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Short communication

Extremely long time stability study of selected antibiotic standards

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ABSTRACT

A study was conducted to investigate the quality of reference substances which were produced long before the introduction of advanced analysis and purification techniques. The quality and antimicrobial activity of WHO and EP oxytetracycline, doxycycline, colistin and spiramycin reference standards were assessed. The oldest substance was stored for 54 years in a freezer. Assay and purity tests were conducted according to Ph. Eur. 6.0. Additionally, antibacterial activity was tested with the microbiological method according to Ph. Eur. 4.0 (agar diffusion method). The results of the study show that several of the tested substances remained stable for over 40 years and one for over 50 years of storage. In most cases, the determined potency is close to the declared one, regardless of the method used (HPLC or microbiological). Composition analysis of multi-substance antibiotics (colistin and spiramycin) showed important differences compared with new reference substances. Results also indicate that no excessive degradation occurred during the entire storage period and impurity levels have not changed significantly.

1. Introduction

Standard reference substances are issued by official organizations like The European Pharmacopoeia (Ph. Eur.) or The United States Pharmacopeia (USP) or The British Pharmacopoeia (BP). As a rule, the validity period of such standards is set by the organization by changing the current (approved) batch. This means that any substances from previous batches must be substituted with new ones [1,2].

The aim of this research was to investigate the antibacterial activity, purity and the potential usefulness for microbiological or chemical analysis (HPLC) of the official reference substances stored for over 40 years in a freezer. It also includes the results presented as a first part of the stability study [3].

Three groups of reference substances were researched during the study. The first group—tetracyclines are represented by doxycycline and oxytetracycline, compounds isolated from the *Streptomyces* strains. Tetracyclines belong to broad spectrum antibiotics active against both Gram-positive (stronger) and Gram-negative (weaker) bacteria [4].

Tetracyclines are rather stable; nevertheless, elevated conditions such as temperature or pH might lead to epimerization, producing impurities [5,6]. They become increasingly labile, especially at high temperatures [7].

The second group—polypeptides represented by colistin, which is a mixture of compounds produced by *Bacillus polymyxa subsp. colistinus*. Their main components are colistin A (polymyxin E1) and colistin B (polymyxin E2). Colistin is active against Gram-negative bacteria. It is rather stable in pH 2–6 but in a pH above 6, its activity decreases. The dry preparation can be stored in high temperatures without being inactivated [8].

As an example of the third group, macrolides, spiramycin (SPM) was chosen. It is produced by *Streptomyces ambofaciens* as a mixture of three major components: Spiramycin I, II and III. Spiramycins are active against Gram-positive bacteria and some Gram-negative rods. In acidic conditions, spiramycin often produces a number of related substances also characterized by antimicrobial activity [4,8]. Although spiramycin consists of 3 compounds, spiramycin I has the highest activity and is regarded as a main component [2,10]. Depending on the site and time of manufacturing, the proportions between spiramycins I, II, and III and the impurity profile can vary considerably [9].

2. Materials and methods

2.1. Instrumentation

Chromatographic system (I) Dionex Ultimate 3000 series (Dionex Softron, Germering, Germany) was equipped with a UV–vis variable wavelength detector and controlled by Chromeleon software. Chromatographic system (II) Shimadzu LC-10AVP series (Shimadzu Kyoto, Japan) was equipped with a DAD detector and controlled by Class-VP software. Both systems also included: a vacuum degasser, an autosampler and a column oven. The diameter of

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Table 1
Compilation of assays of all tested substances performed with HPLC and microbiological methods.

Tested substance	Oxytetracycline						Doxycycline		Colistin		Spiramycin
	1955		1966		1980		1973		1968		1962
	Analysis method										
	HPLC	MICROB.	HPLC	MICROB.	HPLC	MICROB.	HPLC	MICROB.	HPLC	MICROB.	MICROB.
Declared contents [μg/mg] or [I.U./mg][a]		900		880		905		870		20500	3200
Determined contents [μg/mg] or [I.U./mg] (mean of 6)[a,b]	928.96	888.61	876.34	875.81	902.25	880.05	847.97	838.83	20694.79	20361.00	3248.19
% of declared contents	103.22	98.73	99.58	99.52	99.70	97.24	97.47	96.42	100.95	99.32	101.51
Confidence interval (95%)	928.96 ± 8.245	888.61 ± 31.521	876.34 ± 4.153	875.81 ± 13.696	902.25 ± 7.069	880.05 ± 19.704	847.97 ± 3.062	838.83 ± 11.518	20694.79 ± 57.509	20361.00 ± 483.216	3248.19 ± 116.576
SD	7.86	30.04	3.96	13.05	6.74	18.78	2.92	10.98	54.80	460.45	93.89
RSD%	0.85	3.38	0.45	1.49	0.75	2.13	0.34	1.31	0.26	2.26	2.89
Variance	61.73	902.17	15.66	170.33	45.38	352.55	8.52	120.46	3003.08	212016.80	8814.75
t-Test t_{emp}	3.184		0.096		2.726		1.971		1.763		
t-Test t_{tab}	2.228	$t_{emp} > t_{tab}$	2.228	$t_{emp} < t_{tab}$	2.228	$t_{emp} > t_{tab}$	2.228	$t_{emp} < t_{tab}$	2.228	$t_{emp} < t_{tab}$	

[a] [μg/mg] in case of oxytetracycline and doxycycline. "μg" of activity are used. [I.U./mg] in case of colistin and spiramycin.
[b] Mean of 5 in case of colistin.

bacteria growth inhibition zones were measured with the Antibiotic Zone Reader, 3 Fisher-Lilly (Fisher Scientific, Pittsburgh, PA, USA).

2.2. Chemicals and reagents

All reagents were of analytical grade. 2-methyl-2-propanol and tetrabutylammonium hydrogen sulphate were obtained from Sigma–Aldrich (Steiheim, Germany), disodium edeteate was supplied by Fluka Chemie (Buchs, Germany), the medium for microbiological tests PA1 was prepared using components from Difco (Detroit, MI, USA) and Merck (Darmstadt, Germany). Methanol, disodium hydrogen phosphate, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, sodium hydroxide, sodium sulphate, and hydrochloric acid were obtained from POCH S.A. (Gliwice, Poland). All experiments were conducted using highly purified water from Water Pro PS (Labconco, Kansas City, MI, USA).

2.3. Tested substances

Oxytetracycline WHO First Int. Standard Est. 1955; WHO Second Int. Standard Est. 1966, EP RCS standard lot Ph. Eur. 1, 1981; Doxycycline WHO First Int. Standard Est. 1973; Colistin WHO First Int. Standard Est. 1968; Spiramycin WHO First Int. Standard Est. 1962.

The tested substances were kept in a freezer (at about −20 °C) in unopened, original containers for the entire storage period.

2.4. Reference substances

Oxytetracycline LOT J0C084 (USP); Doxycycline LOT J0E174 (USP); Colistin CRS lot. 2a (Ph. Eur.); Spiramycin CRS lot. 4a (Ph. Eur.). For oxytetracycline and doxycycline impurities determination, the CRS standards of impurities were used. The reference standards were stored in the refrigerator (2–8 °C) (colistin was stored at a temperature of about −20 °C) in original containers.

2.5. Chromatographic conditions

All chromatographic conditions had been set precisely in accordance to the corresponding Ph. Eur. monographs. Following are the monograph numbers and the columns used. Oxytetracycline: monograph 01/2008:0198, column: divinyl benzene copolymer 250 mm × 4.6 mm, particle size 8 μm (Polymer Laboratories, Church Stretton, UK). Doxycycline: monograph 01/2008:0272, column: divinyl benzene copolymer 250 mm × 4.6 mm, particle size 8 μm (Polymer Laboratories, Church Stretton, UK). Colistin monograph 01/2008:0320, column: C18 BDS Hypersil 250 mm × 4.6 mm, particle size 5 μm (Thermo scientific, Waltham, MA, USA). Spiramycin monograph 01/2008:0293, column: SymmetryShield RP18 250 mm × 4.6 mm, particle size 5 μm (Waters Corporation, Milford, MA, USA).

2.6. Microbiological tests conditions

2.6.1. Oxytetracycline and doxycycline

Test strain *Bacillus cereus* ATCC 11178; solvent: 0.1 M HCl; diluent: phosphate buffer pH 4.5; medium A pH 4.5 (Ph. Eur.); diffusion 1 h in room temperature. First dilution with 0.1 M HCl achieved 1000 U. and final dilutions with phosphate buffer were 4 and 1 I.U. (two-point assay). Plates were prepared with PA1 medium, 16 ml of the medium and 4 ml of an inoculated medium (0.3 ml of the test strain of a 5 McF density per 80 ml of medium). The above conditions were set in accordance with oxytetracycline Ph. Eur. 4.0 monograph.

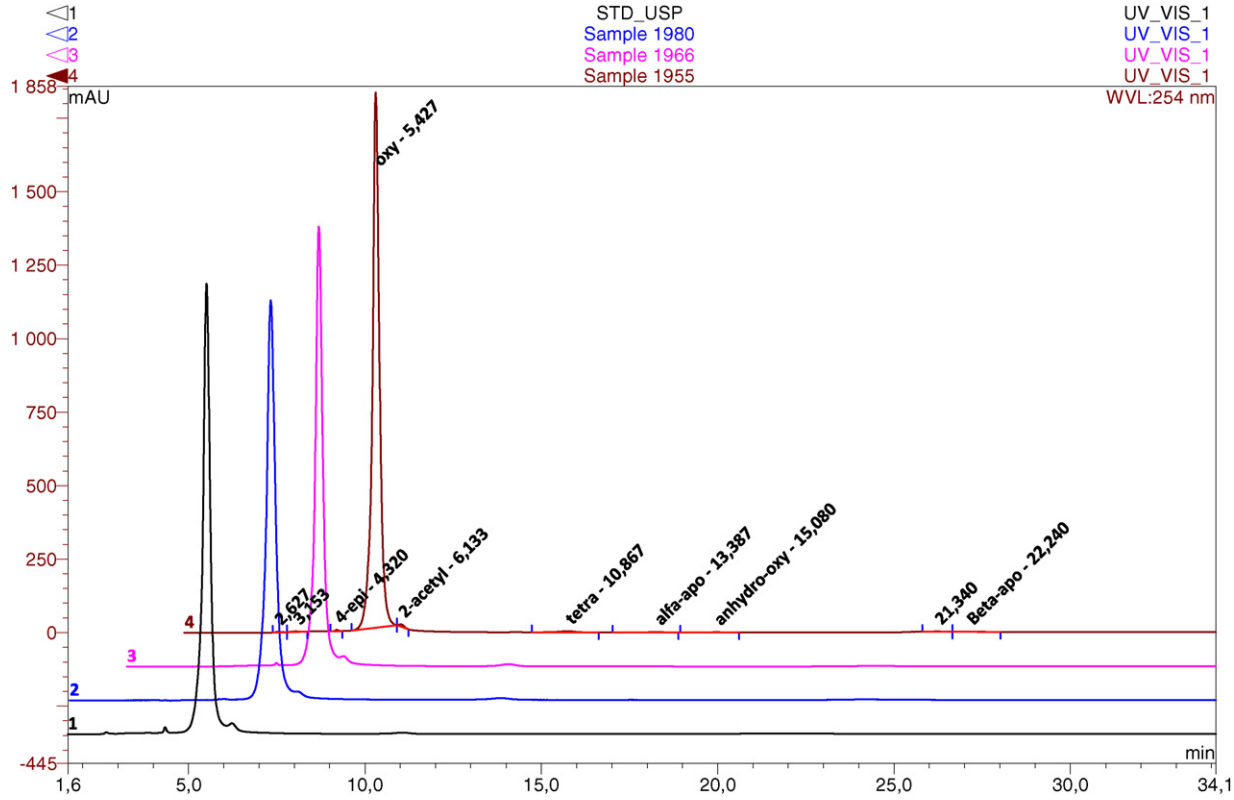

Fig. 1. Comparison of chromatograms obtained with: oxytetracycline reference substance (1), sample from 1980 (2), sample from 1966 (3), and sample from 1955 (4).

2.6.2. Colistin

Test strain *Bordetella bronchiseptica* ATCC 4617; solvent: water; diluent: phosphate buffer pH 6.0 10%; medium B (Ph. Eur.); diffusion 3 h in room temperature. Final dilutions with phosphate buffer were 400 and 100 I.U. (two-point assay). Plates were inoculated with a suspension of the test strain (4.0 ml of a 5 McF density suspension per 80 ml of medium).

2.6.3. Spiramycin

Test strain *Bacillus subtillis* ATCC 6633; solvent: methanol for dissolving the substance to be analyzed and water to dilute this solution to achieve 1st dilution of 1000 I.U.; diluent: phosphate buffer pH 8.0; medium A (Ph. Eur.); diffusion 3 h at room temperature. Final dilutions with phosphate buffer were 40 and 10 I.U. (two-point assay). Plates were inoculated with a suspension of the test strain (0.2 ml of endospores per 80 ml of medium).

2.6.4. Procedures

The same reference standard was used for chemical and microbiological analyses. Chemical and microbiological procedures were started simultaneously ensuring that the tested substance was not stored after first opening. For microbiological procedures, the diffusion time is set in the particular method. Plates were incubated at 37 °C for 18 h. The diameters of bacteria growth inhibition zones were measured and the activity of the antibiotic was calculated by using statistic methods applied in CombiStats software.

3. Results and discussion

3.1. Assay

Unfortunately, no information on the statistical quality and especially the confidence interval concerning a manufacturer potency declaration is available. It is possible that the declared potency was determined with a much higher error; hence, relying solely on that declaration could lead to a lower accuracy of the analyses performed with the tested substance as a primary reference. Additionally, this lack of information makes the interpretation of the results of this study more difficult.

3.1.1. Oxytetracycline

The results of HPLC as well as the microbiological assays are combined in Table 1. In the case of samples from 1955 and 1980, the result – obtained with both methods – differences are statistically significant. A low precision of microbiological assay renders both methods not interchangeable. The results obtained with the 1966 sample show that with better precision, the microbiological assay becomes similar to the HPLC assay. Fig. 1 shows a comparison of sample chromatograms. It can be observed that the peaks on all chromatograms have similar retention times and areas.

In all the obtained results, the highest deviation from the declared value is 103.22% for the 1955 sample—HPLC method, and 97.24% for the 1980 sample—microbiological method. Apart from those, all results are near 100% of the declared value. On the other hand, the interval recommended in European Medicines Agency guidelines on stability [10] is 95–105% (significant change of 5%). Taking this into account, it can be stated that no significant change occurred during storage because the determined values are different from those declared by less than 5%. All but the 1955 sample results are within current Ph. Eur. 6.0 limits (95–102%).

To double-check the system and USP Reference Standard, a comparison had been conducted with the CRS and Pfizer (PZF) working standard. Both showed a similar response factor, namely 470, 457, 458 (peak area for 1 mg/ml) for CRS, PFZ and USP, respectively. The samples from the first run were also tested during the second run to investigate the stability of the prepared sample and standards.

After 1 week of storage at 2–8 °C, no significant decrease of the content of oxytetracycline in the sample solution could be

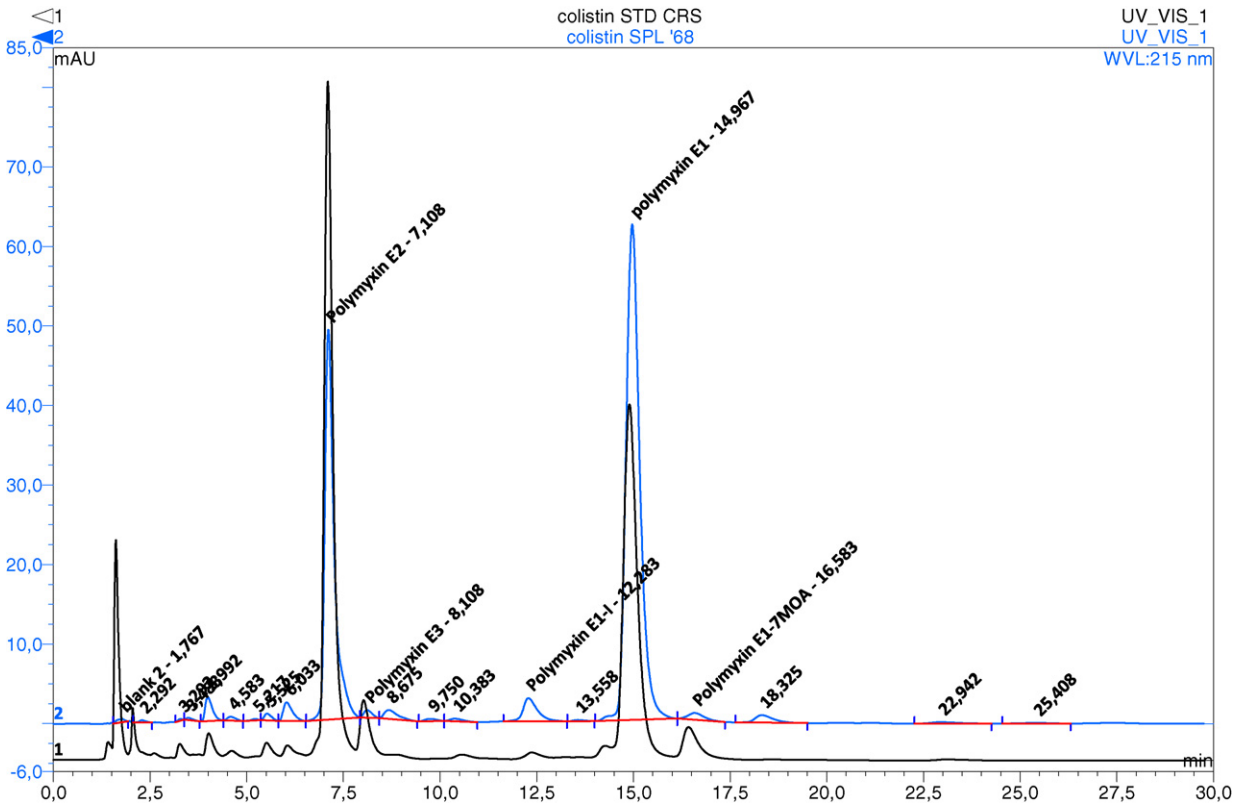

Fig. 2. Comparison of chromatograms obtained with: colistin reference substance (1) and sample from 1968 (2).

observed. Moreover, there were no significant differences between oxytetracycline retention time, the symmetry factor and the theoretical plate number.

3.1.2. Doxycycline

The results of HPLC as well as the microbiological assays are combined in Table 1. The test did not show any significant differences between the values obtained with the microbiological method and HPLC. Nevertheless, HPLC assay results indicate much better precision. Since method comparison with *t*-test is positive, it can be stated that the investigated substance is suitable for both HPLC and microbiological analysis. Also, both methods can be regarded as interchangeable. Similarly to oxytetracycline, it can be stated that no significant change occurred during storage, since assay results fall within the 95–105% interval and current Ph. Eur. 6.0 limits (95–102%).

3.1.3. Colistin

Due to the fact that colistin is a mixture of peptides that show a similar structure and activity (polymyxins), the microbiological assay only gives information about the sum of polymyxins, while during HPLC analysis those polymyxins undergo separation. In order to compare the results from both methods, the area of peaks due to polymyxins was added together. Table 1 combines the assay results. In this case, the microbiological and HPLC methods yielded very similar values, although the precision of the HPLC method (expressed as RSD) is much higher. Since method comparison with *t*-test is positive, it can be stated that the investigated substance is suitable for both HPLC and microbiological analysis. In this case, the obtained assay results are very close to the declared value—the difference is less than 1%. This suggests that no significant change occurred during storage time. The above statements are valid when colistin is treated as a sum of polymyxins. There is no information about the initial composition of the tested WHO standard, thus no conclusions can be formulated about composition changes during storage. Fig. 2 shows sample chromatograms of the tested and reference substances. The figure clearly shows differences in main peak areas. The obtained results for composition are within the Ph. Eur. 6.0 limits, but there are significant differences between the tested WHO standard and the reference USP standard. In particular, the contents of polymyxins E2 (47.71%-USP, 30.65%-WHO) and E1 (37.99%-USP, 57.82%-WHO) differ substantially. Nevertheless, it must be noted that the overall microbiological activity as well as the added contents analyzed by HPLC is similar in both the tested and reference substances and is within Ph. Eur. limits. Since antimicrobial activity of polymyxins is similar [4], the differences in composition do not influence the microbiological assay.

3.1.4. Spiramycin

According to Ph. Eur. 6.0, monograph spiramycin should be assayed by the microbiological method. HPLC was performed only for impurity determination. The results from the microbiological assay are presented in Table 1. The determined activity is very close to the declared value. This suggests that no degradation has occurred during 47 years of storage. The results were calculated using 5 replicates. However, in current Ph. Eur. 6.0 monograph of spiramycin, the overall potency of the substance should not be less than 4100 I.U., which renders the tested substance much below the present requirements for potency.

Table 2 presents the content of spiramycins I, II and III in tested and reference substances. The percentage is calculated on the basis of peak areas in relation to total peak area. The contents of spiramycins in reference to the CRS standard is similar to the values quoted on the CRS batch leaflet, whereas the percentage of spiramycin in the tested WHO standard is quite different. This composition is consistent with literature data [9], which suggests that no major degradation occurred during storage. According to spiramycin monograph in Ph. Eur. 6.0, spiramycin I and II content

Table 2
Comparison of spiramycin composition determined with HPLC.

Spiramycin	Tested WHO standard	Reference CRS
SPM I	54.72%	88.88%
SPM II	23.09%	0.48%
SPM III	13.14%	6.19%
Sum	90.95%	95.54%

is not to be higher than 5.0% and 10.0%, respectively. The tested WHO standard does not meet those requirements, thus it cannot be used as a reference substance for tests in Ph. Eur. 6.0 monograph of spiramycin.

3.2. Impurities

Table 3 combines the results of the impurity determination of oxytetracycline, doxycycline and colistin and the limits set in current Ph. Eur. 6.0. In the case of colistin, Ph. Eur. does not require identification of individual impurities, and for this reason only the total and single biggest impurity are shown. Impurities contents in these 3 substances are well within the limits of Ph. Eur 6.0 monographs.

In the case of 4-epioxytetracycline (oxytetracycline impurity), its contents in the sample from 1966 is different from the samples from 1955 and 1980. This can suggest that different strains and/or different purification were used during the manufacturing process. 3D spectra of oxytetracycline and doxycycline were investigated for absorption at 490 nm. Such absorption measurement for light absorbing impurities was present in the earliest pharmacopoeia monographs for oxytetracycline and doxycycline [11]. No elevated absorbance was detected at this wavelength, which also confirms the purity of the tested substances.

Table 3
Contents of impurities in tested substances: oxytetracycline, doxycycline and colistin.

Impurities	Limit	Determined contents		
		1955	1966	1980
Oxytetracycline				
4-Epioxytetracycline	0.5%	0.08%	0.38%	0.09%
Tetracycline	2.0%	0.26%	0.38%	0.34%
2-Acetyl-2-decarbamoyloxytetracycline	2.0%	0.53%	1.04%	1.02%
Total other impurities	2.0%	0.21%	0.03%	1.62%

Impurities	Limit	Determined contents 1968
Doxycycline		
6-Epidoxycycline	2.0%	1.33%
Metacycline	2.0%	0.27%
4-Epidoxycycline	0.5%	0.19%
Any other impurity	0.5%	<LOQ

Impurities	Limit	Determined contents 1968
Colistin		
Any impurity	4.0%	1.43%
Total impurities	23.0%	7.22%

Fig. 3 presents the impurity profile of spiramycin. Impurity levels were determined on 4 replicates. The profile is compared with

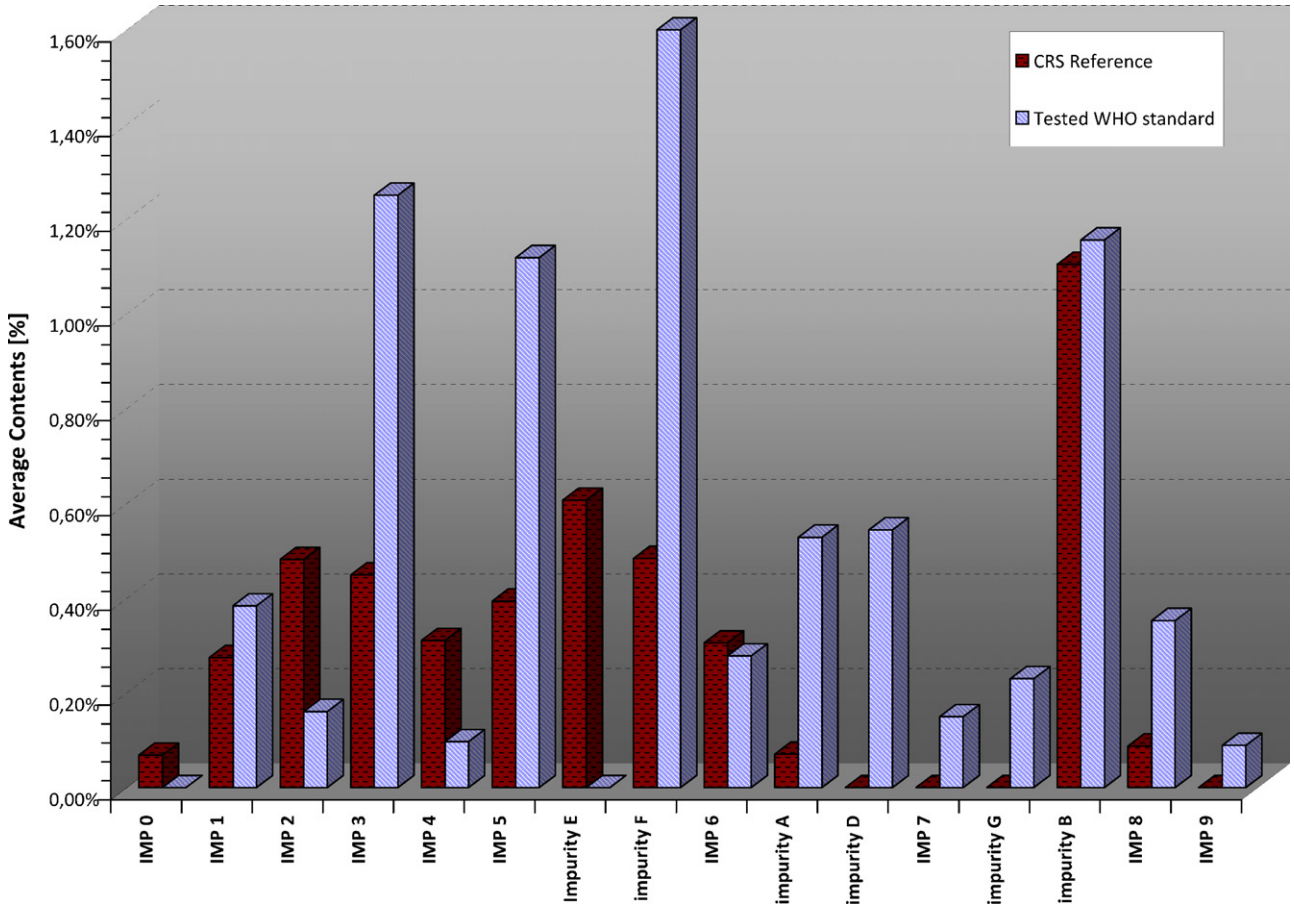

Fig. 3. Comparison of impurities contents determined in spiramycin reference substance and sample from 1962. Impurities identified by Ph. Eur. 6.0 are named with letter corresponding to monograph and unknown impurities are numbered from 0 to 9.

the impurities determined on the reference substance (CRS standard). The contents of all impurities were calculated with reference to the diluted reference substance solution. Some differences can be observed, especially the contents of impurities 3, 5, F and A are significantly different. Moreover, impurities D, 7, G, 9 are present in the WHO sample; whereas, in the CRS standard, they are below the limit of quantitation. This suggests different biosynthesis and/or purification methods used, which is consistent with literature data [9]. The total impurities content is higher than in the reference substance, but all levels still meet the limits of Ph. Eur. 6.0 monograph. These results indicate that no excessive degradation occurred during the whole storage period and impurity levels have not changed significantly. Nevertheless, the tested spiramycin does not comply with current Ph. Eur. monograph because of a different composition.

4. Conclusions

In conclusion, the tested substances remained stable, even after many years of storage in a freezer. Significant changes in physical and chemical properties were not observed. Those parameters are within limits, except spiramycin. In this case, the substance does not comply with current Ph. Eur. Monograph, not because of degradation during storage, but because limits had been changed (and probably the biosynthesis process).

The tested reference standards most probably could be used today in routine work, especially in microbiological tests where composition (in case of multi-substance antibiotics) is not as important as total antimicrobial activity. In modern instrumental methods, those substances could be utilized in qualitative rather than quantitative analysis.

Another conclusion that can be drawn from the comparison of declared and determined potency is that the manufactures of reference materials should provide statistical information concerning their results on the product label.

In regard to the results of this study suppliers of reference materials should consider a very good stability of some reference standards and lengthen the intervals between introducing new batches of such substances. Exchanging good reference material for a new batch causes additional costs, especially for laboratories using many different substances. Many analyses can be performed with appropriate quality results using old reference material, provided it is stored properly and retested on a regular basis using the same modern technique.

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