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Interlaboratory study comparing the microbiological potency of spiramycins I, II and III

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Abstract

An interlaboratory study has been performed to determine the relative potencies of spiramycins (SPMs) I, II and III by diffusion or/and turbidimetric assays with *Bacillus subtilis* or *Staphylococcus aureus* as the test organisms. Six laboratories from three countries participated. Experimental procedures were according to the European Pharmacopoeia, 3rd ed. The activity of SPM I is markedly higher than that of SPM II and III. By diffusion, the activities of SPM II and III relative to SPM I were found to be 57 and 72%, respectively. The interlaboratory relative standard deviations (RSD) varied from 3.6 to 16.3%. By turbidimetry, the activities of SPM II and III relative to SPM I were found to be 45 and 52%, respectively. The interlaboratory RSD values varied from 2.6 to 7.7%. The results of the study were analyzed according to the ISO 5725-2 guidelines to determine the repeatability, the between-laboratory and the reproducibility variances of both methods.

Keywords: Spiramycin; Interlaboratory study; Microbiological potency; Macrolide antibiotic

1. Introduction

Spiramycin (SPM) is a 16-membered macrolide antibiotic produced by *Streptomyces ambofaciens*. It is a complex mixture with three major components, differentiated in the substituent at 3-position, namely I (3-OH), II (3-*O*-acetyl) and III (3-*O*-propionyl). This series of compounds shows gram-positive and gram-negative antibacterial activities.

Some microbiological data on these antibiotics have been presented in previous publications.

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Corbaz et al. [1] reported on the potency of metabolites from actinomycetes in which they used several gram-positive bacteria as test organisms. However, relative potencies of SPMs and their derivatives were not determined. Describing the liquid chromatographic (LC) determination of spiramycin components in pharmaceutical preparations, Horie et al. [2] mentioned that SPMs I and III have higher activity against *Micrococcus luteus* ATCC 9341 than the other related substances. Sanders et al. [3] compared liquid chromatography and bioassay methods for determining SPM in bovine plasma. Moreover, in none of the previous publications samples of the different spiramycins with well-established purity were available. Until now, very little is known about the relative antibacterial activity of spiramycin components.

Recently, a liquid chromatographic method was developed for the determination of the different SPM components in commercial samples of both European and Chinese origin [4]. SPMs I, II and III house standards were well characterized.

With the intention to determine the relative potency of these three compounds, six laboratories from three countries participated in this collaborative study. Each laboratory received SPM I, II and III house standards as samples. SPM I was also used as the reference substance (RS) for the comparison, with an assigned potency of 5535 International Units (IU) mg^{-1}, as determined in the organizing laboratory versus the European Pharmacopoeia (Ph. Eur.) chemical reference substance of spiramycin with a potency of 3750 IU mg^{-1}. The collaborating laboratories were not advised as to the identical nature of the SPM I sample and the RS.

Participants were asked to work according to the Ph. Eur. [5] and to use the agar diffusion method with *Bacillus subtilis* as the test organism and/or the turbidimetric method with *Staphylococcus aureus*. For both methods, a three- or two-dose single assay with randomized block design without replication or Latin square design was used. Experimental procedures and calculations were according to Ph. Eur. guidelines.

For both the diffusion and turbidimetric methods, the concentrations of the solutions were to be chosen to ensure that a linear relationship existed between the logarithm of the dose and the response. Other microorganisms and media amenable to a better sensitivity of SPMs, and other conditions of temperature and pH than those prescribed were allowed.

Each participant was requested to report individual measurements on a prepared data sheet. All results were calculated in IU versus the RS being identical to the SPM I house standard (HS). The precision of the assays had to be such that confidence limits of $\pm 5\%$ ($P = 0.95$) would be expected.

2. Materials and methods

2.1. Materials

Petri dishes 90 mm in diameter, rectangular plates were 30.5×30.5 cm with aluminum frames. Steel cylinders were 6 mm i.d. $\times$ 8 mm o.d. $\times$ 10 mm height. Visualization systems were used for measurement of inhibition zone diameters.

2.2. Samples

Spiramycin I, II and III (labeled A, B and C) were prepared in the organizing laboratory by published procedures [4]. The total mass indicated by the sum of nonaqueous potentiometric titration, the water content determined by Karl–Fischer titration and the amount of residual organic solvents by gas chromatography was 99.18, 99.01 and 98.94% (m m^{-1}), respectively. The purities of the SPMs were calculated to be 95.0, 93.3 and 94.2% (mm^{-1}) respectively, by subtraction of the impurities, determined by LC, from the total base content determined by potentiometric titration.

2.3. Diffusion method

An assay medium containing (in grams): peptone, 6.0; pancreatic digest of casein, 4.0; beef extract, 1.5; yeast extract, 3.0; dextrose monohydrate, 1.0; agar, 15.0 and water to produce 1000 ml (medium A of the Ph. Eur.) was used [5]. The pH of the medium was adjusted to 7.9 with

1 M NaOH before sterilization. *Bacillus subtilis* spores cultivated using the protocol described in the Eur. Ph. were washed off by 10 ml of sterile water. This suspension (suitable amount) was used to inoculate 300 ml of medium A. This medium was distributed in 20-ml amounts in 90-mm Petri dishes (except Lab. 4, they used 14 ml of inoculum medium) or in 30.5×30.5 cm large rectangular plates. Spiramycin samples were dissolved in a minimal amount of methanol and then diluted with 0.05 M phosphate buffer solution pH 8.0 to the desired volume (1000 IU ml^{-1}). Further working dilutions were prepared with the same buffer solution. Suitable amounts of sample solutions (50–200 μl) were applied in wells punched out of the agar, plates were prediffused for 1–3 h at 4°C and incubated for 18 h at 30–37°C. Zone diameters were determined with a visualization system and the potency of each sample was calculated with confidence intervals $P = 0.95$.

General information on experimental conditions used in the different laboratories is presented in Table 1. The numbers assigned to the laboratories do not correspond to numbers assigned to the authors.

2.4. Turbidimetric method

The medium contained (in grams): beef extract, 1.5; yeast extract, 3.0; sodium chloride, 3.5; dextrose monohydrate, 1.0; dipotassium hydrogen phosphate, 3.68; potassium dihydrogen phosphate, 1.32 and water to produce 1000 ml (medium C) [5]. The medium (100 ml) was inoculated with 1.25 ml of *Staphylococcus aureus* suspension in sterile water.

Spiramycin samples were dissolved in a minimal amount of methanol and then diluted with 0.05 M phosphate buffer to around 100 IU ml^{-1} (samples were presumed to be of equal activity as

Table 1
General information on conditions, microorganism and dilutions for the diffusion method

Laboratory	1	2	3	4	5
Medium					
Type (Ph. Eur.)	A	A	A	A	A
pH	7.9 ± 0.2	7.9 ± 0.2	7.9 ± 0.2	7.9 ± 0.2	7.9 ± 0.2
pH of buffer solution (0.05 M)	8.0	8.0	8.0	8.0	8.0
Micro-organism	*Bacillus subtilis* ATCC 6633	*Bacillus subtilis* ATCC 6633	*Bacillus subtilis* ATCC 6633	*Bacillus subtilis* ATCC 6633	*Bacillus subtilis* ATCC 6633
Dose ratio	2	2	1.33	1.33	2
Dilutions examined					
Total number	3	3	3	3	2
Doses (IU ml^{-1})	40–20–10	100–50–25	110–83–62	222–127–72	20–10
Applied volumes (μl)	200	100	50	50	200
Experimental design	Randomized Block	Latin Square	Randomized Block	Randomized Block	Latin Square
Number of replicates	9	6	6	6	6
Incubation					
Temperature (°C)	30–32°C	37°C	30–32°C	30°C	37°C
Time (h)	18	18	18	18	18
Number of assays	5	4	5	5	SPM I:3; SPM II:4; SPM III:4
Precision zone reader (mm)	0.1	0.2	0.1	0.02	0.1

Table 2
General information on conditions, microorganism and dilutions for the turbidimetric method

Laboratory	1	2	3
Medium			
Type (Ph. Eur.)	C	C	C
pH	7.0 ± 0.2	7.0 ± 0.1	7.0 ± 0.1
pH of buffer solution (0.05 M)	7.0	8.0	6.9
Micro-organism	*Staphylococcus aureus* ATCC 6538P	*Staphylococcus aureus* ATCC 6538P	*Staphylococcus aureus* ATCC 6538P
Dose ratio	2	1.33	1.3
Dilutions examined			
Total number	3	4	3
Doses (IU ml^{-1})	40–20–10	7.5–5.6–4.2–3.2	*
Experimental design	Randomized block	Randomized block	Randomized block
Number of replicates	5	6	3
Incubation			
Temperature (°C)	37	37	37
Time	3-3 h 30 min	3 h 30 min	4 h 15 min
Number of assays	5	4	4

* Doses (IU ml^{-1}) for SPM I: 17.7–13.6–10.5; SPM II: 38.7–29.8–22.9; SPM III: 35.5–27.3–21.0.

RS). Further working dilutions were prepared with the same buffer solution. Final dilutions were made using dilution ratios of 2 or 1.33. Sample solutions (1.0 ml) were brought into five test tubes. Inoculated medium (9.0 ml) was added to all tubes. Tubes were incubated in a water bath at 37°C for about 3 h–3 h 30 min. Growth was stopped by adding 0.5 ml of formaldehyde solution (35%). The optical density of the suspension was measured at 532 nm. General information on experimental conditions used in the different laboratories is given in Table 2. The numbers assigned to the laboratories do not correspond to the numbers in Table 1.

3. Results and discussion

Six laboratories participated in the experiment, carrying out the analyses as described above. Five laboratories reported results of the diffusion method and three laboratories reported results of the turbidimetric method. The raw data are shown in Tables 3 and 4. Some laboratories produced less results than required because of lack of sample. The results of the study were treated as described in the ISO (International Organization for Standardization) 5725-2 guidelines [6] to determine the repeatability, the between-laboratory and the reproducibility variances of both methods. For simplicity, the statistic tests applied to the results obtained with the diffusion method are discussed further as an example. Similar procedures were also applied to the turbidimetric method.

First, the results were tested for consistency (graphical technique) and outliers (numerical tests). In order to test for within-laboratory consistency, Mandel's k graphical test (Fig. 1) and the numerical outlier technique of Cochran's criterion with $n = 5$ for $p = 5$ laboratories were applied. Fig. 1 and Cochran's test clearly show that Lab. 4 has a poorer repeatability than the other laboratories, the higher potencies of SPM I and II being outliers (greater than the 1% critical value). Therefore, Lab. 4 has consistently high within-laboratory variation. This can be explained by the fact that they used thinner agar layer which de-

Table 3
Individual activities (IU mg^{-1}) from collaborative tests reported by five laboratories for the estimated potency of spiramycins (SPM) by using the diffusion method

Lab.	Samples														
	SPM I					SPM II					SPM III				
1	5341	5762	5640	5485	5424	2679	2701	2773	2845	2585	3775	3432	3631	3609	3836
2	5610	5228	5410	5816	NP[a]	2699	2715	2611	2689	NP	3530	3396	3148	3342	NP
3	5602	5796	5588	5627	5560	3671	3576	3763	3755	3808	4738	4485	4685	4844	4768
4	5948	5574	6978	5377	6252	3646	3786	3572	3115	2835	4986	4189	3739	4039	4107
5	5546	5589	5924	NP	NP	3649	3987	3933	3540	NP	4299	4281	4940	4237	NP

[a] NP: not performed.

creased the repeatability [7]. For testing between-laboratory consistency, Mandel's *h* graphical test (Fig. 2) and numerical outlier techniques of Grubbs tests were performed on the mean values. The high value in Lab. 4 in Mandel's *h* test is a straggler, but is retained for further analysis. Grubbs' test showed that no single or double stragglers or outliers were found in the mean values and no laboratory was excluded.

Mean values for the potency, limits of confidence expressed as percentages and mean of means are given in Tables 5 and 6. A quantitative estimate of the method accuracy was performed by comparing the SPM I results with the SPM RS values. SPM RS and SPM I are identical, as mentioned above. The accuracy of all the measurements was within 95–105% limits, except for Lab. 4. Here also, the confidence limits for diffusion in the experiments largely exceeded the 95–105% limits.

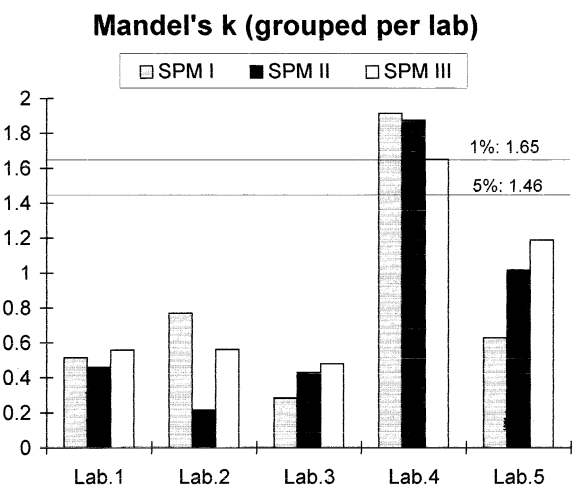

Fig. 1. Graphical consisitency technique for the diffusion method: Mandel's *k* (grouped per laboratory).

Table 4
Individual activities (IU mg^{-1}) from collaborative tests reported by three laboratories for the estimated potency of spiramycins (SPM) by using the turbidimetric method

Lab.	Samples														
	SPM I					SPM II					SPM III				
1	5369	5413	5308	5264	5524	2480	2579	2264	2452	2463	2900	3166	2801	2939	2862
2	5211	5580	5501	5475	NP[a]	2604	2525	2632	2550	NP	2988	3019	2979	3051	NP
3	5617	5707	5702	5568	NP	2348	2393	2365	2250	NP	2581	2632	2609	2559	NP

[a] NP: not performed.

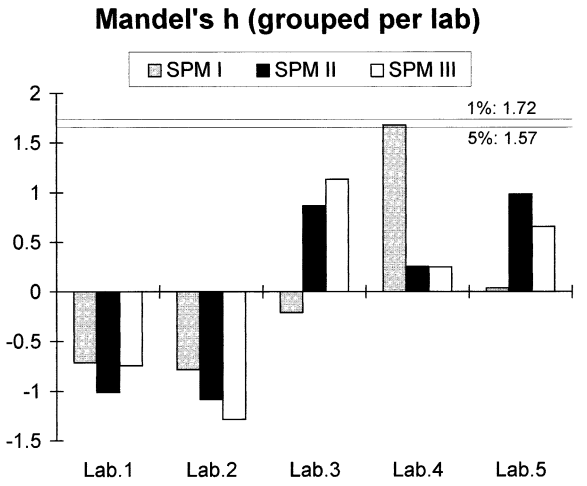

Fig. 2. Graphical consistency technique for the diffusion method: Mandel's *h* (grouped per laboratory).

The laboratory means in Tables 5 and 6 were compared by a two-tailed *t*-test with $(n_1 + n_2 - 2)$ degrees of freedom [8]. The mean values of SPM I are not significantly different in diffusion and turbidimetry (at the 5% level), but the mean values of SPM II and SPM III differ significantly. Variations can be explained by the different methods used (diffusion or turbidimetry) but also by the different microorganisms and culture conditions which are applied.

The variances for both methods are estimated in Table 7. The *F*-ratios of the two methods are compared with the critical values ($P = 0.95$). It is shown that there are no significant variances between diffusion and turbidimetric methods for SPM I and III, but that both methods have significant between-laboratory variances for SPM II.

The results in Table 5 reveal that against *Bacillus subtilis* SPM I has a markedly higher potency than SPM II or SPM III, the latter two having relative activities of 57 and 72%, respectively. Interlaboratory RSDs vary from 3.6 to 16.3%, depending on the samples. In the turbidimetric method with *Staphylococcus aureus* (Table 6), SPM I also exhibits a higher potency and SPM II and III have relative activities of about 45 and 52%, respectively. The RSDs vary from 2.6 to 7.7%.

The present study can contribute to a better standardization of the potencies of different spiramycins.

Table 5
Mean activities (IU mg^{-1}) for the estimated potency of spiramycins by the diffusion method. The limits of confidence ($P = 0.95$), expressed as percentages, are mentioned in parentheses

Lab.	1	2	3	4	5	Mean of means RSD%
SPM RS	5535	5535	5535	5535	5535	5535
SPM I	5530 (96.6–104.2)	5516 (94.8–105.4)	5635 (98.7–102.8)	6026 (89.2–115.8)	5686 (97.5–104.2)	5679, 3.6
SPM II	2717 (95.1–104.7)	2678 (97.5–100.8)	3715 (96.2–102.5)	3391 (86.6–111.6)	3777 (93.7–105.5)	3256, 16.3
SPM III	3657 (93.8–104.9)	3354 (93.8–105.2)	4704 (95.3–103.0)	4212 (88.8–118.4)	4439 (95.4–111.3)	4073, 13.7

Table 6
Mean activities (IU mg^{-1}) for the estimated potency of spiramycins by the turbidimetric method. The limits of confidence ($P = 0.95$), expressed as percentages, are mentioned in parentheses[a]

Lab.	1	2	3	Mean of means RSD%
SPM RS	5535	5535	5535	5535
SPM I	5375 (97.9–102.8)	5442 (95.8–102.5)	5648 (98.0–102.0)	5488, 2.6
SPM II	2448 (92.5–105.3)	2578 (97.9–102.1)	2339 (98.0–102.1)	2455, 4.9
SPM III	2934 (98.0–108.4)	3009 (99.0–101.3)	2595 (98.0–102.0)	2846, 7.7

[a] Paired *t*-test (mean of means in Tables 5 and 6): SPM I: $t = 1.39$; SPM II: $t = 2.50$; SPM III: $t = 3.56$; $t(6df) = 2.447$ two-tailed.

Table 7
Estimation of the repeatability, the between-laboratory and the reproducibility variances for the diffusion and turbidimetric methods

	Variances									
	Diffusion method			Turbidimetric method			*F*-values			$F_{(4,2)}$ critical
	SPM I	SPM II	SPM III	SPM I	SPM II	SPM III	SPM I	SPM II	SPM III	
Repeatability (s^2_{rj})*	118 673	47 721	80 484	13 122	7106	8414	9.04	6.72	9.56	19.25
Between-laboratory (s^2_{Lj})**	20 194	263 083	285 114	17 141	11 624	43 997	1.18	22.63	6.48	19.25
Reproducibility ($s^2_{Rj} = s^2_{rj} + s^2_{Lj}$)***	138 867	310 804	365 599	30 263	18 730	52 411	4.59	16.59	6.98	19.25

* The repeatability variance at each sample *j*.
** The between-laboratory variance at each sample *j*.
*** The reproducibility variance at each sample *j*.

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