ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
17 (1998) 757–766

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

Neomycin: microbiological assay or liquid chromatography?

E. Adams [a,*], L. Liu [a], K. Dierick [b], S. Guyomard [c], P. Nabet [d], S. Rico [e], P. Louis [f], E. Roets [a], J. Hoogmartens [a]

[a] *Katholieke Universiteit Leuven, Faculteit Farmaceutische Wetenschappen, Laboratorium voor Farmaceutische Chemie en Analyse van Geneesmiddelen, E. Van Evenstraat 4, B-3000 Leuven, Belgium*
[b] *Departement Farmaco–Bromatologie, Instituut voor Hygiëne en Epidemiologie, Brussels, Belgium*
[c] *Rhône–Poulenc Rorer Recherche–Développement, Analyse Qualité Pharmaceutique, Antony Cédex, France*
[d] *Roussel Uclaf, Département Central d'Analyse, Analyse Biologique, Romainville Cédex, France*
[e] *Instituto de Salud Carlos III, Centro Nacional de Farmacobiologia, Majadahonda, Madrid, Spain*
[f] *Algemene Pharmaceutische Bond, Dienst voor Geneesmiddelenonderzoek, Brussels, Belgium*

Received 5 November 1997; accepted 10 November 1997

Abstract

In a multicentre study involving six laboratories, a microbiological assay was performed on three neomycin samples containing respectively, 0.12, 2.1 and 11% (m/m) of neomycin C, as well on a pure neomycin C sample. The potency was determined according to the European Pharmacopoeia method but using a neomycin B base standard. The relative standard deviations between laboratories (RSD) on the potencies varied from 4.8 to 50%, depending on the sample examined. The RSD increased with the neomycin C content of the samples and the highest RSD values were observed for the pure neomycin C sample. The activity of neomycin C relative to neomycin B was found to be 62% by diffusion (RSD:41%) and 56% by turbidimetry (RSD: 50%). This confirmed that the presence of neomycin C in a neomycin sample influences the reproducibility of the microbiological assay. To estimate the influence of this effect on official standards, their composition was verified by liquid chromatography. The neomycin C base content of the standards varied between 0.4 and 5.8% (m/m). Based on the results obtained and on formerly published reports discussing problems encountered with microbiological assay of neomycin, it is proposed to introduce liquid chromatography in official monographs to replace microbiological assay.

Keywords: Neomycin B; Neomycin C; Microbiological assay; Liquid chromatography

1. Introduction

Neomycin, which is mainly used as the sulphate, is a widely used broad spectrum, water soluble aminoglycoside antibiotic, produced during the fermentation of *Streptomyces fradiae* [1]. It inhibits the growth of Gram-negative and Gram-positive bacteria. It has a narrow therapeutic range, is potentially toxic, like other aminoglycosides and may cause oto- and nephrotoxicity.

Neomycin sulphate is mainly composed of a mixture of neomycin B and its stereoisomer neomycin C [2]. Small amounts of other con-

* Corresponding author. Tel.: +32 16 323440; fax: +32 16 323448.

0731-7085/98/$19.00
PII S0731-7085(97)00247-1

stituents, such as neamine, paromamine, paromomycin I and II, neomycin LP-A and neomycin LP-B (LP, low potency) may also be present in commercial samples [3]. Neomycins LP-A and LP-B, which are the mono-*N*-acetyl derivatives of neomycins A and B and neamine, possess nearly no antimicrobial activity [4,5]. The antimicrobial potency of neomycin C is lower than that of neomycin B, necessitating a limit for neomycin C in commercial samples [6]. The European Pharmacopoeia (Ph. Eur.) limits the amount of neomycin C to 3–15% (m/m) [7]. Neomycin with a content of less than 3% (m/m) neomycin C is called framycetin. The United States Pharmacopeia (USP) does not distinguish between neomycin and framycetin and therefore does not limit neomycin C in a separate test [8].

Sokolski et al. described the potency values in neomycin bioassay as varying depending on the B:C ratios of the test and standard preparations and on the method used for assay [9]. They also reported that with commonly used methods, neomycin C responses were only 35–50% of neomycin B responses. With modified methods however, it was possible to obtain approximately equal responses [10]. Tsuji et al. reported the neomycin C response against *Staphylococcus aureus* to be one-third that of neomycin B [4]. Robertson et al. found a value of 50% against *Staphylococcus epidermidis* [11]. Lightbown et al. reported that the composition of the first international reference preparation of neomycin, established in 1958, with a content of 21.7% (m/m) of neomycin C and 4.4% (m/m) of neamine, differed too much from the composition of commercial samples [12]. They described the establishment of the second international reference preparation of neomycin. This substance contained 8.6% (m/m) of neomycin C and less than 1.0% (m/m) of neamine. The potency with respect to the first international reference preparation was determined in a collaborative study. The mean laboratory potencies varied over a range of 23%, variation which was attributed to the different composition of the compounds compared [13]. Therefore, an international reference preparation of neomycin B was established to serve as a reference in the assay of purer neomycins [14]. The Ph. Eur. also prescribes the use of chemical reference substances of different purity for use in the monographs neomycin and framycetin. Since the USP only has one monograph for neomycin, it compares samples of all different compositions against the same reference standard [8]. Barzaghi et al. examined this situation and concluded that a reference standard of almost pure neomycin B could be used for the determination of framycetin and neomycin. They proposed that the Ph. Eur. should reconsider the use of different reference substances [15].

In this study, collaborating laboratories were asked to determine the potency of neomycin samples, employing methods conform to the Ph. Eur. One sample was pure neomycin B base, one was pure neomycin C sulphate, one was framycetin sulphate and one was neomycin sulphate. The pure neomycin B base sample also served as a reference substance. The collaborators were not informed about the identity of this sample and reference substance. They were free to choose assay conditions, within the frame of Ph. Eur. prescriptions. The results should show the extent of variation obtained by different laboratories, carrying out the analysis of neomycin samples without taking special precautions, e.g. in a fully detailed protocol indicating the use of a well defined strain and defining all the parameters. This is also the first time that the potency of neomycin C is examined in a collaborative study. The results should allow to verify the proposal by Barzaghi et al. If the B:C ratio indeed has an influence on the potency value obtained, it is interesting to know the composition of the official standards available. A liquid chromatographic (LC) method for the analysis of neomycin sulphate and related substances on poly(styrene-divinylbenzene) (1000 Å) has been described previously [3]. With this method, the composition of the samples used in this study and of 6 official standards was determined.

2. Experimental

2.1. Samples and reference substance

The neomycin C content of the neomycin and

framycetin sulphate samples (Alcon, Puurs, Belgium) was determined by liquid chromatography (LC) and corresponded to 11.0 and 2.1% (m/m), respectively [3]. Neomycin C sulphate was prepared in the laboratory from commercial neomycin as described [5]. Using LC, it was found to contain 54.8% (m/m), expressed as neomycin B base and calculated on the substance as is. The neomycin B base standard was prepared according to the same method [5]. This house standard contained 92.84% (m/m) base, expressed as neomycin B and calculated on the substance as is, as determined by non-aqueous potentiometric titration in glacial acetic acid with 0.1 M perchloric acid. The water content was determined by Karl–Fischer titration and amounted to 6.74% (m/m). The total mass explained by titration and water determination was 99.58% (m/m). The content of neomycin C, determined by LC, was 0.12% (m/m). This standard was therefore accepted to contain 92.7% (m/m) of neomycin B base. For the purpose of this study, a potency of 930 IU mg^{-1} was assigned to this standard.

2.2. Microbiological assay

To determine the potency of the samples, the participating laboratories were asked to employ the zone diffusion and/or turbidimetric method described in the Ph. Eur. [16]. Corresponding to the Ph. Eur. prescriptions, the protocol for the diffusion method prescribed the use of medium E at pH 7.9, an incubation temperature of 30–37°C for about 18 h, a buffer solution of pH 8.0 (0.05 M), water as the solvent to prepare the stock solution and *Bacillus subtilis* (NCTC 10400, CIP 52.62 or ATCC 6633) or *Bacillus pumilus* (NCTC 8241 or CIP 76.18) as micro-organisms. The diameters of the zones had to be measured with a precision of at least 0.1 mm. For the turbidimetric method, the protocol prescribed the use of medium C at pH 8.0, an incubation temperature of 35–37°C for about 3 h 30 min to 4 h, a buffer solution of pH 8.0, water as the solvent to prepare the stock solution and *Staphylococcus aureus* (NCTC 7477, CIP 53.156 or ATCC 6538P) as micro-organism.

For both the diffusion and turbidimetric method, 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 [16]. Other micro-organisms than those described were allowed, provided that they were shown to be sensitive to neomycin and were used in appropriate media and appropriate conditions of temperature and pH. For the neomycin C sulphate sample, the concentrations had to be chosen so as to obtain values of the same size as with the other samples. It was mentioned that in the diffusion method using *Bacillus subtilis*, the potency of neomycin C relative to neomycin B was about 45% and in the turbidimetric method using *Staphylococcus aureus*, about 40%.

All results had to be calculated in International Units (IU) versus the neomycin B house standard. The samples examined were those mentioned above (2.1). The neomycin B house standard was also incorporated in the study as a sample to be examined. Since none of the participants was aware of this, it was a good control for the accuracy of the method. The precision of the assay had to be such that the limits of confidence ($P = 0.95$) were not less than 95%, nor greater than 105% [16].

2.3. LC analysis of official neomycin and framycetin standards

Six standards of neomycin and framycetin sulphate were analysed using a previously described liquid chromatographic system combined with pulsed electrochemical detection [3]. The official standards of neomycin and framycetin sulphate available were: the Ph. Eur. framycetin chemical reference substance (Ph. Eur. Fram. CRS); the Ph. Eur. neomycin sulphate chemical reference substance, Batch no. 2 (Ph. Eur. Neo. CRS); the USP reference standard of neomycin sulphate, Lot L (USP-RS); the WHO neomycin B proposed international reference preparation 68/41 (WHO-PIRP); the WHO neomycin B 1st international standard 1970 68/041 (WHO-1st IS); and the WHO neomycin 2nd international reference preparation 1974 72/406 (WHO-2nd IRP). The WHO-PIRP was stored in the laboratory for

Stationary phase : PLRP-S 1000 Å, 8 μm, 250 mm × 4.6 mm I.D., Polymer Laboratories, Shropshire, UK

Mobile phase :

sodium sulfate	70 g/l
sodium 1-octanesulfonate	1.4 g/l
phosphate buffer pH 3, 0.2 M	50 ml/l
water	up to 1 l

Flow rate : 1 ml/min

Injection volume : 20 μl

Column temperature : 35 °C

Post column addition of 0.5 M NaOH : 0.3 ml/min

Pulsed electrochemical detector :

Working electrode : gold
Reference electrode : Ag/AgCl
Counter electrode : stainless steel

Detector settings :

t (s)	E (volt)
0.00 - 0.40	0.05
0.41 - 0.60	0.75
0.61 - 1.00	-0.15

Integration period : 0.20 - 0.40 s
Sensitivity : 1 μC
The detector was kept at 35 °C

Scheme 1.

many years at room temperature. The other official standards were recently obtained from the different authorities.

The chromatographic conditions are shown in Scheme 1. The column (250 × 4.6 mm) was packed with poly(styrene-divinylbenzene) PLRP-S 1000 Å, 8 μm (Polymer Laboratories, Shropshire, UK). The mobile phase consisted of an aqueous solution containing 70 g l^{-1} of sodium sulphate, 1.4 g l^{-1} of sodium 1-octanesulfonate and 50 ml l^{-1} of a 0.2 M phosphate buffer (pH 3). These were injected in amounts of 10 μg. To allow

pulsed electrochemical detection on a gold electrode, sodium hydroxide was added post-column. This method allows paromamine, LP-A, neamine, paromomycin I and II, LP-B and neomycin C to be separated from the main component neomycin B [3].

3. Results and discussion

3.1. Microbiological assay

Table 1 includes information regarding conditions, micro-organisms and dilutions used by each laboratory for the diffusion method, which was carried out by all six laboratories. The numbers assigned to the laboratories do not correspond to the numbers assigned to the authors. Laboratories 1 and 3 decided to use medium type A, which was prescribed by the Ph. Eur. first edition, whereas the second and third edition prescribe medium E. The results for the determination of the potency and the limits of confidence for the different samples are given in Table 2. Results from the different collaborators were accepted as such, without any further verification of assay conditions such as parallelism and confidence intervals. Laboratory 2 reported a double inhibition zone for the neomycin C sulphate sample. Measurements were performed on the large inhibition zone. The accuracy of the method was examined by comparing the neomycin B base sample with the standard sample. These 2 samples are identical, as mentioned above. The results of Laboratories 1 and 4 differ significantly from the standard value ($P = 0.95$). Information about the turbidimetric method, carried out in three laboratories, is shown in Table 3. Laboratory 1 decided to use *Klebsiella pneumoniae*, which was prescribed by the Ph. Eur. first edition, whereas *Staphylococcus aureus* is prescribed by the second and third edition. Laboratory 2 chose medium type D, which was prescribed by the Ph. Eur. first edition, whereas type C is prescribed by the second and third edition. The results for the determination of the potency and the limits of confidence are given in Table 4. In the three laboratories, there were no problems with the repeatability. Also, for this method the accuracy of the laboratories was tested as above and for Laboratory 1 the difference was significant. In Laboratory 3 the confidence limits for diffusion were much larger than those obtained by turbidimetry.

The differences between the means of means of the different samples for the diffusion and the turbidimetric method are not significant ($P = 0.95$). An F-test was used to compare the standard deviations of the two methods, but no significant difference between the variances was found for any of the samples ($P = 0.95$). The mean activity of neomycin C, calculated for the free base and expressed relative to neomycin B base, was 62 and 56% for the diffusion and turbidimetric method, respectively. For both the diffusion and the turbidimetric method, it is observed that the relative standard deviation for the means of means increases with the content of neomycin C in the samples: 0.12% (m/m) of C in neomycin B base, 2.1% (m/m) of C in framycetin sulphate and 11.0% (m/m) of C in neomycin sulphate. In particular, the relative standard deviation (RSD) values for neomycin C sulphate are high: 41 and 50% for the diffusion and turbidimetric method, respectively. This confirms that the presence of neomycin C in a neomycin sample influences the reproducibility of the microbiological assay. This is in agreement with Sokolski and Lightbown [9,10,12–14] and in disagreement with Barzaghi [15]. It also means that the USP approach, making no distinction between neomycin and framycetin and therefore using only one standard, is not appropriate.

3.2. LC analysis of neomycin and framycetin standards

To determine the neomycin C content of official standards and of the samples used in the microbiological study, the liquid chromatographic method described above was used. The solutions to be analysed (0.5 mg ml^{-1}) were prepared as prescribed by the accompanying leaflets. The percentages of neomycin B were calculated with reference to the neomycin B base standard. The percentages of the other components were calculated using chromatograms obtained with diluted

Table 1
General information on conditions, micro-organisms and dilutions for the diffusion method

Laboratory	1	2	3	4	5	6
Medium						
Type (Ph. Eur.)	A	E	A	E	E	E
PH	8.5	7.9	8.0	7.9	7.9	7.9
PH of buffer solution	8.0	8.0	8.0	8.0	8.0	8.0
Micro-organism	*Bacillus pumilus* NCTC 8241	*Bacillus subtilis* ATCC 6633	*Bacillus subtilis* ATCC6633	*Bacillus subtilis* DSM 618	*Bacillus subtilis* ATCC6633	*Bacillus subtilis* ATCC6633
Dilution ratio	2	1.5	2	1.5	2	1.25
Dilutions examined						
Total number	3	3	3	3	3	3
Concentrations (IU ml^{-1})	4, 2, 1	1.33, 0.89, 0.59	12, 6, 3	9.3, 6.2, 4.1	6, 3, 1.5	3.5, 2.8, 2.2
Applied volumes (μl)	50	150	100	50	200	50
Experimental design	Latin square	Latin square	Latin square	Randomised block	Randomised block	Randomised block
Number of replicates	9	6	6	5	9	6
Incubation						
Temperature (°C)	32	33	37	30	37	37
Time (h)	18	18	18	18	18	17
Number of assays	5	3	4	1	5	5
Precision zone reader (mm)	0.1	0.1	0.2	0.02	0.1	0.1

Table 2
Mean values (IU mg^{-1}) for the potency of the different samples obtained by the diffusion method

Laboratory	1	2	3	4	5	6	Mean of means RSD
Neomycin B base standard	930	930	930	930	930	930	930
Neomycin B base sample	1072 (98.4–101.6)	937 (97.5–102.6)	901 (86.7–113.3)	1069 (91.3–109.5)	933 (94.2–105.8)	948 (95.1–104.9)	977 (7.6%)
Neomycin C sulfate	488 (98.0–102.0)	253 (95.7–104.7)	525 (76.8–123.2)	181 (83.4–119.9)	270 (91.8–109.3)	308 (86.7–113.3)	338 (41%)
Neomycin sulphate	711 (97.9–102.1)	547 (98.0–102.0)	568 (88.6–111.4)	467 (88.9–112.2)	532 (91.8–109.2)	605 (94.5–105.5)	572 (14%)
Framycetin sulphate	623 (94.6–105.4)	605 (96.0–104.0)	582 (94.8–105.2)	598 (86.8–114.9)	589 (94.1–105.9)	683 (97.4–102.6)	613 (6.1%)

The confidence limits ($P = 0.95$), expressed as percentages, are shown in parentheses.

Table 3
General information on conditions, micro-organisms and dilutions for the turbidimetric method

Laboratory	1	2	3
Medium			
Type (Ph. Eur.)	C	D	C
pH	7.0	6.9	7.0
pH of buffer solution	7.0	8.0	8.0
Micro-organism	*Klebsiella pneumoniae* ATCC 9997	*Staphylococcus aureus* ATCC 6538 P	*Staphylococcus aureus* ATCC 6538 P
Dilution ratio	1.1	1.1	1.1
Dilutions examined			
Total number	Samples: 3/standards: 6	4	3
Concentrations (IU ml^{-1})	Samples: 1.55, 1.41, 1.55 Standards: 2.07, 1.88, 1.71, 1.55, 1.41, 1.28	4.65, 4.23, 3.84, 3.49	16.6, 15.0, 13.6
Experimental design	Randomised block	Randomised block	Randomised block
Number of replicates	4	3	5
Incubation			
Temperature (°C)	37	37	37
Time	3 h 20	4 h 45	3 h 30
Number of assays	5	3	4

solutions of neomycin C and expressed in terms of neomycin C base. The results are shown in Table 5. The neomycin C base content varies between 0.36 and 5.77% (m/m) (0.51–8.24% (m/m), expressed as sulphate). The USP-RS is the purest sample. The Ph. Eur. Neo. CRS with its content of 4.45% (m/m) of neomycin C base has a lower content than samples which are usually found on the market and a lower content than the WHO-2nd IRP [12]. However, it will be impossible to consistently have a standard of the same composition as that of the sample. The Ph. Eur. allows a content of neomycin C sulphate in neomycin of up to 15% (m/m). The other components are always present in small amounts ($< 0.6\%$ (m/m)). It is also observed that the WHO-PIRP, which

Table 4
Mean values (IU mg^{-1}) for the potency of the different samples obtained by the turbidimetric method

Laboratory	1	2	3	Mean of means RSD
Neomycin B base standard	930	930	930	930
Neomycin B base sample	1043 (98.0–102.0)	928 (98.1–102.0)	947 (97.6–102.4)	973 (6.3%)
Neomycin C sulphate	487 (93.8–106.2)	215 (96.7–103.3)	223 (96.4–103.6)	308 (50%)
Neomycin sulphate	707 (98.2–101.8)	553 (98.6–101.6)	555 (95.3–104.7)	605 (15%)
Framycetin sulphate	644 (98.9–101.1)	585 (95.4–104.8)	613 (98.4–101.6)	614 (4.8%)

The confidence limits ($P = 0.95$), expressed as percentages, are shown in parentheses.

Table 5
Comparison of the composition of neomycin and framycetin standards (% (m/m))

	Paromamine	LP-A	Neamine	Paromomycin II	Paromomycin I	LP-B	Neomycin C	Neomycin B
Ph. Eur. Fram. CRS 647 IU mg^{-1} (12 anal.)	*	*	0.16 (4.2)	*	*	*	1.06 (3.2)	61.1 (1.0)
Ph. Eur. Neo. CRS 760 IU mg^{-1} (12 anal.)	0.28 (6.8)	0.21 (3.4)	0.48 (2.2)	0.08 (4.5)	0.58 (2.6)	0.38 (2.9)	4.45 (2.0)	57.8 (0.8)
USP-RS 782 μg mg^{-1} (16 anal.)	0.02 (9.1)	*	0.08 (5.5)	*	*	0.04 (11.5)	0.36 (6.8)	66.4 (0.8)
WHO-PIRP 670 IU mg^{-1} (8 anal.)	0.36 (2.4)	*	0.58 (5.1)	*	*	*	0.39 (6.0)	63.6 (1.2)
WHO-1st IS 670 IU mg^{-1} (8 anal.)	0.23 (4.0)	*	0.22 (4.1)	*	*	*	0.37 (4.5)	64.6 (1.0)
WHO-2nd IRP 775 IU mg^{-1} (8 anal.)	0.10 (4.7)	*	0.26 (5.8)	*	0.08 (6.5)	*	5.77 (2.0)	58.8 (1.5)
Neomycin C sulfate (12 analyses) (12 analyses)	1.16 (2.7)	*	0.07 (11.3)	*	*	0.13 (14.2)	54.8 (1.4)	0.37 (0.9)
Neomycin B base (12 analyses)	*	*	*	*	*	*	0.12 (5.4)	92.7 (0.7)

The relative standard deviation (%) is shown in parentheses after each content; *, $<0.02\%$ (m/m).

was stored at room temperature for more than 25 years and which is the same sample as the WHO-1st IS, had only slightly decomposed. The difference in neomycin B content is significant at the 0.05 level, but not at the 0.01 level of significance. This is an indication for the very good stability of this material, even if it is not stored under the prescribed conditions.

It is also to be emphasised that three different systems of units are used in Table 5. One IU system for pure neomycin B (Ph. Eur. Fram. CRS, WHO-PIRP and WHO-1st IS), another IU system for neomycin containing neomycin C (Ph. Eur. Neo. CRS and WHO-2nd IRP) and the USP $\mu g\ mg^{-1}$ system. It is observed that the IU system with the lowest values is used for the purest samples. There is no logical correspondence between these systems and the real content. A separate system of units corresponds to each reference substance. The number of μg in the USP-RS does not correspond to the real mass of neomycin present. Indeed, these micrograms have to be considered as 'micrograms of activity'. This has also been discussed elsewhere [17].

4. Conclusion

The activity of neomycin C relative to neomycin B was 62 and 56%, as found by diffusion and turbidimetry, respectively. The reproducibility obtained for the different samples, expressed as RSD (%), varied between 4.8 and 50%. It was observed that the reproducibility of the microbiological assays became poorer when the content of neomycin C in the sample increased. In order to verify the influence that this may have on the use of official standards, their neomycin C base content was determined by LC. The results varied between 0.36 and 5.77% (m/m). The results confirm that more variation will be observed when the B:C ratios of the test and standard preparations differ more. Since it will never be possible to have a standard with the same B:C ratio as all the samples allowed on the market, it is strongly recommended to replace the microbiological assay of neomycin by liquid chromatography, which allows all the components to be determined accurately, without interference from the standard composition.

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Acknowledgements

The authors wish to thank S. Austruy, T. Cepeda, V. Daix, P. Delaey, N. Gougeard, L. Kretz, C. Ravyts and M.D. Paniagua for technical assistance.

References

[1] S.A. Waksman, H.A. Lechevalier, Science 109 (1949) 305–307.
[2] J.D. Dutcher, N. Hosansky, M. Donin, O. Wintersteiner, J. Am. Chem. Soc. 73 (1951) 1384–1385.
[3] E. Adams, R. Schepers, E. Roets, J. Hoogmartens, J. Chromatogr. A 741 (1996) 233–240.
[4] K. Tsuji, J.H. Robertson, R. Baas, D.J. McInnis, Appl. Microbiol. 18 (1969) 396–398.
[5] P. Claes, F. Compernolle, H. Vanderhaeghe, J. Antibiot. 27 (1974) 931–942.
[6] H. Maehr, C.P. Schaffner, Anal. Chem. 36 (1964) 104–108.
[7] European Pharmacopoeia, 3rd ed., Monograph 197, European Department for the Quality of Medicines, Strasbourg, France, 1997.
[8] United States Pharmacopeia 23, United States Pharmacopeial Convention, Rockville, MD, USA, 1995.
[9] W.T. Sokolski, C.G. Chidester, D.G. Kaiser, J. Pharm. Sci. 53 (1964) 726–729.
[10] W.T. Sokolski, C.G. Chidester, O.S. Carpenter, W.M. Kaneshiro, J. Pharm. Sci. 53 (1964) 826–828.
[11] J.H. Robertson, R. Baas, R.L. Yeager, K. Tsuji, Appl. Microbiol. 18 (1971) 1164–1165.
[12] J.W. Lightbown, P. de Rossi, P. Isaacson, J. Biol. Stand. 7 (1979) 221–226.
[13] J.W. Lightbown, A.H. Thomas, B. Grab, H. Dixon, J. Biol. Stand. 7 (1979) 227–239.
[14] J.W. Lightbown, P. de Rossi, P. Isaacson, J. Biol. Stand. 7 (1979) 241–248.
[15] D. Barzaghi, G. Bella, D. Bizzarri, C. Burinato, M. Carazzone, M. Fava, R. Genova, L. Malesani, D. Piovani, M. Veronese, J. Biol. Stand. 13 (1985) 275–281.
[16] European Pharmacopoeia, 3rd ed., 2.7.2. Microbiological assay of antibiotics, European Department for the Quality of Medicines, Strasbourg, France, 1997.
[17] J. Hoogmartens, J. Pharm. Biomed. Anal. 10 (1992) 845–850.