

Mathematical modeling for determining the potency of bovine tuberculin purified protein derivative (P.P.D.)

Modelarea matematică a determinării potenței derivatului proteic purificat (P.P.D.) de tuberculină bovină

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Cuvinte cheie: *Mycobacterium bovis*, *Soft Combistats*, *potenta estimata*.

Abstract

Bovine tuberculin purified protein derivative (bovine tuberculin PPD) is a preparation obtained from the heat-treated products of growth and lysis of *Mycobacterium bovis*, strain AN₅, purified and solubilized into a liquid preservative, buffered, glycerinate and phenolate, so that the diagnostic product contain at least 20,000 international units. The main characteristic of commercial product is "revelatory power" or potency. The animals infected with *Mycobacterium bovis* develop delayed hypersensitivity which can be revealed *in vivo* by skin allergic test. The validation study aims to demonstrate the determination of the potency of the bovine tuberculin PPD, by comparing the reactions produced in guinea pigs sensitized by the intradermal injection 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 diagnostic reagents. The paper establishes the performance characteristics of the method considered and identify the factors that influence these characteristics. The diameters of the lesions, directly proportional to the logarithm of the concentration of the tuberculin, measured and calculated using statistical methods (*Combistats Soft*). 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 66 per cent and not more than 150 per cent of the stated potency. The stated potency is not less than 20,000 international units. The validation procedure includes details on protocol working to determine the potency of the tuberculin A, validation criteria, experimental results, mathematical modeling for determining the potency, inter-laboratory comparisons.

Rezumat

Derivatul proteic purificat (P.P.D.) de tuberculina bovina este un preparat obținut din produsele tratate termic de creștere a tulpinei *Mycobacterium bovis*, AN₅, purificate și solubilizate într-un lichid de conservare, tamponat, glicerinat și fenolat, astfel încât produsul de diagnostic să conțină cel puțin 20.000 de unități internaționale/ml. Mediul de cultură utilizat pentru cultivarea tuberculinei de tip bovin este un mediu lichid semisintetic Dorset - Henley (BAI). Principala caracteristică a produsului comercial este "puterea revelatoare" sau potența. Animalele infectate cu *Mycobacterium bovis* dezvoltă o hipersensibilitate tardivă, *in vivo*, evidențiată prin testul alergic cutanat. Studiul de validare își propune să demonstreze ca determinarea potenței tuberculinei PPD bovin, prin compararea reacțiilor produse la cobaii sensibilizați prin injectarea intradermică a unei serii de diluții ale produsului, care trebuie examinat cu cele produse de un standard de referință calibrat în unități internaționale, este o metodă analitică adecvată, reproductibilă și îndeplinește cerințele de calitate ale reagentului de diagnostic. Lucrarea stabilește caracteristicile de performanță ale metodei considerate și identifică factorii care influențează aceste caracteristici. Diametrele leziunilor, direct proporționale cu logaritmul concentrației tuberculinelor, se măsoară și se calculează utilizând metode statistice (*Soft Combistats*). Testul este valid dacă limitele de încredere ($p = 0,95$) sunt între 50-200% din potența estimată și potența estimată este între 66-150% din potența declarată. Potența declarată trebuie să fie de minim 20.000 unități internaționale/ml. Procedura de validare include detalii privind protocolul de lucru al determinării potenței tuberculinei B, criteriile de validare, rezultate experimentale, date statistice, comparații inter-laboratoare.

Introduction

Bovine tuberculin purified protein derivative (bovine tuberculin PPD) is a preparation obtained from the heat-treated products of growth and lysis of *Mycobacterium bovis*, strain AN₅, purified and solubilized into a liquid preservative, buffered, glycerinate and phenolate, so that the diagnostic product contains at least 20.000 international units.

The culture medium used for the cultivation of bovine tuberculin is a semi-synthetic liquid medium Dorset - Henley (BAI).

The main characteristic of commercial product is "revelatory power" or potency, [6, 7].

The animals infected with *Mycobacterium bovis* develop delayed hypersensitivity which can be revealed in vivo by skin allergic test, [5, 8].

The quality of reagent guaranteed as long as it is constantly controlled by an analytical mode, as described in registration file [9].

The validation study aims to demonstrate the determination of the potency of the bovine tuberculin PPD it is an appropriate analytical method, reproducible and meets the quality requirements of diagnostic reagents [2, 3, 4].

1. Materials and Methods

The potency of bovine tuberculin purified protein derivative is determined by comparing the reactions produced in sensitized guinea-pigs by the intradermal injection of a series of dilutions of the sample with those produced by known concentrations of a reference preparation calibrated in International Units.

The International Unit is the activity content in a stated amount of the International Standard.

The equivalence in International Units of the International Standard is stated by the World Health Organization [1, 2, 4, 9].

16 albino guinea-pigs were sensitizing, weighing 400-600g, by the deep intramuscular injection with 0.5 ml of the inactivated *Mycobacterium bovis*.

After 4 weeks were injected 3 doses of the reference preparation and 3 doses of the sample.

Dilution of the preparation to be examined and of the reference preparation were prepared using isotonic phosphate-buffered saline (pH = 6.5 – 7.5), containing 0.005 g/L of polysorbate 80.

The dilutions were allocated randomly to the sites. Each dose intradermal was injected in a constant volume of 0.1 ml.

The diameters of the lesions directly proportional to the logarithm of the concentration of the tuberculin, were measured and calculated using statistical methods with CombiStats soft, version 5.0, release date 11 March 213, European Directorate for the Quality of Medicines Health Care, Council of Europe, Invoice no 90054917. CombiStats soft is according to European Pharmacopoeia Monographs 8th Edition – Statistical analysis of results of biological assay and tests [6].

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 dose and the response can be represented by a straight line over the range of doses used.

The design of our assay is randomized blocks because each block (the guinea pig) can be identified as a source of variation. Were used a constant dilution step of a factor 5 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 per cent and not more than 200 per cent of the estimated potency. The estimated potency is not less than 66 per cent and not more than 150 per cent of the stated potency.

The stated potency is not less than 20.000 international units [6, 7].

The validation procedure includes details on protocol working to determine the potency of the tuberculin B, validation criteria, experimental results, mathematical modeling for determining the potency, inter-laboratory comparisons.

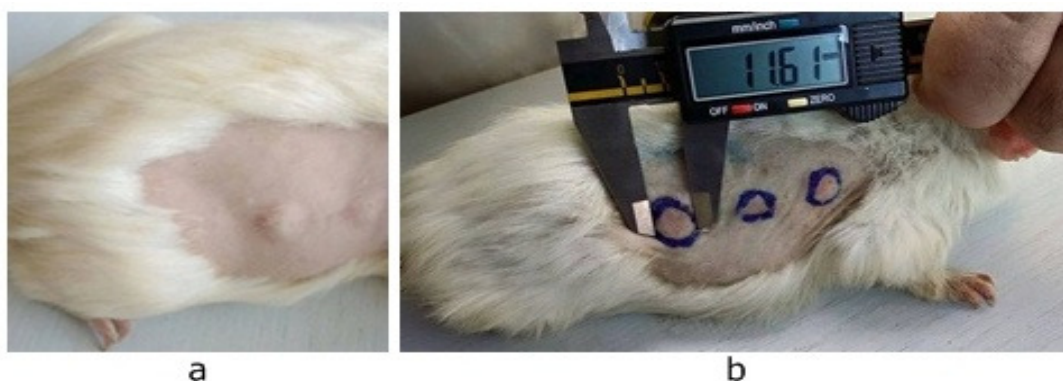


Figure 1. The reactions produced in sensitised guinea-pigs by the intradermal injection of bovine tuberculin.

In image 1.a is the reaction at 4 weeks after the sensitization the guinea-pigs were injected each dose intradermal in a constant volume of 0.1 ml.

Measuring the diameters of the lesions after 24 hours intradermal injection (three injection on each side) are represented in image 1.b.

2. Results and Discussions

Validation Combistats version 5.0 for bovine tuberculin serial number 541

Combistats Version 5.0. Tuesday, 27 October 2015, 12:02:15 [+02:00]. Page 1 of 2

Substance	Bovine tuberculin, No 541	Remarks: validation method
Method	Potency	
Assay number	1	
Technician	Viviana Ciuca	
Date of assay	25.08.2015	

Standard																
Id.	Standard															
Ass. pot.	32500uti/ml															
Doses	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)	(15)	(16)
1/32.5	17.47	17.32	17.88	17.49	17.55	17.80	17.32	17.77	17.52	17.43	17.33	17.37	17.45	17.39	17.59	17.62
1/162.5	14.46	14.78	14.84	14.86	14.00	14.78	14.75	14.59	14.88	14.00	14.81	14.72	14.65	14.74	14.79	14.60
1/812.5	10.65	10.76	10.43	10.40	11.23	10.78	11.46	10.46	11.57	10.78	10.68	10.91	11.2	11.37	10.72	10.90

Sample 1																
Id.	541															
Ass. pot.	20000 uti/ml															
Doses	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)	(15)	(16)
1/20	17.11	17.22	17.48	16.96	17.13	17.39	17.00	16.87	17.00	16.98	17.27	17.38	17.41	16.98	17.10	17.85
1/100	14.75	14.69	14.65	14.94	14.00	13.98	14.28	13.55	13.95	14.83	14.21	14.82	14.76	14.96	14.81	14.69
1/500	10.58	10.53	10.23	10.00	10.46	10.78	10.67	10.32	11.00	10.79	10.71	10.62	10.59	10.76	10.80	10.78

Model: Parallel lines
 Design: Randomised block
 Transformation: $y' = y$
 Variance: Observed residuals
 Dilution step (Increasing): 5

Common slope(factor) = 2.05341 (2.01407 to 2.09276)
 Correlation |r| : 0.991067

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-ratio	Probability
Preparations	1	1.55805	1.55805	16.842	0.000 (****)
Regression	1	699.008	699.008	>1000	0.000 (****)
Non-parallelism	1	0.00375156	0.00375156	0.041	0.841
Non-linearity	2	5.76906	2.88453	31.181	0.000 (****)
Standard	1	2.01260	2.01260	21.756	0.000 (****)
Sample 1	1	3.75646	3.75646	40.606	0.000 (****)
Quadratic curvature	1	5.63413	5.63413	60.903	0.000 (****)
Lack of quadratic fit	1	0.134938	0.134938	1.459	0.231
Treatments	5	706.338	141.268	>1000	0.000 (****)
Blocks	15	1.40204	0.0934694	1.010	0.454
Residual error	75	6.93822	0.0925095		
Total	95	714.679	7.52293		

Sample 1			
Id.	541		
(uti/ml)	Lower limit	Estimate	Upper limit
Potency	16631.0	17666.1	18763.2
Rel. to Ass.	83.2%	88.3%	93.8%
Rel. to Est.	94.1%	100.0%	106.2%

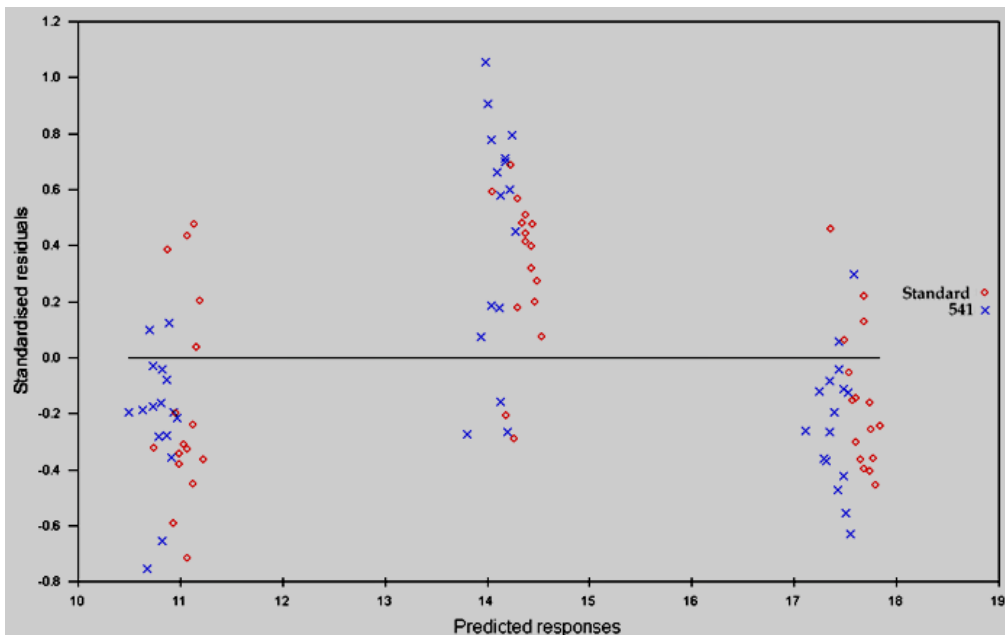
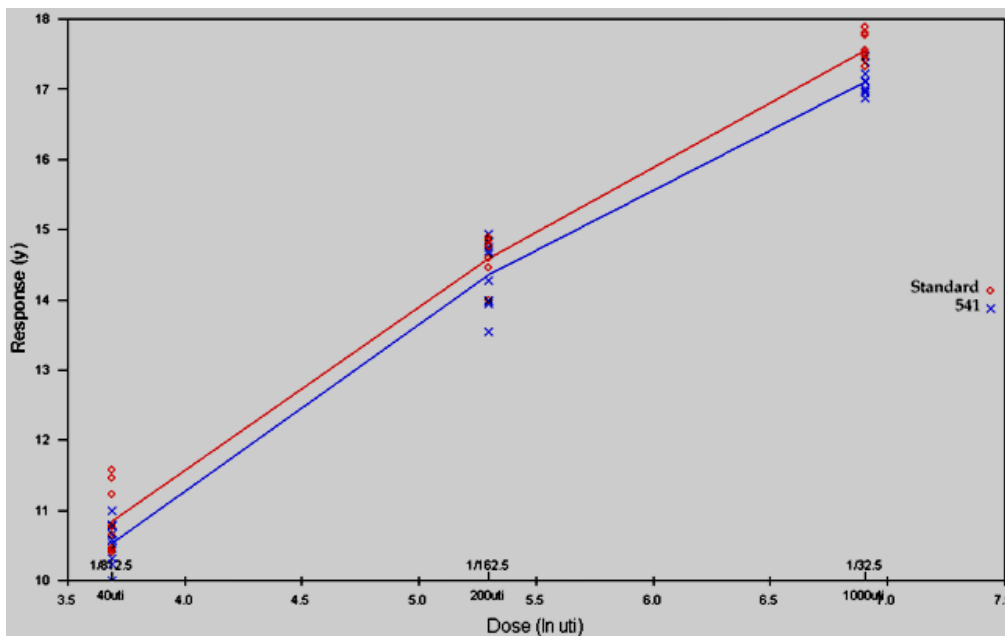


Table 1

Validation of the method for three serial number

SOP.	Product	Serial no.	LIMITS	RESULTS	RESULTS ICPBMUV
051	Bovine tuberculin	551	50%≤LC≤200% 66%≤EP≤150%	LC=70.8 – 135.7% EP = 72.4%	LC=68.4 – 143.4% EP = 69.5% A.C. no 176
051	Bovine tuberculin	521	50%≤LC≤200% 66%≤EP≤150%	LC=78.1 – 93.0% EP = 91.8%	EP = 97.67% A.C. no 223
051	Bovine tuberculin	541	50%≤LC≤200% 66%≤EP≤150%	LC= 83.2– 93.8% EP = 88.3%	EP = 119.23% A.C. no 2

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

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

The estimated potency EP, is not less than 66 per cent and not more than 150 per cent of the stated potency. The stated potency is not less than 20.000 international units.

A.C.= certificate of analysis.

3. Conclusions

The determination of the potency of the bovine tuberculin PPD, by comparing the reactions produced in guinea pigs sensitized by the intradermal injection 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 diagnostic reagents and is considered valid, the results obtained for each validation parameter are within the admissibility criteria, [10].

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