MATHEMATICAL MODELING FOR ERYTHROMYCIN POTENCY DETERMINATION OF MASTIKER

MODELAREA MATEMATICĂ A DETERMINĂRII POTENȚEI ERITROMICINEI DIN MASTIKER

Viviana Ciuca[1], Victorita Burghelea[1], V. V. Safta[2] , Despina Nita[1], Luciana Paraschiv[1]

[1] NS Pasteur Institute SA, [2] Polytechnic University of Bucharest

Key words: *Erythromycin, Microbiological method, Combistats Soft, estimated potency.*

Cuvinte cheie :*Eritromicina, metoda microbiologica, Soft Combistats , potenta estimata.*

Abstract

Mastiker E is an antibacterial suspension for intramammary infusion in cattle mastitis. The active drug is erythromycin, a macrolide antibiotic. The main characteristic of commercial product is erythromycin potency. The potency of erythromycin is estimated by comparing the inhibition of growth of sensitive micro-organisms produced by known concentrations of the antibiotic to be examined and a reference substance. The validation study aims to demonstrate the determination of the potency of erythromycin, it is an appropriate analytical method, reproducible and meets the quality requirements of Mastiker product. The paper establishes the performance characteristics of the method considered and identify the factors that influence these characteristics. The diameters of inhibition zones, directly proportional to the logarithm of the concentration of the antibiotic used for the assay, measured and calculated using statistical methods (Combistats Soft). The assay is designed in such a way that the mathematical model on which the potency equation is based can be proved to be valid. A parallel-line model is chosen. The two log dose response lines of the preparation under examination and the standard preparation are parallel; they are rectilinear over the range of doses used in the calculation. These conditions are verified by validity tests for a given probability (P = 0.05). The test is not valid unless the confidence limits (P = 0.95) are not less than 50% and not more than 200% of the estimated potency. The estimated potency is not less than 95% and not more than 105% of the stated potency. The stated potency is not less than 50.000 IU/g. The validation procedure includes details on protocol working to determine the potency of erythromycin, validation criteria, experimental results, mathematical modeling for determining the potency, inter-laboratory comparisons.

Rezumat

Mastiker E este o suspensie antibacteriana pentru administrare intramamară in mastite la vaci. Substanța activă este eritromicina (erythromycin), un antibiotic din grupa macrolide. Caracteristica principală a produsului comercial este valoarea biologica a eritromicinei. Potența eritromicinei este estimată prin compararea inhibarii creșterii microorganismelor sensibile produse de concentrații cunoscute de antibioticul de examinat și o substanță de referință. Studiul de validare își propune să demonstreze ca determinarea potenței eritromicinei, este o metodă analitică adecvată, reproductibilă și îndeplinește cerințele de calitate ale produsului Mastiker. Diametrele zonelor de inhibiție, direct proporționale cu logaritmul concentrației de antibiotic utilizat pentru testare, se măsoara și se calculeaza folosind metode statistice (Combistats Soft). Testul este conceput în așa fel încât modelul matematic pe care se bazează ecuația potenței poate fi dovedit a fi valid. Metoda aleasă a fost cea a liniilor paralele. În cazul în care un model paralel-line este ales, cele două linii de raspuns, ale preparatului în curs de examinare și preparatului standard, trebuie să fie paralele; acestea ar trebui să fie rectilinii. Aceste condiții sunt verificate prin teste de valabilitate pentru o anumită probabilitate (P = 0,05). Testul este valid dacă limitele de încredere (p = 0,95) sunt intre 50-200% din potența estimată și potența estimata este între 95-105% din potența declarată. Potența declarată trebuie să fie de minim 50.000 UI/g. Procedura de validare include

detalii privind protocolul de lucru al determinării potenței eritromicinei, criterii de validare, rezultate experimentale, date statistice, comparații interlaboratoare.

Introduction

Mastiker E is an antibacterial suspension for intramammary infusion in cattle mastitis.

The active drug is erythromycin, a macrolide antibiotic with an action spectrum larger than penicillin, so:

- Gram-positive cocci (*Streptococcus spp., Stafilococcus spp.*, including penicilin-rezistant, pneumococci, enterococci),
- Gram-negative cocci (gonococci, meningococci – low activity *in vivo* due to low difusion in cephalo-rahidian fluid), clostridia (C. *tetani, perfringens, botulinum*), *B. subtilis, Sarcina lutea*, *Corinebacterium spp.*, also and some Gram-negative bacilli (Brucella, Pasteurella, Haemophylus),
- spirochetes (Leptospira),
- mycoplasma, rickettsia,
- large viruses,
- some protozoa (*Entamoeba, Toxoplasma, Trichomonas*) (1, 3, 5, 6, 10, 12).

Erythromycin has no activity against enterobacteriaceae (*Salmonella, Aerobacter, E. coli, Pseudomonas, Proteus, Klebsiella*), fungi (1, 3, 5, 12).

Erythromycin has dose related bacteriostatic or bactericid action, trough a mechanism consisting of blocking of polipeptidic chains construction in ribosoms and consecutive protein synthesis inhibition (1, 3, 5, 12).

Concentration in product is 50000 IU erythromycin / g.

Estimation of erythromycin potency make through direct comparison between sample Mastiker, and standard erythromycin which is valid, calibrated and used as references.

Correct dosing of antibiotic is a decisive step of final control, critical for ensuring the Mastiker quality (4, 7, 8, 11).

The validation study aims to demonstrate the determination of the potency of erythromycin it is an appropriate analytical method, reproducible and meets the quality requirements of Mastiker product (7, 13).

1. Materials and Methods

The microbiological assay of erythromycin is based upon a comparison of the inhibition of growth of micro-organisms by measured concentrations of the antibiotics under examination with that produced by known concentrations of a standard preparation of the antibiotic having a known activity (7, 8, 11, 13).

The cylinder-plate method (Agar diffusion) depends upon diffusion of the antibiotic from a vertical cylinder through a solidified agar layer in a Petri dish or plate to an extent such that growth of the added micro-organism is prevented entirely in a zone around the cylinder containing a solution of the antibiotic (7, 8, 11, 13).

Petri dish, 20 x 100 mm.and stainless steel cylinder with diameter: outer 8 mm, inside 6 mm, height 10 mm, were used.

Culture media:

- Antibiotic Medium No.IV (Himedia) and
- Antibiotic Medium No. VII (Himedia) were used.

Test microorganism: the following bacterial strains were used:

- *Micrococcus luteus* ATCC 9341-

self-prepared bacterial suspensions prepared as described in the laboratory instruction. Antimicrobial standard was provided by the Sigma.

Potency of erythromycin standard is **916 IU** of activity per mg of dried material, as stated in European Pharmacopoeia, checked to reference substance, Erythromycin CRS.

Potency of erythromycin, raw material for Mastiker, provided by Century Pharmaceutical India, is **998 IU** of activity per mg of dried material.

Solvent used in erythromycin extraction and preparing the stock solution: Methanol: water 4:1.

Buffer solution: 0.05 M. phosphate buffer pH 8.

Preparation of standard:

- pre dilution 40 mg / 25ml,
- working dilutions: 25 IU/ml, 50 IU/ml, 100 IU/ml.

Preparation of samples:

- 3g Mastiker / 100ml extract,
- pre dilution 37,5 mg/25 ml,
- working dilutions: 25 IU/ml, 50 IU/ml, 100 IU/ml.
- Medium and final pH (± 0.1 pH unit) = 7.9.
- Incubation temperature: 30-37 ℃.
- Thermostatic control for diffusion: ± 0.5℃.

In diffusion assay, parameter used is diameter of inhibition formed around the disc, after incubation. Observed the growth of microorganism after incubation.

Measured the diameters of the circular inhibition zones formed, with a corresponding precision (at least 0.1 mm) and calculate the potency using statistical methods (8).

The diameters of inhibition formed on agar, directly proportional to the logarithm of the concentration of the antibiotic, were measured and calculated using statistical methods with CombiStats soft, version 5.0, release date 11 March 2013, European Directorate for the Quality of Medicines Health Care, Council of Europe (2, 9, 11).

CombiStats soft is according to European Pharmacopoeia Monographs 9th Edition – Statistical analysis of results of biological assay and tests (2, 9).

Our experimental data were analyzed by the method of parallel lines for the calculation of the 95 per cent confidence limits. The relationship between the logarithm of the erythromycin concentration and the diameters of the circular inhibition zones formed can be represented by a straight line over the range of doses used.

The design of our assay was "Randomised blocked" because each block can be identified as a source of variation. Were used a constant dilution step of a factor = 2, in increasing.

In assays with quantitative responses, the observed residuals are normally used to estimate the residual variance.

The test is not valid unless the confidence limits (P = 0.95) are not less than 50% and not more than 200% of the estimated potency.

The estimated potency is not less than 95% and not more than 105% of the stated potency.

The stated potency is not less than 50.000 international units/g (2, 9, 13).

The validation procedure includes details on:

- protocol
- working to determine the potency of the erythromycin,
- validation criteria,
- experimental results,
- mathematical modeling for determining the potency,
- inter laboratory comparisons.

2. Results and Discussions

2.1. Validation Combistats version 5.0 for serial No. 3

Version 5.0. Tuesday, 15 November 2016, 10:52:27 [+02:00]. Page 1 of 2

Substance	Erytromycin
Method	agar diffusion
Assay number	1
Technician	Viviana Ciuca
Date of assay	08.11.2016

Remarks: Mastiker, batch no.3

Standard			
Id.	Erytromycin		
Ass. pot.	916 IU/mg		
Pre-dil. 1	40mg/25ml		
Doses	S1	S2	S3
(1)	231	245	276
(2)	235	257	265
(3)	229	255	270
(4)	224	252	268
(5)	230	250	272
(6)	237	248	280

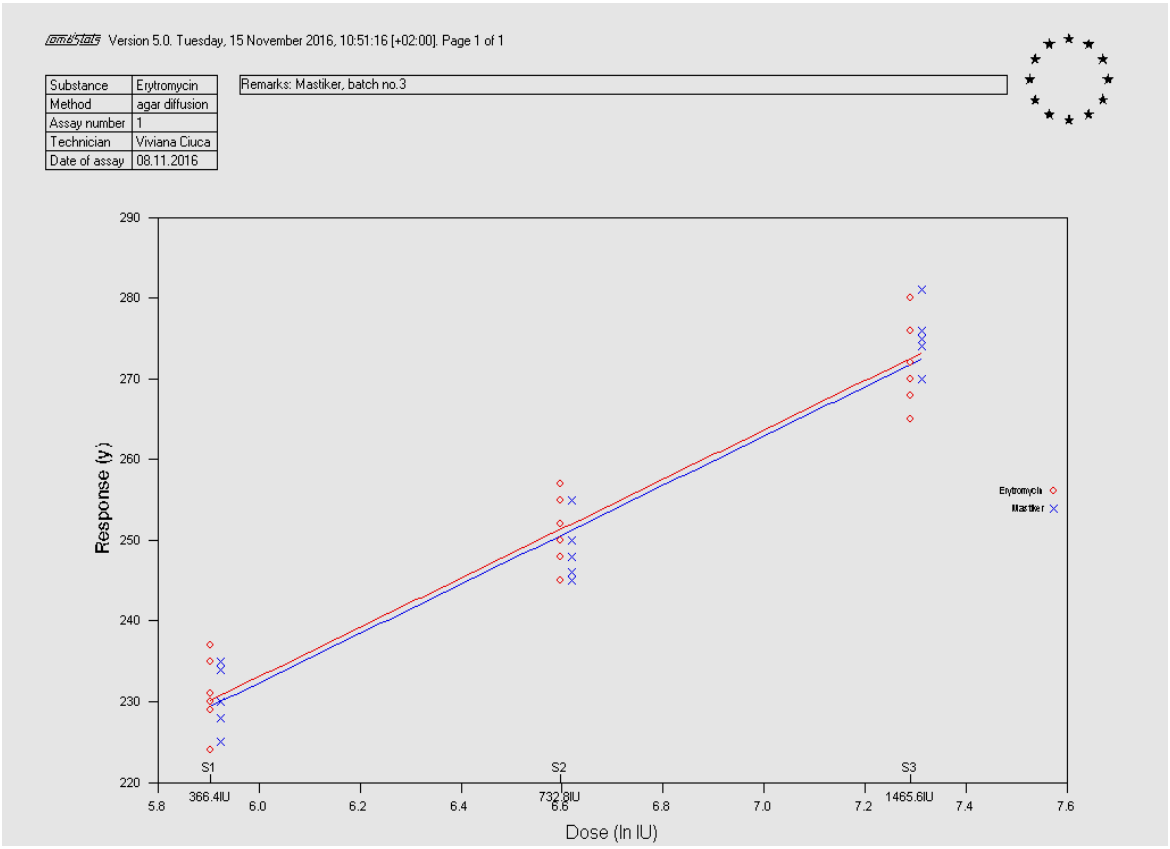
Sample 1			
Id.	Mastiker		
Ass. pot.	998IU/mg		
Pre-dil. 1	37.5mg/25ml		
Doses	T1	T2	T3
(1)	230	250	281
(2)	225	255	270
(3)	228	245	274
(4)	230	250	276
(5)	234	248	275
(6)	235	246	270

Model: Parallel lines
Design: Randomised block
Transformation: $y' = y$
Variance: Observed residuals
Dilution step (Increasing): 2

Common slope(factor) = 30.5972 (28.2627 to 32.9317)
Correlation | r |: 0.973475

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-ratio	Probability
Preparations	1	0.111111	0.111111	0.005	0.943
Regression	1	10795.0	10795.0	501.214	0.000 (***)
Non-parallelism	1	15.0417	15.0417	0.698	0.411
Non-linearity	2	44.6944	22.3472	1.038	0.369
Standard	1	0.250000	0.250000	0.012	0.915
Sample 1	1	44.4444	44.4444	2.064	0.163
Quadratic curvature	1	25.6806	25.6806	1.192	0.285
Lack of quadratic fit	1	19.0139	19.0139	0.883	0.356
Treatments	5	10854.9	2170.98	100.799	0.000 (***)
Blocks	5	33.8889	6.77778	0.315	0.899
Residual error	25	538.444	21.5378		
Total	35	11427.2	326.492		

Sample 1			
Id.	Mastiker		
(IU/mg)	Lower limit	Estimate	Upper limit
Potency	876.836	973.525	1080.81
Rel. to Ass.	87.9%	97.5%	108.3%
Rel. to Est.	90.1%	100.0%	111.0%


Version 5.0. Tuesday, 15 November 2016, 10:51:16 [+02:00]. Page 1 of 1
Substance Erytromycin
Method agar diffusion
Assay number 1
Technician Viviana Ciuca
Date of assay 08.11.2016
Remarks: Mastiker, batch no.3
Response (y)
Dose (ln IU)
S1
366.4IU
S2
732.8IU
S3
1465.6IU
Erytromycin
Mastiker

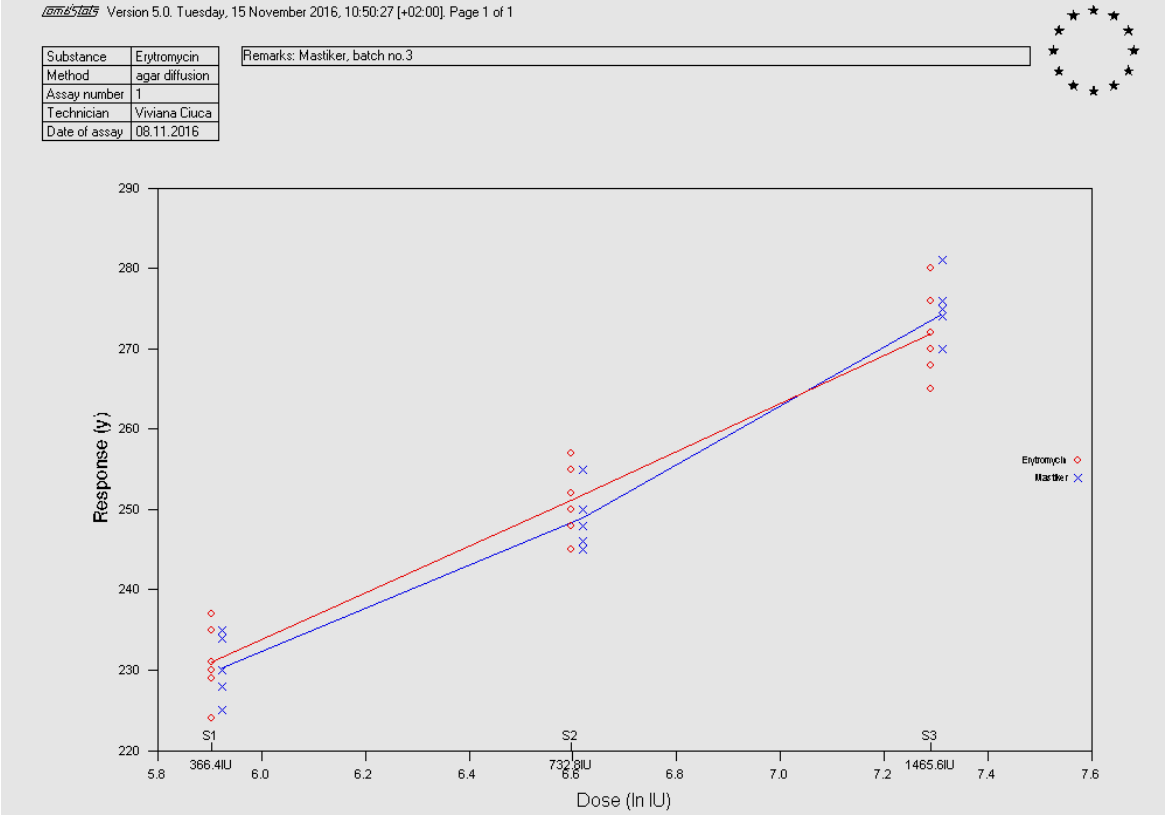

Version 5.0. Tuesday, 15 November 2016, 10:50:27 [+02:00]. Page 1 of 1
Substance Erytromycin
Method agar diffusion
Assay number 1
Technician Viviana Ciuca
Date of assay 08.11.2016
Remarks: Mastiker, batch no.3
Response (y)
Dose (ln IU)
S1
366.4IU
S2
732.8IU
S3
1465.6IU
Erytromycin
Mastiker

Table 1 includes the results of the method for three serial number of Mastiker E.

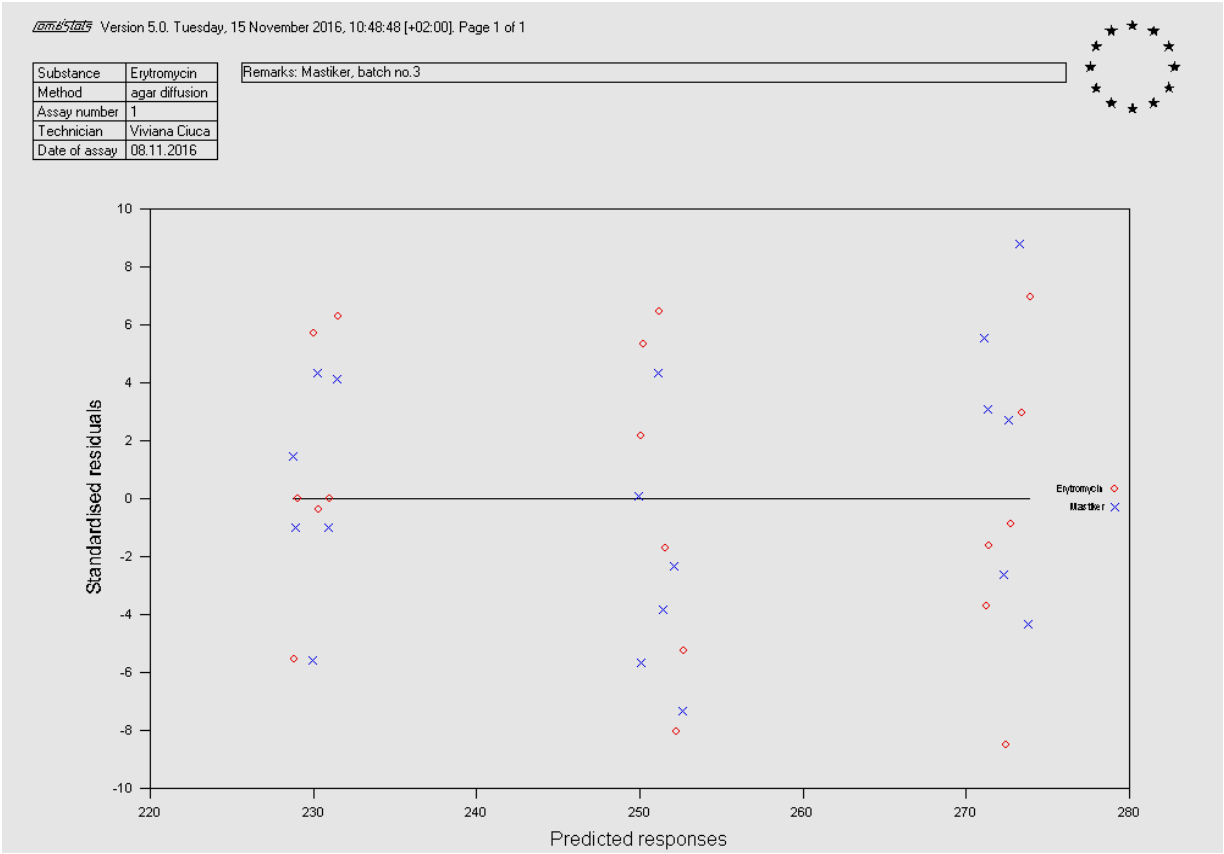
The test is not valid unless the confidence limits (P = 0.95), LC, are not less than 50% and not more than 200% of the estimated potency. The estimated potency, EP, is not less than 95% and not more than 105% of the stated potency.

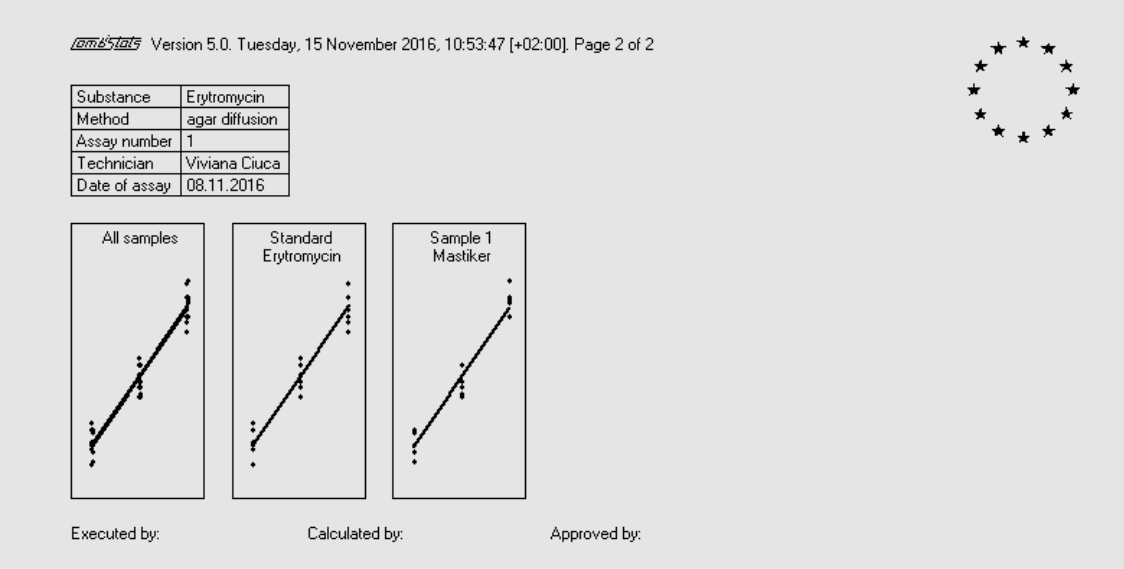
The stated potency is not less than **50.000** international units/g.

Table 1

Validation of the method for three serial number

SOP	Product	Serial no.	LIMITS	RESULTS Quality Control Laboratory-Pasteur Bucharest	RESULTS Quality Control Laboratory-Pasteur Filipesti
065	Mastiker E	3	50%≤LC≤200% 95%≤EP≤105%	**LC = 87.9 – 108.3% EP = 97.5%**	**LC = 85.1 – 110.8% EP = 97.9%**
065	Mastiker E	4	50%≤LC≤200% 95%≤EP≤105%	**LC = 88.1 – 103.0% EP = 96.8%**	**LC = 80.7 – 113.0% EP = 97.5%**
065	**Mastiker E**	**5**	**50%≤LC≤200% 95%≤EP≤105%**	**LC = 84.7– 109.8% EP = 97.3%**	**LC = 86.8– 119.1% EP = 98.1%**





3. Conclusions

The determination of the erythromycin potency of the Mastiker E, by comparing the diameters of the circular inhibition zones produced of a series of dilutions of the product to be examined with those produced by a reference standard calibrated in international units, it is an appropriate analytical method, reproducible and meets the quality requirements of Mastiker product and is considered valid , the results obtained for each validation parameter are within the admissibility criteria (2, 9, 11),

Bibliography

1. **Anghelescu M** (1998). Terapia cu antibiotice. Ed. Medicală, București 70-73.
2. **Daas A** (2013). The Combistats User Manual, EDQM – Council of Europe.
3. **Cristina RT** (2006). Introducere în farmacologia și terapia veterinară, Editura Solness, Timișoara, pp. 385-425
4. **Cristina RT, Teuşdea V** (2008). Ghid de farmacie şi terapeutică veterinară Brumar Timisoara, pp. 23-30.
5. **Crivineanu M** (2005). Farmacologie veterinară. Ed. Fundis, București, pp. 116-117.
6. **Drugs A to Z** - Erythromycin. Available at: https://www.drug.com
7. **Glick D, Kersey RC, Fink FC** (2006). Microbiological assay of antibiotics. In: Methods of Biochemical Analysis, Volume 1 (Ed. D Glick), John Wiley & Sons, Inc., Hoboken, NJ, USA. doi: 10.1002/9780470110171.ch3
8. **European Pharmacopoeia** Monographs 9th Edition – Microbiological assay of antibiotics.
9. **European Pharmacopoeia** Monographs 9th Edition – Statistical analysis of results of biological assays and tests.
10. **Macrolides.** Available at: http://en.wikipedia.org/wiki/Macrolide
11. **Sovasia N, Arshad H** (2016). Microbial Assay of Antibiotics**.** PharmaTutor Magazine (ISSN 2347-7881). On line Article, Reference Id: PHARMATUTOR-ART-1560. Available at: http://www.pharmatutor.org/articles/microbial-assay-antibiotics
12. **Stroescu V** (1998). Bazele farmacologice ale practicii medicale. Ed. Medicală, Bucureşti pp: 1288-1291
13. **The International Pharmacopoeia** (2015). Fifth Edition, Microbiological assay of antibiotics