# MATHEMATICAL MODELING FOR BENZYLPENICILIN POTASSIUM AND STREPTOMYCIN SULPHATE POTENCY DETERMINATION OF ASCOMICIN

# MODELAREA MATEMATICĂ A DETERMINARII POTENȚEI BENZIL PENICILINEI POTASICE SI STREPTOMICINEI SULFAT DIN ASCOMICIN

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**Key words**: *Benzylpenicilin potassium,streptomycin sulphate, microbiological method, Combistats Soft, estimated potency.*
***Cuvinte cheie:*** Benzilpenicilina potasica, streptomIcina sulfat, *metoda microbiologica, Soft Combistats, potenta estimata.*

## Abstract

**Ascomicin is an antibacterial unguent for treatment of local infections of skin, eyes, outer ear, in cattle, sheep, pig, dog and cat. The product contains two active substances: benzylpenicillin potassium (Penicillin G potassium) and streptomycin sulphate. The main characteristic of commercial product is benzylpenicillin potassium and streptomycin sulphate potency. The potency 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 benzylpenicillin potassium and streptomycin sulphate, it is an appropriate analytical method, reproducible and meets the quality requirements of Ascomicin 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 per cent and not more than 200 per cent of the estimated potency. The estimated potency is not less than 95 per cent and not more than 105 per cent of the stated potency. The stated potency is not less than 19400 international units/g benzylpenicillin potassium and 13960 international units/g streptomycin sulphate. The validation procedure includes details on protocol working to determine the potency of benzylpenicillin potassium and streptomycin sulphate, validation criteria, experimental results, mathematical modeling for determining the potency, interlaboratory comparisons.**

## Rezumat

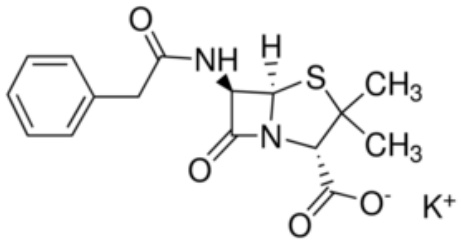
**Ascomicin este un unguent antibacterian pentru tratamentul infecțiilor locale ale pielii, ochilor și urechii externe, la taurine, ovine, porci, câini si pisici. Produsul conține două substanțe active: Benzilpenicilina potasică (Benzylpenicillin potassium/ Penicillin G potassium) și streptomicina sulfat (Streptomycin sulphate). Caracteristica principală a produsului comercial este valoarea biologica a celor doua substante active: benzilpenicilina potasică și streptomicina sulfat. Potența lor este estimată prin compararea inhibarii creșterii microorganismelor sensibile produse de concentrații cunoscute ale antibioticul de examinat și o substanță de referință.Studiul de validare își propune să demonstreze ca determinarea potenței benzilpenicilinei potasice și streptomicinei sulfat, este o metodă analitică adecvată, reproductibilă și îndeplinește cerințele de calitate ale produsului Ascomicin. 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. S-a ales metoda 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 19400 unități internaționale/g benzilpenicilina potasica si 13960 unități internaționale/g streptomycin sulphate. Procedura de validare include detalii privind protocolul de lucru al determinării potenței eritromicinei, criterii de validare, rezultate experimentale, date statistice, comparații interlaboratoare.**

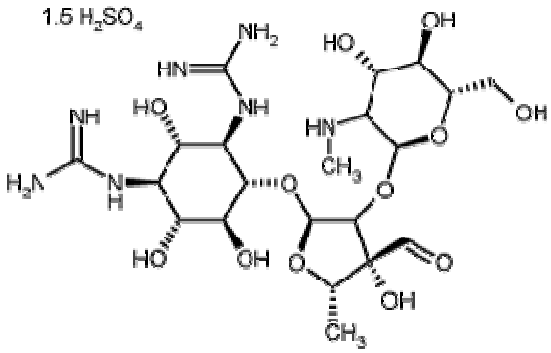
## Introduction

Ascomicin is an antibacterial unguent for treatement of local infections of skin, eyes, outer ear, in cattle, sheep, pig, dog and cat. The product contains two active substances:

- Benzylpenicilin potassium (Penicillin G potassium) and
- Streptomycin sulphate.



**Benzylpenicilin potassium**



**Streptomycin sulphate**

As a beta-lactam antibiotics, Penicillin G is noted to possess effectiveness mainly against Gram-positive organisms: Gram-positive cocci (beta-haemolytic streptococci, beta-lactamase negative staphylococci, aerobic/anaerobic Gram-positive bacilli (except beta-lactamase producing bacteroides), actinomycetes, spirochetes and leptospira. Some Gram-negative organisms such as *Neisseria gonorrhoea* and *N. meningitides* are also susceptible to Penicillin G.

Acts by inhibition of the formation of peptidoglycan cross-links in the bacterial cell wall causing the rapid die of cell (1, 3, 5, 11).

Concentration in product is **20000 I.U.** / 1 gram (8).

As an aminoglycoside antibiotics Streptomycin sulphate is an antibiotic displaying bactericidal activity against Gram-negative aerobes such as Pseudomonas, Acinetobacter and Enterobacter and some anaerobic bacilli where resistance has not yet arisen, but generally not against Gram-positive and anaerobic Gram-negatives (1, 3, 5, 11).

Some mycobacteria, including *M. tuberculosis*, are also susceptible to aminoglycosides. Acts by inhibition of vital protein synthesis in target micro-organisms (1, 3, 5, 11).

Concentration in product is **14500 I.U.** / 1 gram (6).

Ascomicin formula cumulates the antibacterial spectra and takes advantage of synergistic effects of the two antibiotics besides in streptococcal infection.

Correct dosing of antibiotics is a decisive step of final control, critical for ensuring the Ascomycin quality (4, 7, 8, 10, 12).

Estimation of benzylpenicillin potassium / streptomycin sulphate potency make through direct comparison between sample Ascomycin, and standard benzylpenicillin potassium, streptomycin sulphate which is valid, calibrated and used as references.

Correct dosing of antibiotic is a decisive step of final control, critical for ensuring the Ascomycin quality (2, 9, 10).

The validation study aims to demonstrate the determination of the potency of benzylpenicilin potassium and streptomycin sulphate it is an appropriate analytical method, reproducible and meets the quality requirements of Ascomicin product (7, 8, 10, 12).

## 1. Materials and Methods

The microbiological assay of benzylpenicillin potassium and streptomycin sulphate 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, 12).

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, 12).

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),
- Antibiotic Medium No. V (Himedia) were

used for benzylpenicillin potassium and

- Antibiotic Medium No.IV (Himedia),
- Antibiotic Medium No. VII (Himedia) for streptomycin sulphate.

Test microorganism: the following bacterial strains were used:

- *Staphylococcus aureus* ATCC 6538 P - for benzylpenicillin potassium and
- *Bacillus subtilis* ATCC 6633 for streptomycin sulphate,

self-prepared bacterial suspensions prepared as described in the laboratory instruction.

Antimicrobial standard was provided by the Sigma.

Potency of benzylpenicillin potassium standard is 1530 IU of activity per mg of dried material, as stated in European Pharmacopoeia, checked to reference substance (8).

Potency of streptomycin sulphate standard is 720 IU of activity per mg of dried material, as stated in European Pharmacopoeia, checked to reference substance.

Potency of benzylpenicillin potassium raw material for Ascomycin, provided by Sino-Kemmed, is **1560 IU** of activity per mg of dried material (8).

Potency of streptomycin sulphate raw material for Ascomycin, provided by Sino-Kemmed, is **749 IU** of activity per mg of dried material.

- Solvent used in benzylpenicillin potassium extraction was diethyl ether, phosphate buffer pH 8,
- solvent used in preparing the stock solution: phosphate buffer pH 8.
- Solvent used in streptomycin sulphate extraction: dietyl ether, phosphate buffer pH 7,
- Solvent used in preparing the stock solution: phosphate buffer pH 7.

Preparation of benzylpenicillin potassium standard:

- pre dilution 13mg/50ml,
- working dilutions: 1IU/ml, 2IU/ml, 4IU/ml.

Preparation of benzylpenicillin potassium samples:

- 2g Ascomycin/100ml extract,
- pre dilution 13mg/50ml,
- working dilutions: 1IU/ml, 2IU/ml, 4IU/ml.

Preparation of streptomycin sulphate standard:

- pre dilution 20mg/50ml,
- working dilutions: 5IU/ml, 10IU/ml, 20IU/ml

Preparation of streptomycin sulphate samples:

- 2g Ascomycin/100ml extract,
- Pre dilution 19,2mg / 50ml,
- working dilutions: 5IU/ml, 10IU/ml, 20IU/ml.
- 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. It was observed the growth of microorganism after incubation.

Were 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 (3, 9).

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 (3).

CombiStats soft is according to European Pharmacopoeia Monographs 9th Edition – Statisical analysis of results of biological assay and tests (3, 9).

Our experimental data were analyzed by the method of parallel lines for the calculation of the 95 % 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 is "Randomised blocked" because each block can be identified as a source of variation (3).

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 (3).

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 (8, 12).

The stated potency is not less than 19400 international units/g benzylpenicillin potassium and 13960 international units/g streptomycin sulphate (8, 12).

The validation procedure includes details on protocol working to determine the potency of the benzylpenicillin potassium and streptomycin sulphate, validation criteria, experimental results, mathematical modelling for determining the potency, inter laboratory comparisons.

## 2. Results and Discussions

### Validation Combistats version 5.0 for potassium benzyl penicillin, Ascomicin, serial number 15

CombiStats Version 5.0. Friday, 11 November 2016, 15:05:37 [+02:00]. Page 1 of 2

| | |
|---|---|
| Substance | Potassium benzyl penicillin |
| Method | agar diffusion |
| Assay number | 2 |
| Technician | Viviana Ciuca |
| Date of assay | 11.11.2016 |

Remarks: Ascomicin, batch no.15

| Standard | | | |
|---|---|---|---|
| Id. | Potassium benzyl penicillin | | |
| Ass. pot. | 1530 IU/mg | | |
| Pre-dil. 1 | 13mg/50 ml | | |
| Doses | S1 | S2 | S3 |
| (1) | 230 | 241 | 270 |
| (2) | 225 | 258 | 268 |
| (3) | 239 | 255 | 275 |
| (4) | 235 | 254 | 273 |
| (5) | 229 | 248 | 282 |
| (6) | 238 | 242 | 284 |

| Sample 1 | | | |
|---|---|---|---|
| Id. | Ascomicin | | |
| Ass. pot. | 1560 IU/mg | | |
| Pre-dil. 1 | 13mg/50ml | | |
| Doses | T1 | T2 | T3 |
| (1) | 224 | 250 | 278 |
| (2) | 235 | 251 | 280 |
| (3) | 230 | 248 | 266 |
| (4) | 226 | 242 | 265 |
| (5) | 235 | 255 | 283 |
| (6) | 227 | 254 | 279 |

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

Common slope(factor) = 31.8595 (28.7995 to 34.9195)
Correlation | r |: 0.959193

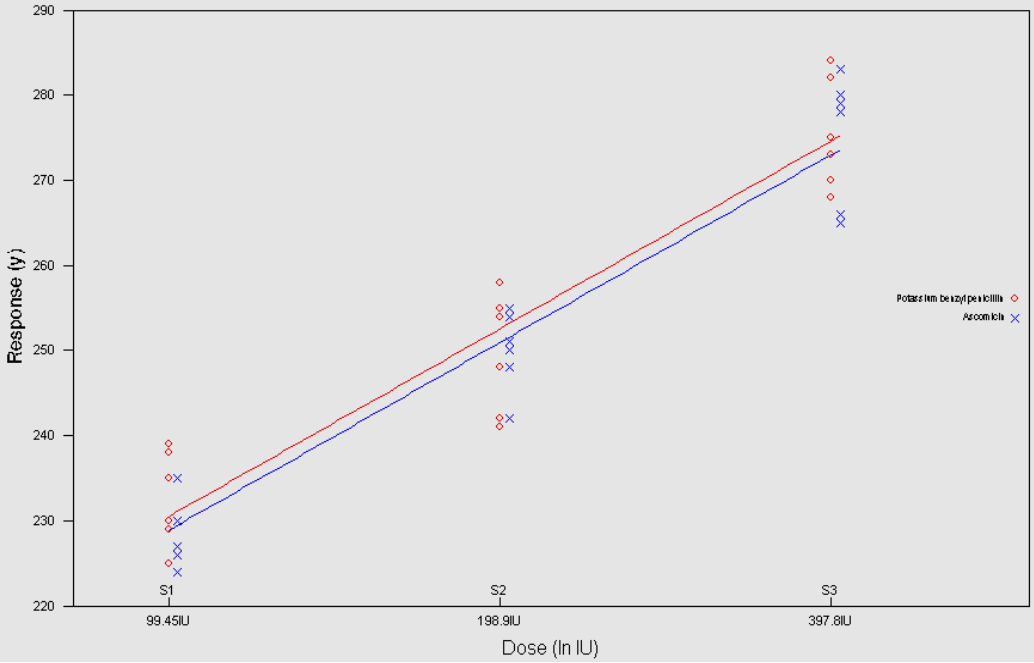
| Source of variation | Degrees of freedom | Sum of squares | Mean square | F-ratio | Probability |
|---|---|---|---|---|---|
| Preparations | 1 | 9.00000 | 9.00000 | 0.243 | 0.626 |
| Regression | 1 | 11704.2 | 11704.2 | 316.291 | 0.000 (***) |
| Non-parallelism | 1 | 13.5000 | 13.5000 | 0.365 | 0.551 |
| Non-linearity | 2 | 96.8889 | 48.4444 | 1.309 | 0.288 |
| Standard | 1 | 75.1111 | 75.1111 | 2.030 | 0.167 |
| Sample 1 | 1 | 21.7778 | 21.7778 | 0.589 | 0.450 |
| Quadratic curvature | 1 | 88.8889 | 88.8889 | 2.402 | 0.134 |
| Lack of quadratic fit | 1 | 8.00000 | 8.00000 | 0.216 | 0.646 |
| Treatments | 5 | 11823.6 | 2364.71 | 63.903 | 0.000 (***) |
| Blocks | 5 | 203.222 | 40.6444 | 1.098 | 0.386 |
| Residual error | 25 | 925.111 | 37.0044 | | |
| Total | 35 | 12951.9 | 370.054 | | |

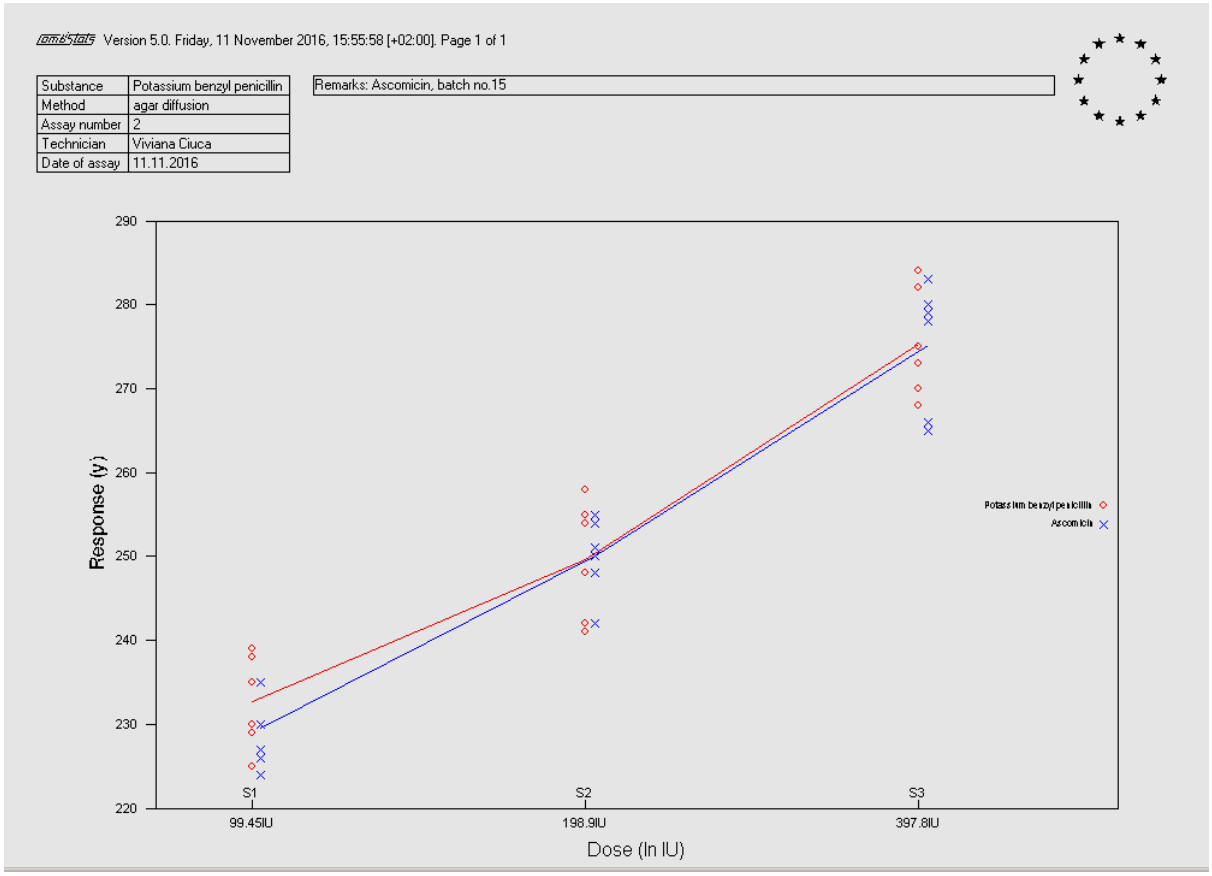
| Sample 1 | | | |
|---|---|---|---|
| Id. | Ascomicin | | |
| (IU/mg) | Lower limit | Estimate | Upper limit |
| Potency | 1298.79 | 1482.72 | 1691.26 |
| Rel. to Ass. | 83.3% | 95.0% | 108.4% |
| Rel. to Est. | 87.6% | 100.0% | 114.1% |

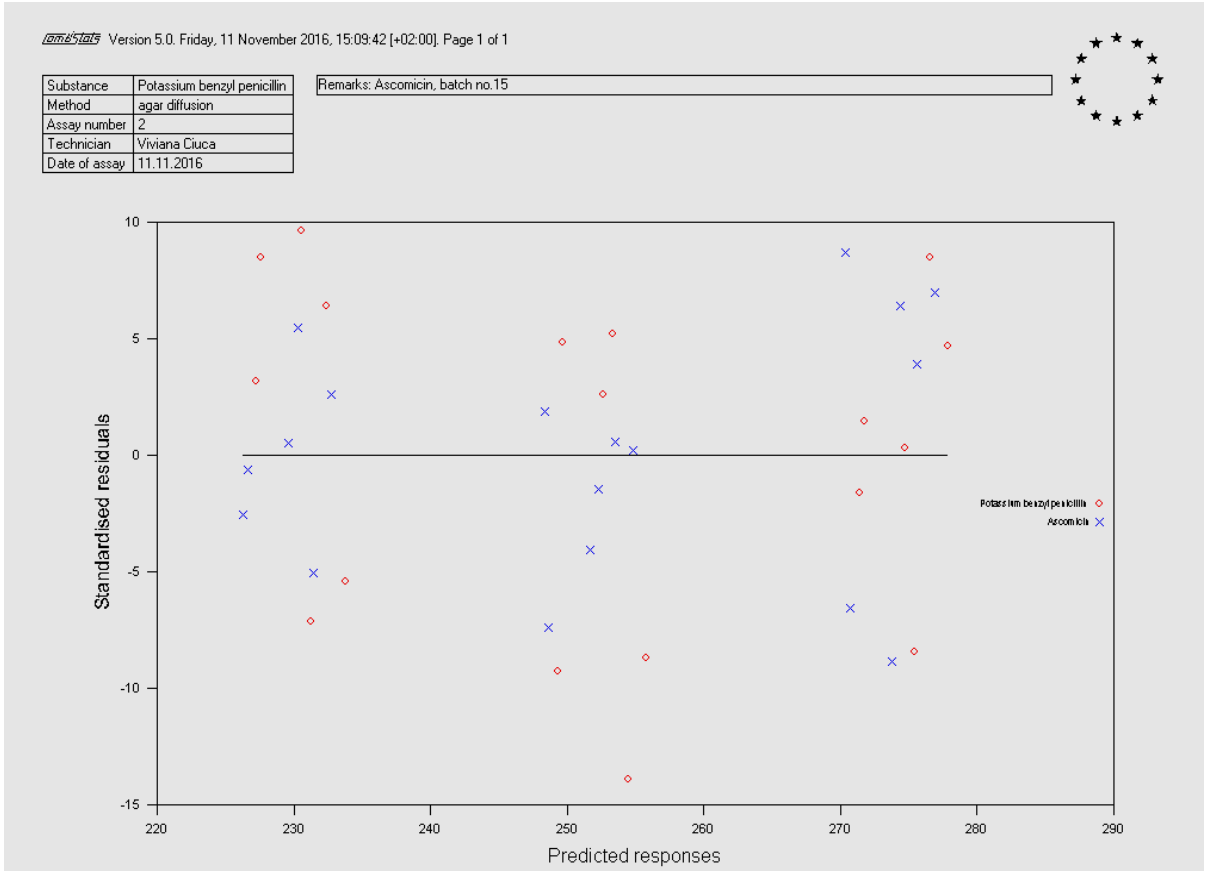
emi'stats Version 5.0. Friday, 11 November 2016, 15:08:37 [+02:00]. Page 1 of 1

| | |
|---|---|
| Substance | Potassium benzyl penicillin |
| Method | agar diffusion |
| Assay number | 2 |
| Technician | Viviana Ciuca |
| Date of assay | 11.11.2016 |

Remarks: Ascomicin, batch no.15

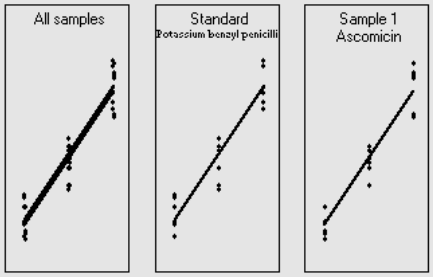



Version 5.0. Friday, 11 November 2016, 15:55:58 [+02:00]. Page 1 of 1
Substance | Potassium benzyl penicillin
Method | agar diffusion
Assay number | 2
Technician | Viviana Ciuca
Date of assay | 11.11.2016
Remarks: Ascomicin, batch no.15
Response (y)
290
280
270
260
250
240
230
220
S1
S2
S3
99.45IU
198.9IU
397.8IU
Dose (ln IU)
Potassium benzyl penicillin
Ascomicin


Version 5.0. Friday, 11 November 2016, 15:09:42 [+02:00]. Page 1 of 1
Substance | Potassium benzyl penicillin
Method | agar diffusion
Assay number | 2
Technician | Viviana Ciuca
Date of assay | 11.11.2016
Remarks: Ascomicin, batch no.15
Standardised residuals
10
5
0
-5
-10
-15
220
230
240
250
260
270
280
290
Predicted responses
Potassium benzyl penicillin
Ascomicin

Combistats Version 5.0. Friday, 11 November 2016, 15:06:55 [+02:00]. Page 2 of 2

| | |
|---|---|
| Substance | Potassium benzyl penicillin |
| Method | agar diffusion |
| Assay number | 2 |
| Technician | Viviana Ciuca |
| Date of assay | 11.11.2016 |

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## 2.2. Validation Combistats version 5.0 for Streptomycin sulphate, Ascomicin, serial No.15

Combistats Version 5.0. Friday, 11 November 2016, 12:45:54 [+02:00]. Page 1 of 2

| | |
|---|---|
| Substance | Streptomycin sulfat |
| Method | agar diffusion |
| Assay number | 1 |
| Technician | Viviana Ciuca |
| Date of assay | 11.11.2016 |

Remarks: Ascomicin, batch no.15

| Standard | | | |
|---|---|---|---|
| Id. | Streptomycin sulfat | | |
| Ass. pot. | 720 IU/mg | | |
| Pre-dil. 1 | 20mg/50ml | | |
| Doses | S1 | S2 | S3 |
| (1) | 128 | 148 | 167 |
| (2) | 126 | 145 | 161 |
| (3) | 130 | 142 | 168 |
| (4) | 133 | 158 | 176 |
| (5) | 137 | 150 | 179 |
| (6) | 130 | 155 | 170 |

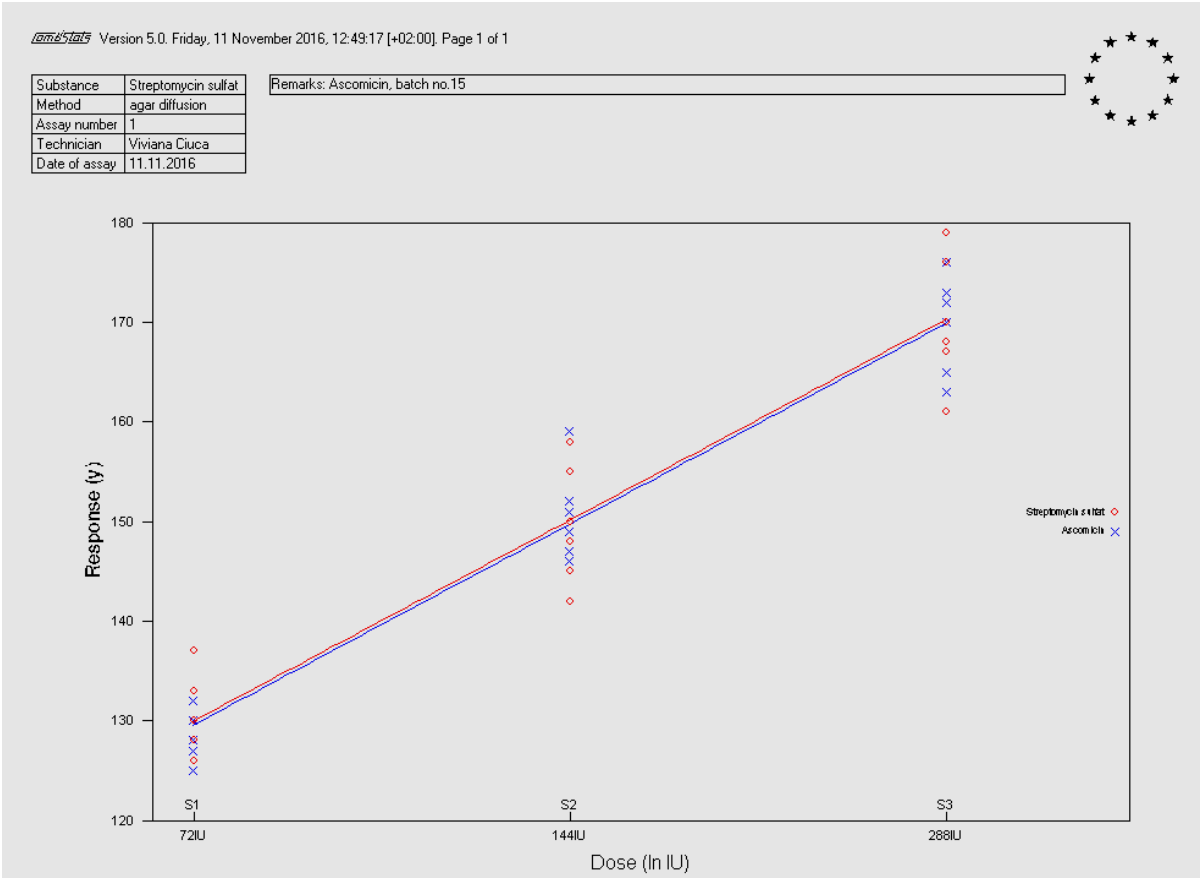
| Sample 1 | | | |
|---|---|---|---|
| Id. | Ascomicin | | |
| Ass. pot. | 749 IU/mg | | |
| Pre-dil. 1 | 19.2 mg/50ml | | |
| Doses | T1 | T2 | T3 |
| (1) | 127 | 146 | 176 |
| (2) | 130 | 149 | 165 |
| (3) | 132 | 152 | 163 |
| (4) | 125 | 159 | 170 |
| (5) | 128 | 151 | 173 |
| (6) | 130 | 147 | 172 |

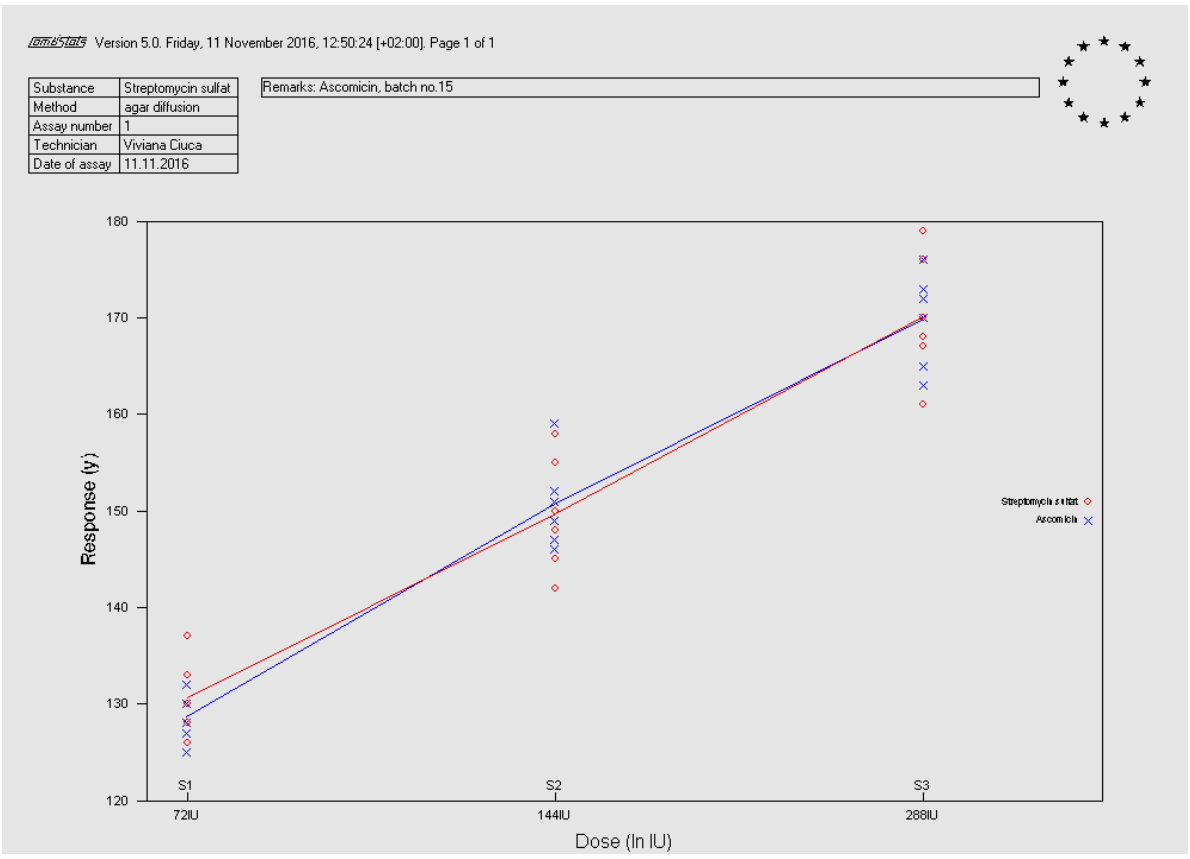
Model: Parallel lines
Design: Randomised block
Transformation: y' = y
Variance: Observed residuals
Dilution step (Increasing): 2

Common slope(factor) = 29.0943 (26.9223 to 31.2664)
Correlation | r |: 0.976866

| Source of variation | Degrees of freedom | Sum of squares | Mean square | F-ratio | Probability |
|---|---|---|---|---|---|
| Preparations | 1 | 1.77778 | 1.77778 | 0.095 | 0.760 |
| Regression | 1 | 9760.67 | 9760.67 | 523.516 | 0.000 (***) |
| Non-parallelism | 1 | 4.16667 | 4.16667 | 0.223 | 0.641 |
| Non-linearity | 2 | 10.2778 | 5.13889 | 0.276 | 0.761 |
| Standard | 1 | 2.25000 | 2.25000 | 0.121 | 0.731 |
| Sample 1 | 1 | 8.02778 | 8.02778 | 0.431 | 0.518 |
| Quadratic curvature | 1 | 0.888889 | 0.888889 | 0.048 | 0.829 |
| Lack of quadratic fit | 1 | 9.38889 | 9.38889 | 0.504 | 0.484 |
| Treatments | 5 | 9776.89 | 1955.38 | 104.877 | 0.000 (***) |
| Blocks | 5 | 264.889 | 52.9778 | 2.841 | 0.036 (*) |
| Residual error | 25 | 466.111 | 18.6444 | | |
| Total | 35 | 10507.9 | 300.225 | | |

| Sample 1 | | | |
|---|---|---|---|
| Id. | Ascomicin | | |
| (IU/mg) | Lower limit | Estimate | Upper limit |
| Potency | 666.714 | 738.630 | 818.099 |
| Rel. to Ass. | 89.0% | 98.6% | 109.2% |
| Rel. to Est. | 90.3% | 100.0% | 110.8% |


Version 5.0. Friday, 11 November 2016, 12:49:17 [+02:00]. Page 1 of 1
Substance Streptomycin sulfat
Method agar diffusion
Assay number 1
Technician Viviana Ciuca
Date of assay 11.11.2016
Remarks: Ascomicin, batch no.15
Response (y)
180
170
160
150
140
130
120
S1
S2
S3
72IU
144IU
288IU
Dose (In IU)
Streptomycin sulfat
Ascomicin


Version 5.0. Friday, 11 November 2016, 12:50:24 [+02:00]. Page 1 of 1
Substance Streptomycin sulfat
Method agar diffusion
Assay number 1
Technician Viviana Ciuca
Date of assay 11.11.2016
Remarks: Ascomicin, batch no.15
Response (y)
180
170
160
150
140
130
120
S1
S2
S3
72IU
144IU
288IU
Dose (In IU)
Streptomycin sulfat
Ascomicin

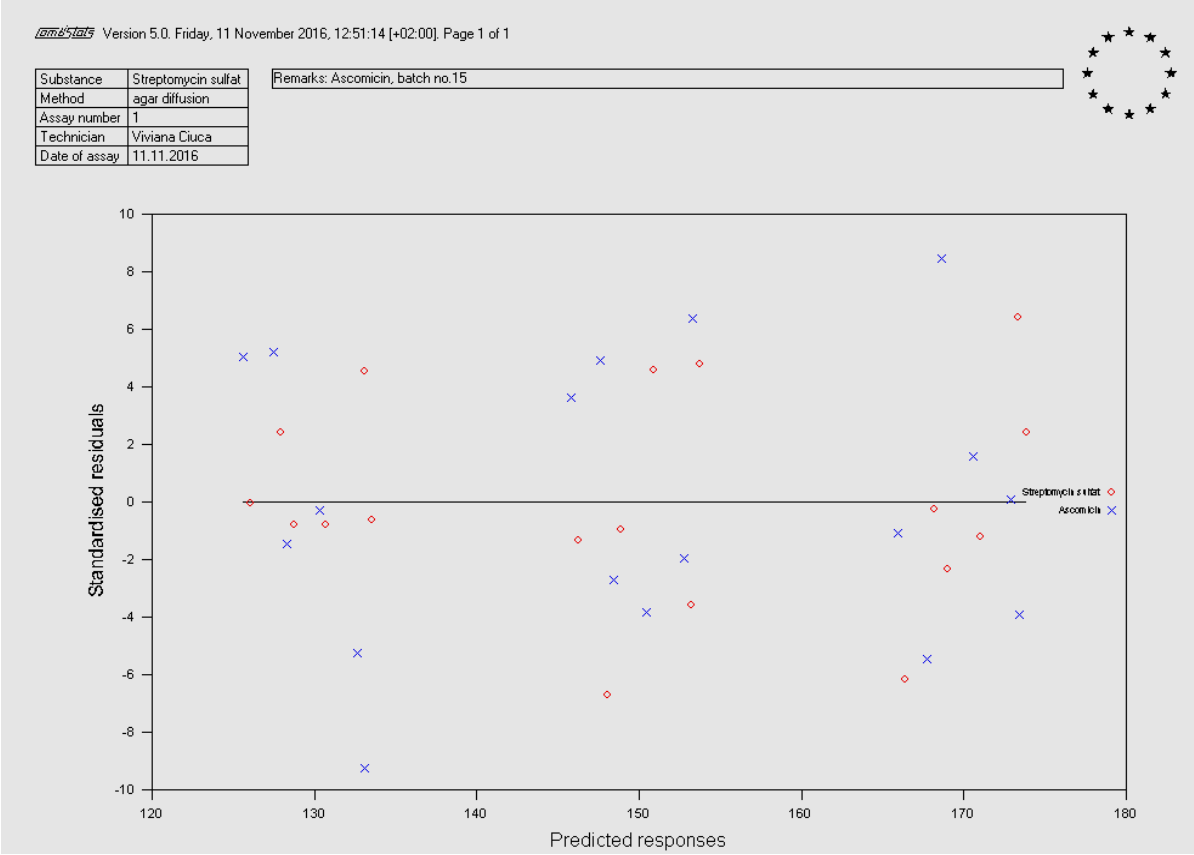



Table 1 includes the results of the method for three serial number of Ascomicin.

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 19400 international units/g benzylpenicillin potassium and 13960 international units/g streptomycin sulphate (8, 12).

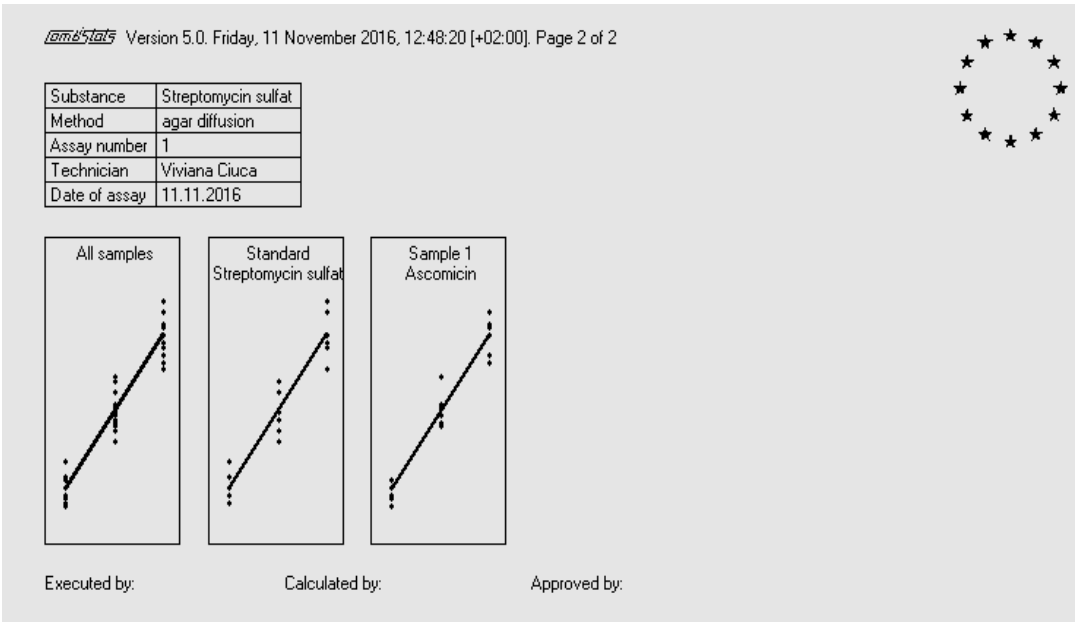


**Table 1**

**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 |
|---|---|---|---|---|---|
| **986 987** | Ascomicin | 13 | 50%≤LC≤200% 95%≤EP≤105% | ***Potassium benzyl penicillin*** **LC = 89.9 – 118.3%** **EP = 97.8%** ***Streptomycin sulfat*** **LC = 89.8 – 114.3%** **EP = 98.1%** | ***Potassium benzyl penicillin*** **LC = 80.7 – 118.1%** **EP = 98.2%** ***Streptomycin sulfat*** **LC = 88.9 – 111.3%** **EP = 98.5%** |
| **986 987** | Ascomicin | 14 | 50%≤LC≤200% 95%≤EP≤105% | ***Potassium benzyl penicillin*** **LC = 83.9 – 118.1%** **EP = 96.8%** ***Streptomycin sulfat*** **LC = 86.3 – 109.5%** **EP = 97.1%** | ***Potassium benzyl penicillin*** **LC = 80.9 – 116.3%** **EP = 97.4%** ***Streptomycin sulfat*** **LC = 79.9 – 121.1%** **EP = 98.7%** |
| **986 987** | **Ascomicin** | **15** | **50%≤LC≤200% 95%≤EP≤105%** | ***Potassium benzyl penicillin*** **LC = 83,3 – 108.4%** **EP = 95%** ***Streptomycin sulfat*** **LC = 89.0 – 109.2%** **EP = 98.6%** | ***Potassium benzyl penicillin*** **LC = 8919 – 109.3%** **EP = 96.4%** ***Streptomycin sulfat*** **LC = 84.7 – 110.2%** **EP = 98.5%** |

## 3. Conclusions

The precision of the assay is such that the fiducial limits of error of the estimated potency (P = 0.95) are not less than 95% and not more than 105% of the estimated potency.

The determination of the benzylpenicillin potassium and streptomycin sulphate potency of the Ascomicin, 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 Ascomicin product and is considered valid, the results obtained for each validation parameter are within the admissibility criteria (6, 7, 10).

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