. With respect to their chemical structures, AGs can be divided into 3 main groups with different cyclitol structure, such as (i) streptidine (streptomycin), (ii) 4,5-di-substituted deoxystreptamine (neomycin, paromomycin) and (iii) 4,6-di-substituted deoxystreptamine (kanamycin, amikacin, tobramycin, gentamicin). Beside main groups, some AGs are characterised by specific, atypical structure pattern, like apramycin, where molecule consists of bis-glycosyl structure (Fig. 1). Additionally,

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AGs are polycationic, weak bases, without chromophores. They are soluble in water and semi- or insoluble in organic solvents. Physico-chemical properties of AGs makes the development of reliable quantitative and qualitative analytical protocols a non-trivial task [6]. The most popular analytical technique for AGs determination is liquid chromatography (LC) [7–9]. For years, the main approach was based on derivatization of AGs and determination of reaction products using reversed phase liquid chromatography (RPLC) with commonly available UV or fluorescence detectors [7,10,11]. However, due to limitations and problems, associated with the sensitivity and selectivity, derivatization-based methods are mainly used to analyse samples characterized by relatively simple matrices e.g. some pharmaceutical products [12]. Problems of chemical nature are not the only drawbacks associated with derivatization. It is a time- and work-consuming process which requires a number of sometimes costly and hazardous chemicals and solvents.

Therefore, new procedures, relying on determination of AGs in their native forms are being developed. The main advantages of direct AGs analysis are the speed, simplicity and cost-effectiveness. Hydrophilic interaction liquid chromatography (HILIC) [13–16] or ion-pairing liquid chromatography (IPLC) [17–19] are the most commonly taken approaches.

HILIC seems to be a very attractive solution for separation and determination of AGs. It is well suited for separation of very polar compounds and does not require any specialized chemicals. In practice however, HILIC suffers from several drawbacks when it comes to separation of AGs. Rather modest effects in terms of separation quality have been reported in recent literature. This in turn necessitates the use of highly selective detection such as mass spectrometry (MS) if a mixture of AGs needs to be analysed. Other problems reported in case of HILIC include badly shaped peaks, long column equilibration times and interferences caused by highly concentrated buffers necessary for obtaining good peaks shapes and satisfactory separation. All of this results in impaired quantitation and poor repeatability of results which makes HILIC impractical for routine control of pharmaceutical formulations [14,15,20,21].

Ion-pairing liquid chromatography is commonly used to separate polar compounds capable of forming, with certain agents, electrostatically-bound ion-pairs. The choice of ion-pairing agent

depends on the nature of analytes and the final determination technique. Since AGs show cationic character an anionic ion-pairing agent has to be used to form ion-pairs. Two popular choices are alkylsulfonic or perfluorinated organic acids. Perfluorinated ion-pairing agents are volatile, therefore they can be used in scenarios where detectors such as MS, ELSD or charged aerosol detector (CAD) will be used [22–24]. IPLC mode of separation is mostly free of problems associated with HILIC. Baseline separation of multiple AGs is possible, the peak shapes are symmetrical, column equilibration times are short and the precision of results is excellent [6,25]. The biggest drawbacks of using IPLC include the shortened lifetime of chromatographic equipment and columns (low pH of mobile phases) as well as possible detection problems due to ionization suppression when mass spectrometry is employed [25].

Most of the published IPLC methods, similarly to HILIC-based ones, rely on MS/MS detection [17,26]. The reason is that, due to selective character of detection, baseline separation of analytes is not required. This makes the multi-analyte method development easier, at the cost of higher probability of matrix effects caused by coeluting compounds, however. Nevertheless, there are also reports on the use of IPLC coupled with less selective detectors, like ELSD. Usually only up to 3 AGs can be simultaneously determined in a single run, however [22,27–30]. To the best of our knowledge, only one published IPLC-ELSD procedure allows for simultaneous determination of 10 AGs (amikacin, apramycin, hygromycin B, neomycin B, ribostamycin, spectinomycin, tobramycin, streptomycin, kanamycin B, gentamicin) [31]. However, the total separation run-time, needed for complete analyte separation was 35 min. Despite some drawbacks of ELSD detection (lower sensitivity, need for baseline separation of analytes) it has several advantages over MS/MS detection which include lower cost of instrumentation, its ruggedness, high availability and lower requirements in terms of personnel qualifications.

Table 1 shows a comparison of IPLC-ELSD methodologies described in scientific literature. The reported LOD values are comparable or slightly lower than those achieved with the method described in this paper. Nevertheless, almost all reported analytical protocols were developed for the determination of a single AG. This makes easier to obtain lower LOD value by fine tuning of mobile phase composition. Such approach isn't possible in the case of multi-analyte methods since

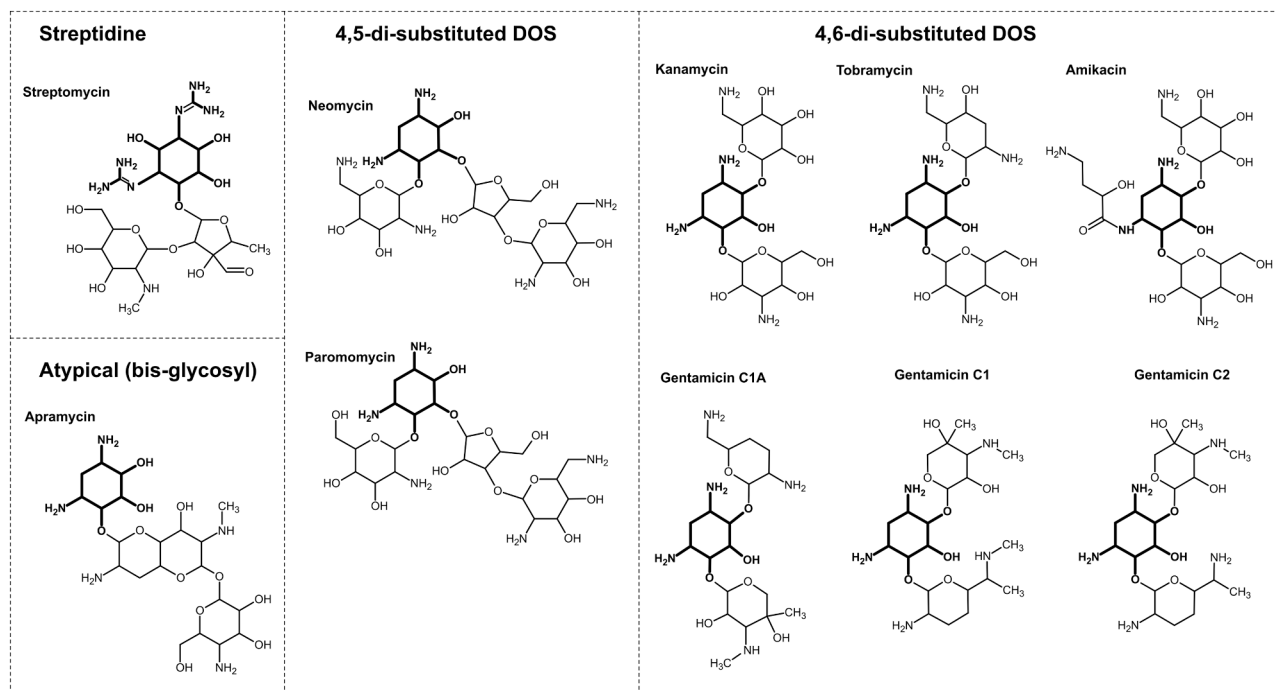


Fig. 1. Structures of investigated aminoglycosides (DOS -DeOxyStreptamine).

**Table 1**  
Comparison of IPLC-ELSD methods used for determination of aminoglycosides.

Aminoglycoside	Matrix	LC column	Mobile phase	Detection	LOD/LOQ	Separation run-time [min]	Ref.
Amikacin, apramycin, gentamicin, kanamycin, neomycin, paromomycin, tobramycin, streptomycin Neomycin B	Veterinary formulations, standard solutions	ZORBAX RX C18 (150 × 4.6 mm, 3.5 μm)	A: H <sub>2</sub> O + 0.1% NFPAB: MeOH/acetone (85:15 v/v) + 0.1% NFPA	ELSD	LOD: 1.2 – 2.8 μg/mL mLLOQ: 2.9 – 4.1 μg/mL	9.5	This work
	Medicated animal feed, aerosol, cream	Spherisorb ODS-2 C18 (250 × 4.6 mm), 5 μm	H <sub>2</sub> O/acetone (50:50 v/v) + 0.15% HFBA	ELSD	LOD: 0.6 μg/mL	10	[22]
Gentamycin C1, C2, C2A, C1	Injections, eye drops, cream	Spherisorb ODS-2 C18 (250 × 4.6 mm), 5 μm	H <sub>2</sub> O + TCA (35.4 μg/mL) + TFA (0.89 μg/mL)/MeOH/ACN (99:0.5:0.5 v/v/v)	ELSD	LOD: 1.2 – 2.4 μg/mL	10	[28]
Tobramycin	Pharmaceuticals	Zorbax SB-C18 (250 × 4.6 mm), 5 μm	A: H <sub>2</sub> O/ACN/HFBA (96:4:1 v/v/v): H <sub>2</sub> O/ACN/HFBA (60:40:1 v/v/v)	ELSD	LOD: 0.75 – 2 μg/mL	23	[29]
Hygromycin, amikacin, kanamycin, ribostamycin, apramycin, tobramycin, gentamycin, neomycin	Animal feeds	Hypersil BDS C18 (250 × 4.6 mm), 5 μm	A: ACN/H <sub>2</sub> O (5:95 v/v) + 20 mM HFBA: ACN/H <sub>2</sub> O (50:50 v/v) + 20 mM HFBA	ELSD	LOD: 0.2 – 0.7 μg/g <sup>a</sup> preconcentration using SPE	35	[31]

proper chromatographic separation has higher priority.

This paper presents a novel, rapid and reliable IPLC-ELSD method for simultaneous determination of 8 AGs in 9.5 min. Impact of mobile phase composition, stationary phase type (C18, C8, graphite column), as well as separation temperature (20 °C – 70 °C) and ion-pairing reagent type (trifluoroacetic acid, pentafluoropropionic acid, heptafluorobutyric acid, perfluoropentanoic acid) was studied. The developed method was validated (according to ICH guidelines) and successfully applied for determination of AGs in commercial veterinary formulations.

## 2. Experimental

### 2.1. Reagents

Standards: amikacin sulphate (Interquim, CAS 39831–55-5, European Pharmacopoeia (EP) Reference Standard), apramycin sulphate (Alfa Aesar, CAS 70560–51-9, 96% purity), gentamicin sulphate as a sum of gentamicin C1, C2 and C1A (Merck, CAS 1405–41-0, ≥ 99% purity), kanamycin sulphate (Sigma-Aldrich, CAS 70560–51-9, ≥ 99.5% purity), neomycin sulphate (VWR, CAS 1405-10-3, Ultra-Pure Grade), paromomycin sulphate (Alfa Aesar, CAS 1263–89-4, ≥ 97% purity), streptomycin sulphate (Sigma-Aldrich, CAS 3810-74-0, ≥ 99% purity), tobramycin sulphate (Alfa Aesar, CAS 79645–27-5, ≥ 99% purity).

Solvents: ultrapure water was prepared using HLP5 system (Hydro-lab), acetonitrile, methanol, n-hexane and acetone (LC grade, Sigma Aldrich), tetrahydrofuran, chloroform, hydrochloric acid and ammonia (99% purity, POCH).

Ion pairing reagents: LC grade trifluoroacetic acid (TFA), pentafluoropropionic acid (PFPA), heptafluorobutyric acid (HFBA) from Sigma-Aldrich and LC grade nonafluoropentanoic acid (NFPA) from Apollo Scientific.

Veterinary drugs:

- udder suspension 1 (paraffin based): streptomycin sulfate (100 mg/5 g), neomycin sulfate (100 mg/5 g), procaine penicillin (100000 IU/5 g), prednisolone (10 mg/5 g),
- udder suspension 2 (paraffin based): kanamycin sulfate (100000 IU/10 g = 125.63 mg/10 g), cefalexin monohydrate (200 mg/10 g),
- powder for making solutions in water or milk: paromomycin sulfate (100 g/kg).
- eye/ear drops: neomycin sulfate (2500 IU/mL = 3.31 mg/mL), gramicidin (25 IU/mL), fludrocortisone acetate (1 mg/mL).

Concentrations given in IU were converted using an on-line calculator. All concentrations are expressed as concentrations of respective sulphate salts.

### 2.2. HPLC conditions

Determination of aminoglycosides was performed using an Agilent 1200 LC system consisting of degasser, binary pump, autosampler, thermostatted column compartment, Hewlett Packard 35900E analog-to-digital converter and Shimadzu ELSD-LT II detector.

During method optimisation, the various chromatographic columns were investigated: (i) Alltima C18, Alltech (250 × 4.6 mm, 5 μm), (ii) Ascentis Express C18, Supelco (150 × 4.6 mm, 2.7 μm), (iii) Hypercarb graphitic carbon LC column, Thermo Fisher (100 × 3.0 mm, 3 μm), (iv) Purospher C18e LiChroCART, Merck (250 × 4.6 mm, 5 μm), (v) Zorbax Eclipse XDB-C8, Agilent (150 × 4.6 mm, 5 μm), (vi) Zorbax RX-C18, Agilent (150 × 4.6 mm, 3.5 μm).

The final chromatographic conditions were as follows: separation of aminoglycosides was carried out using ZORBAX RX C18 (150 × 4.6 mm, 3.5 μm) chromatographic column working at 60 °C. Mobile phase component A consisted of 0.1% v/v aqueous NFPA solution and mobile phase component B consisted of 0.1% v/v NFPA solution in MeOH/acetone (85:15 v/v) mixture. Following elution program was used: 0 min – 55% B, 1 min – 60% B, 6 min – 70% B, 6.5 min – 95% B, 9.5 min – 95% B. Flow rate of 0.75 mL/min was applied. Injection volume was 20 μL. Working temperature of the ELSD drift-tube was 60 °C, the gain 9 and the nebulizing gas (N<sub>2</sub>) pressure 335 kPa.

### 2.3. Standard solutions

Individual stock solutions (1 – 1.6 mg/mL) of each AG were prepared in distilled water and kept in polypropylene screw capped tubes stored at –20 °C. Mixed standard solution (stock solution diluted 10-times) was prepared by adding an aliquot of each individual stock solution to a volumetric flask and making up to the mark with distilled water. Calibration solutions were prepared by diluting the mixed standard solution with distilled water.

### 2.4. Extraction of samples

#### 2.4.1. Preparation of udder suspension samples

50 mg of sample was sonicated with 2 mL of chloroform for 10 min (to dissolve the creamy suspension). Subsequently, 1 mL of 0.1 M HCl

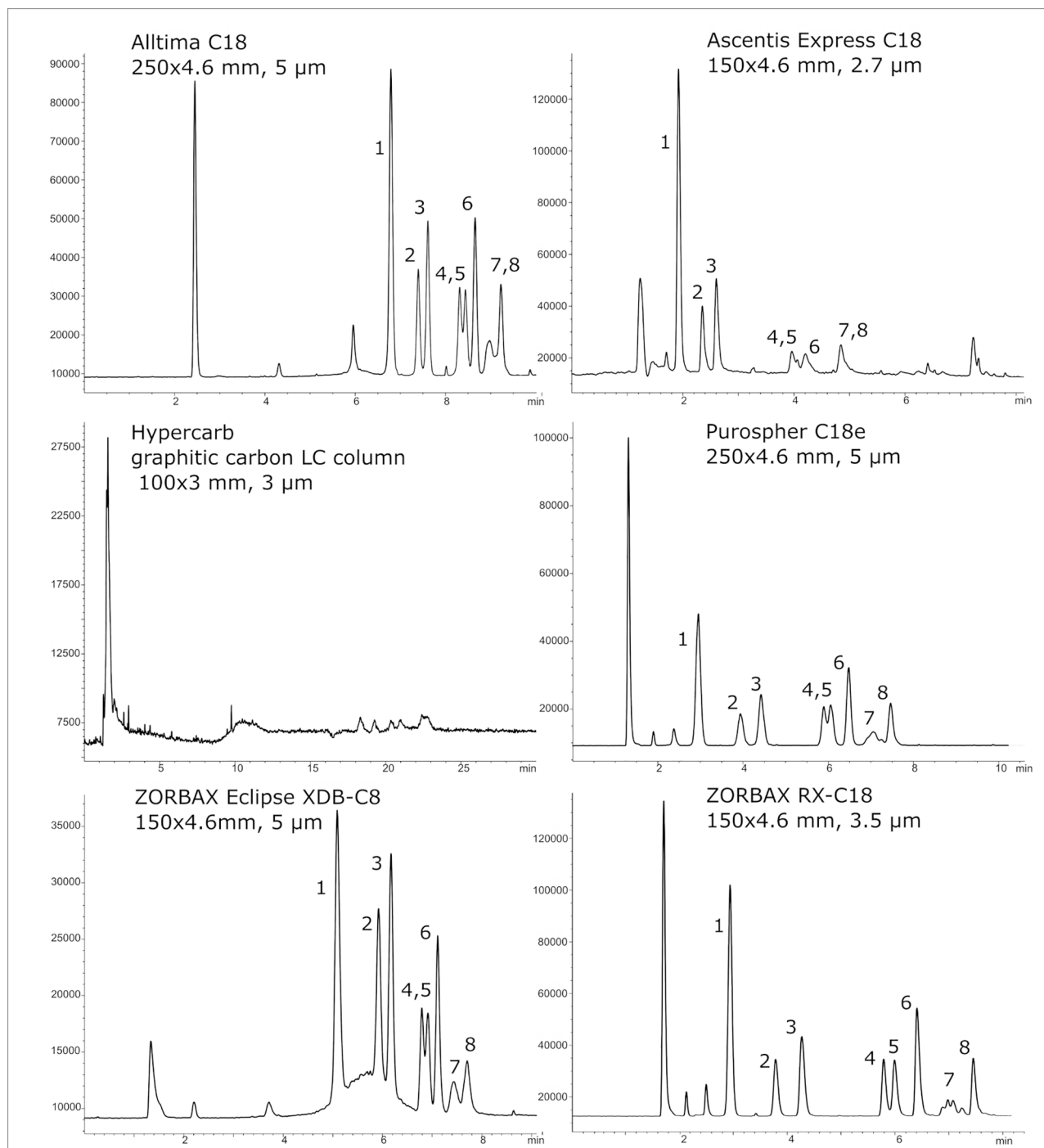
was added and the mixture was vortexed for 5 s. The role of HCl was to enhance protonation of amino moieties within the structures of AGs and promotion of their transfer into aqueous phase. After that, the mixture was placed in a laboratory dryer and heated to 55 °C for 30 min. After cooling down to room temperature, the mixture was centrifuged (2200 rpm, 0.8 rcf, 1 min). Forty (upper suspension 1) or fifty microliters (upper suspension 2) of the upper aqueous layer was collected and diluted to 1 mL with distilled water.

#### 2.4.2. Preparation of powder sample

85 mg of powder was dissolved in 200 mL of distilled water in a volumetric flask.

#### 2.4.3. Preparation of eye/ear drop samples

The 0.1 mL of the sample was introduced into a 10 mL volumetric flask and made up to the mark with distilled water.



**Fig. 2.** Examples of chromatograms obtained using different types of LC columns, note that non-optimal composition of the mobile phase was used: MeOH with water both acidified with 0.1% HFBA. In the case of the Hypercarb column no peaks were registered even after 30 min of analysis. Peak identification (determined by analysis of standards of individual AGs): 1 – streptomycin, 2 – amikacin, 3 – kanamycin, 4 – paromomycin, 5 – apramycin, 6 – tobramycin, 7 – gentamicin (C1, C2, C1A), 8 – neomycin.

### 3. Results

The aim of the study was to develop an IPLC procedure for simultaneous determination of polar AGs, compatible with ELSD detection. During optimisation of chromatographic conditions the most problematic aspects were associated with co-elution of analytes (especially: paromomycin-apramycin, kanamycin-amikacin-tobramycin and gentamicin-neomycin) as well as with peak tailing (mainly: gentamicin and neomycin).

The starting conditions for method development were selected based on literature survey [28,29].

#### 3.1. Type of LC columns

To ensure the best analyte separation and reliable results, various column types were investigated. Despite testing columns of different lengths (100 mm, 150 mm, 250 mm), packed with particles of various sizes (5  $\mu\text{m}$ , 3.5  $\mu\text{m}$ , 3  $\mu\text{m}$ , 2.7  $\mu\text{m}$ ) modified with three different functional groups (C18, C8, graphitized carbon), we were unable to achieve baseline separation of all components (Fig. 2).

Out of all tested columns, the best results in terms of peak shape and separation of analytes were obtained using ZORBAX RX C18 (150  $\times$  4.6 mm, 3.5  $\mu\text{m}$ ) column, hence further investigations were carried out using this column.

#### 3.2. Impact of IPLC mobile phase composition

The choice of an appropriate balance between organic and aqueous solvents ratio in the mobile phase and the establishment of an elution gradient program was also studied. It was noted that the increase in aqueous component content resulted in signal decrease and likewise increase in peak widths, in particular for streptomycin. On the other hand, significant deterioration in selectivity was observed when too much organic component was used (Fig. S2).

Examination of ACN and MeOH as the organic mobile phase components was also performed. It was found that ACN is unsuitable for AGs separation. Its high elution strength leads to almost complete co-elution of AGs, even when only 30% of ACN is used (Fig. S3). MeOH elution strength was significantly lower. Under the same conditions but using MeOH instead of ACN not a single analyte eluted after 45 min. Investigating various elution gradients with ACN as an organic modifier also didn't result in improved separation of analytes. Significantly better results were observed when MeOH was used. Nevertheless, even with MeOH the separation between paromomycin and apramycin wasn't complete. Also the shape of the gentamicin peak was far from perfection, in addition it eluted close to neomycin which may cause further problems during quantitation.

To improve separation selectivity, the impact of additives such as acetone and tetrahydrofuran (THF) was examined (Fig. S4). Following compositions of eluent B were investigated: MeOH/acetone (90:10 v/v) + 0.1% HFBA, MeOH/acetone (85:15 v/v) + 0.1% HFBA, MeOH/THF (95:5 v/v) + 0.1% HFBA and MeOH/THF (90:10 v/v) + 0.1% HFBA.

The composition of eluent A was the same in all cases ( $\text{H}_2\text{O}$  + 0.1% HFBA). The major effect of THF addition to the mobile phase was just a decrease in AGs retention times. Consequently, no improvement in terms of selectivity was observed. The other modifier – acetone was chosen based on our personal experiences and scientific literature survey [22,32]. It was found that acetone addition was beneficial in two aspects. The separation between neomycin and gentamicin was improved, also the increase in signal intensity was noted. Considering the concentration of acetone, the use of 10% provided slightly better signal intensity than 15%. However, the separation between gentamicin and neomycin as well as neomycin peak shape were better when 15% of acetone was used. Additionally, use of 15% of acetone resulted in less noisy signal and better baseline shape. Therefore, for further experiments an organic mobile phase composed of MeOH/acetone (85:15 v/v)

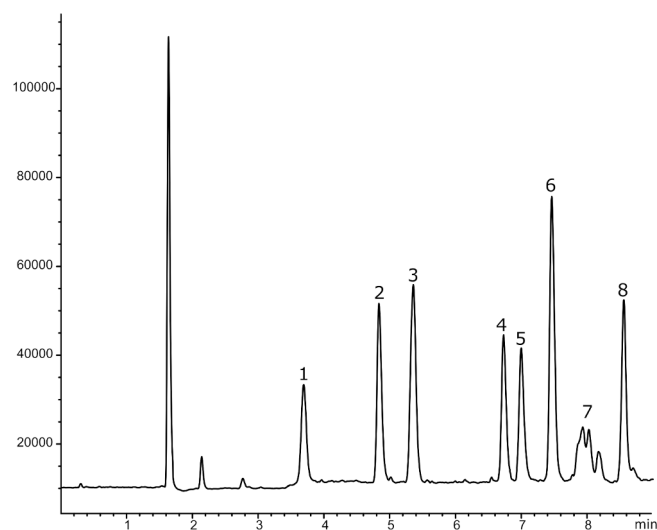
+ 0.1% HFBA mixture was chosen. Despite some improvement in overall separation of analytes, followed by experimenting with various gradient elution programs the separation between paromomycin and apramycin was still not satisfactory. Therefore, it was investigated whether the type and the concentration of ion-pairing (IP) reagent could provide any improvement in this regard.

Different volatile fluorinated organic acids at the concentrations of 0.1% v/v were examined (Fig. S5): trifluoroacetic acid (TFA), pentafluoropropionic acid (PFPA), heptafluorobutyric acid (HFBA), and nonafluoropentanoic acid (NFPA). The AGs ion pairs created with TFA or PFPA were characterized by too low hydrophobic character to provide the retention of the analytes using C-18 stationary phase. The use of HFBA, resulted in the increase of AGs retention and separation selectivity. However, as already mentioned, the complete separation of analytes was still not possible. The best results were obtained using NFPA. With this IP reagent both peak shapes and separation efficiency were satisfactory. Therefore it was finally chosen as the IP reagent.

Concentration of the IP reagent in the range of 0.05% – 0.2% (v/v) was studied (Fig. S6). It was found that too low concentration (0.05% – 0.075%) leads to severe coelution of analytes. On the other hand too high concentration (0.2%) resulted in significant peak tailing and loss of separation efficiency. Finally the reagent IP concentration equal to 0.1% v/v was chosen (Fig. 2) as the optimal (pH of aqueous component of mobile phase was equal to 2.1).

#### 3.3. Impact of column temperature on ion-pairing AGs separation

Separations of AGs were carried out at: 20  $^{\circ}\text{C}$ , 30  $^{\circ}\text{C}$ , 40  $^{\circ}\text{C}$ , 50  $^{\circ}\text{C}$ , 60  $^{\circ}\text{C}$  and 70  $^{\circ}\text{C}$ . For a given temperature each separation experiment was repeated five times. The separations conducted at lower temperatures take longer time without noticeable improvement in quality. Under the final gradient conditions only the first two analytes were eluted in 10 min at 20  $^{\circ}\text{C}$ . Experiments with separations at higher temperatures resulted in shortening the analysis time. The difference in total analysis time between 60  $^{\circ}\text{C}$  and 70  $^{\circ}\text{C}$  was only 0.3 min, however. Therefore, taking into account that increase in separation temperature negatively affects lifetime of column and other components of chromatographic system 60  $^{\circ}\text{C}$  was chosen as fair compromise. At this temperature the analysis time was relatively short as well as the



**Fig. 3.** Chromatogram of AGs mixture after method optimization. Chromatographic conditions: ZORBAX RX C18 chromatographic column working at 60  $^{\circ}\text{C}$ , injection volume: 20  $\mu\text{L}$ , mobile phase A consisted of 0.1% NFPA, mobile phase B consisted of MeOH/acetone (85:15 v/v) + 0.1% NFPA, elution program: 0 min – 55% B, 1 min – 60% B, 6 min – 70% B, 6.5 min – 95% B, 9.5 min – 95% B, flow rate: 0.75 mL/min. For peak identification see Fig. 2.

satisfactory analytes separation and peak shapes were obtained (Fig. 3).

After optimization of separation conditions peak resolution ( $R_s$ ) for two critical pairs (paromomycin – apramycin and gentamicin – neomycin) was improved by around 40%.

### 3.4. Extraction efficiency

Due to the paraffin content, veterinary drugs in the form of udder suspensions required the pre-treatment step before analysis. Preliminary study showed that the sonication of samples in the mixture of MeOH/acetone/H<sub>2</sub>O (mixed in the same ratios as in the initial mobile phase composition) was insufficient for paraffin-based suspensions. Determined concentrations were relatively low (extraction efficiency defined as a ratio of found and expected concentration around 50%). Due to this fact, the simple liquid–liquid extraction systems employing binary polar and non-polar solvents mixtures were investigated. Extraction efficiency (EE) was studied using commercially available veterinary formulation in the form of paraffin suspension (udder suspension 1). Manufacturer's declaration concerning the content of AGs was treated as a reference. External calibration using pure standards dissolved in water was employed for quantitation. During optimisation of extraction conditions, firstly the impact of non-polar solvent type was checked. The non-polar solvent was used to solubilize the matrix of analysed suspensions and as a receiver for hydrophobic components of the formulations. It was found that the use of n-hexane resulted in very low EE values (below 30% of the expected value). Much better results were obtained when chloroform was employed (EE > 96%). Next, the composition of acceptor solution was considered. The extractions were performed using: (i) distilled water, (ii) 0.01% of ammonia and (iii) 0.1 M HCl. Water and ammonia produced rather low EE values (neomycin EE < 70% and EE < 45% using H<sub>2</sub>O and ammonia, respectively). Also an unidentified peak eluting close to streptomycin was observed when water or ammonia were used as acceptor solutions (Fig. S7). These phenomena weren't observed when 0.1 M HCl was used as an acceptor solution. The EE values were close to 100% and lack of artifacts were observed in the chromatograms of extracts. Therefore 0.1 M HCl was selected as an aqueous component of the extraction system.

The extraction temperature (35 °C–65 °C) was also taken into account (Fig. S8). Slight improvement in EE was observed with the increase of temperature from 35 °C to 55 °C. Since there was no significant difference between 55 °C and 65 °C in terms of EE, 55 °C was selected as optimal due to slightly better repeatability of results. Extraction time (the time samples spent in the dryer) was also studied (Fig. S8). In the case of streptomycin no statistically significant differences in terms of EE or repeatability were observed for the extraction times in the range of 5 to 30 min. For neomycin, a modest increase of EE with the increase of extraction time was noticed therefore an extraction time of 30 min was selected as optimal.

Similar results were obtained for udder suspension 1 from other production batches (4 independent samples analysed in triplicate). Extraction efficiencies for neomycin and streptomycin were in the same range, approx. 103%. The same extraction procedure was applied for the other veterinary formulation in the form of paraffin-based suspension (udder suspension 2) containing kanamycin. The extraction efficiency for this compound was 96.8 ± 2%.

### 3.5. Method validation

a) calibration range, linearity, limits of detection and limits of quantitation, precision

Due to non-linear nature of ELSD response the LOD and LOQ values, were determined experimentally (S/N = 3 for LOD and S/N = 10 for LOQ) injecting consecutive dilutions of lowest concentration calibration solution. Obtained LOD and LOQ values were in the range from 1.2 µg/mL to 2.8 µg/mL and from 2.9 µg/mL to 4.1 µg/mL respectively.

Calibration parameters are summarized in Table 2.

Visual examination of calibration curves revealed their linear character in the analysed concentration ranges with  $R^2$  values higher than 0.998 for all investigated aminoglycosides.

Intra- and interday (every three days) precision, expressed as coefficient of variation, of the method was estimated by repeated injections of standard solution of AGs (n = 5, 20 µg/mL). Intraday precision, was in the range of 0.6% – 1.5% and interday precision was in the range of 0.8% – 3.8%.

b) trueness

To assess the presence of possible matrix effects influencing the trueness of the method aqueous extracts of investigated formulations were divided into two identical aliquots. One portion was spiked with a known amount of the respective AG (corresponding to 50%, 75% and 100% of the expected/declared amount), the other with pure water. Both portions were analysed by HPLC-ELSD. All formulations were analysed using three independent samples per spiking level. The trueness was calculated as the ratio of difference between amount found in fortified and in non-fortified samples and the amount of analyte added. The results of this experiment are summarized in Table 3. No statistically important influence of the matrix at three tested spiking levels was observed.

c) recovery

It seemed necessary to verify recoveries of analytes from the paraffin-based formulations which in comparison with other samples were not completely soluble in water. The recoveries of AGs from such samples were tested by addition of spiking solutions before the extraction step. After weighting the samples, 0.2 mL of spiking solution containing analytes was added. Spiking solutions were prepared at three concentration levels corresponding to 50%, 75% and 100% of the expected/declared amount in deionized water. After addition of chloroform and sonication, 0.8 mL of 0.1 M HCl was added. In parallel, non-fortified samples were prepared by addition of deionised water instead of spiking solution. Subsequent sample preparation steps were identical to those used for genuine samples. As in the case of trueness testing the recoveries were calculated as the ratios of difference between amount found in fortified and non-fortified samples and the amount of analyte added. The obtained results are presented in Table 4. Formulations under the testing contained three AGs in total. For all of them recoveries were close to 100% at all spiking levels. The lowest mean recovery value (96.6%) was observed for neomycin. This is probably due to the fact that this compound exhibits the most hydrophobic character out of all tested AGs and its release from the non-polar matrix (paraffin) is slightly less efficient.

d) repeatability and real-world samples analysis

Repeatability of the developed method was verified analysing real-world samples of veterinary formulations (Table 5). In all cases the precision was consistent and better than 4%. In the case of eye/ear drops the result of the analysis was slightly higher than expected. Examples of chromatograms obtained during analysis of real-world samples are shown in Fig. S9.

## 4. Conclusions

The developed IPLC-ELSD method provides a direct, simple and quick means for simultaneous determination of 8 AGs within 9.5 min. The improvement of AGs separation selectivity was achieved due to use of NFPA as an ion-pairing reagent. Acetone as a mobile phase additive improves peak shape and resolution. The obtained LOQ values varied from 2.9 µg/mL to 4.1 µg/mL, which makes the method suitable for

**Table 2**

Calibration parameters for the developed IPLC – ELSD method.

AG	Calibration curve range [µg/mL]	Calibration curve = ax + b*	S <sub>a</sub>	S <sub>b</sub>	R <sup>2</sup>	LOD [µg/mL]	LOQ [µg/mL]	CV (intraday, n = 5)** [%]	CV (interday, n = 5)** [%]
STR	2.6 – 128.0	y = 1.259x + 3.252	0.023	0.048	0.9986	1.3	3.2	1.2	1.2
AMI	2.6 – 133.5	y = 1.2173x + 3.4887	0.0033	0.0088	0.9985	1.3	3.3	0.6	2.5
KAN	2.6 – 130.0	y = 1.278x + 3.493	0.012	0.018	0.9995	1.3	3.3	1.5	2.6
PAR	2.3 – 115.9	y = 1.278x + 3.402	0.013	0.018	0.9991	1.2	2.9	0.7	3.4
APR	2.4 – 118.2	y = 1.256x + 3.398	0.044	0.087	0.9981	1.2	3.0	1.4	2.2
TOB	3.1 – 114.3	y = 1.3085x + 3.457	0.0085	0.017	0.9990	1.5	3.8	0.9	3.8
GEN (C1, CIA, C2)***	4.0 – 160.3	y = 1.297x + 3.223	0.035	0.053	0.9991	2.8	4.1	1.2	1.6
NEO	3.0 – 111.0	y = 1.435x + 3.079	0.032	0.061	0.9982	1.9	3.7	0.8	0.8

Analysis of each calibration solution was performed in triplicate (n = 3)

S<sub>a</sub> – standard deviation of slope of calibration curve, S<sub>b</sub> – standard deviation of intercept of calibration curve, R<sup>2</sup> – coefficient of determination, LOD – limits of detection, LOQ – limits of quantification\* y – log<sub>10</sub>(peak area); x – log<sub>10</sub>(concentration)

\*\* based on peak areas for 20 µg/mL AGs standard solution

\*\*\* gentamicin peak was quantified using total peak areas

**Table 3**

Estimation of matrix effects for veterinary formulations.

Sample	Analyte	Spiking level <sup>a</sup> [%]	Trueness ± SD <sup>b</sup> [%]
Udder suspension 1	streptomycin	50	100.0 ± 3.4
		75	100.2 ± 1.2
		100	100.9 ± 5.1
	neomycin	50	105.3 ± 3.1
		75	101.2 ± 1.2
		100	99.5 ± 3.0
Udder suspension 2	kanamycin	50	98.8 ± 1.6
		75	102.10 ± 0.70
		100	101.4 ± 2.8
Powder	paromomycin	50	103.1 ± 2.3
		75	103.8 ± 1.6
		100	104.1 ± 2.8
Eye/ear drops	neomycin	50	98.3 ± 2.2
		75	97.7 ± 2.9
		100	96.7 ± 1.1

<sup>a</sup> - expressed as % of the expected/declared value, b - n = 3**Table 4**

Recovery of the analytes from spiked samples in the form of paraffin-based suspensions.

Sample	Analyte	Spiking level <sup>a</sup> [%]	Recovery ± SD <sup>b</sup> , [%]
Udder suspension 1	streptomycin	50	101.8 ± 1.8
		75	98.3 ± 4.8
		100	99.9 ± 4.2
	neomycin	50	97.1 ± 1.9
		75	96.02 ± 0.51
		100	96.6 ± 1.8
Udder suspension 2	kanamycin	50	97.1 ± 2.7
		75	99.0 ± 1.6
		100	98.03 ± 0.92

<sup>a</sup> - expressed as % of the expected/declared value, b - n = 3

routine analysis of multi-AGs preparations. Although such formulations are relatively rare the proposed method is also well suited for analysis of simpler mixtures as evidenced by the results of the analysis of exemplary real-world formulations. Chromatographic conditions described here make a good starting point for the development of formulation-specific methods aiming at the determination of single or multiple aminoglycosides. The sample preparation protocol was found suitable for a few different matrices, indicating that it would likely be suitable for other products. Additionally, after inclusion of an efficient sample enrichment step, the developed method can be also an attractive alternative in other

**Table 5**

Aminoglycosides concentrations in tested veterinary formulations.

Veterinary formulation	AGs	Concentration found ± SD <sup>a</sup> [mg/g]	(C <sub>found</sub> /C <sub>declared</sub> ) ± SD <sup>a</sup> [%]
Udder suspension 1	streptomycin	20.07 ± 0.76	100.4 ± 3.8
	neomycin	20.53 ± 0.64	102.6 ± 3.1
Udder suspension 2	kanamycin	12.14 ± 0.26	96.6 ± 2.1
Powder	paromomycin	102.4 ± 3.9	102.4 ± 3.9
	neomycin	3.432 ± 0.058 <sup>a</sup>	103.7 ± 1.7

<sup>a</sup> - n = three independent samples analysed in triplicate; a - concentration expressed as mg/mL.

applications, including clinical trials or food analysis.

**CRedit authorship contribution statement**

**Marta Glinka:** Writing – original draft, Visualization, Investigation, Data curation, Methodology, Validation, Writing – review & editing.  
**Andrzej Wasik:** Conceptualization, Writing – review & editing, Supervision.

**Declaration of Competing Interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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**Appendix A. Supplementary data**

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