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Determination of netilmicin sulfate by liquid chromatography with pulsed electrochemical detection

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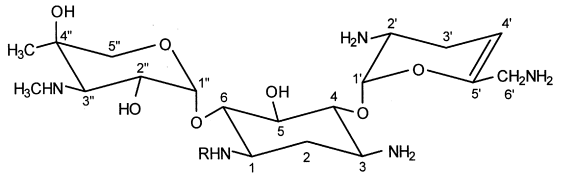
Abstract

The determination of netilmicin sulfate by liquid chromatography using a column packed with poly(styrene–divinylbenzene) and pulsed electrochemical detection on a gold electrode is described. The mobile phase consisted of an aqueous solution containing 35 $g\,l^{-1}$ of sodium sulfate, 0.5 $g\,l^{-1}$ of sodium octanesulfonate, 10 $ml\,l^{-1}$ of tetrahydrofuran and 50 $ml\,l^{-1}$ of 0.2 *M* phosphate buffer (pH 3.0). The total analysis time was not more than 25 min. The effects of the different chromatographic parameters on the separation were also investigated. When a number of commercial samples of netilmicin sulfate was analyzed using this method, eight different components were separated, three of which were of unknown identities.

Keywords: Pharmaceutical analysis; Netilmicin sulfate; Antibiotics; Aminoglycosides; Ethylgaramine; Ethylnetilmicins; Sisomicin

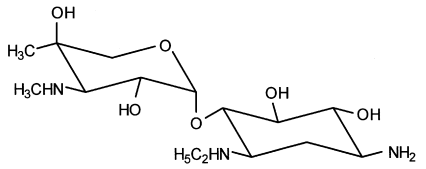
1. Introduction

Netilmicin, which is mainly used as the sulfate, is a semisynthetic, water soluble aminoglycoside antibiotic obtained by chemical modification of sisomicin (Fig. 1) [1]. It is active against both gram-positive and gram-negative bacteria, including strains which are resistant to other aminoglycosides [2–4]. The oto- and nephrotoxicity of netilmicin are substantially lower than those of other aminoglycoside antibiotics [3,4]. Nevertheless, netilmicin still has a narrow therapeutic range and it is necessary to monitor the levels in the blood. For the determination of netilmicin in serum and plasma, gas–liquid chromatography after trimethylsilylation [5] and reversed-phase liquid chromatography (LC) combined with pre-column derivatization with dansyl



netilmicin : R = C_2H_5

sisomicin : R = H



N^1-ethylgaramine

Fig. 1. Structures of some netilmicin components.

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chloride [6], *ortho*-phthalaldehyde (OPA) [7–11], 1-fluoro-2,4-dinitrobenzene [12,13] or OPA-β-mercaptopropionic acid [14] and post-column derivatization with OPA [15–17] have been described. However, no LC method has been described to analyze netilmicin sulfate as a drug substance and to determine possible impurities. The United States Pharmacopeia prescribes a microbiological assay, which is not able to distinguish between the main component and the impurities in the drug [18].

Netilmicin is the N^1-ethyl derivative of sisomicin. Therefore, sisomicin can be expected to be contained as a possible impurity in the samples. The N^3-, $N^{3''}$-, $N^{2'}$- and $N^{6'}$-ethyl derivatives of sisomicin can be also formed during synthesis of netilmicin. The N^3- and $N^{3''}$-ethyl derivatives, which are not active, are normally removed by crystallization [1,19]. Since garamine can be also formed by hydrolysis of sisomicin [20], N^1-ethylgaramine (Fig. 1) may be present in netilmicin sulfate.

In this work an ion-pair LC method using a column packed with poly(styrene–divinylbenzene) is described. The composition of the mobile phase used in this study is based on that previously used for the analysis of two other aminoglycoside antibiotics, neomycin sulfate and kanamycin sulfate [21,22]. Since pre- and post-column derivatization are time consuming and give some problems with quantitation, two other detection techniques were investigated: direct UV detection at 205 nm and pulsed electrochemical detection. The first was chosen because it is mentioned that sisomicin can be detected using direct UV detection at low wavelengths [23] and the latter because of the good results obtained for neomycin and kanamycin [21,22]. Finally, the chosen method has been applied to analyze a number of commercial samples of netilmicin.

2. Experimental

2.1. Reagents and reference substances

Water was distilled twice from glass apparatus. The buffer solution (pH of 3.0) was prepared by mixing 0.2 *M* phosphoric acid and 0.2 *M* potassium dihydrogenphosphate, which were prepared with phosphoric acid 85% (*m*/*m*) (Acros Chimica, Geel, Belgium) and potassium dihydrogenphosphate (BDH, Poole, UK) respectively. Sodium sulfate anhydrous, ethyl acetate, methyl ethyl ketone and tetrahydrofuran, stabilised with 2,6-di-*tert*.-butyl-4-methylphenol were obtained from Merck (Darmstadt, Germany); dimethyl sulfoxide and sodium 1-octanesulfonate, monohydrate 98% from Acros Chimica, acetonitrile and acetone from Rathburn (Walkerburn, UK), 2-methyl-2-propanol from Vel (Leuven, Belgium), and helium from Air Liquide (Machelen, Belgium). The 0.5 *M* sodium hydroxide solution was prepared using 50% (*m*/*m*) sodium hydroxide, aqueous solution (Baker, Deventer, Netherlands).

Sisomicin and N^1-ethylgaramine were obtained from the European Pharmacopoeia laboratory (Ph. Eur., Strasbourg, France) and $N^{2'}$- and $N^{6'}$-ethylnetilmicin from Schering–Plough (Kenilworth, NJ, USA). Commercial samples of netilmicin were provided by the Ph. Eur. and Schering–Plough (Heist-op-den-Berg, Belgium).

2.2. Apparatus

The chromatographic analysis was carried out using a L-6200 Intelligent Pump (Merck–Hitachi, Darmstadt, Germany), a Gilson 234 autoinjector (Villiers-le-Bel, France) with a fixed loop of 20 μl and an electronic integrator HP 3393A (Hewlett–Packard, Avondale, PA, USA). The column (250× 4.6 mm I.D.) was packed with poly(styrene–divinylbenzene) PLRP-S (1000 Å, 8 μm, Polymer Labs., Shropshire, UK). The temperature of the column was maintained at 50°C by immersion in a water bath with a circulator (Julabo, Seelbach, Germany). The UV detector (Spectra Monitor 3100, Milton Roy, Riviera Beach, FL, USA) was set at 205 nm. The PED-1 pulsed electrochemical detector (Dionex, Sunnyvale, CA, USA) was equipped with a gold working electrode, an Ag/AgCl reference electrode and a stainless-steel counter electrode. The 0.5 *M* sodium hydroxide solution was added post-column using a laboratory-made pneumatic device, allowing pulse-free addition of the base. The cell of the pulsed electrochemical detector was placed in a laboratory-made hot air oven to keep the temperature at 35°C.

2.3. Chromatography

The mobile phase consisted of an aqueous solution containing 35 g l^{-1} of sodium sulfate, 0.5 g l^{-1} of sodium 1-octanesulfonate, 10 ml l^{-1} of tetrahydrofuran and 50 ml l^{-1} of 0.2 *M* phosphate buffer (pH 3.0) and was sonicated before use. The flow-rate was 1 ml min^{-1}. All substances to be analyzed were dissolved in the mobile phase. To allow pulsed electrochemical detection, 0.5 *M* NaOH was added post-column (0.3 ml min^{-1}) through a mixing-tee from a helium pressurized reservoir (1.6 bar) and mixed in a packed reaction coil (Dionex, 1.2 m, 500 μl), linking to the electrochemical cell. The flow-rate for the addition of the base is not critical, but it should be reproducible between runs and must be pulse-free. It was necessary to raise the pH of the mobile phase to approximately 13 to improve the sensitivity of the detection [24]. The 0.5 *M* NaOH solution was made starting from a 50% (*m*/*m*) aqueous solution which was pipetted into helium degassed water to avoid carbonates that foul the electrodes. It was advisable to pipette the NaOH solution from the center of the bottle and to use only two-thirds of the bottle [25].

The time and voltage parameters for the pulsed electrochemical detector were the same as previously used for neomycin and kanamycin [21,22] and were set as follows: E_1, E_2 and E_3 were respectively +0.05 V, +0.75 V and −0.15 V with the assigned pulse durations t_1: 0–0.40 s, t_2: 0.41–0.60 s and t_3: 0.61–1.00 s. Integration of the signal was done between 0.20 and 0.40 s.

3. Results and discussion

3.1. Development of the chromatographic method

Poly(styrene–divinylbenzene) was chosen as the stationary phase because of its remarkable stability and batch reproducibility. A chromatogram of netilmicin sulfate, which was obtained with the LC system combined with direct UV detection, gave seven different peaks. However, N^1-ethylgaramine, which has no double bond, could not be detected. A chromatogram obtained by the LC system equipped with pulsed electrochemical detection showed eight peaks, seven corresponding to those observed with UV detection and one corresponding to N^1-ethylgaramine (Fig. 2). Three peaks corresponded to the unknown components which are probably other *N*-ethyl isomers of netilmicin. Further, the method development was examined using the LC system with pulsed electrochemical detection and the influence of the chromatographic parameters on the separation of the netilmicin components was evaluated using the capacity factors (k'). Only one parameter was changed while the others were kept constant. Methanol was used to determine t_0. For the calculation of the k' values, the average retention time of three analyses was used.

As can be seen in Fig. 3, the pH in the range from pH 2.0 to pH 6.0 showed nearly no influence on the k' values of the different netilmicin components, but above pH 4.0, the peak symmetry of the main component was poorer. The influence of the column

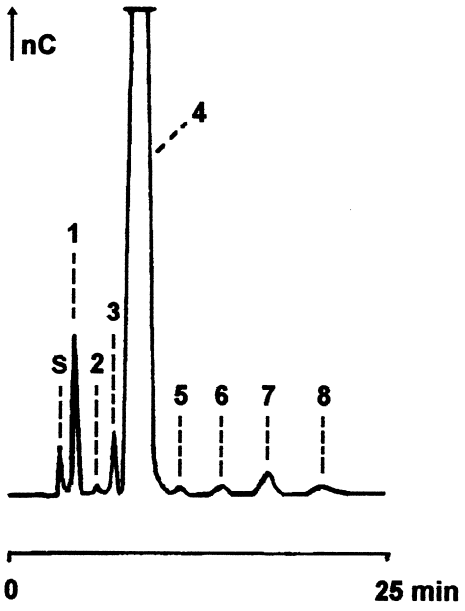

Fig. 2. Typical chromatogram of a commercial sample of netilmicin sulfate obtained with pulsed electrochemical detection. Stationary phase: PLRP-S 1000 Å, 8 μm. Mobile phase: an aqueous solution containing 35 g l^{-1} of sodium sulfate, 0.5 g l^{-1} of sodium octanesulfonate, 10 ml l^{-1} of tetrahydrofuran and 50 ml l^{-1} of 0.2 *M* phosphate buffer (pH 3.0). S=solvent peak; 1=N^1-ethylgaramine; 2=sisomicin; 3=unknown 1; 4=netilmicin; 5=unknown 2; 6=unknown 3; 7=$N^{2'}$-ethylnetilmicin; 8=$N^{6'}$-ethylnetilmicin.

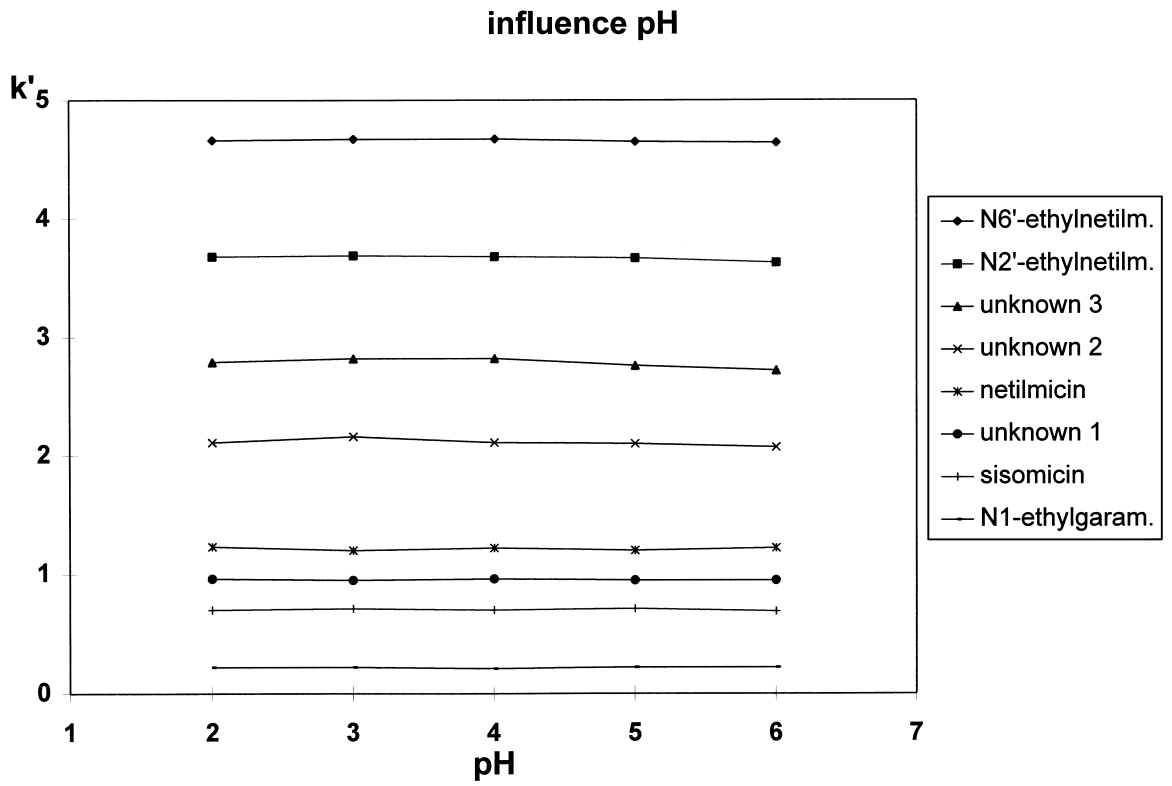

Fig. 3. Influence of the pH of the mobile phase on the k' values.

temperature was examined at 45, 50 and 55°C. As expected, k' values of the components decreased as the column temperature was increased. Sodium octanesulfonate as an ion-pairing agent was added to retain the netilmicin molecules which are positively charged at pH 3.0. The concentration of sodium octanesulfonate in the mobile phase was varied in the range from 0.4 to 0.6 g l^{-1}. As expected, k' values decreased by lowering the concentration of sodium octanesulfonate. The influence of the sodium sulfate in the mobile phase on the k' values of the different netilmicin components was examined in the range from 32 to 38 g l^{-1}. An increase of the sodium sulfate resulted in decreases of k' values. Tetrahydrofuran was added to the mobile phase to improve the peak symmetry of the main peak to 1.8. Without tetrahydrofuran, it was impossible to obtain a peak symmetry of less than 3.0. Other organic modifiers were also investigated. Methanol and ethanol could not be used because they are not compatible with pulsed electrochemical detection. 2-Methyl-2-propanol and acetonitrile gave poor repeatability, probably due to adsorption of the organic solvents to the surface of the gold electrode of the detector [26–28]. Dimethyl sulfoxide caused an unstable baseline and no elution was seen with ethyl acetate. Acetone did not improve the peak symmetry and methyl ethyl ketone decreased the resolution between the impurities.

Using the mobile phase as developed above (see Section 2.3), the chromatographic analysis was also performed by PLRP-S with a smaller pore size of 300 Å. On the narrow pore material the peak symmetry was poor and the retention times were higher. Similar results were observed in the analysis of neomycin [21] and kanamycin [22] as well as in the analysis of macrolide antibiotics like erythromycin [29], tylosin [30] and josamycin [31].

3.2. Robustness

By means of a 4-factorial design, the effect of the individual chromatographic parameters and parameter interactions of the LC method used was studied. The set-up of the applied factorial design was supported by the statistical graphics software system, Statgraphics version 6 (Manugistics, Rockville, MD, USA). The chromatographic parameters examined as variables were the concentration of sodium sulfate and sodium octanesulfonate, the amount of tetrahydrofuran and the column temperature. Since the pH of the mobile phase had nearly no influence on the separation, this variable was omitted in order to reduce the number of experiments. The measured response variables were the retention times of eight peaks on the chromatogram. The values used in the design are shown in Table 1. Two blocks of seventeen experiments were performed. With the measured values of the thirty-four runs, data analysis was performed such as estimation of the effect of each chromatographic parameter, an analysis of variance (ANOVA) table calculation and a standardized pareto chart for each compound. The results showed that the LC system under the examined conditions was principally influenced by the sodium octanesulfonate. Another important chromatographic parameter was tetrahydrofuran. The concentration of sodium sulfate and the column temperature were the third and fourth most important factors which have about the same significance. The interactions between the parameters were not significant. Using the most significant chromatographic parameters as independent variables and the retention times as response variables, a regression model was calculated. From the found regression model, a response surface plot was constructed to help optimizing the parameters, but no further improvement could be made.

3.3. Quantitative aspects of the LC method

For the determination of the impurities a 20 μg sample was examined by injecting 20 μl of a 1.0 mg ml^{-1} netilmicin sulfate solution. The limit of detection for N^1-ethylgaramine was 0.02% (m/m) (4 ng) and for sisomicin 0.03% (m/m) (6 ng), as determined at a signal-to-noise ratio of 3. The limit of quantitation was 0.06% (m/m) for N^1-ethylgaramine (R.S.D.=8.2%; n=4) and 0.1% (m/m) for sisomicin (R.S.D.=10.2%; n=4). The linearity of N^1-ethylgaramine was examined in the concentration range corresponding to 0.06–10% of the sample concentration (1.0 mg ml^{-1}). The following results were found: y =735 907x+541; r=0.9994 and $S_{y,x}$=949, where y=peak area/1000; x= concentration in mg ml^{-1}; r=coefficient of correlation and $S_{y,x}$=standard error of estimate. For sisomicin, the linearity was examined in the range from 0.10 to 10% of the sample concentration (1.0 mg ml^{-1}), giving the following results: y= 364 580x+868; r=0.9991 and $S_{y,x}$=623. Since there is no good linearity in the range from 0.20 to 1.2 mg ml^{-1} for the determination of the netilmicin content, probably due to overloading of the electrodes, the concentration range corresponding to 20 to 120% of the sample concentration 0.10 mg ml^{-1} (2 μg injected) was examined. The following results were found: y=374 695x+1105; r =0.9992 and $S_{y,x}$=716. The repeatability was checked by analyzing a 0.10 mg ml^{-1} solution of netilmicin sulfate six times. The R.S.D. on the area of netilmicin was 1.8%.

3.4. Analysis of commercial samples

Seven commercial samples of netilmicin sulfate were analyzed using the described method. The

Table 1
Factorial analysis: nominal values corresponding to −1, 0 and +1

Chromatographic parameters	Low value (−1)	Central value (0)	High value (+1)
Sodium sulfate (g l^{-1})	32	35	38
Sodium octanesulfonate (g l^{-1})	0.4	0.5	0.6
Tetrahydrofuran (ml l^{-1})	9	10	11
Column temperature (°C)	45	50	55

Table 2
Composition of netilmicin sulfate samples (%), relative to netilmicin

	N^1-Ethylgaramine	Sisomicin	Unknown 1	Unknown 2	Unknown 3	$N^{2'}$-Ethylnetilmicin	$N^{6'}$-Ethylnetilmicin
1	1.71	±0.03	0.69	0.07	0.22	0.65	0.26
2	1.72	±0.03	0.71	0.08	0.17	0.81	0.40
3	1.57	0.04	0.47	±0.05	0.18	0.57	0.37
4	1.72	±0.03	0.58	0.12	0.28	0.64	0.24
5	0.47	0.05	0.62	0.08	±0.04	0.36	0.29
6	0.36	±0.03	0.62	±0.05	±0.04	0.85	0.67
7	3.00	0.06	0.74	0.08	0.33	0.59	0.09

obtained composition of the samples is shown in Table 2. All minor components are expressed as the relative amounts of netilmicin, using chromatograms obtained with a 5% (m/v) dilution (0.05 mg ml^{-1}) of the examined sample. The following contents were found in the seven samples analyzed: 0.36–3.00% of N^1-ethylgaramine, 0.03–0.06% of sisomicin, 0.47–0.74% of unknown 1, 0.05–0.12% of unknown 2, 0.04–0.33% of unknown 3, 0.36–0.85% of $N^{2'}$-ethylnetilmicin and 0.09–0.67% of $N^{6'}$-ethylnetilmicin. N^1-ethyl-garamine was found to be the major impurity, which was not detected using a LC system with direct UV detection. It is also noteworthy that the examined samples contained nearly no sisomicin.

4. Conclusion

The method using poly(styrene–divinylbenzene) as the stationary phase allowed to separate eight components of netilmicin. The total time of analysis was not more than 25 min. Pulsed electrochemical detection suffers from some stability problems and some experience is required to obtain a good reproducibility, but it was preferred to direct UV detection because the latter method showed only seven of the eight peaks, neglecting N^1-ethylgaramine as the major impurity.

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