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Development and validation of a high-performance liquid chromatographic stability-indicating method for the analysis of Synercid® in quality control, stability and compatibility studies

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Abstract

A gradient high-performance liquid chromatographic (HPLC) method was developed for the analysis of Synercid® freeze-dried powder in routine quality control, stability and compatibility studies. This method is suited for a simultaneous assay of drug substances and impurities. The method was validated for precision, reproducibility, linearity, accuracy and limits of detection. The robustness study that was performed according to an experimental design is described. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: HPLC; Method validation; Robustness; Experimental design; Streptogramin; Antibiotic; Quinupristin; Dalfopristin; Synercid®

1. Introduction

Synercid® is a new streptogramin antibacterial agent produced by fermentation-semisynthesis and is available for intravenous administration [1,2]. It was shown to be efficient against a bacterium that fails to respond to vancomycin, a standard treatment [3,4]. The Synercid® sterile formulation contains two semisynthetic [5] pristinamycin derivatives, quinupristin and dalfopristin in the ratio 30:70 w/w. In addition to the drug substances, the formulation contains several related substances that can originate from different sources: natural by-products of fermentation, semisynthesis-related impurities, degradation products generated in bulk drug substances and degradation product arising in the drug product during the manufacturing process or during storage.

Some high-performance liquid chromatographic (HPLC) methods have been previously described in pharmacokinetic studies [6,7] but are directed toward for the analysis of Synercid® in

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biological fluids. The difficulties encountered to develop a quantitative HPLC method for a simultaneous analysis of the drug substances and the related substances arise from the high number of compounds to separate and from the great difference in polarity between quinupristin derivated-compounds and dalfopristin derivated-compounds.

This paper describes the gradient HPLC method developed for the analysis of Synercid® freeze-dried powders in routine quality control and stability studies. This method was slightly extended to study the stability of Synercid® solutions under the clinical conditions of administration as well as the compatibility of Synercid® with other I.V. drugs during *Y*-site simulated injection [8,9]. The method is shown to be precise, accurate, specific, sensitive and robust. The experimental design used to study robustness is described with the selection of the potential critical factors and their levels, the presentation of experiments, the statistical analysis of the main effects of the factors and the interpretation of the results.

2. Experimental

2.1. Equipment

The chromatographic hardware consisted of a Varian Model 9012 pump (Les Ulis, France), an autosampler allowing sample refrigeration (Waters Model 717 plus, St.-Quentin-en Yvelines, France), a Milton Roy (TSP, Les Ulis, France) spectromonitor Model 3100 UV detector, and a Prolabo stabitherm oven (Paris, France) for column temperature control. Integration and data storage were carried out on a VG Multichrom system (Fisons Instruments, Chesline, UK). An Hewlett Packard Model 1050 photodiode-array detector (Les Ulis, France) was used when assessing the specificity of the method and when analyzing mixtures between Synercid® and another drug during compatibility studies. The final choice for the column was a LiChrospher-100 RP18 cartridge (125×4 mm I.D. column) (Merck, catalogue reference 50943, Darmstadt, Germany) with a particle diameter of 5 μm.

2.2. Reagents and chemicals

Potassium dihydrogen phosphate and concentrated phosphoric acid were of analytical reagent grade (Prolabo). Acetonitrile was of HPLC grade (Distrilab, Caen, France). Glucose 5% in purified water was of pharmaceutical grade (Baxter). Purified water was obtained from a Millipore system. Synercid® 500 mg was supplied by Rhône-Poulenc Rorer (Antony, France). In Synercid® 500 mg freeze-dried preparations, the two drug substances quinupristin and dalfopristin are salified by methanesulfonic acid. The pH of the formulation is ~ 4.75. The vials are overfilled so that the mean content is ~ 550 mg per vial, for a mean mass of ~ 650 mg. The solution reconstituted with 5.0 ml of 5% glucose solution contains 500 mg of Synercid® per 5 ml (150 mg of quinupristin + 350 mg of dalfopristin). The working standard is a Synercid® 500 mg freeze-dried preparation.

2.3. Chromatographic conditions

An aqueous buffer was prepared by dissolving 4.08 g (3×10^{-2} mol) of monobasic potassium phosphate in 1 l of purified water and adjusting the pH to 2.9 with phosphoric acid. Mobile phase A was prepared by adding 200 ml acetonitrile to 800 ml buffer solution. Mobile phase B was prepared by adding 650 ml acetonitrile to 350 ml buffer solution. Each mobile phase was degassed before use. The mobile phase program included consecutive linear gradients of 100–34% mobile phase A in 42.5 min, 34–100% mobile phase A in a further 1.5 min and reequilibration of the column with 100% mobile phase A for 5.0 min. The 5 min was validated and the results showed that this time is sufficient for column equilibration.

A flow rate of 1.1 ml min^{-1} was used throughout the analysis. Samples in the autosampler were refrigerated at 4–10°C. The column was regulated at 40 ± 1°C. The injection volume was 10 μl and the wavelength of the UV absorbance detector was 254 nm.

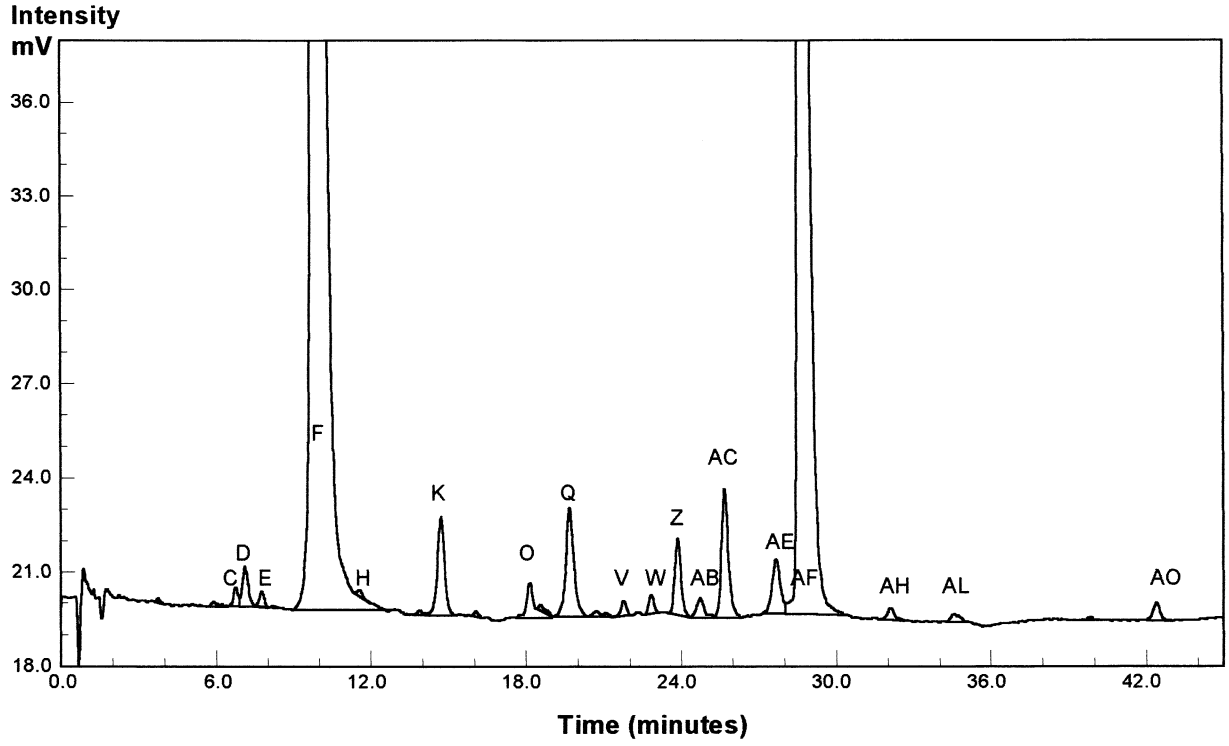


Fig. 1. Typical chromatogram of Synercid®.

2.4. Standard and sample solution preparation

The reference solution was prepared using one vial of Synercid® 500 mg freeze-dried working standard. The vial content was dissolved by adding 5.0 ml of 5% glucose solution. After dissolution, an aliquot was extemporaneously withdrawn and diluted 100 times with mobile phase A. The test solution was prepared using five vials of Synercid® 500 mg freeze-dried preparation. The contents of the vials were each dissolved with 5.0 ml of 5% glucose solution, then pooled and extemporaneously diluted 100 times with mobile phase A. The reference and test solutions were prepared in duplicate. In all cases, the mass of freeze-dried product was precisely calculated by weighing the vials before use and after. For reasons of stability, the total time for preparation and analysis did not exceed 36 h with the solutions stored between 4 and 10°C.

The chromatographic method is suited to analyze diluted infusion solutions of Synercid® but the preparation of the injected solutions differs slightly since the solutions are already diluted. When evaluating the stability of Synercid® solutions diluted at the concentration of 2 mg ml^{-1} in glucose 5% (500 mg in 250 ml soft PVC infusion bag), no dilution is carried out in the mobile phase but the injection volume is reduced to 5 μl in order to obtain the same injected quantity. The reference solution dilutions are modified accordingly. When assessing the compatibility between 2 mg ml^{-1} Synercid® solutions and another I.V. drug, no dilution is carried out in the mobile phase and the injected volume remains 10 μl. A diode-array detector is used instead of a monowavelength UV detector, in order to quantify both co-administered drugs at the appropriate wavelength.

2.5. Assay procedure

A typical chromatogram of Synercid® is shown in Fig. 1. The identification of the peaks (RP code and letter) is given in Table 1.

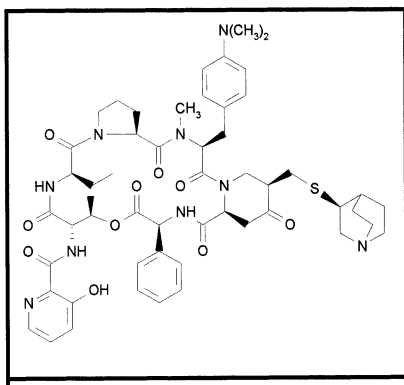
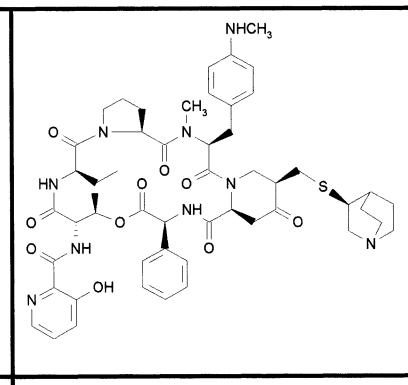
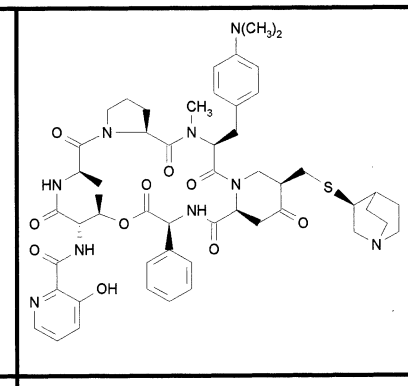
Table 1
Identification of the chromatographic peaks of Synercid®

Name	Peak	Group	Retention time (min)	RRT (relative to dalfopristin)	RRT (relative to AF)	Typical area (%)
	A	PII	5.3	0.67		<0.1
	B	PII	5.6	0.71		<0.1
RP 75645	C	PII	5.7–6.5	0.70–0.75		0.3
RP 54474	D	PII	6.0–6.8	0.74–0.79		0.6
	E	PII	6.7–7.5	0.82–0.84		0.1
RP 54476	F	PII	7.8–9.2	1		71
(shoulder)	G	PII				
RP 51159	H	PII	9.6–10.8	1.17–1.22		0.1
	I	PII	10.5–12.1	1.32–1.35		<0.1
	J	PII	11.6	1.46		<0.1
RP 75646	K	PII	11.8–13.7	1.50–1.56		0.8
(shoulder)	L	PII				
RPR 116368	M	PI	13.5–14.7	1.71	0.52	<0.1
	N	PII	15.1–16.4	1.9	0.57	0.1
RP 60183+RPR 116367	O	PII+PI	14.9–17.4	1.9–2.0	0.58–0.63	0.3
	P	PII	16.3	2.05	0.62	0.2
RP 60182	Q	PII	16.7–18.7	2.0–2.3	0.63–0.68	1.0
	R	PII	18.0		0.68	<0.1
	S	PII	18.4–20.6		0.70–0.73	0.1
(shoulder)	T	PII				
(shoulder)	U	PII				
RPR 122558	V	PII	19.2		0.73	0.2
	W	PII	19.7		0.75	0.2
	X	PII	20.3		0.77	0.1
	Y	PII	20.6		0.78	0.1
RP 12536	Z	PII	20.3–23		0.79–0.84	1.0
(shoulder)	AA	PII				
RP 67648 (PIC)	AB	PI	22.1–24.1		0.85–0.87	0.4
RP 60844 (PIB)	AC	PI	22.9–25.0		0.88–0.90	4
(shoulder)	AD	PI				
RP 69991	AE	PI	25.0–27.3		0.96–0.98	0.7
RP 68888 (PIA)	AF	PI	25.8–28.2		1	22
(shoulder)	AG	PI				
JPB12112A	AH	PI	30.2		1.13–1.15	0.1
JPB12112B (shoulder)	AI	PI				
(shoulder)	AJ	PI				
	AK	PI	33.1		1.25	0.1
RPR 120667	AL	PI	32.0–35.0		1.2–1.4	<0.1
	AM	PI	38.1		1.44	0.1
	AN	PI	38.8		1.47	<0.1
RPR 122663	AO	PI+PII	38.8–43.5		1.5–1.6	0.3

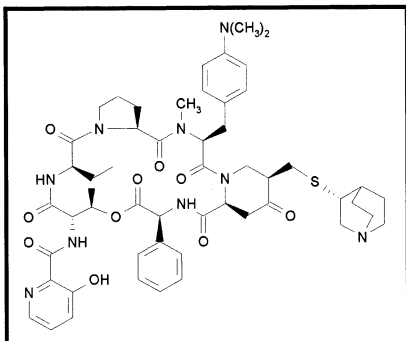
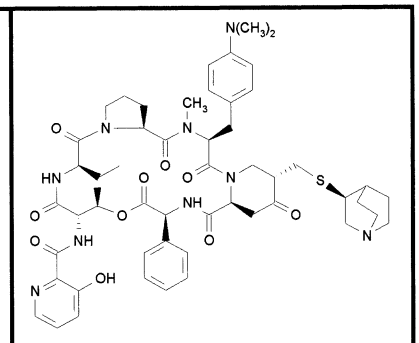
The two active ingredients of Synercid®, dalfopristin and quinupristin are assayed simultaneously by external standard method. The quinupristin content is calculated by taking the sum of its three constituents AC, AB and AF, this latter being the major constituent. Table 2 shows the structures of group I pristinamycin derivatives. Among well known impurities of AF are listed AE and RP 57886, this latter being coeluted with AF. Table 3 shows the structures of

Table 2
Structures of group I Pristinamycin derivatives

Quinupristin (RP 57669) constituents

		
RP 68888	RP 60844	RP 67648
$C_{53}H_{67}N_9O_{10}S$	$C_{52}H_{65}N_9O_{10}S$	$C_{52}H_{65}N_9O_{10}S$
MW = 1022.24	MW = 1008.22	MW = 1008.22

RP 57669 impurities

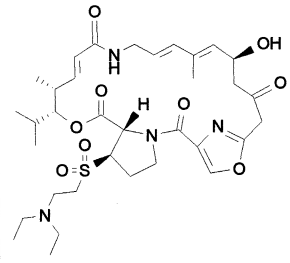
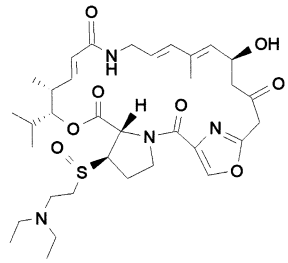
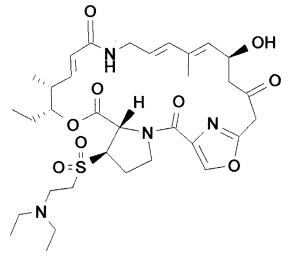
	
RP 57886	RP 69991
$C_{53}H_{67}N_9O_{10}S$	$C_{53}H_{67}N_9O_{10}S$
MW = 1022.24	MW = 1022.24

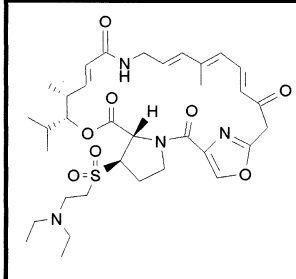
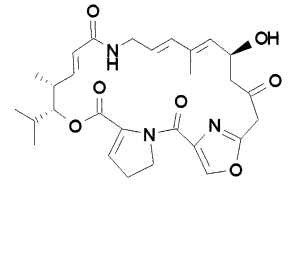
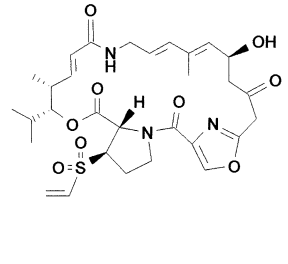
group II pristinamycin derivatives. The main degradation products of dalfopristin are Z (natural pristinamycin IIA) and Q (dehydrated form of dalfopristin). Z mainly increases in solution, whereas Q mainly rises in the freeze-dried form. The temperature and the pH strongly affect the formation of these impurities according to specific mechanisms. The formation of an hetero-dimeric impurity AO occurs during manufacturing by reaction between a dalfopristin impurity and quinupristin.

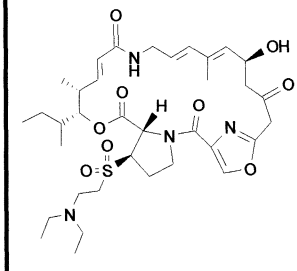
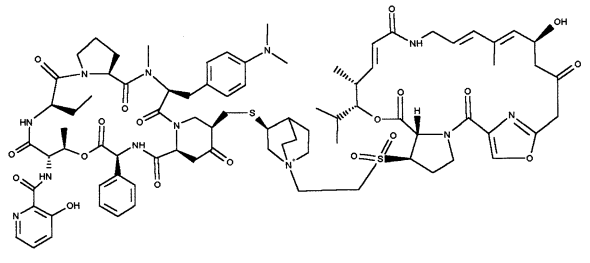
The related substances levels, expressed as a percentage of the total peak area, are calculated by the normalized area percentage method.

For calculations, it is noted that the reconstitution of the freeze-dried powder is performed according to the labeling for clinical use, i.e. adding 5.0 ml of a solution of 5% glucose, instead of

Table 3
Structures of group II Pristinamycin derivatives

Dalfopristin (RP 54476)	RP 54476 impurities	
		
RP 54476	RP 54474	RP 75645
$C_{34}H_{50}N_4O_9S$	$C_{34}H_{50}N_4O_8S$	$C_{33}H_{48}N_4O_9S$
MW = 690.85	MW = 674.86	MW = 676.83

RP 54476 impurities		
		
RP 60182	RP 12536	RP 60183
$C_{34}H_{48}N_4O_8S$	$C_{28}H_{35}N_3O_7$	$C_{30}H_{39}N_3O_9S$
MW = 672.82	MW = 525.61	MW = 617.73

RP 54476 impurity	RP 57669 and RP 54476 impurity
	
RP 75646	RPR 122663
$C_{35}H_{52}N_4O_9S$	$C_{83}H_{107}N_{12}O_{19}S_2$
MW = 704.89	MW = 1640.98

adjusting the final volume to 5.0 ml. The expansion of volume due to the dissolved powder was determined experimentally to be 0.49 ml for 649 mg of freeze-dried powder. Calculations were made using a final working standard volume V_t calculated with the equation, $V_t = 5.0 + (0.000755 \times M)$ where M is the individual mass (mg) of a freeze-dried working standard. In fact, V_t can be rounded to 5.5 ml for freeze-dried preparations where the mass is close to the theoretical value (620–680 mg).

2.6. System suitability

A suitability test was developed for the routine application of the method. Prior to each analysis, the chromatographic system must satisfy suitability test requirements (resolution and repeatability). Peak-to-peak resolution between AF and AE, and between Z and AB, measured on a reference solution, must be above 1.0. The relative standard deviation of the response factor (ratio area/mass) for dalfopristin and AF drug substance peaks, determined on six replicate injections of the reference solutions, must be less than 2.0%.

In order to obtain the satisfactory chromatographic separation when using a new column, it is required to flush the column with acetonitrile and then with a mixture of acetonitrile/water (20/80 v/v) prior to equilibrating the column with phase mobile A.

3. Results and discussion

3.1. Development considerations

The choice of a chromatographic method was naturally oriented towards a reversed-phase technique in aqueous media because Synercid® is soluble in aqueous media. A gradient method was preferred to an isocratic method because Synercid® is a mixture of two substances of widely differing polarity. The gradient made it possible to scan a broad range of polarities by reaching an acceptable compromise between method robustness and a suitable run-time for routine control testing.The final choice for stationary phase was made after testing different types of packing on the basis of following criteria: selectivity, peak symmetry and batch reproducibility. The use of a rather short column length (12.5 cm instead of 25 cm) led to an acceptable run time and improved detection of one late-eluting product, AO.

Optimization of the mobile phase, pH, column temperature and gradient slope was partially computer-aided using Optimix software (Varian SA, France) [10,11]. Initially the gradient system was developed, experimentally, and covered the range of 25–43.25% acetonitrile. Then, after optimization, this range was changed to 20–49.7% acetonitrile. The lower percentage at the start of the chromatogram produced separation of twin peaks D and C which had previously been co-eluted, whereas the higher percentage at the end of the gradient allowed elution of AO. The optimization of other parameters led to a satisfactory resolution of critical peak pairs AE/AF and Z/AB.

Under normal operating conditions, the most critical separation which depends on the quality of the column is that of AE and AF. The resolution most sensitive to the operating conditions is that of Z and AB. Thus, it was decided to include in the suitability test the determination of these resolutions with the minimum specification ($R_s > 1.0$) of the European pharmacopeia [12]. Resolutions close to 1.0 allow to obtain acceptable separation because of the characteristics of the peak pairs considered: AB and Z are always two small peaks, and the small peak AE is eluted before the large peak AF.

A typical chromatogram (Fig. 1) shows the profile obtained using the optimized method described in the experimental section. In order to facilitate the interpretation of chromatograms, the absolute retention times, the relative retention times and the typical area percentages with reference to the total area were determined for each peak (Table 1). Most of the peaks whose levels exceeded 0.1% were identified. Peaks are identified according to their retention time relative to the two major drug substances dalfopristin and AF, in the zone ranging from half to double their respective retention times. It clearly appears that dalfopristin derived compounds are eluted in the first part of the chromatogram while quinupristin derived com-

Table 4
Choice for factors and ranges of variation

Parameters	Units	Nominal	Ranges
Mobile phase			
Accuracy of CH_3CN in (solvent A) (constant amount of aqueous phase)	ml	200	±10
Accuracy of CH_3CN in (solvent B) (constant amount of aqueous phase)	ml	650	±10
Accuracy (slope) of gradient (% B)	final %	66.0	±6.0
pH of aqueous phase	pH unit	2.9	±0.2
Weight of KH_2PO_4	g	4.08	±0.20
Flow rate	ml min^{-1}	1.1	±0.1
Column temperature (temp)	°C	40	±5
Detection: wavelength (λ)	nm	254	±5

pounds are eluted in the second part of the chromatogram. At the stipulated detection wavelength of 254 nm, the two drug substances exhibit similar absorbance and the baseline drift is kept to a minimum. For impurities, the assumption was made that the UV spectrum of each impurity resembles that of its parent drug substance. In the case of the identified impurities, response factors at 254 nm were found to be close to one, except Z which had a response factor close to 1.4.

To confirm the specificity of the method, freshly prepared and degraded solutions were analyzed with a photodiode array detector. The spectra obtained demonstrate the chromatographic purity of the two principal peaks corresponding to dalfopristin and AF. In the latter case, interference caused by a small quantity of a co-eluted impurity cannot be detected because the two substances have the same UV spectrum. These results were confirmed during impurity isolation tests using

Table 5
Taguchi design to test eight factors

Experiment number	Run order	KH_2PO_4 weight	λ	Solvent A	Solvent B	Slope	pH	Flow rate	Temperature
1	16	1	1	1	1	1	1	1	1
2	1	1	1	0	0	0	0	0	0
3	18	1	1	2	2	2	2	2	2
4	19	1	0	1	1	0	0	2	2
5	15	1	0	0	0	2	2	1	1
6	8	1	0	2	2	1	1	0	0
7	10	1	2	1	0	1	2	0	2
8	6	1	2	0	2	0	1	2	1
9	13	1	2	2	1	2	0	1	0
10	4	2	1	1	2	2	0	0	1
11	17	2	1	0	1	1	2	2	0
12	12	2	1	2	0	0	1	1	2
13	7	2	0	1	0	2	1	2	0
14	9	2	0	0	2	1	0	1	2
15	11	2	0	2	1	0	2	0	1
16	14	2	2	1	2	0	2	1	0
17	5	2	2	0	1	2	1	0	2
18	3	2	2	2	0	1	0	2	1
Nominal	2 and 20	0	0	0	0	0	0	0	0
Val. 1	–	3.88 g	249 nm	190 ml	640 ml	60% B	2.7	1.0 ml min^{-1}	35°C
Val. 0	–	4.08 g	254 nm	200 ml	650 ml	66% B	2.9	1.1 ml min^{-1}	40°C
Val. 2	–	4.28 g	259 nm	210 ml	660 ml	72% B	3.1	1.2 ml min^{-1}	45°C

Table 6
Results of experiments for the experimental plan

Criteria	Nominal	Experiment number																	Nominal
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	
Response factor																				
Dalfopristin (RSD %)	0.75	0.62	0.61	1.33	1.28	1.05	1.16	1.38	1.01	1.00	0.68	0.46	1.11	1.05	0.68	0.47	0.75	0.85	0.98	1.16
AF (RSD %)	0.90	0.53	1.01	1.56	1.38	1.81	1.39	1.16	1.21	0.99	0.91	0.72	1.26	0.72	0.96	0.58	0.76	0.91	1.16	1.35
Content (mg 5 ml^{-1})																				
Dalfopristin	327.78	337.79	333.21	338.18	330.81	327.14	338.33	335.59	337.48	331.57	334.7	334.12	338.21	336.61	334.68	331.38	339.21	338.89	335.82	339.45
Quinupristin	141.15	143.19	139.65	144.94	141.19	139.89	142.47	142.39	142.70	139.89	142.59	143.26	143.94	142.12	143.88	140.45	143.48	142.72	142.68	145.31
Ratio dalfopristin/ quinupristin	2.32	2.36	2.39	2.33	2.34	2.34	2.37	2.36	2.37	2.37	2.35	2.33	2.35	2.37	2.33	2.36	2.36	2.37	2.35	2.34
Sum dalfopristin + quinupristin	468.92	480.98	472.86	483.15	472.00	467.04	480.79	477.98	480.18	471.45	477.29	477.38	482.15	478.73	478.56	471.83	482.68	481.60	478.50	484.76
Content (%)																				
C	0.10	0.11	0.08	0.11	0.10	0.09	0.10	0.08	0	0.08	0.10	0.11	0.11	0.10	0.10	0	0.08	0.09	0	0.10
D	0.37	0.40	0.31	0.39	0.36	0.35	0.38	0.30	0.40	0.29	0.40	0.39	0.40	0.37	0.36	0.44	0.29	0.31	0.38	0.36
H	0.15	0.24	0.16	0.30	0.18	0.26	0.18	0.12	0.13	0.15	0.21	0.24	0.27	0.18	0.17	0	0.16	0.14	0.12	0.19
K	0.68	0.75	0.57	0.72	0.66	0.64	0.68	0.54	0.56	0.54	0.75	0.73	0.76	0.68	0.66	0.66	0.53	0.56	0.56	0.67
O	0.20	0.18	0.17	0.19	0.13	0.24	0.21	0.09	0.11	0.13	0.23	0.14	0.28	0.13	0.15	0.26	0.13	0.18	0.11	0.16
Q	0.93	0.90	1.09	1.01	1.00	0.97	1.01	1.08	1.08	1.14	0.87	0.85	0.86	0.96	0.95	0.97	1.10	1.08	1.14	0.94
Z	1.08	1.08	1.34	0.96	1.14	1.06	1.27	1.20	1.53	1.42	1.08	0.98	1.13	1.27	1.21	1.12	1.38	1.59	1.34	1.24
AE	0.54	0.28	0.71	0.46	0.61	0.67	0.56	0.95	0.69	0.93	0.35	0.44	0.33	0.53	0.60	0.57	1.02	0.62	0.74	0.61
AO	0.21	0	0.27	0.23	0.28	0.25	0.23	0	0.26	0.31	0.17	0.22	0.18	0.23	0.17	0.24	0.35	0.30	0.29	0.30
Resolution between																				
C and D	0.92	1.01	0.85	0.92	1.23	0.75	0.76	1.14	0	0.85	0.79	1.00	0.96	1.04	1.09	0	1.10	1.02	0	1.02
Dalfopristin and H	2.62	2.64	2.56	2.61	2.88	2.37	2.61	1.04	2.49	2.51	2.61	2.84	2.70	2.75	2.80	2.4	2.78	2.72	2.48	2.72
Z and AB	2.84	0	3.01	8.80	2.96	6.97	0	7.48	0	4.26	2.39	7.29	0	0	3.49	5.48	7.39	0	1.55	3.38
AB and AC	2.00	3.06	1.85	0.96	1.07	1.08	2.96	1.14	2.23	1.47	1.75	1.13	3.24	1.87	1.62	1.22	0.94	3.26	2.13	1.3
AE and AF	1.69	1.95	1.49	0.92	1.42	0.96	1.90	1.04	0	1.38	1.57	1.07	1.98	1.99	1.57	1.12	0.95	1.94	1.76	1.46
Number of separate peaks >0.1%	15	13	13	13	15	14	15	14	14	15	14	15	12	14	14	12	14	12	15	16
Signal-to-noise ratio																				
K	82.9	103.6	13.1	78.4	46.5	46.2	75.7	33.9	29.8	55.8	133.3	131.2	153.9	61.9	81.2	76.8	41.7	46.2	30.5	69.2
AH	4.0	5.3	6.8	7.5	8.5	7.6	4.7	12.4	4.0	11.0	3.6	7.0	6.5	4.7	7.3	14.2	14.9	4.2	4.7	10.0

Table 7
Expression of models: effects of significant parameters

	Level	R1[a]	R2[a]	R3[b]	R4[b]	R5[c]	R6[b]	R7[d]	R8[d]	R9[d]	R10[d]	R11[d]	R12[d]	R13[d]	R14[d]	R15[d]	R16[e]	R17[e]	R18[e]	R19[e]	R20[e]	R21[f]	R22[g]	R23[g]
m[h]		0.921	1.052	335.22				0.082	0.363	0.182	0.643	0.169	1.000	1.225	0.606	0.219	0.817		3.402	1.818	1.393		69.82	7.496
Weight (g)	3.88	0.134	0.170										0.028		0.037				0.327				−15.21	
	4.28	−0.134	−0.170										−0.028		−0.037				−0.327				15.21	
λ (nm)	249	−0.119						0.022	0.018	0.055	0.071	0.029	−0.070	−0.130	−0.177		0.105						32.43	
	254	0						0	0.017	0	0.024	0	−0.034	−0.056	0		0						0	
	259	0						−0.027	−0.035	−0.045	−0.094	−0.044	0.103	0.185	0.219		−0.132						−30.17	
Solvent A (ml)	190							0.013				−0.021				−0.047	0.235							
	200							0				0				0	0							
	210							−0.015				0.027				0	−0.235							
Solvent B (ml)	640	−0.141	−0.200																					
	650	0.127	0																					
	660	0	0																					
Slope (final %)	60							0				−0.023				−0.067	0		0					
	66							−0.015				0				0	−0.095		0					
	72							0.013				0				0	0.078		0.334					
pH (pH unit)	2.7			2.67										0.087	−0.104				−3.402	0.952	0			−2.596
	2.9			0										0	0				−0.430	−0.212	0			0
	3.1			0										−0.108	0.079				3.833	−0.740	−0.383			3.104
Flow rate (ml min^{-1})	1.0							0.013	0			0					0.143			0				
	1.1							0	0			0.019					0			0				
	1.2							−0.012	0.018			−0.034					−0.118			−0.253				
Temperature (°C)	35	−0.119						−0.032	0.032			0.019			−0.056		−0.392		−0.671					
	40	0						0.015	−0.022			−0.020			0.066		0.149		0.285					
	45	0.184						0.017	0			0			0		0.243		0.386					
Y_{Npr}[i]		0.955	1.125	333.62	143.23	2.33	476.84	0.1	0.365	0.170	0.675	0.180	0.935	1.160	0.575	0.255	0.970	0.267	3.110	1.650	1.575	15.5	76.05	7.0

[a] R1, R2: Relative standard deviation for dalfopristin and AF.
[b] R3, R4, R6: dalfopristin, quinupristin and sum dalfopristin+quinupristin content.
[c] R5: Ratio of dalfopristin/quinupristin contents.
[d] R7, R8, R9, R10, R11, R12, R13, R14 and R15: Levels in C, D, H, K, O, Q, Z, AE, AO.
[e] R16, R17, R18, R19 and R20: Resolution between C/ D, dalfopristin/ H, Z/AB, AB/ AC and AE/ AF.
[f] R21: Number of separate peaks >0.1%.
[g] R22, R23: Signal-to-noise ratio with K and AH.
[h] Model constant.
[i] Mean of each measured response, each input being adjusted for the nominal value.

Table 8
Robustness of the method: normalized effects of significant factors

		R1 (%)[a]	R2 (%)[a]	R3 (%)[b]	R4 (%)[b]	R5 (%)[c]	R6 (%)[b]	R7 (%)[d]	R8 (%)[d]	R9 (%)[d]	R10 (%)[d]	R11 (%)[d]	R12 (%)[d]	R13 (%)[d]	R14 (%)[d]	R15 (%)[d]	R16 (%)[e]	R17 (%)[e]	R18 (%)[e]	R19 (%)[e]	R20 (%)[e]	R21 (%)[f]	R22 (%)[g]	R23 (%)[g]
Weight (g)	3.88	14.0	15.1										3.0		6.4				10.5				−20.0	
	4.28	−14.0	−15.1										−3.0		−6.4				−10.5				20.0	
λ (nm)	249	−12.5						22.0	0.3	32.4	7.0	16.1	−3.9	−6.4	−30.8		10.8						42.6	
	259	0						−27.0	−14.3	−26.5	16.9	24.4	7.4	20.8	38.1		−13.6						−39.7	
Solvent A (ml)	190							13.0				−11.7				−18.4	24.2							
	210							−15.0				15.0				0	−24.2							
Solvent B (ml)	640	−28.1	−17.8																					
	660	0	0																					
Slope (final %)	60							15.0				−12.8				−26.3	9.8		0					
	72							28.0				0				0	17.8		10.7					
pH (pH unit)	2.7			0.8										7.5	−18.1				−95.6	70.5	0			−37.1
	3.1			0										−9.3	13.7				137.1	−32.0	−24.3			44.3
Flow rate (ml min^{-1})	1.0	−12.5						13.0	0			−10.6					14.7			0				
	1.2	19.3						−12.0	4.9			−29.4					−12.2			−15.3				
Temperature (°C)	35							−47.0	14.8			21.7			−21.2	−22.0	−55.8		−30.7					
	45							2.0	6.0			11.1			−11.5	0	9.7		3.3					

[a] R1, R2: Relative standard deviation for dalfopristin and AF.
[b] R3, R4, R6: dalfopristin, quinupristin and sum dalfopristin+quinupristin content.
[c] R5: Ratio of dalfopristin/quinupristin contents.
[d] R7, R8, R9, R10, R11, R12, R13, R14 and R15: Levels in C, D, H, K, O, Q, Z, AE, AO.
[e] R16, R17, R18, R19 and R20: Resolution between C/ D, dalfopristin/ H, Z/AB, AB/ AC and AE/ AF.
[f] R21: Number of separate peaks >0.1%.
[g] R22, R23: Signal-to-noise ratio with K and AH.

Table 9
Robustness of the method: calculated values of response at the extremes of significant factors

		R1 (RSD%)[a]	R2 (RSD%)[a]	R3 (mg 5 ml^{-1})[b]	R4 (mg 5 ml^{-1})[b]	R5 (Ratio)[c]	R6 (mg 5 ml^{-1})[b]	R7 (%)[d]	R8 (%)[d]	R9 (%)[d]	R10 (%)[d]	R11 (%)[d]	R12 (%)[d]	R13 (%)[d]	R14 (%)[d]	R15 (%)[d]	R16 (R)[e]	R17 (R)[e]	R18 (R)[e]	R19 (R)[e]	R20 (R)[e]	R21 (number)[f]	R22 (ratio)[f]	R23 (ratio)[g]
Weight (g)	3.88	1.089	1.295										0.963		0.612				3.437				60.84	
	4.28	0.821	0.955										0.907		0.538				2.783				91.26	
λ (nm)	249	0.836						0.122	0.366	0.225	0.722	0.209	0.899	1.086	0.398		1.075						108.48	
	259	0.955						0.073	0.313	0.125	0.557	0.136	1.072	1.401	0.794		0.838						45.88	
Solvent A (ml)	190							0.113				0.159				0.208	1.205							
	210							0.085				0.207				0.255	0.735							
Solvent B (ml)	640	0.687	0.925																					
	660	0.828	1.125																					
Slope (final %)	60							0.115				0.157				0.188	1.065		3.110					
	72							0.128				0.180				0.255	1.143		3.444					
pH (pH unit)	2.7			336.29										1.247	0.471				0.138	2.814	1.575			4.404
	3.1			333.62										1.052	0.654				7.373	1.122	1.192			10.104
Flow rate (ml min^{-1})	1.0							0.113	0.365			0.161					1.113			1.650				
	1.2							0.088	0.383			0.127					0.852			1.397				
Temperature (°C)	35	0.836						0.053	0.419			0.219			0.453		0.429		2.154					
	45	1.139						0.102	0.387			0.200			0.509		1.064		3.211					
Y_{Npr}[h]		0.955	1.125	333.62	143.23	2.33	476.84	0.1	0.365	0.170	0.675	0.180	0.935	1.160	0.575	0.255	0.970	2.670	3.110	1.650	1.575	15.5	76.05	7.0

[a] R1, R2: Relative standard deviation for dalfopristin and AF.
[b] R3, R4, R6: dalfopristin, quinupristin and sum dalfopristin+quinupristin content.
[c] R5: Ratio of dalfopristin/quinupristin contents.
[d] R7, R8, R9, R10, R11, R12, R13, R14 and R15: Levels in C, D, H, K, O, Q, Z, AE, AO.
[e] R16, R17, R18, R19 and R20: Resolution between C/ D, dalfopristin/ H, Z/AB, AB/ AC and AE/ AF.
[f] R21: Number of separate peaks >0.1%.
[g] R22, R23: Signal-to-noise ratio with K and AH.
[h] Mean of measured response, each input factor being adjusted for the nominal value.

both different chromatographic systems and different detectors.

3.2. Robustness study

The robustness study based on an experimental plan was performed in order to investigate the reproducibility of the method when controlled and limited variations are applied around normal operating conditions. These variations are likely to be encountered in different laboratories for different operators. Practically, the robustness of the method has to be assessed prior to the reproducibility study in order to identify potential critical factors that should be strictly controlled. The essential features of the methodology of robustness testing and practical guidelines when using experimental plan can be found in the published literature [13–15]. Also, different designed experiments for robustness studies of HPLC have been reported [16,17].

3.2.1. Factor choice

The potential critical parameters which are likely to produce unwanted variations and to affect the quality of the results obtained were identified. Thus, eight parameters were tested in the experimental plan. These were related to the mobile phase (accuracy of acetonitrile in solvents A and B, pH, weight of potassium salt) and to the instrument (flow rate, accuracy of gradient, column temperature, detection wavelength). It should be emphasized that two other important parameters previously investigated for their impact on reproducibility were not considered in the study of robustness. Indeed, the equipment is a crucial point and the method remained reproducible if the dead volume in the gradient pump was not excessive and the gradient was correctly programmed. Also, column repeatability was fundamental and was controlled independently. For each parameter or factor, the range of deviation about the nominal values was chosen to represent reasonable variations in experimental conditions (Table 4). However, some variations may have been exaggerated to obtain significant results. The chosen parameters were tested on three levels except the potassium salt weight which consisted of two levels only.

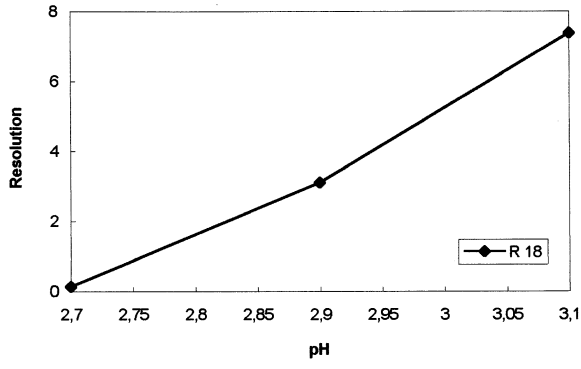


Fig. 2. Effect of pH on the resolution between Z and AB.

3.2.2. Experimental design

A 'screening' matrix, corresponding to a Taguchi matrix L18 ($2^1 \times 3^7$) was prepared [18] and allowed to quantify only direct effects. If interactions occurred between parameters, they were not detected and did not affect the relative importance of each parameter studied. Screening matrixes are designed in such a way that if interaction effects exist, they are divided and distributed on all the main effects. This way, even though bias may exist, it is possible to classify the main effects relatively to their respective importance. The matrix consisted of 18 experiments (Table 5). Moreover, the nominal experiment which corresponds to the normal operating conditions of the method, was carried out once at the start and once at the finish of the matrix. For each experiment, a chromatographic run was obtained and 23 relevant responses were measured from the chromatogram. Resolution between pairs of critical peaks, signal-to-noise ratio with impurities, drug substances content, related substances levels in test solutions, number of separated peaks $> 0.1\%$ and suitability test were chosen as quality criteria.

3.2.3. Calculations of the statistical results

The results of experiments (Table 6) were submitted to calculations and to statistical analysis using Modde software (Umetri, Umeå, Sweden).The effects of factors were estimated for each quality criteria by the following model

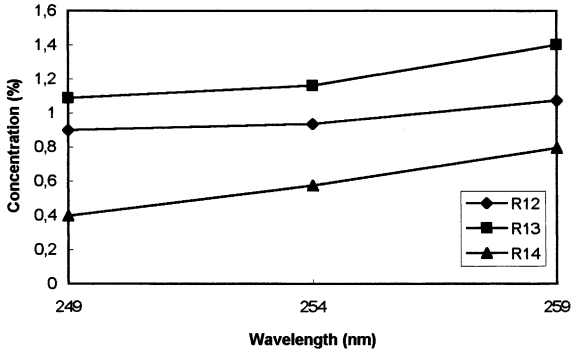


Fig. 3. Effect of wavelength on levels Q, Z and AE.

$$Y = m + [a_1^1;a_2^1]\ \text{weight} + [a_1^2; a_0^2; a_2^2]\ \text{wavelength} + \ldots + [a_1^8; a_0^8; a_2^8]\ \text{temperature}$$

where Y is the response obtained with the considered factors at levels 0 or 1 or 2 and m is a model constant, where a_j^i are the main effects of considered factors, i is in the range 1–8 depending on the factor and j is the level. A model was obtained for each of the quality criteria selected (R1–R23). A F-test was carried out on the models with appropriate degree of freedom to decide if the effect of each factor is significant at the 10% level. This test which compares the variance of the effect with the residual variance allowed to retain or discard an effect. Only the significant effects of the various factors are shown in Table 7. The normalized effects of the various factors are given in Table 8. Normalization of the effects (E_{ij}) was performed relative to the nominal results (Y_{Npr}) using the formula, $E_{ij} = [(a_j^i - a_0^i)] \cdot 100/Y_{\mathrm{Npr}}$, where a_j^i is the effect of factor i at level j and the effect of factor i at level 0. When the effect was zero, the quality criterion considered is robust in terms of the parameter studied over the range of variation adopted. Otherwise, the greater the coefficient in absolute terms, the more marked is the effect of the parameter studied on the quality criterion.

3.2.4. Interpretations

An initial interpretation of results based on statistically significant effects, allowed the identification of the factors that affect the analysis. However, a further interpretation had to be refined in the light of specifications for the considered response (analytical significant effect). With this objective in mind, the theoretical extremes were calculated for each quality criterion using models in which each significant factor was varied in turn from level 1 to level 2 meanwhile the other factors were at level 0 (Table 9). In this paper, discussion will focus on the three main sensitive points that were shown to be pH, temperature and wavelength.The pH had a major effect on the resolution between Z and AB (Fig. 2). The resolution obtained at pH 2.7 was unsatisfactory (0.14) due to co-elution of the two peaks. The two peaks occur at the interface of the elution regions for pH-insensitive group II pristinamycins (including Z) and pH-sensitive group I pristinamycins (particularly AB, AC and AF). It is important that these two peaks be correctly resolved since AB is a constituent of the drug substance and Z is a degradation product of dalfopristin. All the other parameters had negligible effects on their resolution as the calculated resolutions were consistently higher than 1.0. In practice, the pH of the mobile phase will raise no particular problem because the range of variation studied (± 0.2 pH unit) is wide compared to the theoretical accuracy of the adjustment (0.05 pH unit). In addition, any inadequate separation can be improved since the effect of this parameter is understood.

The column temperature is liable to affect the resolution between the peaks of impurities C and D and also the C content. The wavelength had a major effect on the signal-to-noise ratio and hence the limits of detection and quantitation. The wavelength routinely affected the levels of several impurities. It had negligible effect on dalfopristin, H and K levels. In contrast, this wavelength effect led to the levels of Q, Z and AE being overestimated by ~ 0.14, 0.24 and 0.22%, respectively, for corresponding mean contents (Y_{Npr}) of 0.94, 1.16 and 0.58% (Fig. 3). This sensitivity to wavelength might lead to the rejection of a satisfactory batch. However, such a risk is minimal given that the range of wavelength studied (± 5 nm) is wider than the generally accepted specification (± 2 nm). These parameters which have been highlighted can be controlled without any major difficulty. Routine qualification of equipment ensures that equipment-

Table 10
Interlaboratory studies

Test	Site					
	France		USA		Spain	
Equipment	A	B	A	B	A	B
Suitability test						
RSD (%) of dalfopristin	0.4	0.3	0.3	0.3	0.3	0.4
RSD (%) of AF	0.6	0.5	0.3	0.4	0.4	0.5
Resolution AE/AF	1.5	1.6	1.4	1.2	1.3	1.4
Limit of quantitation (%)	≤0.1	≤0.1	≤0.1	≤0.1	≤0.1	≤0.1
Assay of drug substances						
Quinupristin (mg 5 ml^{-1})	148	145	145	147	146	146
Dalfopristin (mg 5 ml^{-1})	341	336	335	340	335	336
Mean (SD) for quinupristin	146 (1.04)					
Mean (SD) for dalfopristin	337 (2.92)					
Impurities						
Z (%)	1.3	1.3	1.5	1.3	1.3	1.3
Q (%)	0.9	0.8	0.9	1.0	0.7	0.8
O (%)	0.2	0.2	0.2	0.3	0.2	0.2
K (%)	0.9	0.9	0.9	0.9	0.8	0.9
AE (%)	0.5	0.5	0.6	0.5	0.6	0.5
Number of peaks >0.5%	0	0	0	0	0	0
Sum of other impurities content (%)	5.1	5.0	5.8	5.3	4.8	4.7

related parameters (wavelength and column temperature) are controlled within the framework of good laboratory practice.

3.3. Method validation

3.3.1. Precision

Precision was evaluated by performing ten replicate injections of the same standard Synercid® solution, ten independent assay of drug substances by the same operator on the same day and six independent assay of related substances by the same operator on the same day. The repeatability of the chromatographic system measured by peak area was satisfactory with relative standard deviation (RSD) less than 1% for both drug substances and allowed a specification of 2% for suitability test to be established. For related substances where results were expressed in normalized area percentages, the repeatability was acceptable with RSD ranging from 2.0 to 8.8%. The repeatability of the assay method was also satisfactory for both drug substances (RSD = 0.74% for dalfopristin and 0.95% for quinupristin). For related substances, the RSD ranged from 0.0 to 9.5% showing that the preparation of the sample did not contribute to increasing dispersion of the results.

3.3.2. Reproducibility

The reproducibility of the method was evaluated in a collaborative study involving three sites and two different chromatographic systems per site, i.e. a total of six different systems. Each system involved one analyst, a different chromatographic column and a set of reagents and preparations. The results are shown in Table 10. The results of the drug substances assays and impurity levels correspond to the mean of duplicate injections of three samples from five pooled vials. The means and the standard deviations (SD) were calculated before rounding off the figures. The suitability test results showed that the systems were suitable in all cases. The results obtained from each site for the assay and evaluation of impurities were similar from an analytical point of view. The results of the assay were statistically evaluated using the software statistical analysis system (SAS). No statistically significant intra- or inter-site differences within the 95%

confidence interval were demonstrated by analysis of variance. Regardless of the conditions, the method yielded reproducible, quantitative results which were not significantly different.

3.3.3. Linearity and accuracy

The linearity of the detector response to drug substances was determined for Synercid® solutions containing 50, 75, 100, 125 and 150% of the theoretical content. The solutions were prepared in triplicate by dissolving the quantities of Synercid® to be tested in 5 ml glucose. The responses were linear throughout the range of masses investigated and the y-intercepts were not significantly different from zero. Thus, the dalfopristin and quinupristin contents can be determined for an injected Synercid® quantity ranging from 5 to 15 μg.

The linearity of the detector response to the major impurities (Z, Q, AE and O) was assessed for low concentrations by spiking Synercid® standard solutions with isolated impurity samples. Four spikes ranging from 0.1 to 10% (w/w relative to the nominal quantity of Synercid®) were chosen to be consistent with the initial quantity of each impurity. The peak area corresponding to the spike was reported as a function of the added quantity. The results show that the responses are linear for each evaluated impurity. For these impurities, response factors at 254 nm were found to be close to their parent drug substances, except for Z.

The recovery of drug substances was evaluated from 50 to 150% of the nominal quantity. The mean recovery was 99.5% (RSD = 1.1%, $n = 15$) for dalfopristin and 100.5% (RSD = 1.4%, $n = 15$) for quinupristin.

3.3.4. Limit of detection and limit of quantitation

The limits of detection based on a signal-to-noise ratio of 3 were determined to be 0.01% for early-eluting peaks (dalfopristin derivated compounds) and 0.05% for late-eluting peaks (quinupristin derivated compounds). Accounting of the quantitation method used, the limit of detection for the related substances should be considered to be close to 0.05% independent of their eluting time.

The limits of quantitation based on a signal-to-noise ratio of 10 were determined to be 0.05% for dalfopristin, 0.12% for quinupristin and 0.1% for the related substances. The quantitation of peaks below 0.1% seemed possible in some cases but was not pursued because of the increasing probability of interference from small unidentified neighboring peaks that might mask the investigated peak.

4. Conclusion

A gradient HPLC method was developed for simultaneous assays of drug substances and impurities in Synercid® formulation. During the development studies, it was proven, that the method is robust. None of the parameters tested is likely to significantly affect the selectivity, repeatability or accuracy of drug substance assays. The method is equally robust for the determination of degradation products levels since the use of qualified equipment renders the risk of overestimation negligible. In conclusion, the proposed method is reliable and convenient for routine control, for stability assays and compatibility studies.

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