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Enhancement of evaporative light scattering detection in high-performance liquid chromatographic determination of neomycin based on highly volatile mobile phase, high-molecular-mass ion-pairing reagents and controlled peak shape

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Abstract

In the frame of the development of a novel HPLC–ELSD (evaporative light scattering detection) method for the determination of the aminoglycoside antibiotic neomycin sulfate, the influence of mobile phase composition and peak broadening on ELSD response was evaluated. ELSD response was enhanced by: (a) increase of mobile phase volatility (solvents examined: water, acetonitrile, methanol and acetone), (b) increase of molecular mass of ion-pairing species [acidic reagents tested: formic, acetic, trifluoroacetic, trichloroacetic and heptafluorobutyric acid (HFBA)], and (c) decrease of peak width and asymmetry obtained by controlling the concentration of the ion-pairing acidic reagent (HFBA). Utilizing a Waters ODS-2 C_{18} Spherisorb column, evaporation temperature of 45 °C and nitrogen pressure of 3.5 bar, the optimized mobile phase was water–acetone (50:50), containing 11.6 mM HFBA, in an isocratic mode at a rate of 1.0 ml/min. Neomycin was eluted at 4.9 min, with asymmetry factor 1.3. Logarithmic calibration curve was obtained from 2 to 50 μg/ml ($r > 0.9997$). Limit of detection (LOD) was 0.6 μg/ml and R.S.D. = 1.7% ($n = 3$, 3.3 μg/ml). In raw materials, the simultaneous determination of sulfate (LOD = 3 μg/ml, R.S.D. = 1.7%, $r > 0.9998$) and of minor impurities was feasible. The developed method was also applied for the determination of neomycin in pharmaceutical formulations (powder, aerosol and cream) without any interference from excipients (recovery from spiked samples ranged from 99 to 102%) and a %R.S.D. of <2.1 ($n = 3$). The HPLC–ELSD method was also found applicable in the determination of neomycin in animal feeds (LOQ = 0.2%) without any interference from the feed matrices.

Keywords: Evaporative light scattering; Neomycin; Sulfate; Aminoglycosides; Pharmaceuticals; Feeds

1. Introduction

Evaporative light scattering detection (ELSD) is increasingly being used in LC during the last decade, as a quasi-universal detector, with very successful applications, especially for non-absorbing analytes (sugars, lipids, amino acids, polymers, etc.) [1,2]. Its ability to perform quantitation of substances with lack of standard material, since it shows nearly equal response factors for molecules with about equal mass and similar formula, is well-established [3]. ELSD operation principle consists of three main successive processes: (a) nebulization of chromatographic eluent using nitrogen or air, (b) evaporation of mobile phase at relatively low temperature and (c) light scattering by the residual particles, which ideally consist of analytes molecules. Although the ELSD response factor varies with the scattering mechanism (Rayleigh, Mie and reflection–refraction), in most applications it is proven that in a wide range of analyte concentrations and polychromatic light beam, the measured peak area (A) is correlated to the analyte mass (m) by the exponential relationship [4,5]:

$$A = a \times m^b \Rightarrow \log A = b \log m + \log a \quad (1)$$

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where *a* and *b* are coefficients depending on instrumental parameters, nature and concentration of analyte, gas and liquid flow rates, evaporation temperature, etc.

Among other parameters, the composition of mobile phase has been reported to affect ELSD response factor in a dual way: firstly, it controls the aerosol mean droplet diameter produced by the nebulization process [6,7] and secondly, it affects the extent of droplets condensation on the walls of the nebulization chamber [1,8,9]. Besides mobile phase composition, peak shape (width and asymmetry) is expected to influence ELSD response factor, as a result of the exponential correlation between peak area and analyte mass (Eq. (1)) [10].

In this study, the dependence of ELSD response factor (peak area/analyte mass) on mobile phase composition, as well as, on peak shape was thoroughly examined. In the frame of the development of a novel HPLC–ELSD method for the determination of the aminoglycoside antibiotic neomycin sulfate, the enhancement of ELSD response was accomplished by using increased mobile phase volatility, ion-pairing reagent of high-molecular-mass, and decreased peak width and asymmetry. The developed HPLC–ELSD method, characterized by low detection limit, high linearity, precision and accuracy, and short analysis time, was successfully applied for the determination of neomycin sulfate in raw materials, pharmaceutical formulations and animal feeds.

Aminoglycosides (neomycin, gentamicin, tobramycin, kanamycin, amikacin, etc.) consist of a very interesting group of drugs (antibiotics) with very difficult but challenging analytical requirements. They are polar polyamine compounds with low UV-absorptivity, requiring time consuming and not always reproducible derivatization in order to develop spectrophotometric or HPLC methods. The existence of similar related substances as minor impurities in raw materials renders their analysis even more difficult. Neomycin (sulfate) is an aminoglycoside antibiotic, effective against a wide spectrum of Gram-negative and Gram-positive bacteria (Fig. 1). It is administrated in the form of powder, aerosols, creams, etc., alone or in combination with other drugs (bacitracin, dexamethasone, fluocinolone, fluorometholone, flurandrenolide, gramicidin, hydrocortisone, methylprednisolone, polymyxin, etc.) and is also used in veterinary medicine [11].

The official method for the assay of neomycin in pharmaceuticals (raw material and formulations), food and tissues is a microbiological one [12–14] and therefore it is time consuming, with low detectability and precision, while it appears no specificity against other antibiotics with similar action.

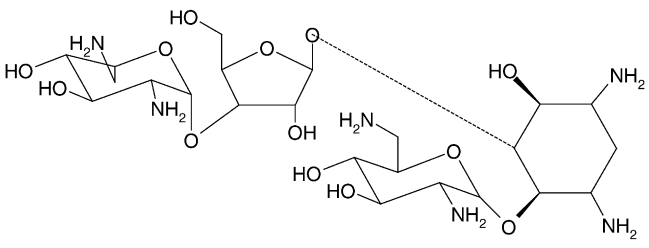

Fig. 1. Structure of neomycin.

Various HPLC methods have been proposed for the determination of neomycin, but since it appears low UV absorptivity pre-column or post-column derivatization is required [15–17]. HPLC methods based on indirect fluorometric [18], pulsed electrochemical [19] and electrospray ionization/ion-trap tandem mass spectrometric detection [20], have also been reported.

2. Experimental

2.1. Instrumentation and software

Chromatographic separations were carried out on a Shimadzu VP Series HPLC (Duisburg, Germany) modular system consisting of: a DGU-14A Online Vacuum-Degasser, a LC-10 AD VP micro double piston pump, a 7725i Rheodyne manual sample injector equipped with a 20 μl loop, a Waters Spherisorb ODS-2 C_{18} analytical column (250 × 4.6 mm, spherical particles of 5 μm and 80 Å pore size), a ss-420x A/D converter board and a Class VP 4 data processing software for the recording and integration of the chromatograms.

The detector used was a Sedex 75, S.E.D.E.R.E. low temperature evaporative light scattering detector. The nebulizer gas was nitrogen of industrial purity grade. Separations were carried out using isocratic elution at controlled room temperature (22–25 °C).

A pH meter (Metrohm Herisau) equipped with a glass combination electrode was used for pH measurement of mobile phase.

2.2. Reagents and standards

All chemicals were of analytical reagent grade unless otherwise stated. HPLC-grade water (specific resistance >17.8 MΩ cm) was obtained by a Milli-Q water purification system (Millipore). For mobile phases preparation, heptafluorobutyric acid (HFBA) (Fluka, ≥99.0%), trichloroacetic acid (TCA) (Merck, >99.5%), trifluoroacetic acid (TFA) (Sigma, >99%, spectrophotometric grade), acetic acid (Panreac, ≥99.7%), formic acid (Merck, ≥98.0%), acetone, acetonitrile and methanol (Lab-scan, HPLC grade) were used.

Neomycin sulfate pure substance (neomycin base 660 mg/g), raw materials and formulations (powder, aerosols and creams) were provided by local pharmaceutical companies. Pure material was tested for identification, assay and impurities according to European Pharmacopoeia procedures [12]. A 5.00 mg/ml standard stock solution was prepared in water and stored protected from light in the refrigerator. Working standard neomycin solutions in the range 2.0–50.0 μg/ml (as neomycin base) were daily prepared in mobile phase.

For sulfate calibration, a 1.00 mg/ml (SO_4^{2-}) standard stock solution was prepared from potassium sulfate. Working standard solutions in the range of 9.0–40.0 μg/ml were daily prepared by appropriate dilution in mobile phase.

For the evaluation of the selectivity of the method, mixed solutions of neomycin and coexisting drugs (gramicidin, naphazoline, phenylephrine, triamcinolone, nystatin, betamethazone valerate, catalase, bacitracin), obtained from local pharmaceutical companies, were prepared and analyzed.

2.3. Procedures

The selected analytical column is not compatible with pH below 1.5. Since the applied mobile phases contained strong acids, pH was measured before usage and analytical column was very carefully washed with acetonitrile at the end of each day and stored in the same solvent. Also, mobile phase was filtered through HVLP Millipore filters (diameter 47 mm, pore size 0.45 μm) under vacuum for removing particles and dissolved air. Before measurements, flow path was rinsed with mobile phase for about 30 min, until baseline noise became negligible (less than 3 mV at detector gain 12).

2.3.1. Study of influence of mobile phase composition and peak shape on ELSD response factor

For the evaluation of the influence of peak shape on ELSD response factor, the chromatographic column was used along with a flow rate of 1.0 ml/min, nitrogen pressure 3.5 bar, evaporation temperature 45 °C and detector gain 12. Examined mobile phases were aqueous solutions of formic acid 4.25–7.50 ml/l, acetic acid 150–300 ml/l or TFA 0.3–0.9 ml/l.

For the evaluation of the influence of mobile phase composition on ELSD response factor a flow injection set-up was utilized by replacing the analytical column by a stainless steel coil (200 mm length, 1/16 in. i.d.). Flow rate was 0.5 ml/min, nitrogen pressure 3.5 bar, evaporation temperature 45 °C and detector gain 9. Examined mobile phases were pure water or mixture of water-polar organic solvent (acetone, methanol, acetonitrile) 50:50 or 5.0 mM aqueous solutions of various volatile organic acids (HFBA, TCA, TFA, acetic or formic acid).

Using the above experimental configurations and mobile phases, logarithmic calibration curves were established using at least five different concentration levels in the range of 5–50 μg/ml (of neomycin base) and three injections per concentration level. From the constructed calibration curves the coefficients *a* and *b* of Eq. (1) were calculated.

2.3.2. Routine determination of neomycin in raw material, formulations and animal feeds

Using the C_{18} chromatographic column the optimized mobile phase was water–acetone (50:50) containing 1.50 ml HFBA per liter. Flow rate was 1.0 ml/min (isocratic mode), nitrogen pressure 3.5 bar, evaporation temperature 45 °C and detector gain 12. Standard and sample working solutions, in the range of 2–50 μg/ml neomycin base in mobile phase, were injected in the HPLC system in triplicate. For sampling and treatment of samples the general procedures described in USP [13], with the appropriate modifications for the new method, were followed.

Raw materials of neomycin sulfate were simply dissolved in mobile phase to obtain a concentration level within the working range.

Powder formulation and animals feeds were dissolved in mobile phase and the sample was filtered through HVLP Millipore 0.45 μm for removing the undissolved particles.

Cream formulations (quantity equivalent to 2.5 mg of neomycin base) were slightly heated and mixed with 2 ml of dichloromethane to dissolve. Neomycin was extracted successively with two 2.5-ml portions of 0.1% (v/v) HFBA aqueous solution, the layers being separated with centrifugation (4000 rpm for 2 min). The extract was further diluted with mobile phase.

For the analysis of aerosol formulations, the content of one package was completely collected in a volumetric cylinder. The remaining propulsive gasses were removed with slight heating into a water bath at 40 °C. The residue was dissolved in mobile phase and filtered through HVLP Millipore 0.45 μm.

Sulfates in raw materials were determined by external calibration curve using potassium sulfate standard after sample dissolution in mobile phase and appropriate dilution to obtain a concentration level within the range of 9–40 μg/ml.

3. Results and discussion

3.1. Influence of mobile phase composition on ELSD response

Since neomycin, like all aminoglycoside antibiotics, is a polar polyamine compound the ion-pair reversed phase chromatographic approach was selected using the non-polar C_{18} column.

The first step of the study was to investigate the influence of mobile phase composition on ELSD response to neomycin sulfate. Since peak shape (width and asymmetry), produced by the retention process in the chromatographic column, may also influence ELSD response factor, a direct flow injection set-up with constant injection volume was utilized. Two components of mobile phase were evaluated: (a) solvents and (b) ion-pairing reagents. The four more common polar solvents in reversed-phase HPLC (water, acetonitrile, acetone and methanol) and a number of volatile, and thus compatible with the ELSD, ion-pairing agents (formic, acetic, trifluoroacetic, trichloroacetic and heptafluorobutiric acid) were examined. The results (peak area of the lowest concentration and coefficients of the exponential calibration curve (Eq. (1)) of neomycin sulfate) for each mobile phase are presented in Table 1, from which the following observations/conclusions

Table 1
Influence of mobile phase composition on ELSD response to neomycin sulphate, in a flow injection set-up

Mobile phase	Peak area[a] ($\times 10^5$) 5.0 μg/ml	a ($\times 10^3$) (±S.D.)	b (±S.D.)	r^2 ($n = 5$)[b]
Water	1.00	10.914 (±0.064)	1.377 (±0.019)	0.9998
Water–acetonitrile (50:50)	1.94	19.248 (±0.017)	1.4358 (±0.0062)	0.99997
Water–methanol (50:50)	1.77	20.183 (±0.062)	1.364 (±0.022)	0.9995
Water–acetone (50:50)	2.58	35.156 (±0.035)	1.245 (±0.012)	0.9998
HCOOH 5.0 mM in water	1.44	20.56 (±0.10)	1.208 (±0.030)	0.9993
CH_3COOH 5.0 mM in water	2.15	36.057 (±0.092)	1.110 (±0.027)	0.9993
TFA 5.0 mM in water	2.16	43.15 (±0.13)	1.001 (±0.039)	0.998
TCA 5.0 mM in water	2.40	43.35 (±0.16)	1.064 (±0.048)	0.998
HFBA 5.0 mM in water	3.65	61.94 (±0.13)	1.102 (±0.039)	0.998

[a] Arbitrary units.
[b] Each point is the average of three replicates, 5–50 μg/ml.

can be obtained:

(a) ELSD response factor was highly dependant on the nature of the mobile phase solvents. Coefficient a increased according to the order: acetone > methanol > acetonitrile > water, which is identical to the order in which solvents volatility increases (Table 2). Therefore, the reduction of droplets condensation on the walls of the nebulization chamber, which is favored by the increase of mobile phase volatility, is probably the reason for the ELSD response enhancement by the presence of organic solvent. The increase of coefficient a, due to the presence of volatile organic solvent, was also accompanied by a slight decrease of coefficient b (the response curve tends to be linear), except of acetonitrile and water for which the order was inverted. The peak area increases also with solvent volatility, except of acetonitrile and methanol for which the order was inverted.

(b) According to Nukiyama and Tanasawa empirical equation [21], which has been reported to be valid for ELSD nebulizers [22], the decrease of mobile phase viscosity results in decrease of mean diameter of aerosol droplets at the nebulization step. Droplets with smaller diameter are less prone to condensation on the walls of the nebulization chamber. Therefore, the observed ELSD response enhancement was possibly attributed, besides mobile phase volatility, to the reduction of mean droplets diameter, caused by the decrease of mobile phase viscosity. The order in which viscosity decreases is identical to the order in which solvents volatility increases, with the exception of the order of acetonitrile and methanol, which is inverted (Table 2). The smaller viscosity of acetonitrile comparing to that of methanol is a reasonable explanation for the higher peak area of acetonitrile, which is in contrast to the volatility order.

(c) The presence of organic anions in the (acidified) mobile phase resulted in increase of peak area and coefficient a associated with a slight decrease of coefficient b. ELSD response enhancement was probably caused by the inclusion of anions in the neomycin sulfate particles, possibly due to electrostatic interactions with neomycin cationic amino groups. This statement is strengthened by the fact that the ELSD response enhancement was proportional to the molecular mass of the mobile phase organic anion. The order of enhancement was: heptafluorobutyric ($M_r = 213.1$) > trichloroacetic ($M_r = 162.4$) > trifluoroacetic ($M_r = 113.0$) > acetic ($M_r = 59.1$) > formic ($M_r = 45.0$) (Fig. 2).

Table 2
Physicochemical properties of mobile phase solvents

	Vapor pressure, 25 °C (kPa)	Viscosity, 20 °C (mPa s)
Water	3.2	1.00
Acetonitrile	11.8	0.34
Methanol	17	0.55
Acetone	31	0.30

3.2. Study of the influence of peak shape on ELSD response

A consequence of the exponential correlation between peak area and analyte mass (Eq. (1)) is the fact that the peak area is strongly dependant on peak broadening and asymmetry [10]. Evidently, this rule is not valid for the conventional linear HPLC detectors (e.g. UV–vis detector).

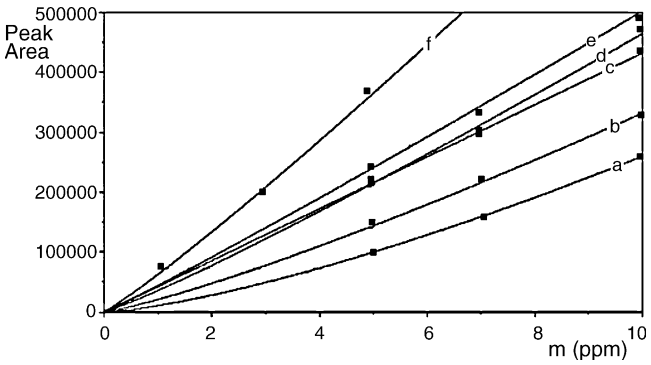

Fig. 2. Influence of molecular mass of mobile phase anions on ELSD response to neomycin in a flow injection system: (a) pure water, (b) formic acid ($M_r = 45.0$), (c) acetic acid ($M_r = 59.1$), (d) TFA ($M_r = 113.0$), (e) TCA ($M_r = 162.4$), (f) HFBA ($M_r = 213.1$).

In the cases that the analyte concentration distribution is considered to be Gaussian, Eq. (1) becomes equivalent to [10]:

$$A = \alpha^* \sqrt{\frac{2\pi}{b}} \frac{\sigma^{(1-b)}}{(F\sqrt{2\pi})^b} m^b \quad (2)$$

where F is the mobile phase flow rate, σ the standard deviation of concentration distribution and α^* is a coefficient independent of the peak shape. In most ELSD applications $b > 1$ and therefore, it is derived that ELSD response is enhanced by the decrease of peak broadening (standard deviation). However, since peak shape is controlled, besides the analytical column, by mobile phase composition, this behavior may be considered as another aspect of the influence of mobile phase composition on ELSD response.

In order to obtain differing shapes of neomycin, aqueous acidic mobile phases of various concentrations of TFA, formic and acetic acid, and so various (acidic) pH, were utilized in conjunction with C_{18} column. Representative results from TFA mobile phases are shown in Table 3. HFBA was not suitable in this study due to its very intense effect on retention time, because of the high lipophilicity of its ion pair. Retention time, width and asymmetry of neomycin peak were controlled by the acidity of the mobile phase through the deprotonation of column silanols and therefore the cation-exchange interactions with the positively charged neomycin amino groups.

From the experimental results presented in Table 3, it is confirmed that increase of TFA concentration in mobile phase (increase of acidity) results in decrease of neomycin retention time, width and asymmetry, which is accompanied by ELSD response enhancement (increase of peak area and coefficient a). These results were reconfirmed with formic and acetic acid mobile phases (data not shown).

ELSD has been reported to appear uniform response factor for molecules with similar structure formula and therefore a universal calibration curve for many analyte classes, such as lipids and sugars, can be obtained [2]. However, according to our observations, peak shape, besides structure of molecule, should be taken into consideration, especially in cases that peak widths increase remarkably with retention time.

3.3. Development and validation of HPLC–ELSD method for the determination of neomycin and sulfates in raw materials

Taking advantage of the conclusions drawn from the previous steps of the study, a novel HPLC–ELSD method for the simultaneous determination of neomycin and sulfates was developed based on ion-pairing mechanism.

The selected mobile phase was water–acetone (50:50) containing 1.50 ml HFBA per liter (11.6 mM). HFBA was selected, since it has the highest molecular mass amongst the examined volatile organic acids and therefore it induces the greatest ELSD response enhancement. For the same reason, amongst the examined volatile polar organic solvents acetone was selected since it appears the highest volatility and the smallest viscosity. The proportion of acetone to water was selected to be 50:50 in order to achieve a retention time of neomycin close to 5.0 min (at smaller retention times neomycin peak may overlap with potentially interfering inorganic anions, while at greater retention time, peak broadening and asymmetry would increase resulting in decrease of ELSD response factor).

Table 4 summarizes the chromatographic and analytical characteristics of neomycin and sulfate for their determination in raw material and commercial formulations. A typical chromatogram of neomycin sulfate raw material is shown in Fig. 3a. The results of the assay of a raw material were in very good agreement with those obtained with reference methods (HPLC with pre-column derivatization for neomycin, indirect EDTA complexometric for sulfate). Furthermore, utilizing the aforementioned chromatographic conditions, the simultaneous direct determination of three minor impurities of neomycin sulfate can be achieved with good resolution ($R_s > 1.5$, Fig. 3b).

3.4. Application to pharmaceutical formulations

The proposed HPLC–ELSD method was further applied for the determination of neomycin in commercial formulations (powder, creams and aerosols). Retention time of co-existing active substances and excipients was examined in order to assure that they do not interfere (peak

Table 3
Influence of chromatographic peak broadening and asymmetry on ELSD response to neomycin

Aqueous mobile phase TFA (ml/l)	Peak area[a] ($\times 10^5$) 17.25 μg/ml (%R.S.D.)	t_R (min)	Width (min)	Asymmetry factor	a ($\times 10^3$) (±S.D.)	b (±S.D.)	r^2 ($n = 5$)[b]
0.3 (3.9 mM)	2.65 (2.9%)	8.6	0.31	3.5	0.950 (±0.108)	1.977 (±0.025)	0.9996
0.4 (5.2 mM)	5.16 (1.9%)	4.8	0.16	2.1	1.534 (±0.170)	2.045 (±0.045)	0.9990
0.5 (6.5 mM)	21.0 (1.6%)	4.6	0.14	1.9	35.810 (±0.076)	1.410 (±0.026)	0.9994
0.6 (7.8 mM)	19.8 (1.3%)	4.2	0.14	1.3	54.575 (±0.119)	1.306 (±0.041)	0.998
0.7 (9.1 mM)	19.4 (1.1%)	4.0	0.13	1.3	43.451 (±0.046)	1.344 (±0.015)	0.9998
0.8 (10.4 mM)	20.6 (0.5%)	3.7	0.12	1.1	57.676 (±0.060)	1.271 (±0.021)	0.9996
0.9 (11.7 mM)	24.2 (0.9%)	3.6	0.10	1.4	52.119 (±0.145)	1.342 (±0.056)	0.998

[a] Arbitrary units.
[b] Each point is the average of three replicates.

Table 4
Characteristics of neomycin and sulfates chromatographic peaks and logarithmic regression of peak areas towards analyte mass concentration. Comparison between ELSD and reference methods for the assay of a raw material

	Sulfates	Neomycin
Retention time (t_R) (min)	2.1	4.9
Width at half height (min)	0.11	0.18
Retention factor[a]	0.62	2.8
Asymmetry factor (at 5% of peak height)	1.7	1.3
Theoretical plates (N)	2.0×10^3	4.1×10^3
Resolution	7.6	
Intercept of logarithmic calibration log a (±S.D.)	3.188 (±0.052)	4.144 (±0.026)
Slope of logarithmic calibration b (±S.D.)	2.438 (±0.042)	1.780 (±0.021)
Correlation coefficient ($n = 5$)	0.9998	0.9997
Range (μg/ml)	9–40	2–50
Detection limit (μg/ml)[b]	3	0.6
R.S.D. ($n = 3 \times 3$)[c]	1.7% (10 μg/ml)	1.7% (3.3 μg/ml)
Assay of raw material		
Reference method (mg/g)	29[d]	660[e]
HPLC–ELSD method (mg/g) ($n = 3 \times 3$)[f]	30 (±0.4)	659 (±5)

[a] Void volume = 1.3 min.
[b] Corresponds to analyte concentration producing a peak area equal to 3.3 times the standard deviation of the most dilute standard and is practically equal to the concentration having S/N ratio equal to 3.3.
[c] Three working days (within a week), three injections per day.
[d] Indirect EDTA complexometric method.
[e] HPLC with pre-column derivatization method.
[f] Three independent sub-samples with three injections per working sample solution.

overlaping) with neomycin. Most of them (gramicidin, naphazoline, phenylephrine, triamcinolone, nystatin, betamethazone valerate, catalase), are lipophilic or of high-molecular-mass and therefore not eluted within a reasonable time with the mobile phase selected from the C_{18} column. Only bacitracin (a polypeptide antibiotic) is eluted with neomycin (with very good resolution $R_s = 12.2$, Fig. 4). A periodical washing of the column with acetonitrile must be performed. The results obtained from the assays of commercial formulations (Table 5) reveal conformance to the Pharmacopoeia requirements for content with R.S.D. 0.4–3% ($n = 3 \times 3$).

The accuracy of the new method was evaluated performing recovery experiments by spiking sample working solutions. Mean recoveries of 98.7–102.4% (Table 5) reveal sufficient accuracy. Further study of the matrix effect on the determination was carried out by dilution experiments (determination of neomycin content in commercial formulations using a varying dilution factor D ($V_{initial}/V_{final}$) at four different levels). The correlation curves of the concentration found (in the diluted solution) versus D were linear ($r > 0.998$) with a slope equal to the content of the formulation and a statistically (proven by t-test) zero intercept. Similarly, the correlation curves of formulation content found versus D were very linear with statistically (proven by t-test) zero slopes. These results reveal the absence of any constant or proportional determinate error due to matrix (excipients) effect.

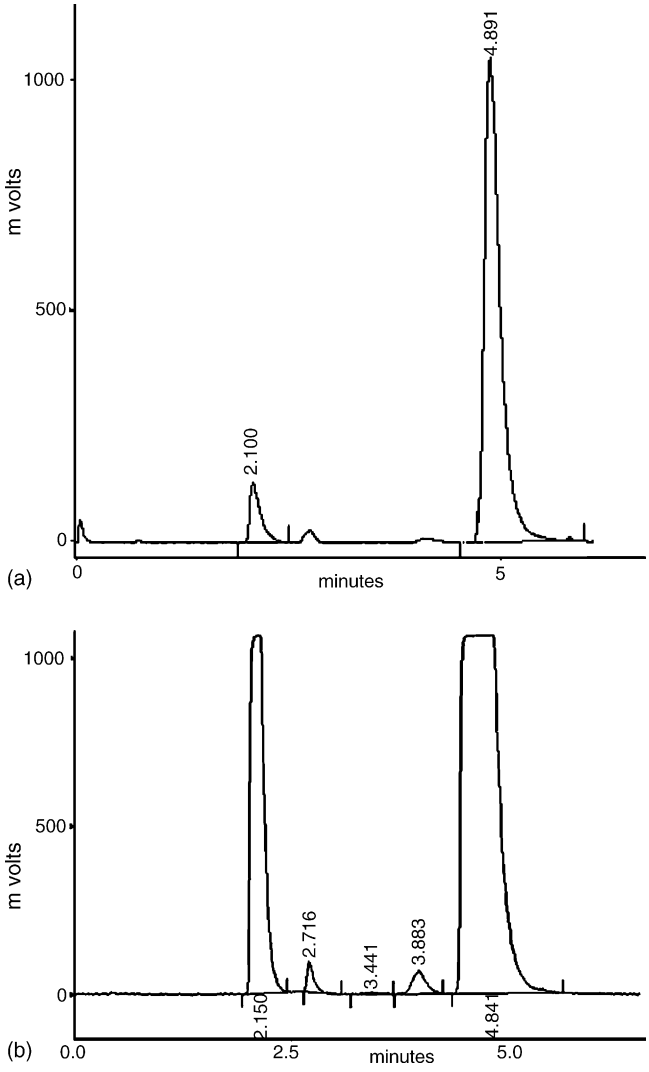

Fig. 3. Typical chromatogram of neomycin sulfate raw material: (a) 75 μg/ml (sulfate: 2.1 min, neomycin: 4.9 min) and (b) 1000 μg/ml [impurity A: 2.7 min (0.73%, w/w), impurity B: 3.4 min (0.30%, w/w) and impurity C: 4.0 min (0.95%, w/w)].

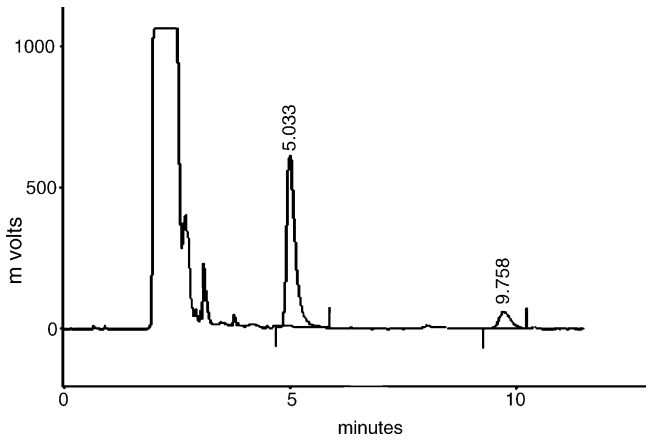

Fig. 4. Typical chromatogram of neomycin sulfate–bacitracin formulation (neomycin: 5.0 min, bacitracin: 9.8 min).

Table 5
Assay of content and recovery results of neomycin (base) commercial formulations

Formulation/label content (co-existing active substances)	Determined content (mg/ml or mg/g) ±S.D. ($n = 3$)	Recovery range (mean recovery, $n = 5$)
Powder 3.35 mg/g (bacitracin)	3.317 ± 0.087	95.3–102.6% (98.7%)
Nasal spray 0.75 mg/ml (gramicidin, naphazoline, phenylephrine)	0.861 ± 0.023	92.9–105.2% (99.1%)
Cream in unibase 2.5 mg/g (triamcinolone, nystatin)	2.71 ± 0.05	95.1–104.6% (99.9%)
Cream/ointment 5 mg/g (betamethazone valerate)	4.738 ± 0.034	97.2–104.6% (100.8%)
Aerosol A 2.23 mg/g (horse liver catalase)	2.393 ± 0.072	95.5–104.3% (100.8%)
Aerosol B 1.05 mg/ml (bacitracin)	0.945 ± 0.004	97.4–107.6% (102.4%)

3.5. Application to medicated animal feeds

The developed HPLC–ELSD method was further applied for the determination of neomycin in medicated animal feeds. Synthetic mixtures of dried molasses, pig feed, and milk replacer containing 0.8–20% were analyzed by dispersion in the mobile phase, adequate mixing and filtration. Eluted compounds were well separated from neomycin and the recovery was 98–101%. The quantitation limit of the method for this application was 0.2%.

4. Conclusions

From the above experimental results, using neomycin, a polyamine polar compound, it is concluded that ELSD response factor is highly dependent on mobile phase composition. In particular, ELSD response is enhanced by: (a) increase of mobile phase volatility, which reduces droplets condensation on the walls of the nebulization chamber and (b) volatile ionic species, which form ion pair with analytes and therefore increase the mass of the detected particles. Furthermore, due to the logarithmic relation between peak area and analyte mass, ELSD response factor decreases with increasing peak width and asymmetry factor.

Based on these observations, the optimum mobile phase for the HPLC–ELSD quantitation of neomycin is a mixture of water–acetone, with high volatility and low viscosity, containing HFBA as an acidic ion-pairing reagent of high-molecular-mass. The proposed HPLC–ELSD method does not require any derivatization step and also enables the simultaneous determination of the inorganic co-ion (sulfates). The relationship of ELSD signal to the analyte concentration is logarithmic, while sufficient detectability, precision and accuracy were obtained. The proposed method was applied successfully for the determination of neomycin and sulfate in raw materials, pharmaceutical formulations (powder, aerosols and creams) and medicated animal feeds with simple pretreatment and with sufficient recoveries.

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