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Liquid chromatography of spiramycin on poly(styrene–divinylbenzene)

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Abstract

An isocratic liquid chromatographic method is described using as stationary phase the very stable poly(styrene–divinylbenzene) PLRP-S (8 μm 1000 Å). The mobile phase is acetonitrile–0.2 *M* potassium phosphate buffer pH 9.0–water (37:5:up to 100, v/v/v), delivered at a flow-rate of 1.0 ml/min. UV detection is performed at 232 nm. Full factorial design was applied to evaluate the robustness of the method. Quantitative results of a number of commercial samples are presented.

Keywords: Mobile phase composition; Pharmaceutical analysis; Stationary phases, LC; Spiramycin; Antibiotics

1. Introduction

Spiramycin (SPM), which belongs to the group of macrolide antibiotics, is produced by fermentation [1–3]. Commercial samples consist mainly of three closely related constituents, differentiated by the substituent at the 3-position, namely I (3-OH), II (3-*O*-acetyl) and III (3-*O*-propionyl), and of several minor components. Structures of these substances are shown in Fig. 1.

Several microbiological methods are available for the determination of spiramycin: direct techniques [4] or combined with a clean-up procedure by thin-layer chromatography [5]. Although these methods are sensitive, they are not selective. A reversed-phase liquid chromatographic (RPLC) method for the analysis of spiramycin was first described by Mourot et al. [6]. Bens et al. [7,8] used both normal- and reversed-phase chromatography to separate and determine the components of spiramycin in bulk powders and in pharmaceutical preparations. These methods mainly provided analytical separation of the major components of spiramycin: SPM I, II and III, and gave no information on differentiating the minor related substances and degradation products present in spiramycin. A suitable method for the analysis of spiramycin should allow the determination of the three main compounds and the separation of related substances such as the neospiramycins (NSPM) and other minor components such as the recently described methylenespiramycin (MSPM) and deoxydihydrospiramycin (DSPM) [9]. Until now, the best results were obtained by Horie et al., who reported on the separation of SPM I, II and III and NSPM I, II and III on a reversed-phase with an acidic mobile phase, but the separation of the main component (SPM I) was incomplete [10]. Some other papers described the separation of acetylspiramycins. Acetylspiramycin corresponds to SPM II [11,12].

The method described here uses a wide-pore

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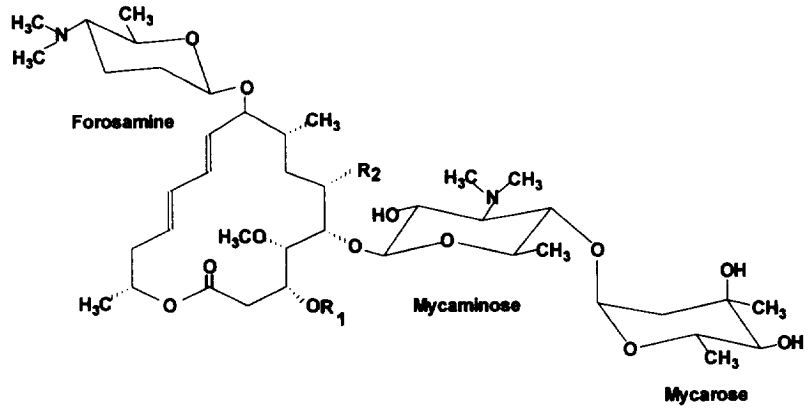

	R_1	R_2	Mycarose
Spiramycin I (SPM I)	H	CH_2CHO	+
Spiramycin II (SPM II)	$COCH_3$	CH_2CHO	+
Spiramycin III (SPM III)	$COCH_2CH_3$	CH_2CHO	+
Spiramycin IV (SPM IV)	H	CH_2CH_2OH	+
Neospiramycin I (NSPM I)	H	CH_2CHO	-
Neospiramycin II (NSPM II)	$COCH_3$	CH_2CHO	-
Neospiramycin III (NSPM III)	$COCH_2CH_3$	CH_2CHO	-
Methylenespiramycin (MSPM)	H	$C(CH_2)CHO$	+
Deoxydihydrospiramycin (DSPM)	H	CH_2CH_3	+

+ = sugar present
- = surgar not present

Fig. 1. Structures of spiramycins and related substances.

poly(styrene–divinylbenzene) (PS–DVB) stationary phase and a mobile phase at pH 9. This method is derived from those previously developed for the analysis of other macrolides: erythromycin, tylosine and josamycin [13–15].

2. Experimental

2.1. LC apparatus and operating conditions

LC analyses were performed using a L-6200 Intelligent pump (Merck-Hitachi, Darmstadt, Germany) equipped with a Valco injector Model CV-6-UHPa-N60 (Houston TX, USA) and a 20 μl loop or with a 655 A-40 autosampler (Merck-Hitachi) and a 20 μl loop, a L-4200 UV–Vis detector (Merck-Hitachi) set at 232 nm and a 3396 A integrator (Hewlett-Packard, Avondale, PA, USA). The column was immersed in a water-bath, heated by a Julabo EM thermostat (Julabo, Seelbach, Germany). Columns (250×4.6 mm I.D.) were packed with PLRP-S 8 μm 1000 Å, 300 Å or 100 Å (Polymer Labs., Church Stretton, Shropshire, UK). The flow-rate was 1.0 ml/min. The column dead volume was determined by injecting potassium nitrate. All chromatographic parameters were calculated using formulas from the European Pharmacopoeia [16].

2.2. Reagents and mobile phase

Dipotassium hydrogen phosphate, acetone, 2-methyl-2-propanol and tetrahydrofuran were of analytical-reagent grade from Acros Chimica (Geel, Belgium). Acetonitrile, diethyl ether and methanol were of chemical grade from the same source. Diethyl ether and methanol were distilled before use. Water was distilled twice before use. The following mobile phase was finally used: acetonitrile–0.2 *M* potassium phosphate buffer (pH 9.0)–water (37:5:up to 100, v/v/v). Mobile phases were degassed by purging with helium gas for 1 min before use.

2.3. Reference substances and samples

SPM I HS (house standard) was obtained by open column chromatography of spiramycin on silica gel (60 H, 15 μm) using diethyl ether–methanol–25% ammonium hydroxide in different ratios (97:3:0.3, 95:5:0.5 and 90:10:1, v/v/v) as mobile phase. The collected fractions were evaporated, redissolved in acetone, evaporated again and dried in vacuo. SPM II HS and SPM III HS were purified in part from spiramycin of Chinese origin by open column chromatography on silica gel. Further purification was performed with medium-pressure LC (Büchi 688, Flawil, Switzerland) on a 22 cm×4.5 cm glass column packed with silica gel which was buffered with 0.1 *M* potassium phosphate (pH 9.0) [17]. The mobile phase was diethyl ether–methanol (98.5:1.5, v/v). The collected fractions were treated as described above. The label HS was only given to the reference substances of the major components, for which the composition was determined (see Section 2.4). SPM IV was prepared by selective reduction of the aldehyde group of SPM I with sodium borohydride in pH 7.5 phosphate buffer solution. The mixture was extracted with chloroform, the organic layers evaporated, and the residue was treated as mentioned above.

Neospiramycins were prepared by mild acid hydrolysis of the corresponding spiramycins at pH 2.0 and room temperature for 48 h. The mixture was extracted with chloroform after adjusting the pH to 8.0 with solid $NaHCO_3$ and Na_2CO_3, and the organic layers were evaporated. The residue was treated as mentioned above. MSPM and DSPM were obtained as described previously [9].

The structures of all the reference substances mentioned above were confirmed by mass and NMR spectrometry.

The commercial grade samples (samples 1-6) of European origin were obtained from Rhône-Poulenc (Paris, France). Commercial grade samples 7 and 8 of Chinese origin were obtained from Shandong Jining Antibiotic Factory (Shandong, China) and Shaoxing Pharmaceutical factory (Hunan, China), respectively. The reference and test solutions for the quantitative analysis were prepared by weighing 25.0 mg of sample and diluting with acetonitrile–water (3:7) to 25.0 ml.

A spiked commercial sample of spiramycin was used for the purpose of optimisation. To prepare the spiked sample a commercial sample (10 mg) was dissolved in 10 ml of acetonitrile–water (3:7) and volumes of reference solutions containing 1 mg/ml each of spiramycin in acetonitrile–water (3:7) were added: 500 μl MSPM, 500 μl mixture of NSPM II and III, 1.0 ml DSPM, 300 μl SPM II and 200 μl SPM IV.

2.4. Analysis of the house standards

The compositions of the house standards of SPM I, II and III are shown in Table 1. The base contents of the house standards were determined by non-aqueous titration. SPM (150 mg) was dissolved in 50 ml of glacial acetic acid and titrated with 0.1 *M* perchloric acid in acetic acid using potentiometric end-point detection. Karl Fischer titration was used to obtain the water content. The content of the residual acetone was determined by gas chromatography (GC). The samples were dissolved in 0.033 *M* phosphoric acid. The separation was carried out on a column packed with ethylvinylbenzene–divinylbenzene (1.5 m×4 mm I.D.) 150 μm to 180 μm using propanol as internal standard and nitrogen as carrier gas, maintaining the temperature of the column at 135°C and that of the injector and detector at 150°C. The content of related substances present in the house standards was determined by the described LC method. The related substances for which sufficiently pure house standards were available (SPM I, II and

Table 1
Composition (%, m/m) of the house standards

	SPM I HS	SPM II HS	SPM III HS
A: Non-aqueous titration	96.92	97.09	97.32
	(n=7, 0.5%)	(n=3, 0.6%)	(n=3, 0.5%)
B: Karl Fischer titration	1.41	1.21	1.25
	(n=4, 13.3%)	(n=3, 0.75%)	(n=3, 5.6%)
C: GC (acetone)	0.85	0.71	0.37
	(n=3, 0.6%)	(n=3, 5.0%)	(n=3, 9.3%)
D: Related substances	1.92	3.79	3.09
Total mass explained (A+B+C)	99.18	99.01	98.94
Content (A−D)	95.00	93.30	94.23

III) were determined using dilutions of these house standards. Other impurities were expressed as SPM I.

3. Results and discussion

3.1. Method development

Wide-pore PS–DVB PLRP-S (8 μm 1000 Å) combined with alkaline mobile phases has shown excellent selectivity in the separation of other macrolide antibiotics [13–15]. This stationary phase, kept at a temperature of 60°C, and a mobile phase consisting of acetonitrile–0.2 *M* phosphate buffer (pH 9.0)–water (x:5:95−x, v/v/v) were chosen as the starting conditions for method development.

The influence of the pH on the selectivity was investigated between 6.0 to 10.0 (Fig. 2). At pH values below 8 the separation was poorer, and the column efficiencies decreased by 40% relative to values above pH 8. The results indicated that in the pH range 8.5 to 9.5, small pH changes did not significantly affect the separation and therefore pH 9.0 was chosen for further work. The effect of the concentration of 0.2 *M* potassium phosphate buffer (pH 9.0) was negligible in the range 2.5–7.5% (Fig. 3). The intermediate value of 5% was retained for further work. When the potassium phosphate buffer was replaced with an ammonium phosphate buffer, overlapping of SPM I and NSPM II occurred.

The influence of column temperature was examined in the range from 50°C to 65°C. The retention times increased regularly with increasing temperature. A temperature of 60°C was selected because elevated temperature allows for better efficiency. Increase of retention time with increasing temperature has also been observed in reversed-phase chromatography of erythromycin [18]. The solubility in water of both erythromycin and spiramycin decreases with increasing temperature. However, in the analysis of erythromycin on PS–DVB, a slight decrease in retention time with increasing temperature was observed [13].

Several organic modifiers were investigated. The influence of the concentration of acetonitrile (CH_3CN) in the mobile phase on the selectivity is shown in Fig. 4. A concentration of 37% acetonitrile was chosen to be as optimal in terms of selectivity and analysis time. Tetrahydrofuran was also examined. However, it proved to be less suitable for the separation of SPM I and its closely eluted impurities. 2-Methyl-2-propanol was also found to be less suitable than acetonitrile in that SPM I and MSPM coeluted.

The influence of the age and pore size of the PS–DVB stationary phase was examined as illustrated for PLRP-S 300 Å and 1000 Å in Fig. 5. The selectivity of the PS–DVB material was found to depend on the pore size of the particles. All the columns packed with 1000 Å material gave very similar separations, although their ages ranged from 4 to 6 years. With the 300 Å material overlapping changes in elution order are observed. With 100 Å material much longer retention times and very poor separations were observed (results not shown). It is therefore concluded that 1000 Å PS–DVB must be used. Similar phenomena due to pore size have also been observed for other macrolides [13–15].

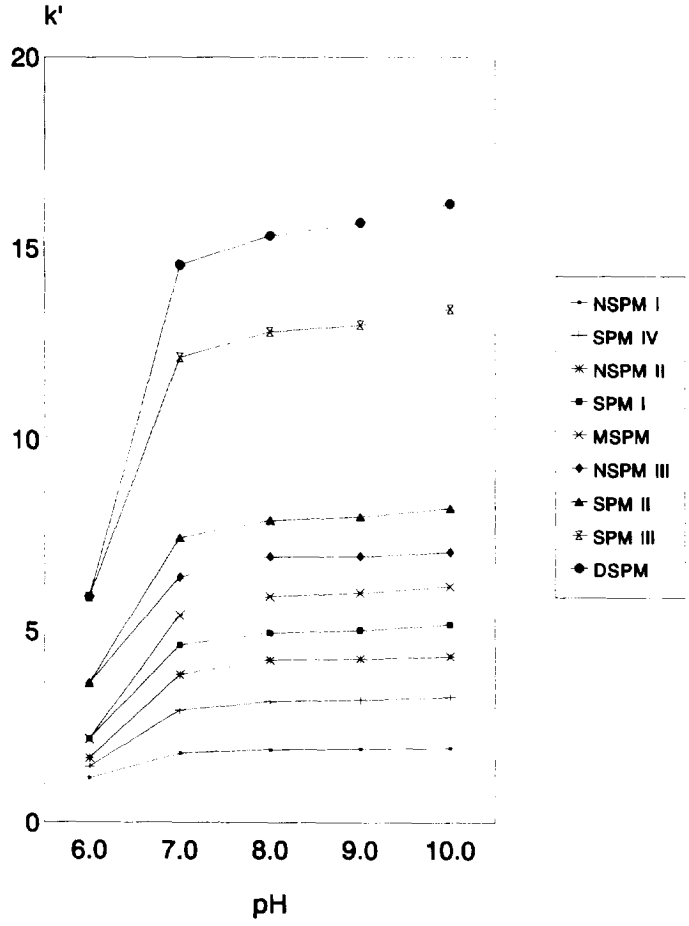

Fig. 2. Influence of the pH of the mobile phase on the capacity factors (k') of spiramycin I and its related substances. Mobile phase: CH_3CN–0.2 *M* phosphate buffer (pH *x*)–water (38:5:up to 100, v/v/v); stationary phase: PLRP-S 8 μm 1000 Å (250×4.6 mm I.D.); flow-rate: 1.0 ml/min; temperature: 60°C; detection: UV at 232 nm.

The final composition of the mobile phase chosen is acetonitrile–0.2 *M* potassium phosphate pH 9.0–water (37:5:up to 100, v/v/v) with nearly baseline separation of all related substances. A typical chromatogram of a spiramycin sample of European origin is shown in Fig. 6.

The robustness of the method was evaluated by performing a full factorial design experiment. The influence of each of the three chromatographic parameters that governed the separation most was studied (low and high values are mentioned in parentheses): the concentration of acetonitrile (36%, 40%); the pH of the mobile phase buffer (8.0, 10.0) and the column temperature (55°C, 65°C). The concentration of phosphate buffer was not considered as variable because it only very slightly influences the separation. The response variables of the experimental design were the resolutions between SPM IV and I, I and II, II and III which are the most important components of spiramycin. The application of this factorial design, analysis of the measured response variables and multivariate regression calculation, were carried out using the statistical graphic software STATGRAPHIC (Version 6.0, STSC, Rockville, MD, USA). This software package allows one to obtain estimated parameters for main effects, and to produce analysis of variance (ANOVA) tables and standardised Pareto charts for the resolutions and response surface plots. A standardised Pareto chart for the resolution between SPM I and II is presented

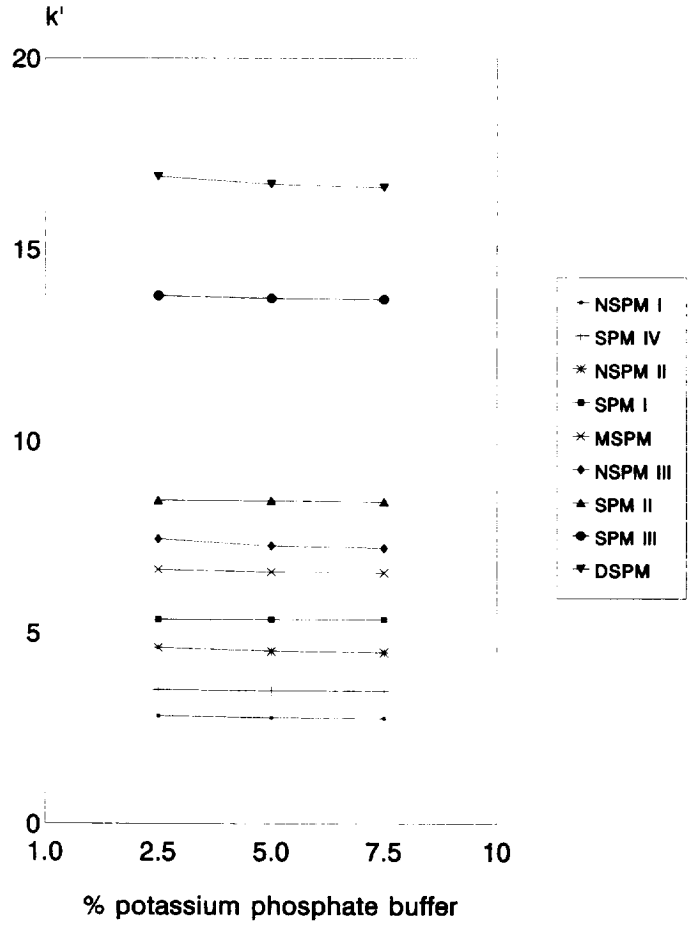

Fig. 3. Influence of the concentration of potassium phosphate buffer (pH 9.0) on the capacity factors (k') of spiramycin I and its related substances. Mobile phase: CH_3CN–0.2 *M* phosphate buffer (pH 9.0)–water (38:*x*:up to 100, v/v/v). See Fig. 2 for other conditions.

in Fig. 7. The Pareto charts for the other resolutions examined are very similar. The standardised Pareto chart consists of bars that are displayed in the size order of the effects, and of a vertical line at a critical *t*-value ($\alpha=0.05$). The codes A, B and C correspond to the effects on the resolution between SPM I and II of the amount of acetonitrile, the pH and the column temperature, respectively. The two-code combinations correspond to the two-way interactions between parameters. Parameter effects for which the bars are smaller than this critical *t*-value line are not statistically significant relative to the response variables. All the results reveal that the pH of the mobile phase buffer, the organic modifier (CH_3CN) in the mobile phase and the column temperature are the most important chromatographic factors that affect the resolution among the adjacent compounds. An increase of A has a negative effect, while an increase of B or C has a positive effect. There is some interaction between pH and column temperature.

The response surface plots were constructed, with the retention times as a function of the most important chromatographic parameters: i.e., mobile phase pH and concentration of acetonitrile. For all conditions examined there were no overlapping peaks. The results of this study show that the

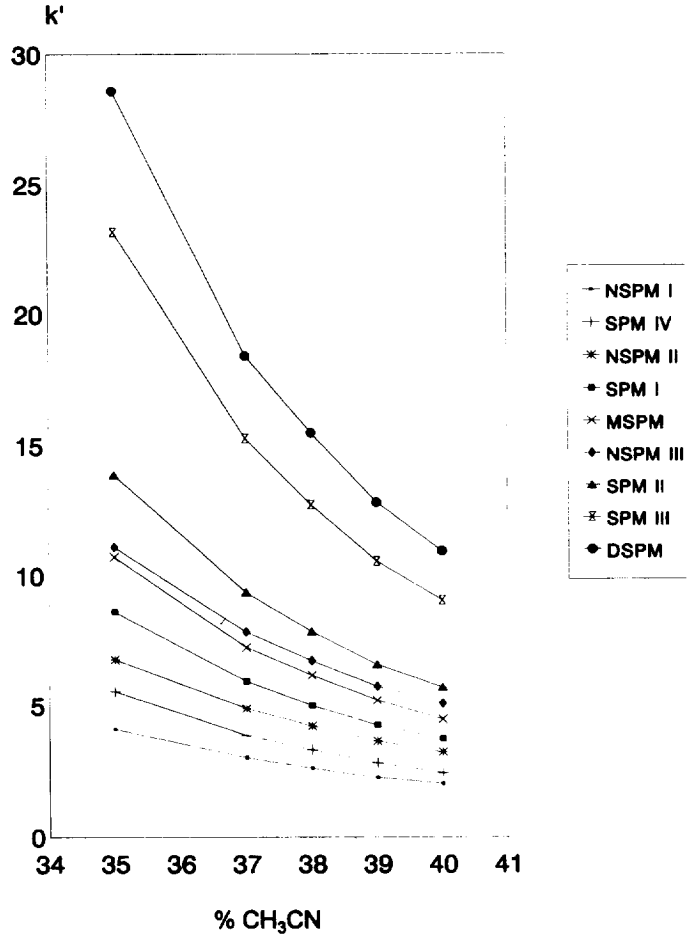

Fig. 4. Influence of the concentration of acetonitrile (CH_3CN) in the mobile phase on the capacity factors (k') of spiramycin I and its related substances. Mobile phase: CH_3CN–0.2 *M* phosphate buffer (pH 9.0)–water (*x*:5:up to 100, v/v/v). See Fig. 2 for other conditions.

developed separation is robust: small changes of the parameters tested do not significantly alter the separation pattern of the spiramycins.

3.2. Quantitative aspects of the LC method

For six consecutive injections of a solution of SPM I HS (20 μg injected on the column) the relative standard deviation (R.S.D.) on the peak area of the main component was 0.8%. The linearity of the method was checked at six points, with a total number of 18 analyses, corresponding to 1%, 2%, 5%, 10%, 50% and 100% of the normally injected mass. The following relationship was found by linear regression analysis: $y=1.282x+0.124$, where $y=$ peak area$\cdot 10^{-6}$, $x=$mass (μg) of sample injected; correlation coefficient $r=0.9999$, standard error of estimate $S_{y.x}=0.191$. Concentrations above 100% of SPM I were not examined, since commercial samples contain less than 80% of SPM I.

The limit of detection (LOD) with signal-to-noise ratio of 4 was 0.04% calculated for an injection of 20 μg of SPM I. The limit of quantitation (LOQ) with signal-to-noise ratio of about 10 was 0.08% ($n=6$,

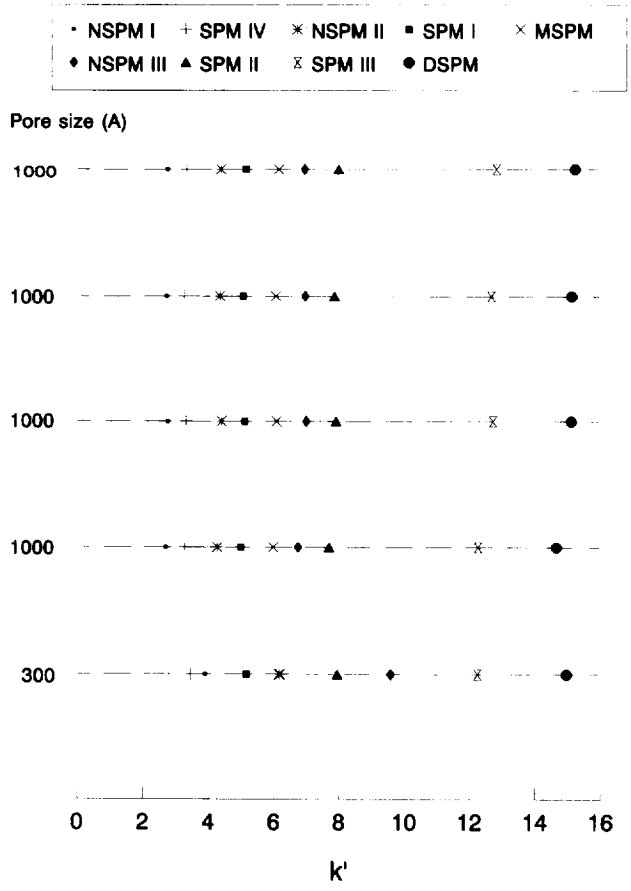

Fig. 5. Capacity factors (k') of spiramycin I and its related substances using PLRP-S columns with different pore sizes. Mobile phase: CH_3CN–0.2 *M* phosphate buffer (pH 9.0)–water (38:5:up to 100, v/v/v). See Fig. 2 for other conditions.

R.S.D.=11%). The stability of the sample solution was evaluated by analysing the same solution of SPM I for 72 h at room temperature of 20 to 22°C, and no additional peaks were observed in the chromatograms. The R.S.D. of the area of the SPM I peak was 0.58% (n=16).

For SPM III, with a retention time more than twice that of SPM I, the LOD with signal-to-noise ratio of 4 was 0.1% calculated for an injection of 20 μg of SPM III. The LOQ with signal-to-noise of about 10 was 0.2% (n=6, R.S.D.=9.0%).

3.3. Analysis of commercial samples

Bulk samples were analysed four times, using two individually prepared solutions. The content of SPM I, SPM II and SPM III in bulk samples (m/m) was determined by comparison with the corresponding SPM HS reference solutions. The other substances were calculated with reference to a 1% dilution of the SPM I HS reference solution (Table 2). The composition of spiramycin is complex. The so-called polar impurities, eluted before NSPM I are counted as a group. The R.S.D. for the main component is always smaller than 1%. Old European samples (e.g., Sample 1 that is more than 10 years old) contain much more SPM II and III and more of the so-called polar impurities. The other more-recently-produced European samples (Samples 2–6) contain mainly SPM I, some SPM III and very little SPM II; whereas in the Chinese samples (Samples 7, 8) SPM II and III are the main components. This observation

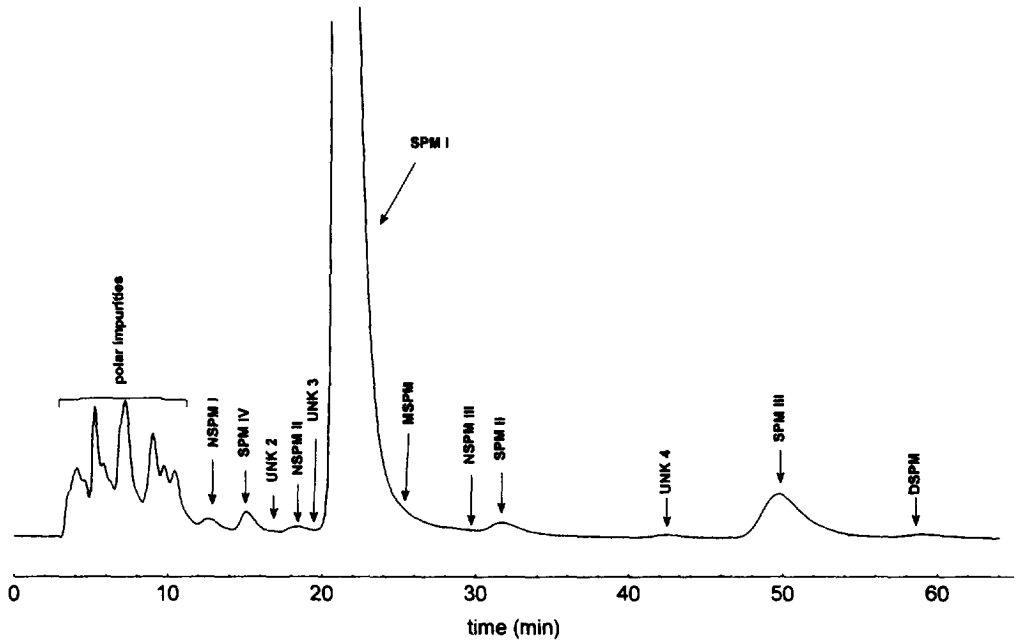

Fig. 6. Typical chromatogram of commercial spiramycin (sample 3) of European origin. Mobile phase: CH_3CN–0.2 *M* phosphate buffer (pH 9.0)–water (37:5:up to 100, v/v/v). See Fig. 2 for other conditions.

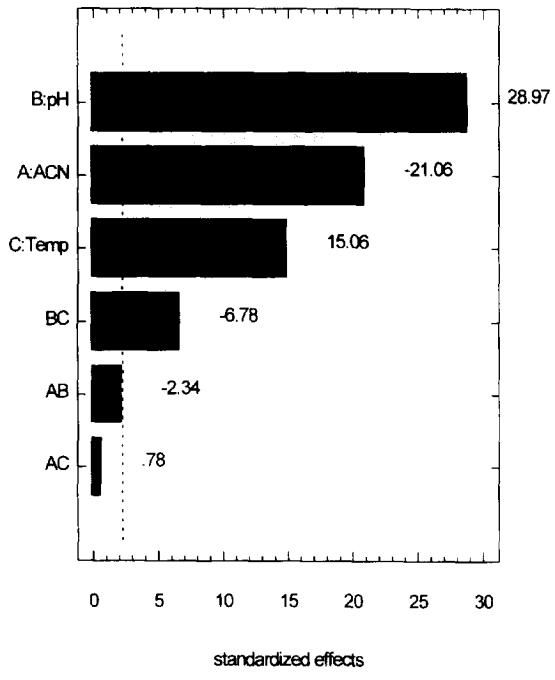

Fig. 7. Standardized Pareto chart showing the estimated effects of parameters and parameter interactions on the resolution between SPM I and II.

is most probably due to the use of different strains of *Streptomyces* for the fermentation. A typical chromatogram of a spiramycin sample of Chinese origin is shown in Fig. 8. The Chinese samples contain less of the so-called polar impurities and more of the neospiramycins. In these samples SPM I is less well separated from the surrounding impurities. The Chinese samples do not comply with the European Pharmacopoeia definition, which stipulates that SPM I be the main component and which limits SPM II and III at 5% and 10%, respectively [19].

The mass balance explained by LC corresponds quite well to that found by non-aqueous titration of total base, calculated as SPM I. The water content determined by Karl Fischer titration is always less than 2.5%. The loss on drying at 80°C in high vacuum (values not shown) was always somewhat lower than the KFT water content. This finding excludes the presence of considerable amounts of organic solvents. The European Pharmacopoeia allows 3.5% of volatile impurities as determined by loss on drying [19]. The total mass explained by the sum of LC results and water content is close to 97%, except for the old European sample 1.

Table 2
Composition (%, m/m) of bulk samples of spiramycin

Sample number	1	2[a]	3	4	5	6	7	8
Polar impurities	16.37	9.63	12.44	10.19	11.45	9.37	4.31	6.13
NSPM I	1.49	1.36	0.68	0.53	0.55	0.48	ND	2.14
UNK 1	ND	ND	ND	ND	ND	ND	0.98	0.76
SPM IV	2.61	0.90	0.87	0.84	0.84	1.05	ND	0.64
UNK 2	0.38	ND	ND	ND	ND	ND	0.57	0.73
NSPM II	0.71	0.22	0.44	0.38	0.40	0.33	1.0	3.34
UNK 3	ND	ND	ND	ND	ND	ND	0.63	0.79
SPM I	49.44	79.55	75.43	78.48	78.24	78.65	9.57	7.69
	(0.7)	(0.7)	(1.0)	(0.6)	(0.7)	(0.4)	(2.2)	(1.1)
NSPM III	ND	ND	ND	ND	ND	ND	0.96	4.20
SPM II	5.82	0.20	0.40	0.51	0.56	0.45	40.24	33.88
	(0.9)	(9.5)	(9.0)	(1.8)	(2.6)	(6.9)	(0.6)	(0.3)
UNK 4	ND	ND	0.21	ND	ND	ND	0.17	0.37
SPM III	12.22	1.51	3.75	3.62	3.18	4.49	36.86	34.84
	(3.0)	(8.0)	(5.4)	(3.7)	(0.7)	(0.6)	(0.2)	(0.6)
DSPM	ND	ND	0.44	ND	ND	ND	ND	ND
E: Total	89.04	93.37	94.66	94.55	95.22	94.82	95.29	95.50
Base titration	91.20	95.94	94.60	95.00	95.38	95.02	95.76	97.08
	(0.5)	(0.1)	(0.5)	(0.2)	(0.4)	(0.5)	(0.3)	(0.2)
F: Water	2.19	2.03	1.35	1.52	1.86	2.33	1.81	1.81
	(4.5)	(4.8)	(11)	(5.1)	(5.4)	(2.6)	(7.2)	(7.3)
Total mass explained (E+F)	91.23	95.40	96.01	96.07	97.08	97.15	97.10	97.31

R.S.D. (%) is given in parentheses. Water was determined by Karl Fischer titration. Total number of analyses=4; number of solutions analysed=2. ND: not detected.

[a] Spiramycin adipate salt, results recalculated on basis of molecular mass.

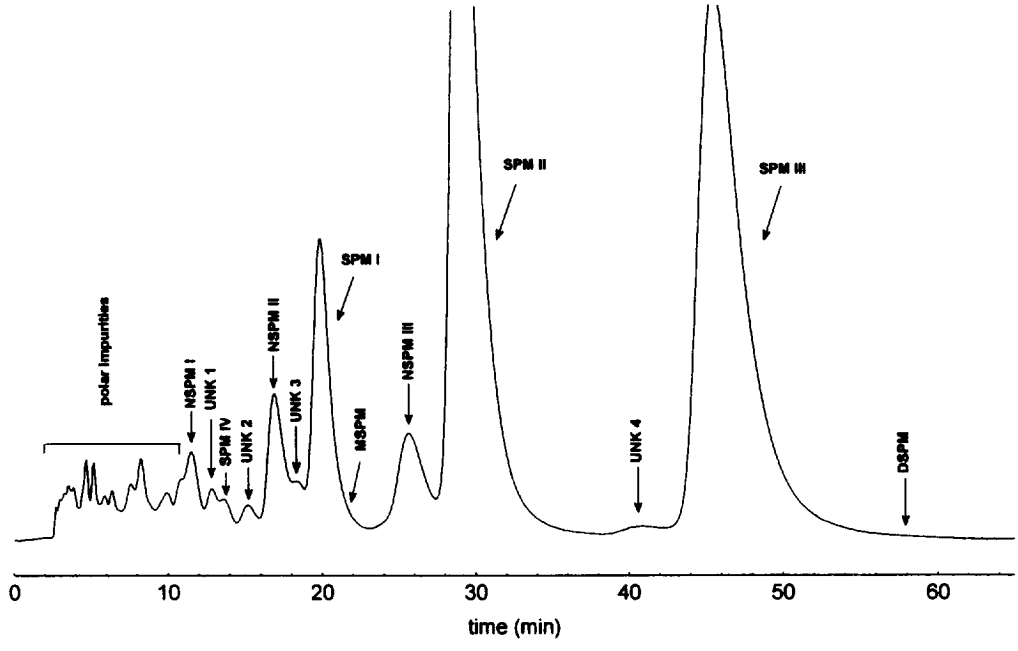

Fig. 8. Typical chromatogram of commercial spiramycin (sample 8) of Chinese origin. See Fig. 5 for chromatographic conditions.

4. Conclusions

The wide-pore PS–DVB stationary phase (PLRP-S 8 μm 1000 Å) shows good selectivity toward spiramycins and related substances. The isocratic method presented here is suitable to separate spiramycin I, II, III from the potential impurities present in samples of European origin, which comply with the European Pharmacopoeia. The method is precise, sensitive and robust.

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