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Analysis of a formulation containing lincomycin and spectinomycin by liquid chromatography with pulsed electrochemical detection

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Abstract

A reversed phase ion-pair liquid chromatographic method using a base deactivated column and pulsed electrochemical detection on a gold electrode is described. It allows the separation of a mixture of spectinomycin sulfate, lincomycin hydrochloride and their related substances. A step gradient was necessary to obtain a good separation together with a reasonable analysis time of 40 min. The mobile phases consisted of an aqueous solution of 3.3 or 0.55 g/l pentanesulfonic acid, 10 mM acetic acid and 20 ml/l tetrahydrofuran. Both mobile phases were adjusted to pH 4.0 with diluted sodium hydroxide. The influence of the different chromatographic parameters on the separation was investigated. Two commercial samples were analyzed using the described method. In total 12 components could be separated. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Lincomycin; Spectinomycin; Liquid chromatography; Pulsed electrochemical detection

1. Introduction

A combination of two antibiotics, lincomycin and spectinomycin (in a ratio 1:2), is used for veterinary purposes. The water soluble mixture is added to the drinking water of the animals. It is mainly indicated in the prevention and treatment of chronic respiratory disease of growing poultry.

Lincomycin is a medium spectrum antibiotic, which is produced by *Streptomyces lincolnensis*. Common impurities which are formed during biosynthesis and which are found in lincomycin samples are lincomycin B and 7-epilincomycin [1]. A principal degradation product of lincomycin is methyl-1-thiolincosamide (MTL) which can be formed by cleavage at the amide group (Fig. 1). For the determination of lincomycin, capillary zone electrophoresis in combination with amperometric detection [2] as well as reversed phase liquid chromatography (LC) combined with UV detection at low wavelengths [1,3–5] and electrochemical detection [6] have been described. Only

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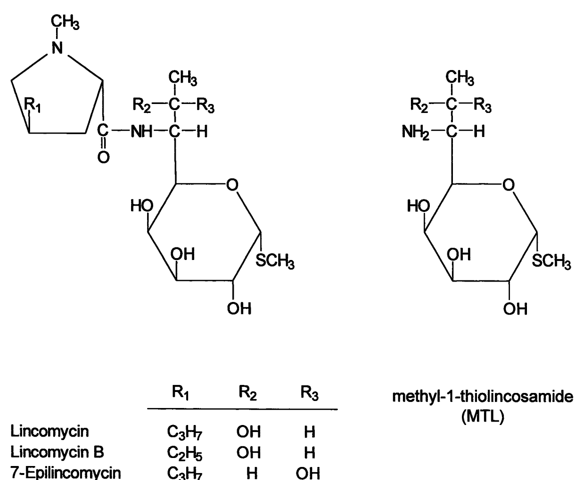
one of these methods reported the simultaneous separation of lincomycin, lincomycin B and 7-epilincomycin [1].

Spectinomycin is an aminoglycoside like antibiotic produced by *Streptomyces spectabilis*. In solution, spectinomycin will undergo a ring opening and closing of the hemiketal function, resulting in an equilibrium mixture of four possible anomers. Hydrolysis with acid produces actinamine and in basic solutions actinospectinoic acid (ASA) is formed. Important fermentation impurities are dihydrospectinomycin and dihydroxyspectinomycin (Fig. 2) [7,8]. Since spectinomycin and its related substances lack a significant UV absorbing chromophore, LC combined with pre- or post-column derivatization [9,10] as well as electrochemical detection has been described [8,11]. Since the repeatability of the results using controlled potential coulometry was poor, pulsed electrochemical detection (PED) was preferred [8].

In this work, an ion-pair LC method allowing the simultaneous determination of lincomycin, spectinomycin and their related substances is described. As stationary phase, a column packed with base deactivated reversed phase silica gel is used, but also other types were examined. Since lincomycin and spectinomycin have only a weak or no UV absorbing chromophore, PED was chosen. This approach allows the detection of both antibiotics, eliminating the difficulties associated with pre- and post-column derivatization. Finally, the chosen method has been applied to analyze some commercial mixtures of lincomycin and spectinomycin.



	R_1	R_2	R_3
Lincomycin	C_3H_7	OH	H
Lincomycin B	C_2H_5	OH	H
7-Epilincomycin	C_3H_7	H	OH

Fig. 1. Structure of lincomycin and some related compounds.

2. Experimental

2.1. Reagents and samples

Water was distilled twice from glass apparatus. Acetic acid was obtained from Riedel-de-Haën (Seelze, Germany); 1-pentanesulfonic acid, sodium salt monohydrate and trifluoroacetic acid from Acros Organics (Geel, Belgium); methanesulfonic acid and tetrahydrofuran (THF) stabilized with 2,6-di-tert-butyl-4-methylphenol from Merck (Darmstadt, Germany); heptafluorobutyric acid (HFBA) from Sigma-Aldrich (Steinham, Germany) and helium from Air Liquide (Machelen, Belgium). The 0.5 M sodium hydroxide solution was made using 50% (m/m) sodium hydroxide, aqueous solution (Baker, Deventer, the Netherlands).

Spectinomycin sulfate, ASA sulfate, actinamine hydrochloride, dihydrospectinomycin dihydrochloride, lincomycin hydrochloride, lincomycin B hydrochloride, 7-epilincomycin hydrochloride and MTL as well as commercial samples were obtained from a pharmaceutical company.

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 3393 A (Hewlett-Packard, Avondale, PA). The Supelcosil LC-ABZ + Plus column (250 mm × 4.6 mm I.D.), packed with base deactivated reversed phase silica gel (5 μm), was obtained from Supelco (Bellefonte, PA). The temperature of the column was maintained at 45 °C by immersion in a water bath with a heating circulator (Julabo, Seelbach, Germany). Other base deactivated columns (250 mm × 4.6 mm I.D.) used were: Hypersil BDS, 5 μm (Shandon, Eng-

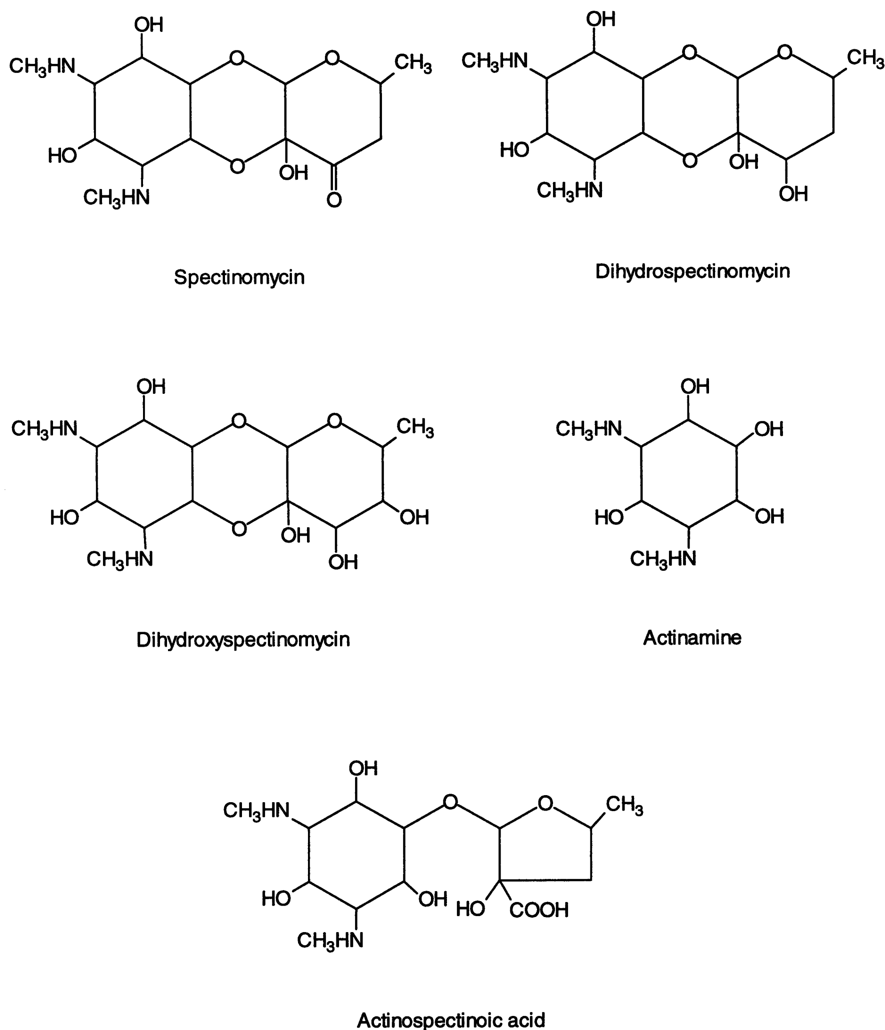


Fig. 2. Structure of spectinomycin and some related compounds.

land) and Spherisorb S5-ODS-B, 5 μm (PhaseSep, Queensferry, UK). Sodium hydroxide was added post-column using a laboratory-made pneumatic device. The PED-1 pulsed electrochemical detector from Dionex (Sunnyvale, CA) was equipped with a gold working electrode with a diameter of 3 mm, a Ag/AgCl reference electrode and a stainless steel counter electrode. 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

An overview of the chromatographic conditions is given in Scheme 1. A step gradient was necessary to obtain a good separation between the first eluted compounds and to elute the others within a reasonable analysis time. Mobile phase A consisted of an aqueous solution containing 10 mM acetic acid, 3.3 g/l pentanesulfonic acid and 20 ml/l THF. Mobile phase B consisted of an aqueous solution containing 10 mM acetic acid,

0.55 g/l pentanesulfonic acid and 20 ml/l THF. Before bringing up to volume, both solutions were adjusted to pH 4.0 using diluted sodium hydroxide. The mobile phases were degassed with helium and sonicated before use. The gradient chosen is shown in Scheme 1. The flow rate was 1 ml/min. All substances to be analyzed were dissolved in a mixture of mobile phases A and B (70:30). For method development a solution of a mixture of lincomycin and spectinomycin (1:2), spiked with the related substances available, was used to be sure that no potential impurity was overlooked. The solution was also left for several hours so that the anomers of spectinomycin could be formed. However, the solutions used to determine the quantitative aspects and the commercial samples were dissolved into the mixture of mobile phases A and B (70:30) just before injection to reduce the formation of spectinomycin anomers. Through a mixing tee 0.5 M sodium hydroxide was added post-column from a helium-pressurized reservoir (1.6 bar) and mixed in a packed reaction coil (1.2

Scheme 1. LC conditions

Stationary phase : Supelcosil LC-ABZ + plus, 5 μm, 250 × 4.6 mm I.D.

Mobile phase :

	A	B
Acetic acid (mM)	10	10
Pentanesulfonic acid (g/l)	3.3	0.55
THF (ml/l)	20	20
Adjust the pH to	4.0	4.0
Water up to	1 liter	1 liter

Gradient :

0 – 12.0 min.	70 % A – 30 % B
12.1 – 32.0 min.	100 % B
32.1 – 40.0 min.	70 % A – 30 % B

Flow rate : 1 ml/min

Injection volume : 20 μl

Column temperature : 45 °C

Post-column addition of 0.5 M NaOH : 0.3 ml/min

Pulsed electrochemical detection :

Working electrode : Au (with a diameter of 3 mm)
Reference electrode : Ag/AgCl
Counter electrode : stainless steel

Detector settings :

t (s)	E (volt)
0 – 0.40	0.05
0.41 – 0.60	0.75
0.61 – 1.00	– 0.15

Integration period : 0.20 – 0.40 s
Sensitivity : 1 μC
The detector was kept at 35 °C.

m, 500 μl) from Dionex, which was linked to the electrochemical cell. The post-column addition of the base must be pulse-free and is necessary to raise the pH of the mobile phase to approximately 13 to improve the sensitivity of the detection [12]. The base was added at a flow rate of 0.3 ml/min. Although this flow rate is not critical, it should be constant. The sodium hydroxide solution was made starting from a 50% (m/m) aqueous solution, which was pipetted into helium degassed water because it is very important to avoid carbonates that foul the electrodes. For this reason it is advisable to pipette the sodium hydroxide solution from the center of the bottle and to use only two thirds of the bottle [13].

The time and voltage parameters for the detector are also shown in Scheme 1 and were the same as used for aminoglycoside antibiotics [14–18]. Although the sequence of the potentials theoretically cleans the electrode surface, it is necessary to polish the gold working electrode after about 60 analyses to obtain a good repeatability. Dionex provides for this purpose a special kit containing an eraser, a fine and a coarse polishing compound. In most cases the use of a wet tissue, the eraser or the fine polishing compound is sufficient. Only when this is not effective, the coarse polishing compound is used. The cleaning of the gold electrode must be done carefully to avoid pits or scratches. After the electrode is cleaned with polishing compound, it is rinsed with deionized water and sonicated for 10 min to remove trace particles from the surface. It takes about 2 h to obtain a stable baseline with a freshly polished electrode. It is also advisable to wipe the counter and reference electrode at the same time with a wet tissue to remove deposited substances.

3. Results and discussion

3.1. Chromatographic method

Since most of the methods published for the determination of lincomycin [1,3,6] and spectinomycin [10,11] used ion-pair reversed phase LC, this type of chromatography was also examined for the analysis of the mixture of both compounds. Although acetonitrile was mostly used as organic modifier [1,3,6,11], it gave poor repeatability in combination with PED, probably due to adsorption of the organic solvent to the surface of the gold electrode of the detector [19–21]. Based on the good results obtained for THF in combination with PED, THF was chosen as organic modifier [16,18]. Since the retention of the lincomycin components on the column was much stronger than that of the spectinomycin components, a gradient was necessary. A gradient using different concentrations of organic modifier was not satisfactory. The best results related to overall separation and baseline shift were obtained using a gradient based on different concentrations of sodium pentanesulfonate. Other ion-pairing agents were examined too: HFBA, trifluoroacetic acid and methanesulfonic acid. However, the selectivity was worse and/or important baseline disturbance after the application of the gradient step occurred, making the quantitation of lincomycin and 7-epilincomycin difficult. A gradient using a combination of two different ion-pairing agents, a long and a short chain, resulted also in a big baseline shift.

A column packed with base deactivated reversed phase silica gel was chosen because it was mentioned that the separation of lincomycin and 7-epilincomycin was only possible on such a column [1]. This material was found to give a good selectivity and peak symmetry. Besides the Supelcosil LC-ABZ + Plus column finally chosen, the chromatography was also performed using two other base deactivated columns: Hypersil BDS, 5 μm and Spherisorb S5-ODS-B, 5 μm. The retention times, peak symmetry and resolution obtained on these columns were similar to those obtained on the Supelcosil column. However, the latter showed the best overall resolution and less disturbances in the baseline as a result of the gradient.

A typical chromatogram of a mixture of lincomycin and spectinomycin, spiked with their related substances, obtained under the selected chromatographic conditions is shown in Fig. 3. Two peaks correspond to components of unknown identity. One of them probably corresponds to dihydroxyspectinomycin.

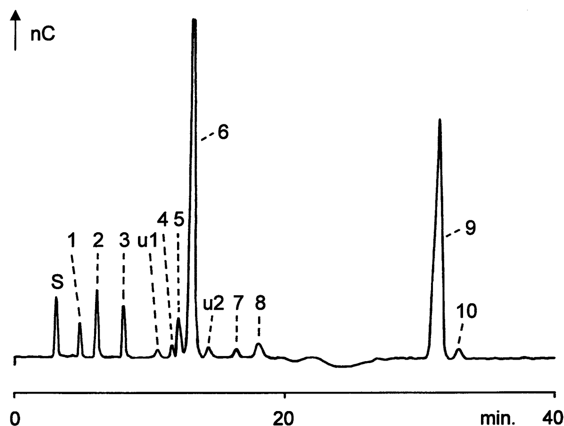


Fig. 3. Typical chromatogram of a mixture of lincomycin and spectinomycin, spiked with their related substances, 48 h after dissolution in a mixture of mobile phases A and B (70:30). S – solvent; 1 – actinospectinoic acid (ASA); 2 – methyl-1-thiolincosamide (MTL); 3 – actinamine; u1 – unknown 1; 4 and 5 – anomers of spectinomycin; 6 – spectinomycin; u2 – unknown 2; 7 – dihydrospectinomycin; 8 – lincomycin B; 9 – lincomycin; 10 – 7-epilincomycin.

Other time and voltage parameters, proposed for the detection of spectinomycin, were also examined: $E_1 = +0.12$ V, $E_2 = +0.70$ V and $E_3 = -0.60$ V with: t_1: 0.00–0.72 s, t_2: 0.73–0.90 s, t_3: 0.91–1.26 s. Integration of the signal occurred between 0.52 and 0.72 s [22]. These settings increased slightly the signals measured, but the noise also increased and a discoloration of the gold electrode was observed after a few analyses. Consequently, the settings mentioned in Scheme 1 were preferred.

The influence of the different chromatographic parameters on the separation was evaluated using the retention factors (k'). Only one parameter was changed while the others were kept constant. Methanol was used to determine t_0. The pH of the mobile phase was varied in the range from 3 to 4.5 (Fig. 4). ASA is the component which is most influenced by changements in pH. The best overall resolution between the peaks was obtained at pH 4.0. The influence of the column temperature was examined at 35, 45 and 55 °C. As expected, the k' values of the components decreased as the column temperature was increased. At 35 °C, poor peak symmetry for lincomycin and lincomycin B was obtained. At 55 °C the anomers of spectinomycin were formed faster so that a temperature of 45 °C was preferred. The concentration of acetic acid was varied in the range from 8 to 12 mM. Nearly no influence on the k' values of the different components was observed. The influence of THF was examined at 15, 20 and 25 ml/l. An increase in THF resulted in a decrease of the k' values. At a concentration of 25 ml/l THF, the selectivity was insufficient, while at 15 ml/l the total time of analysis was unnecessary long.

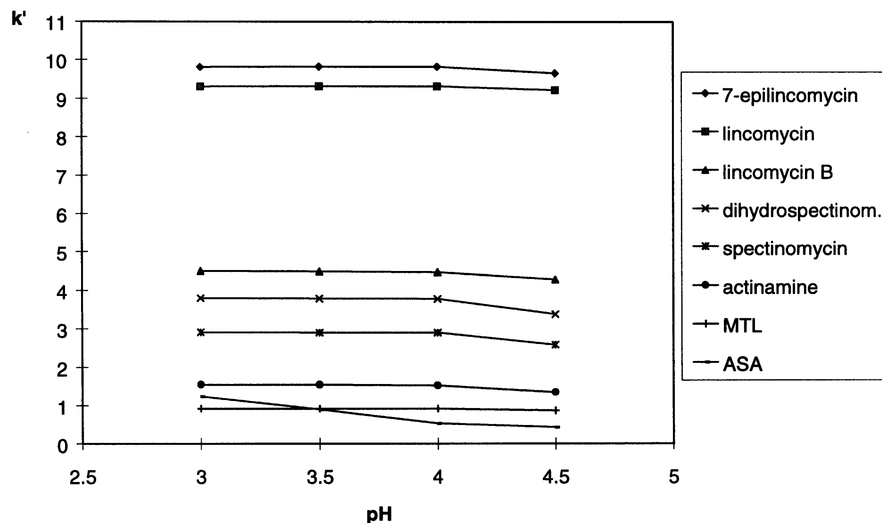


Fig. 4. Influence of the pH of the mobile phase on the k' values.

Table 1
Quantitative aspects of the system

	LOD (ng)	LOQ (ng)	Linearity			
			Range (μg)	y	r	$S_{y,x}$
ASA	3.5	10	0.01–0.35	$53115x+49$	0.9995	243
Actinamine	1.4	4.2	0.004–0.28	$181475x-567$	0.9999	167
MTL	0.7	1.8	0.002–0.18	$151095x-72$	0.9999	192
Lincomycin			0.14–0.84	$68365x+920$	0.9994	1165
Spectinomycin			0.28–1.68	$47535x+2484$	0.9990	1531
Spectinomycin			0.01–0.10	$48765x+111$	0.9995	281

3.2. Quantitative aspects of the LC method

For the analysis of a mixture lincomycin–spectinomycin (1:2), an amount corresponding to 0.7 μg lincomycin hydrochloride and 1.4 μg of spectinomycin sulfate was injected. For these quantities the limit of detection (LOD, S/N = 3) and the limit of quantification (LOQ) for ASA sulfate, actinamine hydrochloride and MTL were determined. The results are shown in Table 1. Linearity results for several components are also shown in Table 1, where y = peak area/1000, x = amount of sample injected (μg), r = coefficient of correlation and $S_{y,x}$ = standard error of estimate. The repeatability was checked by analyzing the mixture six times. The R.S.D. on the peak area was 1.1% for lincomycin and 1.7% for spectinomycin. The accuracy was checked at 100 and 50% of the nominal amounts injected (0.7 μg for lincomycin hydrochloride and 1.4 μg for spectinomycin sulfate). The biases were −0.5% (100% lincomycin), −2.1% (100% spectinomycin), +3.2% (50% lincomycin) and −0.6% (50% spectinomycin).

3.3. Analysis of samples

Since it is mentioned that spectinomycin can give rise to four possible anomers, their formation after dissolution in the mobile phase (70% A–30% B) was examined. The content of the main peak after 24 h was 98% and after 48 h 96.5%. Consequently, it is preferable to dissolve the sample just before injection.

Two commercial products containing both lincomycin and spectinomycin (1:2) were analyzed using the described method. Assay of the samples (content of lincomycin and spectinomycin against label) was carried out using a reference mixture containing both a lincomycin hydrochloride and a spectinomycin sulfate reference sample (1:2). In order to avoid the injection of known amounts of standards for all related substances, those related to lincomycin (MTL, lincomycin B and 7-epilincomycin) are expressed as lincomycin and the other as spectinomycin. To be able to calculate the low content of impurities more precisely, a 5% (v/v) dilution of the lincomycin–spectinomycin reference mixture was used. The results are shown in Table 2. As can be noticed, the formulations analyzed contain only a few impurities. However, since lincomycin and spectinomycin are both fermentation products, they may contain higher amounts of impurities than those found in the commercial samples analyzed in this study. The amount of impurities may also vary with the origin (manufacturer) of the product. It is therefore useful to have a selective method with the ability to detect trace contaminants when present.

4. Conclusion

The method described, using a base deactivated column as stationary phase and PED, allows the separation of lincomycin and spectinomycin from their potential impurities. The total time of analysis is 40 min. The method shows a good selectivity, repeatability and linearity.

Table 2
Content (%) of lincomycin, spectinomycin and their related substances in commercial products

Sample	Actinamine[a]	Unknown 1[a]	Unknown 2[a]	Lincomycin[b]	Spectinomycin[b]
1	1.9	3.1	1.3	101.0	101.1
2	0.9	1.6	0.9	113.3	96.0

[a] Expressed as spectinomycin.
[b] Against label.

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