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Short communication

Capillary electrophoresis assay of netilmicin sulphate

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Abstract

An effective method based on capillary electrophoresis (CE) for the determination of netilmicin sulphate in commercial ophthalmic formulations was developed and validated. The use of a polymer-coated capillary and a non-absorbing running buffer permitted the elution of netilmicin in cationic mode at pH 3.0 and with direct UV detection at $\lambda = 195$ nm. Since pre-treatment of the samples is not required, this procedure may be straightforwardly applied to the other aminoglycosides provided that their extinction coefficient is not too low.

Keywords: Netilmicin; Aminoglycosides; Capillary electrophoresis

1. Introduction

Capillary zone electrophoresis is a suitable method for the separation and quantitative assay of aminoglycosides [1,2]. Efficient separation methods have been developed employing standard silica capillaries, provided that the attractive forces among the positively charged test items and the negatively charged capillary walls are suppressed by the addition of suitable complexing agents to the background electrolyte [3]. Using a standard capillary electrophoresis (CE) instrument equipped with a UV detector, those aminoglycosides that do not contain UV-adsorbing chemical functions may still be detected in the indirect UV mode. However, other components that are likely present in the samples (e.g., electrolytes) may interfere with the analysis [4]. Therefore, the indirect UV detection could require a procedure of purification of the sample. To overcome these limitations, either electrochemical detection methods have been applied [5] or the aminoglycosides have been derivatised with substances containing a chromophore [6,7]. With these treatments, the separative capability of different aminoglycosides may likely be reduced in biological samples [8] and the analysis in pharmaceutical formulations will suffer from the interference of the added components that have to be present. Moreover, although derivatisation will increase sensitivity, the stability of the complex substrate–chromophore is rather limited, originating inconveniences, especially in the case of numerous sample sequences [9].

Thus, all the aforementioned methods did not appear fully satisfactory for the quantitative detection of the aminoglycosides in ophthalmic and injectable solutions for quality control and stability testing purposes and we set out to develop a method derived from previous literature on aminoglycosides and suitable for the analysis of netilmicin sulphate in an ophthalmic solution.

Netilmicin, a semisynthetic aminoglycoside antibiotic derivative of sisomicin, is a wide spectrum antibiotic even more effective than the other compounds of the same class such as tobramycin and gentamicin and used as active substance in several ophthalmic and injectable products. It is particularly active against most of the Gram-negative bacteria and many Gram-positive bacteria including *Staphylococcus aureus*. Chemical name of netilmicin is 4-*O*-(2,6-diamino-2,3,4,6-tetradeoxy α-D-glycero-hex-4-enopyranosyl)-1-*N*-ethyl-6-*O*-[4-C-methyl-3-(methylamino)-3-deoxy-β-L-*arabino*-pyranosyl]-2-deoxy-D-streptamine and its structure is reported in pharmacopoeias [10,11]. Netilmicin, similarly to other aminoglycosides, is available in a soluble form complexed with five molecules of H_2SO_4.

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We reasoned that netilmicin's double bond (also present some other aminoglycosides) should be directly detectable in the 190–220 nm wavelength region (provided that the background absorbance is low) without derivatization. Moreover, since conventional fused silica capillaries produce bad peak shapes, we adopted polymer-coated capillaries for the quantitative determination of netilmicin sulphate and its separation from other aminoglycosides. These capillaries contain a chemically bonded and hydrolytically stable layer of polymer that minimizes hydrophobic and electrostatic solute–wall interactions and suppresses the electro-osmotic flow (EOF) [12].

2. Experimental

2.1. Materials

Netilmicin sulphate chemical reference substance (CRS) was purchased from Promochem (Germany). Commercial samples of netilmicin sulphate were purchased from Zhejiang Zhenyuan Pharmaceutical Co. Ltd. (China), streptomycin sulphate from Sigma (Italy). Amikacin sulphate, kanamycin monosulphate were kindly supplied by Deafarma (Milano, Italy). All chemicals used were of analytical grade. Monobasic sodium phosphate·H_2O and disodium phosphate dodecahydrate were purchased from Merck (VWR-International, Germany). Imidazole and glycolic acid are Sigma (Italy) reagents. Chromatography grade water was produced by Milli-Q water purification system (Millipore Corporation, USA). Buffers were filtered through 0.22 μm PVDF filters Millex®-GV Durapore (Millipore Corporation, USA).

2.2. Methods

Capillary electrophoresis experiments were carried out on an HP^{3D}-CE (Agilent Technologies, Germany) equipped with a diode array detector (DAD) and a Chemstation 09.03 Software. The capillary used were polyvinyl alcohol (PVA)-coated, 50 μm internal diameter and 56 cm effective length (total length 64.5 cm) and cross-linked polyethylene glycol (CEP)-coated capillaries, 75 μm internal diameter and 72 cm effective length (total length 80.5 cm) purchased from Agilent Technologies.

For use on the coated capillaries, a 50 mM phosphate running buffer was prepared by dissolving monobasic sodium phosphate·H_2O in water and adjusting pH to 3.0 with H_3PO_4 (85% (w/v)).

Some experiments were carried out with indirect UV detection using PVA-coated capillary and a pH 4 imidazole running buffer (15 mM of imidazol, 25 mM of glycolic acid).

Samples were introduced into the capillary by applying pressure 4 bar for 0.6 s on the anode side and detection was performed at the cathode side with direct UV at a wavelength of 195 nm or with indirect UV at wavelength of 240 nm. Electrophoretic runs were carried out at 40 °C by applying a voltage of 30 kV unless differently stated. After each run, the capillary was thoroughly rinsed with running buffer.

Microbiological assay was carried out on ophthalmic solution according to the European Pharmacopoeia [11].

2.3. Method validation

Validation of CE netilmicin assay was performed on an ophthalmic solution, prepared by dissolving 4.55 mg/ml of netilmicin sulphate, 1.0 g of disodium phosphate dodecahydrate, 0.147 g of monobasic sodium phosphate·H_2O and 2.1 g of sodium citrate·$2H_2O$ and diluted in water to 0.25 mg/ml. CE was carried out with a PVA-coated capillary, phosphate buffer at pH 3.0 and temperature 40 °C, according to the ICH guidelines [13]. Netilmicin concentration in sample solutions was calculated comparing peak areas to the corresponding reference (EP) netilmicin standard solutions, prepared at three concentrations near to the theoretical one (±20%) without using an internal standard.

The *specificity* was assessed by comparing the blank of the ophthalmic solution with the samples in order to demonstrate that the blank did not contain any peak with migration time and absorption coefficient comparable to those of the tested substance.

For the *linearity assay*, concentrations of netilmicin ranged from 2.4 to 3.6 mg/ml: five solutions were prepared solubilising the amounts of netilmicin sulphate and then filling up to the volume with the blank. The assay was repeated three times at each concentration.

Instrumental precision was tested by running 10 injections of the same standard solution, then *repeatibility* or *intra-assay precision* was assessed by carrying out nine injections on three different concentrations (2.4, 3.0 and 3.6 mg/ml).

The *intermediate precision* was obtained re-analyzing the same three solutions previously used to assess repeatability by a second analyst on a different day with independently prepared running buffer and samples.

Accuracy of the method was assessed on the same nine injections necessary to determine repeatability by comparing the mean concentrations found with the theoretical ones and calculating the relative error.

3. Results and discussion

3.1. Method development

In order to determine the separative power of capillary zone electrophoresis in a PVA-coated capillary, a 250 μg/ml mixture of the sulphates of netilmicin, amikacin, streptomicin and kanamicin, where the last is a non-UV adsorbing substance, was tested with the imidazole running buffer and indirect UV detection. The electropherogram obtained is reported in Fig. 1A. In Fig. 1B, the same mixture was eluted on the same capillary with the phosphate running buffer and direct UV detection.

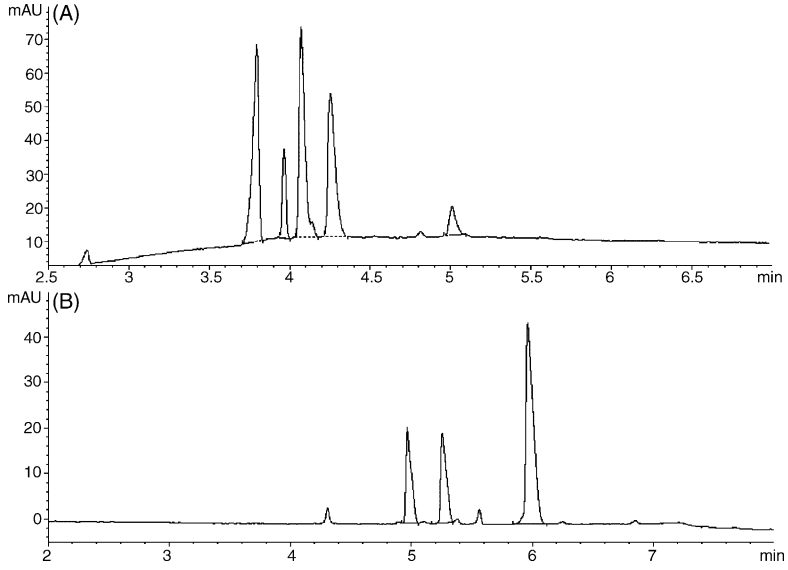

Fig. 1. Electropherograms showing the effects of running buffer on the separation of a mixture of aminoglycosides at a concentration of 250 μg/ml. The peaks correspond to: (1) netilmicin; (2) streptomycin; (3) kanamicin; and (4) amikacin. In (A) an impurity of streptomycin is detected at 5.1 min and in (B) the peak of kanamicin is weakly detected at 5.6 min. Separation medium, voltage and detection: (A) 15 mM imidazole; 25 mM glycolic acid buffer, pH 4.0, 30.0 kV and indirect UV at λ = 240 nm; (B) 50 mM phosphate buffer, pH 3.0, 30 kV and direct UV detection at λ = 195 nm.

The electrophoretic conditions were sufficient for the rapid and complete separation of the mixture of aminoglycosides whereas the direct UV detection is satisfactory only for those substances that contain double bonds.

The use of CEP and PVA capillary coatings was employed for EOF suppression and to control surface adsorptivity.

The verification that the electrostatic interactions among netilmicin and capillary walls were actually suppressed was carried out by comparing the separation parameters of netilmicin sulphate using PVA-coated and CEP-coated capillaries.

The apparent mobility $\mu_a$was calculated from the migration time t by knowing the applied voltage V, the effective capillary length l and the total capillary length L (see Eq. (1)).

$$\mu_a = \frac{lL}{tV} \quad (1)$$

It is worth mentioning that the apparent mobility of netilmicin was different for both the capillaries, indicating that solute–wall interaction may occur. As the pH of the running buffer was increased, the netilmicin sulphate mobility varied differently in the PVA and CEP capillary systems as shown in Fig. 2.

In the case of PVA-coated capillary, the effect of pH on mobility is such that netilmicin mobility increases to some extent with the increase of pH until pH 4 although the net positive charge of the molecule is not expected to increase correspondingly, thereafter netilmicin mobility decreases. When CEP-coated capillary is employed, netilmicin mobility decreases monotonically with pH.

Another observation supporting the presence of specific interactions between the aminoglycoside and the walls of differently coated capillaries is the measurement of amikacin mobility that is reported in Table 1. Amikacin mobility was reduced when processed through PVA capillary with respect to CEP capillary indicating that different aminoglycosides may bear specific interactions with the capillary polymeric coatings.

The interpretation of the above experimental data is complex and the justification of the origin of the solute–wall interactions falls beyond the scope of the present communication. Anyway, the PVA-coated capillary was chosen for method development because peak symmetry of netilmicin was better and the separation between netilmicin and amikacin, gained from the difference of their mobility, was higher than CEP-coated capillary (see Table 1).

For both the capillaries, optimal peaks in terms of width and symmetry were obtained in the range of pH 2.5–4.0. Peak width was measured at half height and peak symmetry (Sf)

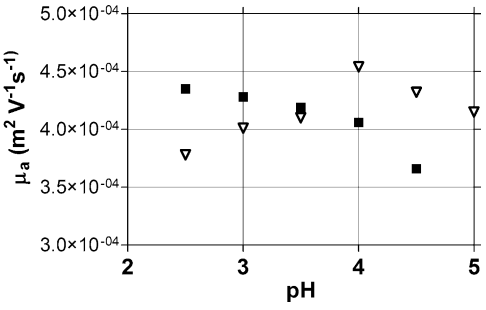

Fig. 2. Dependency of netilmicin sulphate mobility on pH for differently coated columns. Filled squares are referred to CEP-coated capillary and empty triangles are referred to PVA-coated capillary. Conditions: phosphate buffer 50 mM, voltage 30 kV, temperature 40 °C.

Table 1
CE parameters of netilmicin and amikacin sulphates of two polymeric coated capillaries at 40 °C and pH 3.0

	PVA-coated			CEP-coated		
	μ_a (cm^2/(V s))	Peak width (min)	Sf	μ_a (cm^2/(V s))	Peak width (min)	Sf
Netilmicin	4.01×10^{-4}	0.1133	0.40	4.28×10^{-4}	0.1178	0.23
Amikacin	3.42×10^{-4}	0.1533	0.33	4.10×10^{-4}	0.1293	0.23

Table 2
CE parameters of netilmicin sulphate on PVA-coated capillary

pH	μ_a (cm^2/(V s))	Peak width (min)	Sf	μ_a (cm^2/(V s))	Peak width (min)	Sf
	$T = 20$ °C			$T = 30$ °C		
2.5	2.57×10^{-4}	0.1383	0.47	3.15×10^{-4}	0.1227	0.45
3.0	2.74×10^{-4}	0.1347	0.40	3.37×10^{-4}	0.1200	0.38
3.5	2.79×10^{-4}	0.1280	0.37	3.41×10^{-4}	0.1173	0.34
4.0	3.03×10^{-4}	0.1107	0.31	3.71×10^{-4}	0.1067	0.30
	$T = 40$ °C			$T = 50$ °C		
2.5	3.78×10^{-4}	0.1110	0.40	4.47×10^{-4}	0.0967	0.42
3.0	4.01×10^{-4}	0.1133	0.40	4.78×10^{-4}	0.0933	0.42
3.5	4.10×10^{-4}	0.1067	0.33	4.83×10^{-4}	0.0978	0.34
4.0	4.59×10^{-4}	0.0973	0.31	5.54×10^{-4}	0.0610	0.02

Mobility is calculated with the Eq. (1), peak width measured at half height, symmetry (Sf) is calculated by comparing the peak half-widths at 5% of the peak height.

Table 3
Validation results of the netilmicin assay in the ophthalmic solution

Linearity		Accuracy		Intermediate precision		Repeatibility	
Slope:	0.0559	Relative error (%):	0.1%	R.S.D. (%)		R.S.D. (%)	
Intercept:	−10.374	Limit of quantitation		Lower level:	0.48	Lower level:	0.48
Instrumental precision		(μg/ml)	2.5	Central level:	0.15	Central level:	1.14
R.S.D. (%):	0.98			Upper level:	0.70	Upper level:	0.50

was calculated by comparing the peak half-widths at 5% of the peak height as recommended by the FDA.

The effect of the electrostatic interactions with the counter-ions of the buffer electrolyte [14] that affect the analyte mobility was also tested. The higher the ionic strength of the electrolyte solution, the more the counter-ions will shield the analyte ions, thereby reducing their effective charge as shown by measuring netilmicin mobility at constant pH and increasing PO_4 ions concentration. At pH 3.0, 40 °C and 100 mM phosphate buffer, the mobility of netilmicin through the PVA-coated capillary resulted 3.52×10^{-4} cm^2/(V s) corresponding to a change higher than the range under which the mobility varied with pH.

The temperature effect was also investigated at each pH in the range of 20–50 °C. The electrophoretic parameters obtained are reported in Table 2.

The values shown in Table 2 are such that the analysis may be carried out in a satisfactory way in all the range of conditions investigated. The actual method reported in the experimental section was set up also taking into consideration other factors as the capillary stability and buffer preparation.

3.2. Method validation

Examining the data reported in Table 3, it was possible to establish that the method is specific for netilmicin sulphate with no interferences observed from the other components of the formulation. The method is linear in the range considered with the correlation coefficient equal to 0.9997. Recovery does not statistically differ from 100%. A limit of quantitation (peak-threshold ratio 10:1) equal to a dilution of 1200 from the original ophthalmic solution was attained.

Table 4
Comparative CE and microbiological assays of netilmicin sulphate ophthalmic solutions stored in their primary polyethylene containers at 25 °C and 15% relative humidity

	Batch 1			Batch 2		
	Initial[a]	6 months[a]	36 months[a]	Initial[a]	6 months[a]	24 months[a]
CE assay	3.003	3.036	3.069	3.066	3.114	3.036
Microbiology assay	3.051	3.093	2.973	2.964	3.027	3.042

Netilmicin concentration is reported in mg/ml units.
[a] Time after preparation.

The comparison of the CE with the microbial assay on a series of samples put on a stability program showed relative differences between the two methods always of less that 3.5%, thus demonstrating that the two methods are equivalent (see Table 4).

4. Conclusions

The method described in this communication establishes the use of CE for the quantitative assay of netilmicin sulphate in pharmaceutical formulations. The method ruggedness was demonstrated providing evidence that the technique is practicable over a range of capillary, buffer, pH and temperature conditions. Moreover, the sensitivity is sufficiently high for cleaning validation applications. The method does not require sample treatments and allows standard UV detection, thus, permitting easy access to the assay of netilmicin sulphate as well as of those aminoglycosides that bear one or more double bonds.

Although the results of the CE quantitative assay are equivalent to the microbial assay, it should be pointed out that the latter requires a much longer time of analysis and that its reliability is not maintained when the sample concentration is significantly different from the standard ones.

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