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Analysis of neomycin sulfate and framycetin sulfate by high-performance liquid chromatography using evaporative light scattering detection

I. Clarot [a,*], A. Regazzeti [b], N. Auzeil [b], F. Laadani [a], M. Citton [a], P. Netter [c], A. Nicolas [a]

[a] *Laboratoire de Chimie Analytique, UMR 7561 CNRS-UHP, Faculté de Pharmacie, 5 rue Albert Lebrun, F-54000 Nancy Cedex, France*
[b] *Laboratoire de Chimie Analytique, Faculté des sciences pharmaceutiques et biologiques, 4, Av. de l'Observatoire, F-75270 Paris, France*
[c] *UMR 7561 CNRS-UHP, Faculté de Médecine, 9 Avenue Forêt de Haye, F-54500 Vandoeuvre les Nancy, Nancy Cedex, France*

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Abstract

A rapid and simple method for the determination of main components and related substances of both neomycin sulfate and framycetin sulfate by HPLC and evaporative light scattering detection (ELSD) is described. The method was also used to determine the neomycin B and the sample sulfate content. Detection and quantitation of aminoglycoside antibiotics are problematic because of the lack of UV absorbing chromophore. The use of a universal detector avoids the need for sample derivatization or use of specific detector based on pulsed amperometry described to be difficult in routine assays. Separation was performed with a Polaris C18 150 mm × 4.6 mm i.d., 3 μm reversed-phase column with a solution of 170 mM trifluoroacetic acid (TFA) mobile phase at a flow rate of 0.2 mL/min. The chromatographic parameters were optimized with the help of experimental design software. Mass spectrometry (MS) was employed to confirm the ELSD profile. The final method was validated using methodology described by the International Conference of Harmonization in the field of Active Pharmaceutical Ingredients. Commercial samples of different sources were analyzed and results were in good agreement with specifications of the European Pharmacopoeia.

Keywords: Neomycin; Framycetin; Antibiotics; Pharmaceutical analytes; Sulfate; LC; ELSD

1. Introduction

Neomycin sulfate and framycetin sulfate belong to a class of compounds known as aminoglycoside antibiotics. Neomycin and framycetin are broad spectrum antibiotics produced by fermentation of *Streptomyces fradiae* [1,2] or *Streptomyces decaris* [3]. Neomycin sulfate and framycetin sulfate are mixtures of two major stereoisomers components: neomycin B (main product) and neomycin C [4]. The European Pharmacopoeia (EP) limits the neomycin C content to 3.0–15.0% for neomycine sulfate [2] and to less than 3.0% for framycetin sulfate [3]. The United States Pharmacopoeia (USP) does not make this distinction [5] and does not limit the content of neomycin C. Other minor components could be found in both compounds as neomycin A (neamine [6]) and neomycins D (paromamine), E (paromycin I) and F (paromycin II) [7]. Neomycin LP-A (3-acetylneamine) and LP-B, mono-*N*-acetyl derivatives of neomycins A and B can also be found [8]. Structures and molecular masses of the different neomycins are shown in Fig. 1.

These structures are closely related making the chromatographic separation quite difficult. Several chromatographic methods have been described to determine neomycin such as thin-layer chromatography [9] or gas–liquid chromatography [10]. High-performance liquid chromatographic methods were preferred for the determination of neomycin sulfate composition. Normal-phase systems with pre-column derivatization were initially developed [11] but reversed-phase methods were introduced and gave more suitable results

* Corresponding author. Tel.: +33 3 83 68 23 47; fax: +33 3 83 68 23 64.
E-mail address: igor.clarot@pharma.uhp-nancy.fr (I. Clarot).

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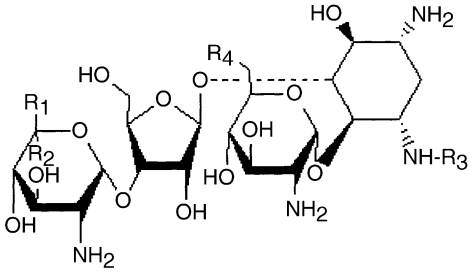
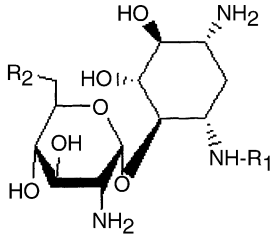


Neomycin	MW	R_1	R_2	R_3	R_4
B	614.6	H	CH_2-NH_2	H	NH_2
C	614.6	CH_2-NH_2	H	H	NH_2
LP-B	656.6	H	CH_2-NH_2	CO-CH_3	NH_2
E	615.6	H	CH_2-NH_2	H	OH
F	615.6	CH_2-NH_2	H	H	OH



Neomycin	MW	R_1	R_2
A	322.4	H	NH_2
LP-A	364.4	CO-CH_3	NH_2
D	323.4	H	OH

Fig. 1. Structures and molecular masses of the neomycin components.

than post-column derivatization with *ortho*-phthalaldehyde [12] or ion pair chromatography [13]. To avoid sample derivatization, a universal mode of detection could be used as refractive index [14] or mass spectrometry (MS) [15]. Recently, pulsed amperometric detection (PAD) has been employed for the determination of the neomycin sulfate composition [16]. This detection is actually prescribed by the European Pharmacopoeia but was shown to be difficult in a routine use because of problematic signal stability.

ELSD is described as a universal detection mode suitable for non-absorbing analytes [17]. This detection mode has been recently used in the field of aminoglycoside antibiotics with gentamicin sulfate [18,19]. The response does not depend on the solute optical properties, any compound less volatile than the mobile phase could be detected. The detector response is now well described [20] and allows all molecules of the sample to give a proportional signal (same sensitivity), principle in good agreement with the search of impurities in pharmaceutical products. A linear relationship between the signal and the analyte concentration was obtained when a double logarithmic representation is used.

The aim of this work was to develop a rapid and simple chromatographic method with a direct sample introduction (no derivatization). The method was validated using validation criteria according to ICH guidelines [21]. The chromatographic parameters were optimized with specific experimental design software and mass spectrometry was employed to confirm the analyte ELSD chromatographic profile. The method was used to evaluate the composition of samples from different sources and to determine their sulfate content.

2. Experimental

2.1. Chemicals

Trifluoroacetic acid (TFA) was purchased from Acros Organics (Geel, Belgium) and was flushed with nitrogen after each use. Ultrapure water was obtained in a Milli-Q system from Millipore (Bedford, MA, USA).

Neomycin sulfate, framycetin sulfate and neamine standards were generous gift from the European Department for the Quality of Medicines (Strasbourg, France). Commercial samples were generous gift from Vetoquinol (Lure, France), Parke-Davis (Angers, France) and Aventis (Paris, France). Ammonium sulphate was obtained from Merck (Darmstadt, Germany).

2.2. Instrumentation

2.2.1. LC apparatus

The isocratic HPLC system consisted of an 515 HPLC pump (Waters, Milford, Ma, USA), a TSP model AS100 autosampler (Thermoseparation Products, Fremont, CA, USA) set to inject 20 μL and an electronic integrator Spectra-Physics SP4290 coupled with a chromatographic data system Spectra-Physics Winner on Windows (Spectra-Physics, San Jose, CA, USA). The evaporative light scattering detector was a Sedex 75 model from SEDERE (Alfortville, France) equipped with a normal or a low flow nebulization head (SEDERE, Alfortville, France). The analytical column was a Varian Polaris C18-A (Ansys Technologies, Lake Forest, CA, USA) 150 mm × 4.6 mm i.d., 3 μm reversed phase column which allows the use of a 100% aqueous mobile phase with a pH near 1.5. The column temperature was controlled with a Croco-Cil external oven (Thermoseparation Products, Fremont, CA, USA). Mobile phases consist of TFA solutions and were filtered with Millipore filter model HVLP 0.45 μm (Millipore, Molsheim, France) before use. Optimization of chromatographic parameters was obtained with the help of the experimental design software, Modde 5.0 (Umetrics, NJ, USA).

2.2.2. Electrospray mass spectrometry

Mass spectrometry was used to confirm the chromatographic profile obtained with the ELSD detection. The HPLC apparatus consists in a Surveyor system (ThermoElectron, Courtaboeuf, France) at a flow rate of 0.2 mL/min and an injection volume of 20 μL. Mass spectrometry, LC–MS and MS–MS experiments were carried out on a LCQ Advantage mass spectrometer (ThermoElectron, Courtaboeuf, France)

equipped with an electrospray interface and an ion-trap analyzer. The mass spectrometer was monitored and data were analysed using Xcalibur software (ThermoElectron, Courtaboeuf, France). High purity nitrogen was used as nebulisation and desolvation gas at 25 °C. The sheath and auxiliary gas flow rate were 60 and 20 in arbitrary units, respectively. High purity helium was used damping gas and collision activation partner in the mass analyzer cavity. The instrument was tuned by direct infusion (5 μL/min) of a neomycin sulfate solution using a 500 μL SGE syringe pump (ThermoElectron, Courtaboeuf, France). The following optimized parameters for the positive ESI mode were retained for optimum neomycin detection: electrospray voltage, 4.0 kV; capillary voltage, 3.0 V; capillary temperature, 200 °C. In MS and LC–MS studies the mass range used was *m/z* 100–1000. In MS–MS experiments, fragments were detected using a two-stage full scan mode. In the first stage, the ions formed are stored in the mass analyzer. Then, ions with a determined value of *m/z* are selected, excited and fragmented to produce one or more ions. In the second stage, the obtained ions are stored and sequentially scanned out of the mass analyzer to produce a full product ions mass spectrum. Elution profiles and retention times were evaluated by choosing the appropriate molecular ion ($m/z \pm 0.5$).

2.3. Sample preparation

Concentrations of neomycin sulfate and framycetin sulfate test solutions were those specified by the European Pharmacopoeia [2,3]: 0.500 and 0.005 mg/mL were respectively used for the quantitation and signal-to-noise ratio determination. For MS analysis, 1.000 mg/mL solutions were used. All dilutions were made in the mobile phase.

2.4. Peak assignment

Peak assignment was made by injection of a 0.500 mg/mL neomycin sulfate reference solution. LC–MS data (Section 3.3) confirmed the estimated elution order.

3. Results and discussion

3.1. Method development

3.1.1. Chromatographic optimization

The composition of the mobile phase used is based on that previously developed for the analysis of gentamicin sulfate [18]. The critical parameters of ELSD are the temperature of the tube (T_{tub}) and the gas pressure (Pressure). Air was chosen for the nebulization as no differences were previously observed between three gas types [18]. The detector signal is also very dependent of the gain factor (Gain) and the nebulization head type: low flow (Head low) or normal flow (Head normal). Separation factors as TFA mobile phase concentration (TFA), column temperature (T_{col}) and flow rate (Flow) were also studied to optimize the chromatographic method. A screening factorial design using seven factors at three levels was employed to maximize the resolution between the two critical stereoisomers (neomycins B and C) and the signal-to-noise ratio. Three replicates of the central point were included in the design to give a final experimental matrix of eleven experiments. The values of this design are given in Table 1.

Table 1
Factors and nominal values used for the method optimisation

	Low value	Central value	High value
Detection parameters			
Gas pressure (bars)	3.2	3.5	3.8
Gain	6	7	8
Drift tube temperature (°C)	40	60	80
Nebulization head type	Low flow		Normal flow
Chromatographic parameters			
Phase mobile TFA concentration (mM)	170	200	230
Column temperature (°C)	30	35	40
Flow rate (mL/min)	0.2	0.5	0.8

Table 2
Optimized values of the LC-ELSD method

Gas pressure	3.2 bars
Gain	8
Drift tube temperature	60 °C
Nebulization head type	Low flow
TFA concentration	170 mM
Column temperature	30 °C
Flow rate	0.2 mL/min

Maxima were obtained with software optimization and lead to the values described in Table 2. A typical chromatogram using these chromatographic conditions is shown in Fig. 2. The observed elution order corresponds to those described by the European Pharmacopoeia [2,3] and relative retention to neomycin B were shown in Fig. 2. A resolution of 1.9 between neomycin B and neomycin C and a signal-to-noise ratio of 15 (0.005 mg/mL) were obtained.

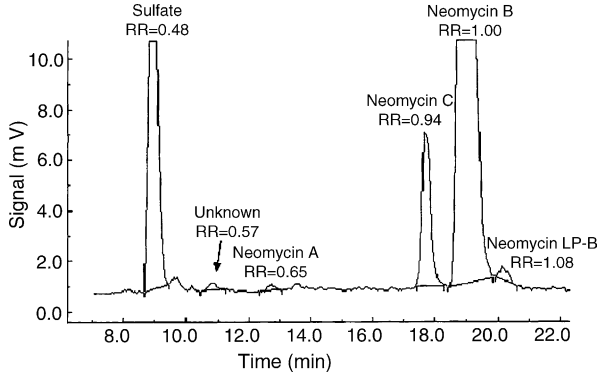


Fig. 2. Chromatogram example of a 0.500 mg/mL neomycin sulfate solution. Relative retentions (RR) indicated were in reference to the neomycin B peak.

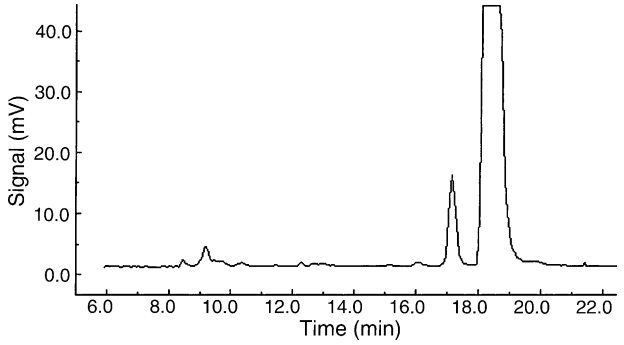


Fig. 3. Chromatogram obtained for a neomycin sulfate sample when sulfates were removed with an anion exchanger column.

3.1.2. Sample treatment

ELSD could detect all the non volatile components of the sample. Neomycins were detected as well as sulfate present in great amount (27.0–31.0%, m/m) [2,3]. The sulfate peak is problematic because of a possible co-elution of neomycin components. To check the specificity of the method sulfates, were eliminated with the help of a specific anion exchanger column (Chromafix PS-OH, Macherey-Nagel Gmbh, Düren, Germany). A 2.000 mg/mL neomycin sulfate prepared in water was used the eluate was further diluted with the mobile phase prior to the chromatographic analysis to obtain a concentration of neomycin equivalent to that of the test solution (0.500 mg/mL). A chromatogram is given in Fig. 3. and demonstrate the absence of the sulfate peak.

3.1.3. Mass spectrometry

3.1.3.1. Direct infusion. The preceding sample treatment procedure was used for mass spectrometry experiments in order to avoid detection of adducts with sulfate ions [M + 98].

Fig. 4 shows the mass spectrum obtained by direct infusion of both the mobile phase and a neomycin solution (1.000 mg/mL). This spectrum presents four major peaks at *m/z* 615.4 (P1), 455.2 (P2), 308.3 (P3) and 161.1 (P4). P1 is attributed to the protonated molecular ion of neomycin $[M + H^+]$, P3 to $[M + 2H^+]/2$, P1 and P4 result from the fragmentation scheme proposed in Fig. 5.

3.1.3.2. LC–MS experiments. To confirm the LC-ELSD peak attribution and to search for other detectable related compounds, a neomycin solution (1.000 mg/mL) was injected in LC–MS full scan mode. A chromatogram was obtained by plotting the total ion current as a time function for $[M + H^+]$ selected ions of all neomycin compounds. This chromatogram was similar to those obtained with the LC-ELSD method (Fig. 3). Using data obtained in full scan mode, chromatograms corresponding to each ion mass of neomycin components $[M + H^+]$ were individually plotted in order to detect possible co-eluted analytes. Chromatograms (A_{ch} to H_{ch}) and associated mass spectra (A_{sm} to H_{sm}) were presented in Fig. 6. Results were summarized in Table 3. LC–MS experiments lead to the identification of neomycin

Table 3
Results and assignment of fragments in LC–MS experiments

Chromatogram	RR	Spectrum	*m/z*	Molecular ion	Identity
A_{ch}	0.57	A_{ms}	324.16	$[M + H^+]$	Neomycin D
B_{ch}	0.66	B_{ms}	323.14	$[M + H^+]$	Neomycin A
C_{ch}	0.68	C_{ms}	365.06	$[M + H^+]$	Neomycin LP-A
D_{ch}	0.94	D_{ms}	308.32	$[M + H^+]/2$	Neomycin B or C
			615.37	$[M + H^+]$	
E_{ch}	1.00	E_{ms}	308.31	$[M + H^+]/2$	Neomycin B or C
			615.37	$[M + H^+]$	
F_{ch}	0.94	F_{ms}	308.33	$[M + H^+]/2$	Neomycin E or F
			616.36	$[M + H^+]$	
G_{ch}	1.00	G_{ms}	308.31	$[M + H^+]/2$	Neomycin E or F
			616.40	$[M + H^+]$	
H_{ch}	1.07	H_{ms}	657.39	$[M + H^+]$	Neomycin LP-B

Relative retention (RR) in reference to the neomycin B peak.

D (RR = 0.57), neomycin A (RR = 0.66), neomycin LP-A (RR = 0.68) and neomycin LP-B (RR = 1.06). The two peaks observed at RR = 0.94 and 1.00 could correspond to neomycin B, C, E or F. It is well known that neomycins E and F were not eluted at these retention times [16], but to confirm the general peak assignment, MS–MS experiments were carried out on these two peaks.

3.1.3.3. MS–MS experiments. The fragmentation of the molecular ion *m/z* 615.6 observed at RR = 0.94 and 1.00 lead to fragments *m/z* 455.22, 323.13 and 293.13. Isomers neomycins B and C could not be identified by the MS–MS method. Therefore, their relative proportion in the neomycin mixture indicates that neomycin C was eluted first (RR = 0.94). The fragmentation of the molecular ion *m/z* 616.6 led to fragments *m/z* 456.22, 324.13 and 294.11. These fragments show a difference of 1 amu face to the fragmentation of the molecular ion *m/z* 615.6 corresponding to the isotopic products of neomycins B and C. Neomycins E and F were not evidenced in the neomycin mixture.

In conclusion to the MS study, related substances observed with the LC-ELSD method were confirmed by the MS detection. The LC-ELSD method was demonstrated suitable to control the main components of neomycin and framycetin sulfates components as well as the related substances. MS detection allows to confirm the elution order observed in Fig. 2.

3.1.4. Quantitation of sulfates with ELSD

Quantitation of sulfates could be made by the use of external standardization. The choice of this standard is a critical point because all inorganic ions could be detected.

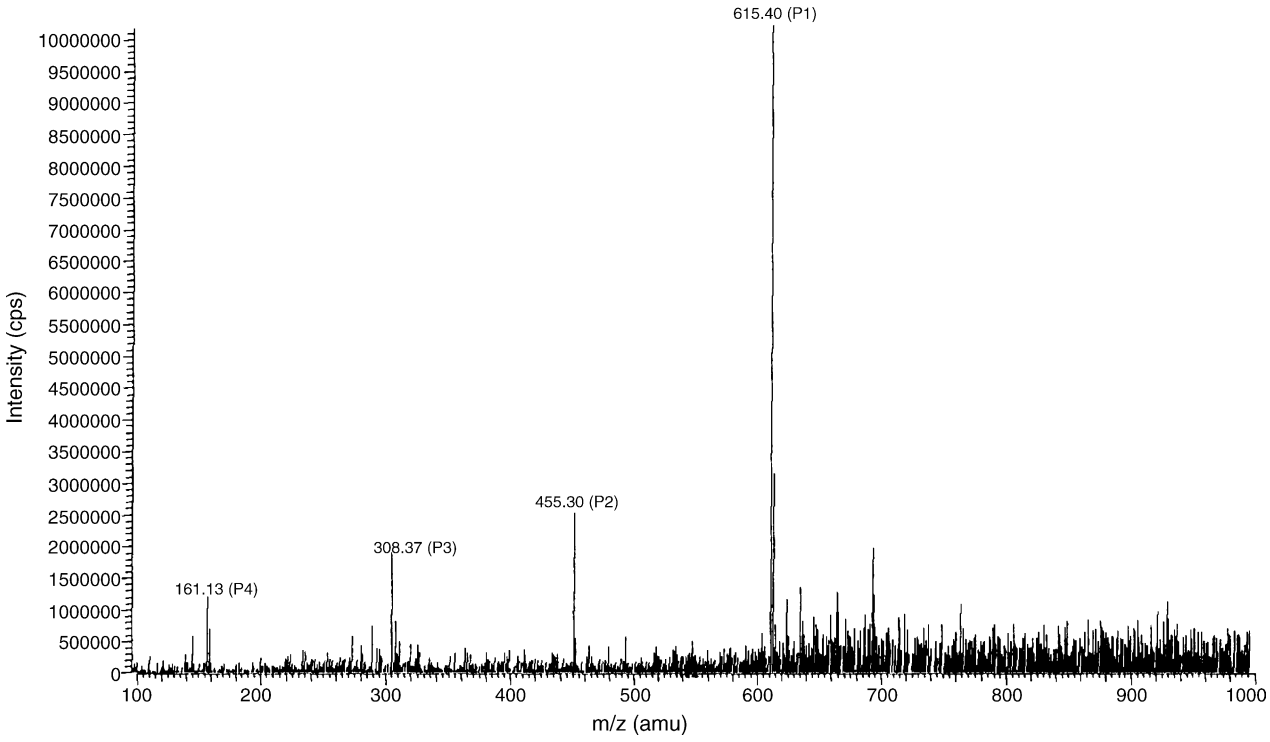


Fig. 4. Mass spectrum obtained by direct infusion of a 1.000 mg/mL neomycin solution (sulfates were removed from the sample).

For example, potassium or sodium sulfate solutions lead to a problematic integration of a double chromatographic peak corresponding to the anionic and cationic part of the solute. In this report, sulfate quantitation was made by the use of ammonium sulfate solutions because of the volatility of the ammonium moiety leading to a single sulfate peak easily usable for quantitation. Standard ammonium sulfate solutions were daily prepared in the range of 0.120–0.250 mg/mL (70.0–170.0% of the theoretical value) of sulfates.

3.2. LC-ELSD method validation

The method was validated using methodology described by the International Conference of Harmonization (ICH)

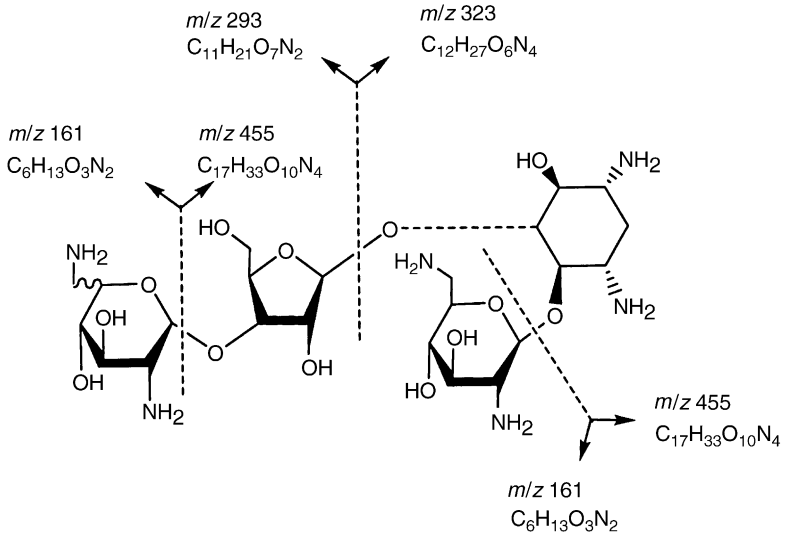


Fig. 5. Fragmentation scheme proposed for neomycin B and neomycin C.

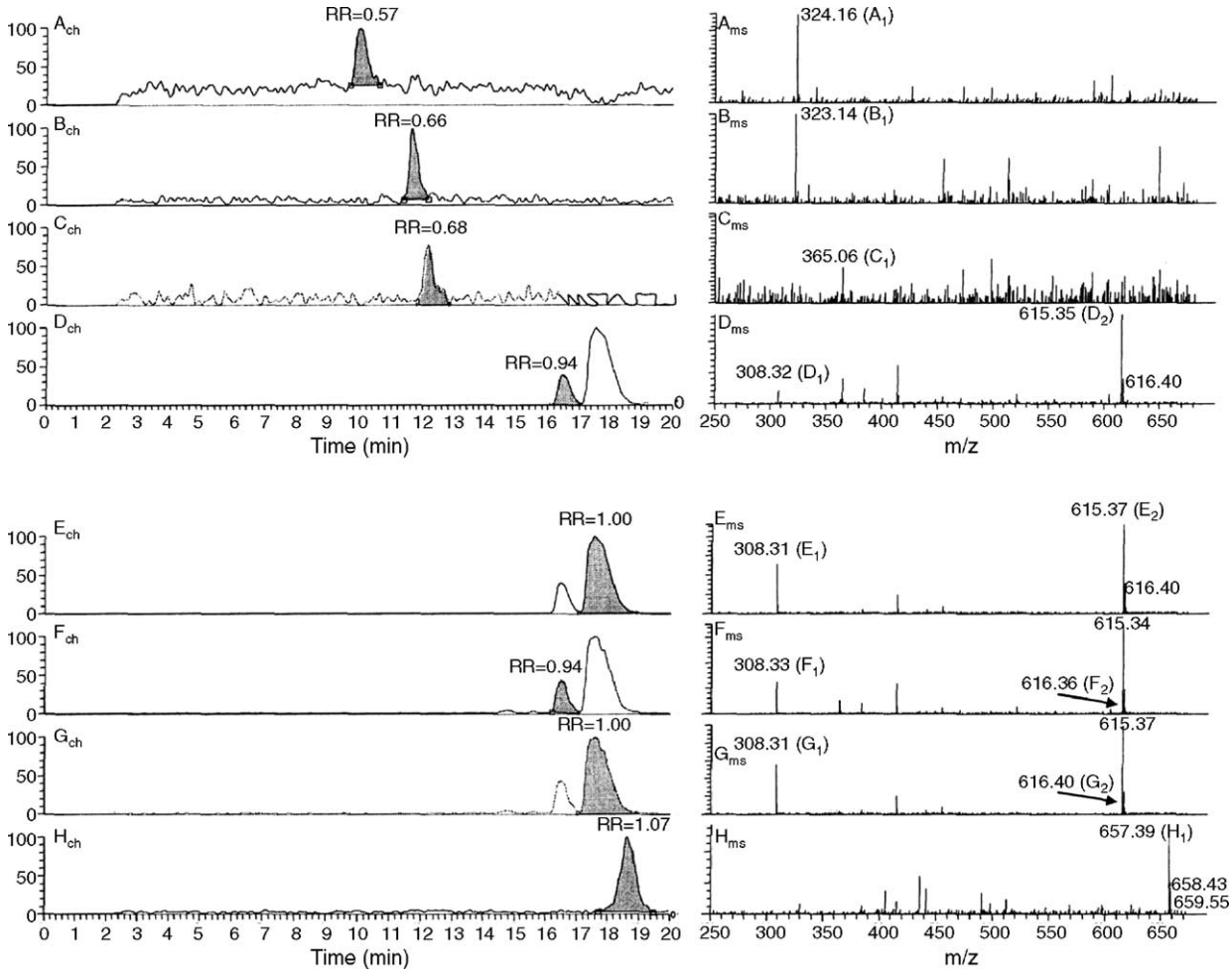


Fig. 6. On the left, chromatograms of a 1.000 mg/mL neomycin solution obtained in LC–MS choosing, after detection in full scan mode, the following *m/z* ratio: A_{ch}: 324.4—B_{ch}: 323.4—C_{ch}: 365.5—D_{ch}: 615.6—E_{ch}: 615.6—F_{ch}: 616.6—G_{ch}: 616.6—H_{ch}: 657.7. Relative retention (RR) in reference to the neomycin B peak. The right part of the figure illustrate the mass spectra (A_{ms} to H_{ms}) associated with each RR.

[21]. Specificity, precision, accuracy and linearity were evaluated. Limits of detection were also calculated.

3.2.1. Specificity

A chromatogram of the mobile phase shows no interferences in the range of retention times under study. The MS study confirmed that no impurities co-elute with the neomycin products.

Neomycin sulfate and framycetin sulfate solutions stored in specific stress conditions for 12 h were also tested. Light (366 nm), heat (80 °C), acidic (0.1 M HCl) or basic (0.1 M NaOH) hydrolysis and oxidation (H_2O_2 3%, v/v) conditions were used. All degradation products formed were identified as neomycins and no new unidentified compounds were observed. Neomycin sulfate and the framycetin sulfate samples were completely described by the LC-ELSD method.

3.2.2. Repeatability and intermediate precision

The repeatability was determined on three days using 6 determinations at 100 and 5% of the test concentration (0.500 mg/mL), results are shown in Table 4. Relative standard deviation (RSD) values are given for sulfates, neomycins B and C. In every case, RSD values were better than 3.0%.

The intermediate precision was determined by 6 injections of the 100 and 5% test solution on three days. Results are given in Table 4 and were satisfactory (<7.5%).

3.2.3. Accuracy

The accuracy of the method was evaluated each day by injection of five neomycin sulfate solutions (5 concentrations, $n = 3$) covering the entire linearity range. Results are shown in Table 4 at the $\alpha = 0.05$ level and are satisfactory.

3.2.4. Linearity

It is now well known that ELSD gives non direct linear response [22]. Light scattering is a complex process involving several mechanisms. It is usually described as a mixture of Rayleigh scattering, Mie scattering, diffraction and reflexion phenomena. The intensity of the scattered light I is a function of the mass of the scattering particles and generally follows an

Table 4
Precision obtained for 6 injections of 0.025 mg/mL (I) and a 0.500 (II) of a test neomycin sulfate solution (0.5 mg/mL) (repeatability per day) and 18 injections on three days (intermediate precision inter-days)

Neomycin B			
	(I) Precision (RSD%)	(II) Precision (RSD%)	Accuracy (%)
Day 1	2.59	1.68	100.33 ± 1.75
Day 2	3.00	1.87	100.19 ± 1.57
Day 3	1.77	1.30	100.36 ± 2.60
Intermediate precision (RSD%)	7.46	4.91	
Neomycin C (II)			
		Precision (RSD%)	Accuracy (%)
Day 1		2.22	100.49 ± 2.44
Day 2		2.80	100.30 ± 2.67
Day 3		2.94	102.72 ± 3.96
Intermediate precision (RSD%)		5.44	
Sulfate (II)			
		Precision (RSD%)	Accuracy (%)
Day 1		1.06	101.26 ± 2.69
Day 2		2.38	101.70 ± 3.49
Day 3		2.17	102.21 ± 3.97
Intermediate precision (RSD%)		4.70	

RSD values for neomycin C at concentration (I) were not calculated as the corresponding peaks were lower than the LOQ. The accuracy was evaluated each day on five concentrations ($n = 3$) covering the entire linearity range and was given as a mean ($n = 15$, $\alpha = 0.05$).

exponential relationship described by the following equation:

$$I = km^b \quad (1)$$

with I the intensity of light, m the mass of the scattering particles, k and b were constants determined principally by the nature of the mobile phase and the detector parameters [23]. b generally varies between 1 and 2 depending on the apparatus conception [24]. If it is equal to 1, the relation becomes linear. Over 2 orders of magnitude, the Eq. (1) is no longer valid and more complex models have to be used [25]. The theoretical Eq. (1) allows the ELSD to give equivalent responses for related structure substances [26], but also whatever the classes of compounds studied [27].

A plot of $\log I$ versus $\log m$ provides a linear response as a plot of the peak area versus the sample concentration in double logarithmic co-ordinates. Such mathematical transformation is allowed by the ICH validation description [21].

The neomycin sulfate linearity study was made by preparing five calibration samples covering the concentration range of 0.005–0.750 mg/mL (1.0–150.0%). The neomycin A linearity study was made between 0.005 and 0.050 mg/mL (1.0–10.0%). A sulfate linearity study was also realized with ammonium sulfate solutions in a concentration range of 0.120–0.250 mg/mL of sulfates. All linearities were determined on three days. The validity of linear models was assessed using classical statistical tests ($n = 3$, $\alpha = 5\%$). Results of regression curves were summarized in Table 5. Good linearities were obtained whatever the solute studied. The response factors for neomycin B, C and A were demonstrated equivalent leading to a possible quantitation of neomycins C and A with dilutions of neomycin B solutions. Regression equations obtained for sulfates content in the neomycin sample and with ammonium sulfate solutions were very closed to each other indicating that the chosen standard is highly adequate for sulfates quantitation.

3.2.5. Limit of detection (LOD)

The limit of detection is defined as the lowest concentration of analyte that can be clearly detected. Its determination could be made by the determination of the signal-to-noise ratio [21]. A ratio of 3 was selected and successive dilutions of the test solution gave a LOD relative to the neomycin B peak of 0.20% (m/m). Such limit is in good agreement with that specified by the European Pharmacopoiea [2,3].

3.3. Analysis of commercial samples

Six commercial samples, five neomycin sulfate (A–E) and one framycetin sulfate (F), were analyzed using the methodology described in this report and with EP methods [2,3]. Each dried sample is described by:

- the sulfates content (ELSD versus EP titrimetric method),
- the related substances content (ELSD versus EP pulsed amperometry method),
- the neomycin B content (ELSD).

Table 5
Regression data for the linearity study of (a) neomycins B, C and sulfate in 0.005–0.75 mg/mL (1.0–150.0%, m/m) neomycin sulfate solution, neomycin A (b) between 0.005 and 0.050 mg/mL (1.0–10.0%, m/m) and (c) sulfate as ammonium salt in a concentration range of 0.120–0.250 mg/mL (20.0–50.0% (m/m) of the test solution) of sulfates

Component	Regression equation	r^2
Neomycin B (a)	$y = (1.473 \pm 0.010)x + (7.729 \pm 0.020)$	0.9994
Neomycin C (a)	$y = (1.435 \pm 0.020)x + (6.416 \pm 0.020)$	0.9982
Sulfates (a)	$y = (1.470 \pm 0.040)x + (7.229 \pm 0.010)$	0.9978
Neomycin A (b)	$y = (1.386 \pm 0.030)x + (7.786 \pm 0.050)$	0.9972
Sulfates (c)	$y = (1.472 \pm 0.040)x + (8.070 \pm 0.030)$	0.9958

The regression curves were obtained by plotting the logarithm of the concentration in mg/mL vs. the logarithm of the peak area on three days. r^2 is the corresponding coefficient of determination.

Table 6
Composition of neomycin sulfate and framycetin sulfate samples determined by the LC-ELSD method and the EP method

		Assay	Related substances				Sulfates
		Neomycin B	Neomycin C	Neomycin A	Any other	Total other	
EP specifications for neomycin sulphate [2]		–	3.0–15.0%	<2.0%	<5.0%	<15.0%	27.0–31.0%
Sample A	ELSD	88.8%	6.0%	0.7%	4 imp	3.0%	30.7%
	EP	–	6.2%	<LOD	4 imp	5.9%	29.6%
Sample B	ELSD	83.8%	8.6%	0.7%	4 imp	2.3%	30.9%
	EP	–	8.8%	<LOD	4 imp	6.2%	29.1%
Sample C	ELSD	89.7%	7.7%	<LOD	4 imp	3.0%	30.9%
	EP	–	7.6%	<LOD	4 imp	6.7%	29.0%
Sample D	ELSD	89.4%	11.9%	0.3%	3 imp	1.9%	30.8%
	EP	–	13.5%	0.5%	3 imp	3.1%	27.7%
Sample E	ELSD	85.9%	11.8%	0.8%	3 imp	2.1%	29.7%
	EP	–	14.0	0.7%	4 imp	4.5%	27.3%
EP specifications for framycetin sulphate [3]			<3.0%	<1.0%	–	<3.0%	27.0–31.0%
Sample F	ELSD	88.8%[a]	1.9%	0.6%	1 imp	0.7%	30.9%
	EP	–	2.4%	0.6%	1 imp	1.7%	28.7%

[a] Used as reference for neomycin B content calculation.

Each sample was first diluted at 0.500 mg/mL for the sulfates and the related substances assay (test solutions). Quantitation of the related substances was made by the application of the double logarithmic regression obtained with the neomycin B peak surface when the sample solution was diluted between 1.0 and 20.0% (v/v). Determination of the sulfates percentage was realized by ammonium sulfate standard solutions corresponding to 20.0–50.0% (m/m) of the test solution.

As illustrated in Table 6, the LC-ELSD method is in good agreement with the European Pharmacopoeia and gave similar results for the related substances test and the sulfates determination for the six samples under study.

Quantitation of the neomycin B content is also proposed. The European Pharmacopoeia indicates a microbiological assay [2,3] which take into account both major neomycins B and C. It could be less time consuming to realize such evaluation with the developed LC-ELSD method. As no commercial reference neomycin B standard exists, we used the framycetin sulfate as reference (neomycin C content <3.0% [3], 88.8% on anhydrous substance). For neomycin B sample quantitation, 10.0% (v/v) dilution of the test concentration was used (0.05 mg/mL). Neomycin B calculation was made by the application of the double logarithmic regression obtained with the neomycin B peak surface for the reference standard in the range of concentration 1.0 and 20.0% (v/v) of the test solution (0.005–0.100 mg/mL). The neomycin B content determined for each sample is indicated in Table 6. As neomycins B and C differs from their biological activities (neomycin C has an activity of 30–50% of that of neomycin B [28]), the neomycin sulfate must be defined only as neomycin B, the neomycin C content has to be determined in the related substances test.

The method described in this report was shown to be suitable for the determination of neomycin B content, specifications of neomycin C and other impurities and sulfates content with a single chromatographic method.

4. Conclusion

The use of ELSD is demonstrated to be suitable to describe a complex pharmaceutical product as well as its related substances and the sulfates content without any derivatization in less than 25 min. The LC–MS–MS study confirms the LC-ELSD chromatographic profile. A methodology for the chromatographic determination of the neomycin B content was also proposed. Such calculation associated with validation criteria obtained led to a possible definition of framycetin sulfate and neomycin sulfate by chromatographic LC-ELSD evaluation. The analysis of commercial samples led to results in good agreement with those obtained by European Pharmacopoeia methodologies.

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