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Analysis of spectinomycin by liquid chromatography with pulsed electrochemical detection

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Abstract

Until now, no LC method is described to determine the purity and content of spectinomycin without prior derivatization. A reversed-phase ion-pair LC method using a base deactivated column and pulsed electrochemical detection is described. The mobile phase consisted of an aqueous solution containing 5.8 g/l pentafluoropropionic acid, 1.25 g/l potassium dihydrogen phosphate and 5.5 ml/l tetrahydrofuran. The pH was adjusted to 6.25 using dilute NaOH solution. An experimental design was used to optimize the chromatographic parameters and to check the robustness. The quality of separation was investigated on different stationary phases. The method allows the separation of spectinomycin from its related substances as well as some other components of unknown identity. The total time of analysis is 65 min. A number of commercial samples were examined using this method.

Keywords: Electrochemical detection; Detection, LC; Pharmaceutical analysis; Spectinomycin; Antibiotics

1. Introduction

Spectinomycin is an antibacterial compound produced by *Streptomyces spectabilis*, *Streptomyces flavopersicus* and other related *Streptomyces* species [1]. It is active against a wide variety of Gram-positive and Gram-negative organisms [2]. It is mainly used in veterinary medicine and in the treatment of gonorrhea.

In solution, spectinomycin undergoes a ring opening and closure of the hemiketal function as shown in Fig. 1. This results in an equilibrium mixture of four possible anomers, which are not necessarily co-eluted in a chromatographic system. Acid hydrolysis produces actinamine and basic degradation leads to actinospectinoic acid. Important fermentation impurities are the dihydrospectinomycins and dihydroxyspectinomycin [1,3]. As can be seen, neither spectinomycin nor its related substances contain a significant UV absorbing chromophore. Ion-pair liquid chromatography with post-column derivatization with *o*-phthalaldehyde and fluorometric detection has been described [4]. In 1985, a normal-phase liquid chromatographic method was described using pre-column derivatization with 2-naphthalene-

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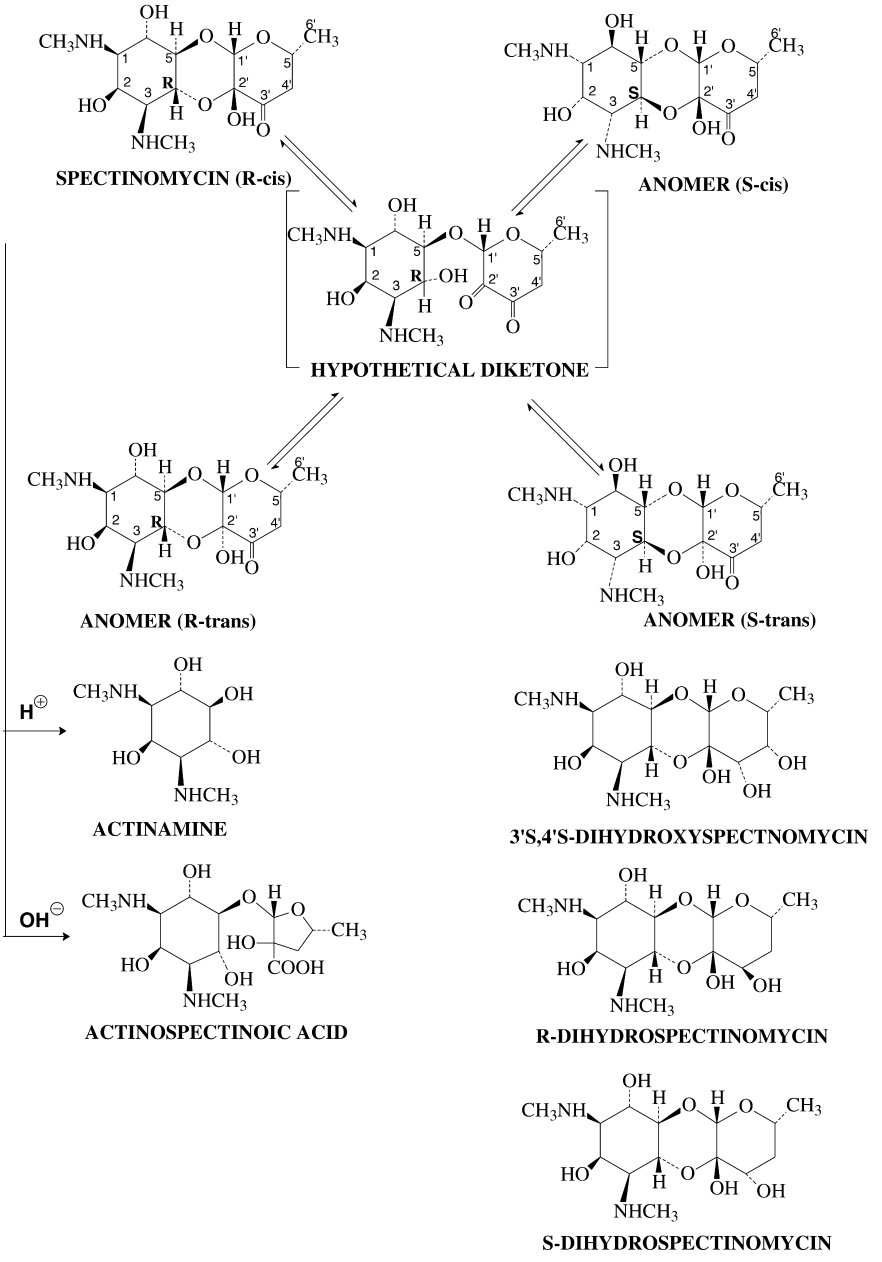

Fig. 1. Structure of spectinomycin, its anomers and its major impurities.

sulfonyl chloride [5]. Since pre- and post-column derivatization are cumbersome, electrochemical detection has also been investigated following reversed-phase [6,7] or cation-exchange chromatography [8]. Since the repeatability of the results using controlled potential coulometry was poor [6], pulsed electrochemical detection (PED) was preferred [8]. The European Pharmacopoeia (Ph. Eur.) [9] as well as the United States Pharmacopeia (USP) [10] prescribe gas chromatography after silylation as

assay method [11]. Thin-layer chromatography is prescribed in the Ph. Eur. for the determination of the related substances [9].

In this paper, reversed-phase ion-pair chromatography combined with PED is described. The two mobile phases that were investigated are based on a method for the analysis of a mixture of lincomycin and spectinomycin [7] and on a method proposed by a pharmaceutical company. The better method was further optimized using an experimental design and the robustness was tested. The quality of separation on different stationary phases was compared. Finally, the method chosen has been applied to analyze some commercial samples of spectinomycin.

2. Experimental

2.1. Reagents and samples

Water was distilled twice from glass apparatus. Acetic acid was obtained from Riedel-deHaën (Seelze, Germany), sodium 1-pentanesulfonate monohydrate and pentafluoropropionic acid from Acros Organics (Geel, Belgium), tetrahydrofuran (THF) stabilized with 2,6-di-*tert*.-butyl-4-methylphenol and potassium dihydrogenphosphate from Merck (Darmstadt, Germany) and helium from Air Liquide (Machelen, Belgium). The 0.5 *M* NaOH solution was made using 50% (m/m) NaOH, aqueous solution from Baker (Deventer, Netherlands).

Spectinomycin dihydrochloride, actinospectinoic acid sulfate, actinamine hydrochloride, (*R*)- and (*S*)-dihydrospectinomycin as well as some commercial samples were obtained from Abbott (North Chicago, IL, USA) and Pharmacia and Upjohn (Puurs, Belgium and Kalamazoo, MI, USA). The European Pharmacopoeia Chemical Reference Substance (Ph. Eur. CRS) of spectinomycin dihydrochloride (79.2%, m/m, $C_{14}H_{26}Cl_2N_2O_7$, as is) was used as a standard.

2.2. Apparatus

Chromatographic analyses were 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 3395 A (Hewlett-Packard, Avondale, PA, USA). The chosen Hypersil BDS column (250×4.6 mm I.D.), packed with base deactivated reversed-phase silica gel (5 μm) was obtained from Thermo Hypersil (Runcorn, UK). The temperature of the column was maintained by immersion in a water bath with a heating circulator (Julabo, Seelbach, Germany) or, when necessary, with a cooling system Julabo F-10 (Julabo). Other base deactivated columns (250×4.6 mm I.D.) used were Supelcosil LC-ABZ+ plus and Supelcosil LC-ABZ (Supelco, Bellefonte, PA, USA), Chromspher 5B (Chrompack, Middelburg, Netherlands), Inertsil ODS-2 (Alltech, Deerfield, IL, USA) and Sperisorb S5-ODS-B (Phase Sep, Queensferry, UK). Sodium hydroxide was added post-column using a laboratory-made pneumatic device. The PED-1 pulsed electrochemical detector from Dionex (Sunnyvale, CA, USA) was equipped with a golden working electrode with a diameter of 3 mm, an 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. Sample preparation and chromatography

All substances to be analyzed were dissolved (0.5 mg/ml) in mobile phase to avoid interfering system peaks during analysis. The spectinomycin solutions were left for 2 h at room temperature so that equilibrium between the main compound and its anomers was reached. An overview of the LC conditions finally chosen is given in Table 1. Before bringing up to volume, the mobile phase was adjusted to pH 6.25 using dilute NaOH after which it was degassed with helium. Through a mixing tee, 0.5 *M* NaOH was added post-column from a helium-pressurized reservoir (1.6 bar) and mixed in a packed reaction coil (length 1.2 m, internal volume 500 μl) from Dionex, which was linked to the electrochemical cell. The post-column addition of the base (0.3 ml/min) 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]. Although the flow-rate for the addition of the base is not critical, it should be constant. The NaOH solution was prepared starting from a 50% (m/m) aqueous solution, which was pipetted into helium

Table 1
LC conditions finally chosen

Stationary phase	Hypersil BDS, 5 μm, 250×4.6 mm I.D.	
Mobile phase		
Tetrahydrofuran (ml/l)	5.5	
Pentafluoropropionic acid (g/l)	5.8	
Potassium dihydrogen phosphate (g/l)	1.25	
Add 900 ml of water and adjust the pH (NaOH) to	6.25	
Water up to	1 l	
Flow rate	1 ml/min	
Injection volume	20 μl	
Column temperature	17.5 °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* (V)
	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		

degassed water to avoid carbonates that foul the electrode. For this reason, it is advisable to pipette the NaOH solution from the center of the bottle and to use only two thirds of the bottle.

The time and voltage parameters for the detector are also shown in Table 1 and were the same as used before for similar components, like aminoglycoside antibiotics [13–17]. Although the sequence of the potentials theoretically cleans the electrode surface, it is necessary to polish the golden working electrode after about 60 analyses to obtain a good repeatability. After the electrode is cleaned with fine polishing compound, it is sonicated in water for 10 min. It is advisable to wipe the counter and reference electrodes at the same time with a tissue to remove deposited substances. It takes about 2 h to obtain a stable baseline with a freshly polished electrode.

2.4. Software

The experimental design and optimization were performed by multivariate analysis using Modde 4.0 software (Umetri, Umeå, Sweden).

3. Results and discussion

3.1. Choice of the initial chromatographic method

Based on the good results obtained in house for the analysis of a mixture of lincomycin and spectinomycin [7], method 1 was developed. Method 2 was proposed by a pharmaceutical company. Both methods used base deactivated reversed-phase silica gel as the stationary phase and PED for detection.

The mobile phase of method 1 contained 20 ml/l THF, 2.47 g/l sodium pentanesulfonate and 10 m*M* acetic acid. Before bringing to volume, the solution was adjusted to pH 4.0 using dilute NaOH. Using this type of mobile phase, four columns were examined at 45 °C: Supelcosil LC-ABZ+ plus, Supelcosil LC-ABZ, Sperisorb S5-OBS-B and Hypersil BDS. The latter gave clearly better overall resolution and was the only column that allowed the separation of spectinomycin from two of its anomers. In the case of co-elution, the anomers appear as shoulders on the front of the main peak. This method was improved by increasing the amount of sodium pentanesulfonate to 2.8 g/l and by decreasing the column temperature to 20 °C.

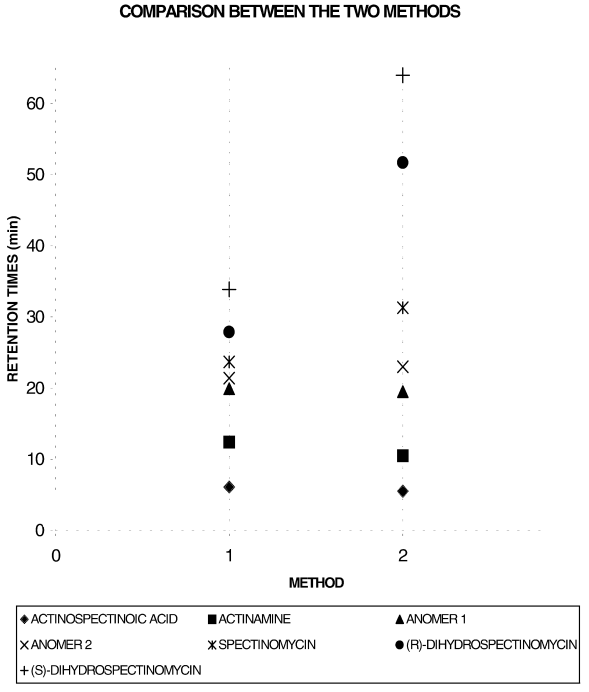

Fig. 2. Comparison between method 1 and method 2 for the components that could be identified. Stationary phase: Hypersil BDS, 5 μm, 250×4.6 mm I.D. at 20 °C (method 1) and 17.5 °C (method 2). Mobile phase: an aqueous solution containing 20 ml/l of tetrahydrofuran, 2.8 g/l of sodium pentanesulfonate and 10 m*M* acetic acid, adjusted to pH 4.0 with dilute NaOH (method 1) and an aqueous solution containing 5.5 ml/l of tetrahydrofuran, 5.8 g/l of pentafluoropropionic acid and 1.25 g/l of potassium-dihydrogen phosphate, adjusted to pH 6.25 with dilute NaOH (method 2). The other chromatographic parameters are the same for both methods and are presented in Table 1.

To test the mobile phase proposed by the company, the Hypersil BDS column, maintained at 20 °C, was used. The mobile phase was composed of 92 volumes of an aqueous solution containing 1.36 g/l of potassium dihydrogen phosphate and 4.0 ml/l (=6.3 g/l) of pentafluoropropionic acid and eight volumes of acetonitrile. The pH of the mobile phase was adjusted to 6.0 with ammonium hydroxide. Originally, this mobile phase was coupled with refractive index detection, but was here combined with PED. However, acetonitrile gives poor quantitative repeatability in combination with PED, probably due to adsorption of the organic solvent to the electrode surface of the detector [18–20]. Based on the good results obtained for THF in combination with PED [15,17], acetonitrile was replaced by THF in the mobile phase. The amount of THF was chosen so that it gave a retention time for the main peak similar to that obtained with method 1. The ammonium hydroxide used to adjust the pH gave a problem similar to that of acetonitrile and therefore NaOH was used instead. This gave a more stable baseline and higher responses. So, the adapted mobile phase of method 2 consisted of an aqueous solution containing 1.25 g/l potassium dihydrogen phosphate, 5.5 ml/l THF and 5.8 g/l pentafluoropropionic acid. Before bringing to volume, the solution was adjusted to pH 6.0 using dilute NaOH.

A comparison between method 1 and method 2 for the peaks that could be identified is shown in Fig. 2. It can be seen that, although the analysis time of method 2 is longer, it gives a clearly better separation of the known compounds. Method 2 shows also an additional advantage because it allows separating a supplementary peak just before the main peak. As a conclusion, method 2 was preferred above method 1.

3.2. Method optimization

Method 2 was further optimized. A chromatogram of a typical commercial spectinomycin sample, obtained under the optimized chromatographic conditions is shown in Fig. 3. Five of the 12 peaks correspond to components of unknown identity. Dihydroxyspectinomycin was not available as a reference substance and therefore it could not be identified in the chromatogram.

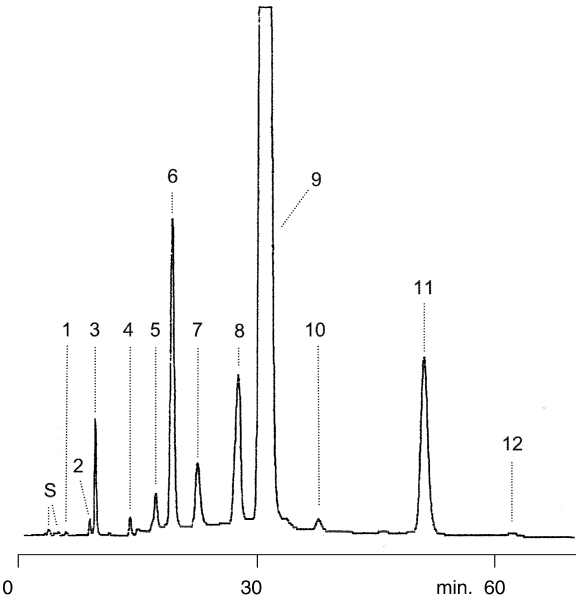

Fig. 3. Chromatogram of a commercial spectinomycin sample (see Table 1 for chromatographic conditions). *S* = Solvent, 1 = actinospectinoic acid, 2 = unknown 1, 3 = actinamine, 4 = unknown 2, 5 = unknown 3, 6 = anomer 1, 7 = anomer 2, 8 = unknown 4, 9 = spectinomycin, 10 = unknown 5, 11 = (*R*)-dihydrospectinomycin, 12 = (*S*)-dihydrospectinomycin.

In a first step of the optimization process, the influence of the chromatographic parameters on the separation was investigated using an experimental design. The principal idea of an experimental design is to vary all relevant parameters or factors simultaneously over a number of previously planned experiments. The data obtained are used to build a mathematical model that allows predicting, interpreting and optimizing the results. In this study, the importance of four chromatographic parameters was examined: the concentration of THF and pentafluoropropionic acid, the column temperature and the pH of the mobile phase. The values used in the design are shown in Table 2. The concentration of the buffer was not taken into account, because it was found in screening experiments that the effect of small deviations from the nominal value on the separation was negligible. As response variables, the selectivity factors for the most critical peak pairs were used: unknown 2, anomer 1 (α_{u2-a1}); anomer 1, anomer 2 (α_{a1-a2}); and unknown 3, spectinomycin (α_{u3-sp}). Although the ranges between the levels examined were rather small (Table 2), it has to be mentioned that it was not always easy to determine the retention times of the unknown components in the chromatogram under the varying chromatographic conditions of the experimental design.

A central composite design was used, requiring 27 experiments in total ($2^k + 2k + 3$; with k the number of factors), including three times the central level. The collected experimental data were fitted by a partial least square (PLS) model. The statistical relationship between the measured values y and the parameters x_i, x_j, ... can be described by a Taylor expansion (see [21]): $y = \beta_0 + \beta_i x_i + \beta_j x_j + \beta_{ij} x_i x_j + \beta_{ii} x_i^2 + \beta_{jj} x_j^2 + \cdots + E$ where β is the regression coefficient and E the overall experimental error. The single term of each factor describes the linear effect on the response, the square term the non-linear effect and the cross term of two different variables the effect of their interaction. Fig. 4 illustrates the regression coefficient plots for $\alpha_{u2-a1,}$ α_{a1-a2} and α_{u3-sp}. The 95% confidence interval is expressed as an error bar over the coefficient bar. If the coefficient bar is smaller than the interval, the variation of the response caused by varying the parameter is smaller

Table 2
Range of the chromatographic parameters studied in the experimental design to investigate their importance and their robustness

	Chromatographic parameter	Low level	Central level	High level
– ∘ –	Tetrahydrofuran (ml/l)	5.0	5.5	6.0
	Pentafluoropropionic acid (g/l)	5.6	5.8	6.0
	pH of the mobile phase	5.75	6.00	6.25
	Column temperature (°C)	17.5	20.0	22.5
—	pH of the mobile phase	6.00	6.25	6.50
	Column temperature (°C)	15.0	17.5	20.0

– ∘ –, Parameter importance; —, Parameter robustness.

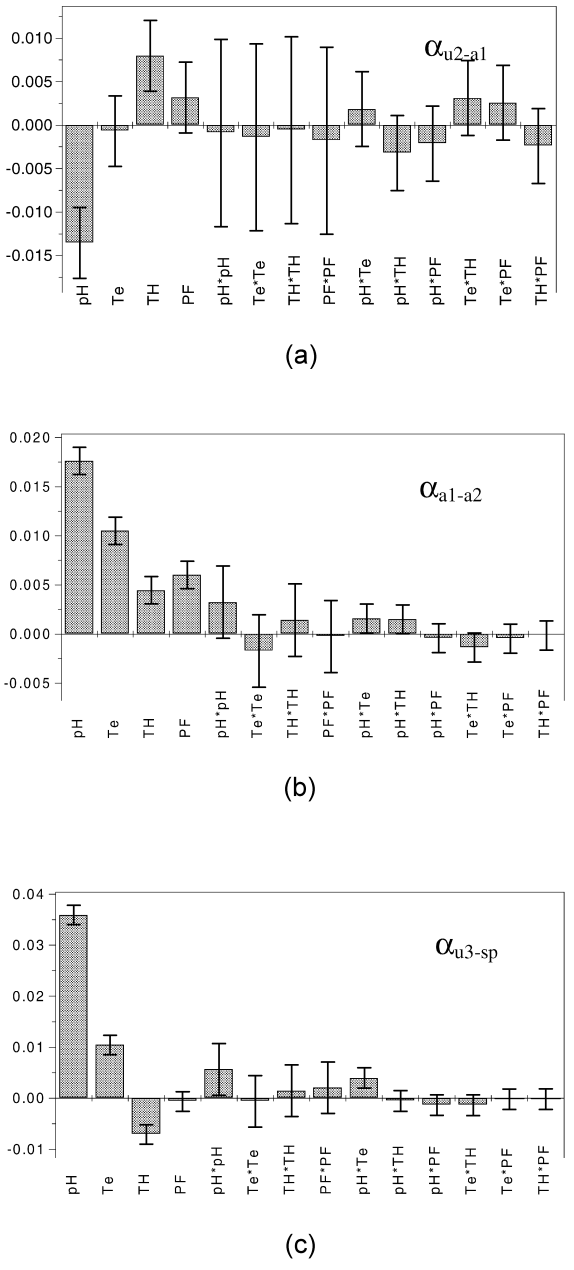

Fig. 4. Regression coefficient plots for the selectivity factors: (a) α_{u2-a1}, (b) α_{a1-a2} and (c) α_{u3-sp}. Te: column temperature, TH: tetrahydrofuran, PF: pentafluoropropionic acid.

than the experimental error. Therefore the variable is considered insignificant.

Under the experimental conditions examined, α_{u2-a1}, α_{a1-a2} as well as α_{u3-sp} are mostly influenced by the pH of the mobile phase. However, the pH has a positive effect on α_{a1-a2} and α_{u3-sp}, but a negative on α_{u2-a1}. This means that the separation between the two anomers and between unknown 3 and spectinomycin will improve when the pH is increased, but at the same time the separation between unknown 2 and anomer 1 will become poorer. The second most important factor is the column temperature, which has only a significant positive effect on α_{a1-a2} and $\alpha_{u3-sp.}$ THF has a small positive effect on α_{u2-a1} and α_{a1-a2} and a small negative effect on α_{u3-sp}. The amount of pentafluoropropionic acid has only a small significant positive effect on α_{a1-a2}. No important non-linear effects or interactions were noticed. Response surface plots can be constructed by plotting the selectivity factor as a function of the most important factors. Fig. 5 shows the response surface plots for $\alpha_{u2-a1,}$ α_{a1-a2} and for α_{u3-sp} as a function of the pH and the column temperature. The concentration of the other factors was kept constant at the nominal values.

Compared to pH 6.00, a better overall separation is obtained at pH 6.25: the selectivities between the two anomer peaks and between unknown 3 and spectinomycin improve as mentioned above, while the peaks corresponding to unknown 2 and anomer 1 are somewhat less good, but still baseline separated (Fig. 3). Although the column temperature has a positive effect on α_{a1-a2} and α_{u3-sp}, it was noticed that at higher column temperatures (30 °C was also examined in an additional experiment), the baseline increased considerably between anomer 1 and the main peak. This is probably caused by a faster equilibration rate of the anomers during analysis on the column. Since baseline shifts make accurate quantitative work more difficult and since the separation at the column temperature of the lower level of the experimental design was still sufficient, 17.5 °C was used in further experiments. The concentration of THF and pentafluoropropionic acid were maintained at 5.5 ml/l and 5.8 g/l, respectively, because their influence on the separation was small in the range examined.

3.3. Robustness

Since the newly chosen values for pH (6.25) and column temperature (17.5 °C) corresponded to the

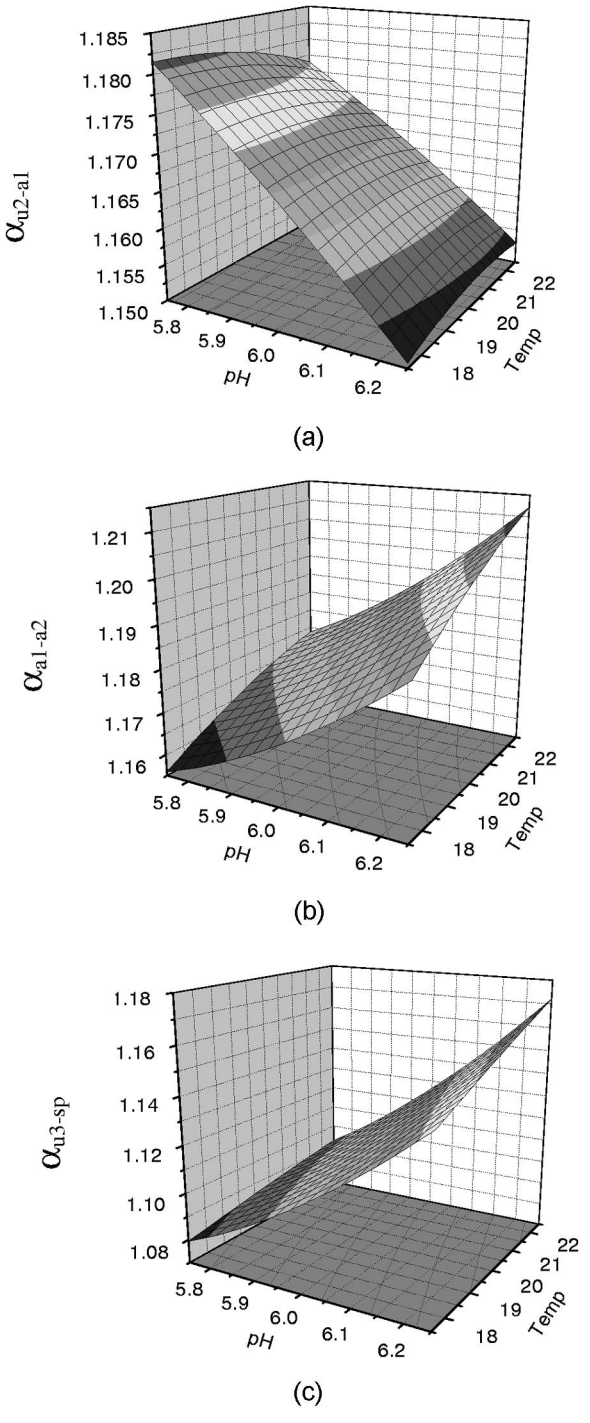

Fig. 5. Response surface plots showing how the selectivity factors (a) α_{u2-a1}, (b) α_{a1-a2} and (c) α_{u3-sp} vary as a function of the pH and the column temperature. The concentration of tetrahydrofuran and of pentafluoropropionic acid were kept constant at 5.5 ml/l and 5.8 g/l, respectively.

limits examined in the experimental design, a second experimental design was set up to check the robustness of these two chromatographic parameters and eventually to improve them further. The ranges examined are shown in Table 2. As expected, the regression coefficients and response surface plots were similar to those obtained in the first design. It was observed that at pH 6.50 the separation between unknown 2 and anomer 1 was only just acceptable. An additional experiment at pH 6.75 revealed that there was no baseline separation anymore between these two peaks. In comparison to the pH, the column temperature has a smaller, positive influence on the three selectivity factors studied. More important however is its influence on the baseline. Therefore, 17.5 °C was accepted as a good compromise between separation and baseline disturbance. The finally chosen experimental conditions are summarized in Table 1. The method was proven to be robust in the following ranges: 6.0–6.5 for the pH, 15–20 °C for the column temperature, 5.0–6.0 ml/l for THF and 5.6–6.0 g/l for pentafluoropropionic acid. Using the optimized method conditions, the quality of separation on four other base deactivated stationary phases was examined: Supelcosil LC-ABZ+ plus, Supelcosil LC-ABZ, Sperisorb S5-OBS-B and Inertsil ODS-2. The overall separation on the first three columns was not sufficient and peak symmetry was poor. Only Inertsil ODS-2 gave a result similar to the Hypersil column, but the analysis time was 75 min. By increasing the THF concentration to 10 ml/l, comparable retention times and selectivities as on Hypersil are obtained.

3.4. Quantitative aspects of the LC method

For the determination of the impurities in spectinomycin a 10 μg sample was used by injecting 20 μl of a 0.5 mg/ml solution. For this quantity the limit of detection (LOD, $S/N=3$) and the limit of quantification (LOQ, $S/N=10$) for actinospectinoic acid, actinamine, spectinomycin, (*R*)-dihydrospectinomycin and (*S*)-dihydrospectinomycin were determined. The results are shown in Table 3, 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 a 0.5 mg/ml solution of spectinomycin six

Table 3
Quantitative aspects of the method

	LOD (μg) (100% = 10 μg)	LOQ (μg) (100% = 10 μg)	Linearity			
			Range (μg)	y	r	$S_{y,x}$
Spectinomycin	0.0010 (0.01%)	0.0030 (0.03%)	6–12.5 (60–125%)	$41990x + 11558$	0.99946	3648
			0.0050–0.5 (0.05–5%)	$47671x + 146$	0.99943	336
Actinospectinoic acid	0.0010 (0.01%)	0.0030 (0.03%)	0.0030–0.5 (0.03–5%)	$54430x - 46$	0.99995	115
Actinamine	0.0005 (0.005%)	0.0015 (0.015%)	0.0015–0.5 (0.015–5%)	$172629x + 63$	0.99998	193
(*R*)-Dihydrospectinomycin	0.0015 (0.015%)	0.0050 (0.05%)	0.0050–0.5 (0.05–5%)	$37379x + 163$	0.99982	150
(*S*)-Dihydrospectinomycin	0.0050 (0.05%)	0.0075 (0.075%)	0.0075–0.5 (0.075–5%)	$33305x + 226$	0.99967	179

times. The RSD on the sum of the areas of the main peak and its two anomers was 1.3%. The linearity of different spectinomycin compounds was examined in the following concentration ranges, relative to the sample concentration (0.5 mg/ml as 100%): 0.03–5% for actinospectinoic acid, 0.015–5% for actinamine, 0.05–5% for (*R*)-dihydrospectinomycin and 0.075–5% for (*S*)-dihydrospectinomycin. For spectinomycin, including anomers the linearity was examined in two concentration ranges: 0.05–5% and 60–125%. In the investigated ranges six concentration points ($n = 2$) were included for all the compounds. The developed method shows a good linearity in the ranges examined. For spectinomycin, it can be seen that the slope varies with the concentration range. This is probably due to saturation effects on the golden working electrode. It was also observed that the slope values for the different compounds are strongly dependent on the detector conditions. Therefore, when performing analyses using PED, standard solutions and solutions to be analyzed should be injected alternately.

3.5. Analysis of samples

Commercial spectinomycin samples, obtained from two different manufacturers, were analyzed using the described method. The composition of two commercial samples is shown in Table 4. All the contents (%, m/m) are expressed as spectinomycin dihydrochloride on the substance as is and calculated with reference to spectinomycin dihydrochloride Ph. Eur. CRS (79.2% m/m $C_{14}H_{26}Cl_2N_2O_7$, as is). The value of 79.2% was attributed to the spectinomycin peak, including the anomers of the CRS. The content of the minor components was calculated using reference chromatograms obtained with a 1% dilution (0.005 mg/ml) of the spectinomycin CRS. As can be seen, the composition of spectinomycin samples is dependent on the origin. Different batches from the same manufacturer had a similar composition.

4. Conclusion

A liquid chromatographic method for the analysis of spectinomycin has been developed. The method was optimized and verified as being robust, using an experimental design. The pH was found to be the most influencing factor, which has to be carefully controlled. The total time of analysis is 65 min. It is

Table 4
Composition of commercial spectinomycin samples (%, m/m) from different origins, expressed as spectinomycin dihydrochloride on the substance as is[a]

Sample	Content (%, m/m)									
	Act.ac.	Unk. 1	Act.	Unk. 2	Unk. 3	Unk. 4	Sp. + an.	Unk. 5	(*R*) DHS	(*S*) DHS
A	<LOQ	0.06	0.55	0.14	0.39	1.94	70.4	0.20	3.52	0.11
B	0.18	ND	0.41	ND	0.91	0.42	69.5	ND	0.55	ND

[a] Act.ac. = actinospectinoic acid; Act. = actinamine; Sp. + an. = spectinomycin including anomers; (*R*) DHS = (*R*)-dihydrospectinomycin; (*S*) DHS = (*S*)-dihydrospectinomycin; Unk. = unknown; ND = not detected (<LOD).

the first time that quantitative results are reported for so many spectinomycin components. Pulsed electrochemical detection requires some experience to obtain a good repeatability, but it allows for sensitive detection without derivatization. This method will be proposed for use in the European Pharmacopoeia and in the United States Pharmacopeia.

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