STUDIES OF METHOD FOR DETERMINING THE PROTEIN CONCENTRATION OF "MALEIN PPD" BY THE KJELDAHL METHOD

STUDII DE VALIDARE PENTRU METODA DE DETERMINARE A PROTEINEI DIN "MALEINA PPD" PRIN METODA KJELDAHL

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Key words: *Burkholderia mallei, Kjeldahl method, nitrogen from protein molecule.*

Cuvinte cheie: *Burkholderia mallei, Kjeldahl method, azot proteic.*

Abstract

Glanders is a contagious and fatal disease of horses, donkeys, and mules, caused by infection with the bacterium Burkholderia mallei. The pathogen causes nodules and ulcerations in the upper respiratory tract and lungs. Glanders is transmissible to humans by direct contact with diseased animals or with infected or contaminated material. In the untreated acute disease, the mortality rate can reach 95% within 3 weeks Malein PPD - the diagnostic product contain max 2mg/ml *Burkholderia mallei.* The amount of protein in the biological product "Malein PPD" is measured as nitrogen from protein molecule, applying the Kjeldahl (method determination of nitrogen by sulphuric acid digestion). The validation study aims to demonstrate the determination of the protein of the Malein PPD, by sulphuric acid digestion, 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 method for determining the concentration of protein, by the Kjeldahl method is considered valid if the results obtained for each validation parameter are within the admissibility criteria.The validation procedure includes details on protocol working to determine the protein of the Malein PPD, validation criteria, experimental results, mathematical calculations.

Rezumat

Maleina PPD este un derivat proteic purificat de *Burkholderia mallei Cal S.* Produsul de diagnostic contine max 2mg/ml *Burkholderia mallei.* Principala caracteristică a produsului comercial este concentratia in proteina. Morva este o boală contagioasă și fatală de cai, măgari și catâri, cauzate de infecția cu bacteria Burkholderia mallei. Agentul patogen produce noduli și ulcerații la nivelul tractului respirator superior și plămâni. Boala este transmisibila la om prin contactul direct cu animalele bolnave sau cu un material infectat sau contaminat. In boala acuta netratata, rata mortalitatii poate ajunge la 95% în decurs de 3 săptămâni. Cantitatea de proteine din produsul biologic "Maleina PPD" este măsurată ca azot din molecula proteica, aplicând metoda Kjeldahl (determinarea azotului prin digestie cu acid sulfuric). Studiul de validare își propune să demonstreze ca determinarea proteinei din Maleina PPD, 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. Procedura de validare include detalii privind protocolul de lucru al determinării proteinei din Maleina PPD, criterii de validare, rezultate experimentale, calcule matematice.

1. Introduction

Malein PPD is a purified protein derivative (P.P.D.) *Burkholderia mallei*, Cal S.symbol. The diagnostic product contain max 2mg/ml *Burkholderia mallei.* The main characteristic of commercial product is the concentration of the protein (1, 2).

Glanders is a contagious and fatal disease of horses, donkeys, and mules, caused by infection with the bacterium *Burkholderia mallei*. The pathogen causes nodules and ulcerations in the upper respiratory tract and lungs. Glanders is transmissible to humans by direct contact with diseased animals or with infected or contaminated material.

In the untreated acute disease, the mortality rate can reach 95% within 3 weeks (Neubauer et al., 1997).

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

The validation study aims to demonstrate the determination of the protein of the Malein PPD it is an appropriate analytical method, reproducible and meets the quality requirements of diagnostic reagents, (1, 2, 4, 5, 6).

2. Materials and Method

The amount of protein in the biological product "Malein PPD" is measured as nitrogen from protein molecule, applying the Kjeldahl (method determination of nitrogen by sulphuric acid digestion (2).

In figures 1 to 3 phases of samples preparation are presented.

Precipitation of proteins from the biological product

- Pipette in centrifuge cups 2 ml sample and 2.5 ml 40% trichloroacetic acid
- Shake and leave to cool for 24 hours for complete precipitation.
- Centrifuge for 30 minutes at 3000-4000 rpm.
- The supernatant was decanted carefully and the precipitate was dissolved with 0.5 mL of 5N NaOH solution.
- After complete dissolution of the precipitate is transferred into a 200 ml flask mineralization. Cup washed 3 times with a minimal amount of distilled water.

Ammonia distillation

Ammonia release by decomposition of ammonium sulphate and ammonia distillation distiller is in VELP, according to instructions.

The mineralized product vial add distilled water up to a volume of 30 ml, then distilled with 60 ml of 33% NaOH.

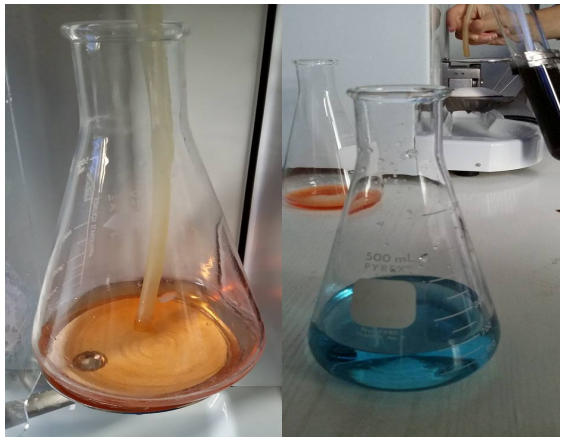
The distillate is taken up in 20 ml H2SO4 N/50 and 0.3 ml indicator Cooper.

The proteins mineralization

- The mineralization sample vial, add 5 ml of H2SO4 concentrate and approximately 3 g of a mixture of catalysts, dipotassium sulfate and copper sulfate pentahydrate in a ratio of 5:1.
- Put the vials mineralization in VELP digester, for 23 minutes at 420 ºC.

Dosing of ammonia

The excess of N/50, H2SO4, unreacted ammonia, is titrated with N/50 NaOH to the blue color.



2.1. Results calculation

The difference between the number of ml of N/50 H2SO4 put and the initial titration to find the solution of NaOH, N/50 represents the number of ml of sulfuric acid react with ammonia (4, 5, 6).

Calculation formula:

- ml of H2SO4 N/50 corresponding to 0.28 mg of N2 mg protein / ml sample = (V1F1 - V2F2) x 0.28 x 6.25 / 2
- V1 = volume of H2SO4 N/50 taken in work ml
- F1 = factor solution H2SO4 N/50
- V2 = volume of NaOH N/50 used for titration
- F2 = factor solution of NaOH N/50

Equipments:

- analytical balance,
- centrifuge,
- Velp digester,
- Velp distiller,
- Kjeldahl flasks,
- flasks, pipette,
- Erlenmeyer flasks,
- Burette semiautomatic.

Reagents and materials used:

- Concentrated sulfuric acid (H_2SO_4, M = 98.08, d = 1.84).
- Blend catalysts: dipotassium sulfate (K_2SO4) and copper sulfate pentahydrate ($Cu\ SO_4 \times 5H_2O$) in a ratio of 5: 1.
- Sodium hydroxide, 33% solution (NaOH, M =40): 33 g of sodium hydroxide, washed in advance with 60-70 ml water dissolves in fresh water boiled and cooled and completed to 100 ml with the same solvent.
- Sulphuric acid, solution N / 50 (H_2SO4; M = 98.08).
- Sodium hydroxide solution N / 50 (NaOH, M = 40).
- Sodium hydroxide solution, 0.02 N NaOH in 1000 ml containing 0.800g.
- Indicator Cooper: 0.4 g brome-cresol green in 100 ml ethanol and 0.04 g methyl red in 100 ml ethanol mix in equal parts.
- Trichloroacetic acid ($C_2HCl_3O_2$ M = 163.4), 40% solution: 40 g trichloroacetic acid is dissolved in 70 ml distilled water and bring to the mark with the same solvent in 100ml volumetric flask.
- Sodium hydroxide (NaOH, M = 40), 5N solution: 5 N sodium hydroxide solution containing 200 g NaOH in 1000 ml distilled water.
- Substance Reference: L-Tryptophan 99%.
- Substance Reference: Acetanilide 99%.
- Ammonium chloride NH_4Cl 99%.
- Sucrose, indicating rotation 66.3-670 (3, 4, 5).

2.2. Method's validation

Validation parameters:

- Reproducibility and accuracy inter-tests
- Accuracy by reference material
- Uncertainty method
- The value domain of method

VALIDATION OF THE METHOD

Criteria of validation	Criteria of admissibility	Results
Repeatability and accuracy intra-testing	SD repeatability ≤ 10% CV repeatability ≤ 10%	X average =1.833 SD repeatability=0,0760 CV repeatability=4,1492
Reproducibility and accuracy inter-tests	SD reproducibility ≤ 10% CV reproducibility ≤ 10%	X average =1,822 SDreproducibility=0,10042 CV reproducibility=5,511
Accuracy by reference material	Bias ± 10% Accuracy.in 80-120% the range required.	Acuratetea%= (2,142/2)*100 = 107.1 Bias%=[(2,142-2) /2] * 100= 7.1
The sensitivity	0.2 mg N / ml	0.14 mg N / ml
Uncertainty k=2, confidence level 96.64%	±30%	$U_{extinsa}$ = 2 x 12.5 %=25 %

2.2.1. Repeatability and accuracy intra-testing

- Repetability protein mg/ml,
- Malein PPD, series 1, availability 20.04.2017
- Malein PPD
- Analyst 1/ 14.12.2015

ml NaOH 0.02 N		Concentratie proteina mg/ml
17.80		1.92
17.90		1.83
18.00		1.75
17.90		1.83
17.80		1.75
18.00		1.92
Media	17.9	1.833333333
Stdev	0.089442719	0.076070143
CV	0.499679995	4.149280531

According to ISO 5983-1 / 2006,

2.2.2. Reproducibility and accuracy intra-testing

Repeatability limit

r = 0.3% + 0.008 x M (media independent results)

r = 0.3%+ 0,008 x **1.83** = 0.314

- Protein reproductibility mg/ml,
- Maleina PPD, seria 1,
- valabilitate 20.04.2017
- Malein PPD

Analist 1 si 2 10 si 11.12.2015		Analist 1 si 2 15 si 16.12.2015
17.80		1.92
17.90		1.83
18.00		1.75
17.90		1.83
17.80		1.75
18.00		1.92
18.02		1.73
18.06		1.69
17.89		1.84
17.96		1.78
17.86		1.87
17.78		1.94
18.17		1.6
18.01		1.74
17.84		1.89
17.80		1.92
17.90		1.83
17.95		1.79
17.87		1.86
17.97		1.77
17.81		1.91
18.11		1.65
18.03		1.72
Media	17.914	1.822
Stdev	0.115296719	0.100421335
CV	0.643612363	5.511599045

According to ISO 5983-1 / 2006,

limit of reproducibility, **r** = 1,3+0,027 x M (media independent results)

r = 1,3+ 0,027 x **1,82**= **1,34**

It finds that value's **bias** is within the range ± 10%, **accuracy** is within the range: 80-120%, in the range imposed.

2.2.3. Exactignes (acuracy) trough the reference material

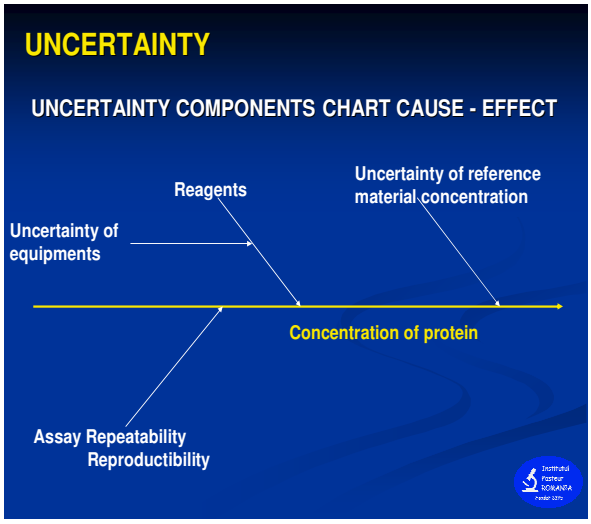
Calculation file for acuracy BIAS		
Accuracy BIAS mg/ml, MRC: L-Tryptophane, 99%, Lot: 10182432 / exp 09.2016 Analyst 1 14.12.2015		
ml NaOH 0.02 N		**Concentratie proteina mg/ml**
12.20		2.184
12.30		2.156
12.40		2.128
12.40		2.128
12.30		2.156
12.50		2.100
Average	17.9	2.142
StDev	0.089442719	0.029366648
CV	0.499679995	1.370991958
Accuracy %=(2.142/2) = 1.071 * 100 = 107.64%		
Bias%=[(2.142-2)/2] * 100 = 0.07 * 100 = 7.1%		
Accuracy % = 107.64% - included in the interval 80-120%		
Bias% = 7.1% - included in 10%.		

Method's values domain

Calculation file for method's sensibily limit	
Nitrate dosing mg/ml, MRC: L-Tryptophane, 99%, Lot: 10182432 / exp 09.2016	
ml NaOH 0.02 N	**Nitrogen concentration mg/ml**
18.20	0.50
18.50	0.40
18.90	0.30
19.30	0.20
19.50	0.14
19.50	0.14
Dozare azot mg/ml, Maleina PPD, Seria 1, valabilitate 20.04.2017	
ml NaOH 0.02 N	**Nitrogen concentration mg/ml**
19.00	0.14
18.90	0.15
19.00	0.14
18.90	0.15
19.00	0.14
19.00	0.14

The sensitivity of the method is 0.14 mg N / ml.

2.2.4. Uncertainty



Uncertainty is the result of a measurement parameter that characterizes the spread values that reasonably could be attributed to the measurement.

It is envisaged to establish a clear, unambiguous, quantitative expression of the measured value and the parameters on which it depends.

Performance criteria:

- identify sources of uncertainty;
- uncertainty components are converted into standard deviations either by direct observation or by indications of calibration certificates etc.
- the combined standard uncertainty is calculated;
- extended uncertainty is calculated for a probability of 96.64% (with the coverage factor k = 2).

According to Ishikawa diagram, the formula for calculating the uncertainty is:

U=

$$\sqrt{U^2_{reproductibility} + U_{BIAS}{}^2 + U^2_{nas.volume} + U^{\ 2}_{weighting}}$$

To calculate uncertainty were introduced in formulas mentioned values obtained to evaluate reproducibility and bias's calculation.

$U^2_{reproducibility}$ = dev. Std^2 = 0.10^2 = ***0.01% N***

Bias% = 0.071, So bias^2 is = ***0.005***

$U_{easuring\ volume:}$ when measuring the volume of 100 ml volumetric flask occurs:

- **U_{flask}** = 0.1/$\sqrt{3}$ = 0.0577;
- **U_{temp}** = 25 * (± 4℃) * 2.1 * 10^{-4} = 0.0084; 0.0084 / $\sqrt{6}$ = 0.00343
- Uncertainty due to variations in filling the flask: 0.02 ml
- calibration flask was done at 20 ℃, while the laboratory temperature varies within ±4 ℃.
- The uncertainty due to this effect can be calculated from the interval for changing the temperature (±4 ℃), and volume expansion coefficient of 2.1 × 10-4 ℃.
- **$U_{pipette}$** = 0.05/$\sqrt{3}$ =0,0288 ml;

U_v = $\sqrt{0{,}0577^2 + 0{,}0343^2 + 0.02^2}$ = $\sqrt{0{,}0033 + 0{,}0012 + 0.0004}$ = $\sqrt{0{,}0049}$ =0,07 ml

U_v / V = 0,07 / 10 = **0.0007**

- When measuring pipette 25 ml of the deviation it occurs:
- the quality specification of the pipette (± 0.1 ml);
- pipette calibration was done at 20 ℃, while the laboratory temperature varies within ±4 ℃.
- The uncertainty due to this effect can be calculated from the interval for changing the temperature (±4 ℃) and water expansion coefficient of 2.1 x 10-4 ℃.

$U_{pipette}$ = 0.1 / $\sqrt{3}$ = 0.057 ml;

U_{temp} = 25 * (±4 ℃) * 2.1 * 10^{-4} = 0.0084, 0,0084 / $\sqrt{6}$ = 0.00343

U_v = $\sqrt{0.057^2 + 0.00343^2}$ = $\sqrt{0.0032 + 0.000012}$ = $\sqrt{0.0032}$ = 0.057 ml

U_v / V = 0.057 / 25 = **0.0023**

When measuring the volume of 10 ml pipette deviation occurs in:

- specification of the quality of the pump (±0.05 ml);

$U_{pipette}$ = 0.05 / $\sqrt{3}$ = 0.0288 ml

- calibration of the pipette was done at 20 ℃, while the laboratory temperature varies within ± 4 ℃. The uncertainty due to this effect can be calculated from the interval for changing the temperature (± 4 ℃) and water expansion coefficient of 2.1 x 10-4 ℃.

U_{temp} = 10 * (±4 ℃) * 2.1 * 10^{-4} = 0.0084;
The resulting uncertainty on the rectangular distribution of temperature: 0.0084 / $\sqrt{6}$ = 0.00343

$$U_v = \sqrt{0{,}0288^2 + 0{,}00343^2} = \sqrt{0{,}00082 + 0{,}00001} = \sqrt{0{,}00084} = 0.029 \text{ ml}$$

U_v / V = 0.029 / 10 = **0.0029**

When measuring pipette 5 ml of the deviation occurs:
- the quality specification of the pipette (± 0.03 ml);

$U_{pipette}$ = 0.03 / $\sqrt{3}$ = 0.0173 ml;

- calibration of the pipette was done at 20 ℃, while the laboratory temperature varies within ± 4 ℃.
- The uncertainty due to this effect can be calculated from the interval for changing the temperature (± 4 ℃) and water expansion coefficient of 2.1 x 10-4 ℃.

U_{temp} = 10 * (±4 ℃) * 2.1 * 10^{-4} = 0.0084;
The resulting uncertainty on the rectangular distribution of temperature: 0.0084 / $\sqrt{6}$ = 0.00343

$$U_v = \sqrt{0{,}0173^2 + 0{,}00343^2} = \sqrt{0{,}00029 + 0{,}000012} = \sqrt{0{,}00031} = 0{,}018 \text{ ml}$$

U_v / V = 0.018 / 5 = **0.0035**

$U_{burette}$ = 0,03/ $\sqrt{6}$ = 0,0122 ml;

- burette calibration was done at 20 ℃, while the laboratory temperature varies within ±4 ℃.
- uncertainty due to this effect can be calculated from the interval for changing the temperature (±4 ℃) and water expansion coefficient of 2.1 × 10-4 ℃.

U_{temp} = 18 * (±4 ℃) * 2.1 * 10^{-4} = 0.00151;
The resulting uncertainty on the rectangular distribution of temperature: 0.00151 / $\sqrt{6}$ = 0.0061

$$U_v = \sqrt{0{,}0122^2 + 0{,}0061^2} = \sqrt{0{,}00014 + 0{,}000037} = \sqrt{0{,}00031} = 0.0133 \text{ ml}$$

U_v / V = 0.0133 / 18 = **0.00073**

The substances to be weighed using a balance with a calibration certificate mentioned in the deviation value of ± 0.10 mg.

Uncertainty associated calibration balance is:

$U_{weighing}$ =.0.10 / $\sqrt{3}$ = 0.0578 mg;

This value is calculated twice (home and the actual weighing).

$U_{weighing}$ = $\sqrt{2 * 0{,}0578^2} = 0.0817$

$U_{weighing}$ / V = 0.0817 / 3000 = **0.00002**

- the combined uncertainty becomes

$$U_c = \sqrt{0{,}1^2 + 0{,}071^2 + 0{,}0007^2 + 0.0023^2 + 0.0029^2 + 0.0035^2 + 0.00073^2 + 0.00002^2} = 0{,}125$$

= 12.5 %

$U_{extended}$ = k x U_C,
K = 2,
confidence level results 95%.

The uncertainty extended U_{ext} = 2 x 12.5% = **25%**

So, the concentration of protein sample of malein PPD tested, with extended uncertainty associated is: 1.82 ±0.45 or uncertainty is ± 25% (3, 7, 8, 9).

Conclusions

- Determination of Malein PPD protein, as measured by nitrogen in the protein molecule, applying the Kjeldahl method (determination of nitrogen by digestion with sulfuric acid) is a suitable analytical method, reproducible quality and meet the requirements of a diagnostic reagent.
- The test is considered valid, results obtained for each parameter validation meet the criteria of admissibility (9).

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