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STABILITY AND PRODUCTION OF CLINICAL REAGENTS

DG/CUB/86/015/11-51

CUBA

Technical report: Findings and recommendations*

Prepared for the Government of Cuba
by the United Nations Industrial Development Organization

Based on the work of Dr. Gabor Szepesi, expert in production of clinical reagents

Backstopping officer: Ms. M. Sanchez, Chemical Industries Branch

* This document has not been edited.

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Note

Some enclosures to this report are in Spanish because they are technical descriptions of different reagents at Finlay Institute ("Laboratory Prescription").

Abstract

Title: Stability and Production of Clinical Reagents Project No: OC/CUB/86/015/11-51/J 13422

Objective: To advise and give concrete technical recommendations on the production and stability testing of clinical reagents listed in Job Description (Annex I).

Duration of my activity: 1 month

Most important conclusions and recommendations based on my personal experiences obtained at Finlay Institute can be summarized as follows:

I. Conclusions

(1) The colleagues at Finlay Institute are well-experienced and are able to solve the problems which we discussed during my stay. Most of my advises and recommendations where the raw materials were available were realized, and based on the instructions given to them they will succesfully carry out the others after arrival of the necessary materials.

(2) The stability testing of the reagents produced at Finlay Institute in the future will be well organised and will meet with the international regulations based on the very intensive work performing together.

(3) Regarding the basic GMP and GLP regulations the situation is not so simple. The most important rules are used at the Institute, to satisfy some of them require additional efforts, such as:

- the work of QC Laboratories should be improved ;
- the process and method validation should be performed;
- necessary cleaning instructions are needed.

All works have been started and will be finished soon.

(4) The quality of packaging materials should be established, re-evaluated and standardized. Necessary instructions have been given.

(5) The new building built for production of diagnostic reagents after validation of the equipments, energy supply system, etc, provides a very good possibility to produce products in good quality meeting with the international standards.

II. Recommendations

(1) QA-QC manager of Finlay Institute should be sent to participate on an intensive GMP-GLP training course.

(2) One of the basic problems disadvantageously affecting on the quality of diagnostic reagents (and of pharmaceutical product, too) is connected to the unsatisfactory quality of direct packaging materials (glass bottles, vials, rubber stoppers, caps, etc.). I highly recommend to UNIDO to support the development of glass industry to improve the quality of direct packaging materials.

(3) I can recommend to support Finlay Institute by UNIDO to get equipments (reaction vessels, filling instrument) applicable for larger scale of production compared to the presently available ones.

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INTRODUCTION

This report is written by Dr.G.Szepesi after finishing the mission entitled "Assistance the technical staff of Finlay Institute (Havanna, Cuba) on the production and quality control of clinical reagents" (details can be found in Annex I - Job Description). My activity has been started at 16 October 91, and finished at 12 November 91.

The original objectives were: to give assistance in the preparation of reference solutions, buffers, substrates, control sera and colorants for microbiological and hematological tinctures, to give recommendations on the quality of raw and packaging materials; to advise on design of technical processes, stability studies as well as on basic GMP and GLP rules, quality control techniques, etc.

The original objectives were revised at two points. In point No.1. the project was extended with the preparation of the reagents to give complete description of each individual topic. In point No.3, as it was requested by Finlay Institute, instead of synthetic preparation of the three substrates (G-GP-Na, butysil thiocholme iodide and benzoylarginine-p-nitroanilide) the preparation of the following kits have been discussed: glucose, creatinine kinase, cholinesterse, lactate dehidrogenoze, acidic and alkaline phosphatase. The Institute has no synthetic background and the colleagues were more interested in the preparation of these enzyme containing reagents.

These objectives after making this revision were mostly realized. Those parts of the work where the raw materials, ingredients and/or packaging materials were available, the kit preparations were made,and stability studies have been started requiring more time to complete them (3 month studies), where the materials were not available, the Institute ordered them and will start with the work after their arrival based on the instructions, recommendations given to them and discussions with the colleagues, as well.

I. ASSISTANCE IN THE PREPARATION OF REFERENCE SOLUTIONS OF PROTEINS, URIC ACID, IRON, LITHIUM, BILIRUBIN AND ALBUMIN

This topic was extended with the preparation of reagent solutions for bilirubin, uric acid, and iron, because I could give full information about the kits. Finlay Institute developed kits for the determination of albumin, total iron binding capacity and proteins, which are in the market. In these case we discussed the preparation of standard solutions, providing some information and recommendations for the reagents, too.

A./ Preparation of Bilirubin reagents and reference solution

The objectives were as follows:

(1) To prepare two kits differing in the chemical reactions and compare their selectivity and detection possibilities.

(2) To stabilize the bilirubin in solution used as calibrator.

(3) Using different chemicals (dimethylsulfoxide and cetyltrymethylammonium bromide) to the liberation of bilirubin from peptide adsorption bound.

1. Study of Bilirubin stability in solution

Bilirubin is not stable in aqueous solution, therefore the preparation of Bilirubin calibrator is difficult. In order to stabilize the bilirubin in solution the effects of the following parameters on bilirubin decomposition have been investigated:

(a) effect of nature of organic solvent on the stability

(b) effect of orgainic solvent concentration using the selected solvent(s) found as optimum

(c) effect of pH (acetic acid concentration) on the stability

(d) using human albumin and BSA in the solution optimized

During my stay point (a) has been completed based on two week stability test results. Point (b) to (d) will be made by the Institute using a stability program which we have recently discussed.

When the optimum solvent composition has been selected the following experimental conditions were used:

(a) Bilirubin stock solution

77 mg bilirubin was dissolved in 500 ml 0.1 M sodium carbonate solution using nitrogen and light protection.

(b) Bilirubin calibrator solution

50 ml Bilirubin solution was mixed with 60 ml organic solvent and 90 ml water-acetic acid mixture (88:2).5 ml of this solution was filled into 10 ml glass bottle.

(c) Organic solvents

Used for this study: Isopropanol, dimetrylsulfoxide, ethanol, ethylenglycol, propyleneglycol, acetonitrile

(d) Stability test

Treatments: $4^{o}C$, room temperature, $37^{o}C$, $60^{o}C$

Testing periods: zero time,5 hours, 1,2,3,4,6,10,14 days

Analytical investigations: UV - spectrum

Assay

pH

After finishing the test (14 days) the test compositions were investigated with sulfanilic acid-sodium nitrite test.

(e) Results

Based on the results obtained in this study isopropanol was found as best solvent and selected for further stability test.

(f) Stability tests for selection of optimum isopropanol concentration

Bilirubin stock solution: same as (a), Bilirubin calibrator solution: 50 ml solution (a) is mixed with 0, 40, 60, 80 and 100 ml isopropanol diluted to 200 ml with water-acetic acid mixture (88:2), and 5 ml portions are filled into 10 ml glass bottle.

Stability test is similarly organised, using $4^{0}C$ and $60^{0}C$ for heat treatment, and zero time, 1 week, 2 weeks testing periods.

Analysis: same as (d)

(g) Stability tests for selection of optimum pH (acetic acid consentration)

Bilirubin test solution: same as (a)

Bilirubin calibrator solution: 50 ml solution (a) is mixed with "A" ml (found as optimum) isopropanol and "150-A" ml water-acetic acid mixture. The ratios of water and acetic acid are as follows: 90-0; 88-2; 86-4; 83-7; 90-10.

Testing periods, treatements and analysis same as in (f).

(h) Stability tests by using human albumin and BSA in the calibrator solution

Bilirubin stock solution: 77 mg Bilirubin is dissolved in 100 ml 0.1 M sodium carbonate, Human albumin stock solution: 400 ml 5% human albumin sol is mixed with acetic acid (calculated on basis of results of (f) experiments - acetic acid ratiox 11.11) and filled up with distilled water to 1000 ml.

BSA stock solution: 400 ml 5% BSA solution is treated similarly than Human Albumin stock solution.

Solutions for stability testing:

"A" 10 ml Bilirubin stock solution + "A" ml isopropanol + 100 ml HUman Albumin stock solution + 90-"A" ml water

"B" Same as "A" but 400 ml BSA stock solutions are used

"C" Optimum composition obtained in test (f)

Treatments: -20^{O}C, 4^{O}C, reflected light, 25^{O}C, 37^{O}C, 60^{O}C

Testing periods: zero time, 2 weeks, 1,2,3 months.

Tests are continued with - -20^{O}C, 4^{O}C, RT for 6, 9, 12, 18, 24 and 36 months.

Assay: colour reaction based on sulfanilic acid-sodium nitrite test, pH.

2. Preparation of Bilirubin test reagents

(a) Sulfanic acid - sodium nitrite reaction

Reagent "A" (for total bilirubin)

Sulphanilic acid 32.2 mmol/l

Hydrochloric acid 165 mmol/l

Dimethyl sulfoxide 7 mmol/l

Dissolve 5.57 g sulfanilic acid in 600 ml 0.165 N hydrochloric acid, then add 0.5 ml dimethylsulfoxide. Fill up to 1 litre with 0.165 N hydrochloric acid. Shake well, homogenize and filter using 0.8-0.2 µ pore size filter.

Reagent "B" (for direct bilirubin)

Sulphanilic acid 32.2 mmol/l

Hydrochloric acid 165 mmol/l

Preparation is the same, but without dimethylsulfoxide.

Reagent "C"

Sodium nitrite 29 mmol/l

Dissolve 2.0 g sodium nitrite in distilled water and bring the volume to 1 litre.

Homogenize and filter.

Reagent "D"

Sulfanilic acid 32.2 mmol/l

Hydrochloric acid 165 mmol/l

Cetyltrimethylammmonium bromide 30 g/l

(CTMA) (~0.1 mmol/l)

Dissolve 5.57 g sulfanilic acid in 300 ml 0.165 N hydrochloric acid. Separately dissolve 30 g CTMA in 500 ml 0.165 N hydrochloric acid. Transfer the CTMA solution to the sulfanilic acid one and mix with gentle moving of the flask. Fill up to 1.0 litre with 0.165 N hydrochloric acid and carefully homogenize and filter.

Measurement

Temperature: 20-25°C

Wavelength: 550 nm

Cuvette: 1 cm

Total Bilirubin	A		B	
	BLANK	STD	BLANK	STD
Reagent "A"	1.5 ml	1.5 ml	-	-
Reagent "D"	-	-	1.5 ml	1.5 ml
Reagent "C"	-	50 µl		50 µl
Bilirubin cal	100 µl	100 µl	100 µl	100 µl
Direct Bilirubin				
Reagent "B"	1.5 ml	1.5 ml	-	-
Reagent "C"	-	50 µl		
Bilirubin cal	100 µl	100 µl		

Results

Direct Bilirubin test goes well. Total Bilirubin deesnot work due to dimethylsulfoxide, possibly stabilizer is used which hinderes the reaction. CTMA containing reagent works very well. The results are much better than direct bilirubin test.

Further experiments have been decided with CTMA to clarify the optimum CTMA concentration, using Reagent "D" in 1,2,3,4 and 5% concentration. Necessary instructions have been given to continue the experiments.

Quality of raw materials

Dimethyl sulfoxide: (has to be clarified)

Other ingredients: analytical grade

Sulfanilic acid: could be analysed by UV-test (total spectrum and assay at the maximum, colour of solution).

Bilirubin: highly purified quality (min. 98.0%) UV-assay + decomposition product according to the description which I gave.

Quality of packaging materials

Bilirubin calibrator sol: ambered glass vials + stopper + caps (vial in 1st class quality) or ambered glass bottle + insert (plastic) (the glass is 2nd class surface treated quality).

For reagents: ambered 2nd class surface treated quality glass bottle.

Quality of formulated products

Reagents: visual inspection

pH

Refractive index (for dimethylsulfoxide and CTMA)

Sulfanilic acid content (Reagent "A" and "B")

Sodium nitrate content (for reagent "C")

Bilirubin calibrator: Bilirubin content (UV)

pH

Visual inspection

Refractive index

Stability testing of reagents (Reagent "A", "B", "C" and "D")

Samples: 3 pilot batches from each Reagent

Treatments: -20°C, 4°C, 25°C, reflected light, 37°C

Testing period: zero, 3, 6, 9, 12, 18, 24 and 36 months

Analysis: according to the Quality Specification used for release

Laboratory prescription (in spanish): see Annex II.

(b) Dichloroaniline - sodium nitrite test

Reagent "E" (for total Bilirubin)

2.4-dichloroaniline	1.5 mmol/l
Hydrochloric acid	40 mmol/l
CTMA	30 g/l

Dissolve 2.43 g dichloroaniline in 200 ml 0.08 N hydrochloric acid. Separately dissolve 30 g CTMA in 200 ml 0.08 N hydrochloric acid and after mixing as proceed at Reagent "D" fill up to 500 ml with 0.08 N hydrochloric acid.

Reagent "F"

2.4 dichloroaniline	1.5 mmol/l
Hydrochloric acid	40 mmol/l
Dimethylsulfoxide	7 mmol/l

Prepase as described at Reagent "E", but instead of CTMA, add 0.5 ml Dimethylsulfoxide.

Reagent "G"

Sodium nitrite	1.5 mmol/l

Dissolve 0.1035 g sodium nitrite in 500 ml distilled water.

Reagent

Reagent "H" (Sample blank)

Mix Reagent "E" or "F" (Reagent EH or Reagent FH) with distilled water in 1:1 ratio.

Reagent "J" (for direct Bilirubin)

2.4 dichloroaniline	1.5 mmol/l
Hydrochloric acid	40 mmol/l

Preparation same as Reagent "E" without CTMA.

Reagent "K"

Mix Reagent "J" with Reagent "G" in 1:1 ratio. Allow to stand at least 15 min before use.

Reagent "L"

Dilute Reagent "J" with distilled water to double volume.

Measurement

Before use mix

I./ Reagent "E" with Reagent "G" (Reagent "EG") in 1:1 ratio

II./ Reagent "F" with Reagent "G" (Reagent "FG") in 1:1 ratio

and allow to stand at least 15 min at room temperature protected from light.

Total Bilirubin	I.		II.	
	BLANK	STD	BLANK	STD
Reagent "EG"	-	1000/µl	-	-
Reagent "FG"	-	-	-	1000/µl
Reagent "EH"	1000/µl	-	-	-
Reagent "FH"	-	-	100/µl	-
Bilirubin cal	100/µl	100/µl	100/µl	100/µl

Direct Bilirubin	I.		II.	
	BLANK	STD	BLANK	STD
Reagent "K"	-	1000/ul	-	-
Reagent "L"	1000/ul	-	-	-
Bilirubin cal	100/ul	100/ul	-	-

Results

Because 2.4-dichloroaniline has not been available during my stay, experiments will start after arrival of this material.

Quality control of raw materials, packaging materials and finished products

Will be established after the stability tests are completed.

Stability tests of the Reagents (Reagent "E", Reagent "F")

Will start after the solutions are prepared using similar principles and organisation than at sulfanilic acid sodium nitrite test.

Laboratory prescription (in spanish): See Annex II.

B./ Preparation of uric acid reagents and reference solution

Finlay Institute developed a method for the production of uricase and just is dealing with the development of peroxidase enzyme. We started with the development of the diagnostic kits.

Barically three methods can be used for the determination of uric acid:

(a) Direct method by UV-spectrometry;

(b) Uricase / peroxidase method and

(c) Uricase / catalase method

Because the Institute has not been interested in the development of uricase / catalase method we dealt only with the first two methods.

1. Direct method by UV-spectrophotometry

Reagent "A" (Buffer solution-1)

Sodium dihydrogen phosphate		20.4	g
Sodium hydroxide		4.95	g
EDTA		1.86	g
Sodium azide		0.1	g
Triton-X-100		1	ml
Distilled water	up to	1	litre

20.4 g sodium dihydrogenphosphate and 4.95 g sodium hydroxide are dissolved in 800 ml distilled water. The pH of the solution is adjusted to 7.5 $\pm$ 0.1 (20^{o}C). In this solution the materials are dissolved in the following order: EDTA, sodium azide and Triton-X-100. The volume is completed to 1 litre and the pH is controled (7.5 $\pm$ 0.1). Filter!

Reagent "B" (Buffer solution-2)

Boric acid	12.5	g
Sodium carbonate	20	g
Distilled water ad	1	litre

Dissolve 12.5 g uric acid and 20 g sodium carbonate in 800 ml distilled water at about 40°C. Cool down and measure the pH of solution (pH= 9.5 ± 0.1) and adjust if necessary. Fille up to 1 litre with distilled water and filter.

Reagent "C"

Uricase 1 U/ml

Dissolved in 50% glicerin in water.

Reagent "D" (Uric acid standard)

Uric acid 180 mg

Lithium carbonate 10.8 mg

Sodium azide 150 mg

Distilled water ad 1 litre

Firstly the lithium carbonate (10.8 mg) is dissolved in distilled water at 90°C. The warm lithium carbonate solution is added to the uric acid. Rigorously mix and after perfect dissolution in the cooled solution (25°C) 1.5 g sodium azide is dissolved. Complete the volume to 1 litre with distilled water.

(The solution is stored at 4°C, under light protection.)

Measurement

Wavelength: 293 nm

	I.	II.
Reagent "A"	3 ml	-
Reagent "B"	-	3 ml
Uric acid	150/ul	150/ul
Determine the absorbance at 293 nm (A_I)		
+ Uricase	50/ul	50/ul

After 5-6 min, determine the absorbance at the same wavelength (A_{II})

Calculation

Uric acid /umole/l = $(A_I - A_{II})$ x 4.92

Results

The reagents were prepared and tried with two different uricase, with one prepared by Finlay Institute and with the reagent of Boehringer Manheim. Both uricase provide similar results, but the activity of BM uricase is higher giving a shorter time within the reaction is completed. The uricase can oxidase uric acid at both pH (7.5 and 9.5). Based on these results, the reagents are suitable for the measurement. The general problem is that the sensitivity (detection limit) is low and cannot measure the uric acid in plasma in the normal range.

Laboratory prescription (in Spainsh): See Annex III.

2. Uricase / peroxidase method

Originally I suggested to use dichlorophenolsulphonate Na as reagent (DHBS), however, this material was not available. Until it arrives we tried to use 2.4-dichlorophenol and phenol for this purpose instead of DHBS in the same amount.

Reagent "E"

The same composition than Reagent "A", but containing 0.523 g/l 2.4-dichlorophenol. It is dissolved in 1 litre Reagent "A".

Reagent "F"

1 litre Reagent "A" contains 0.523 g phenol.

Reagent "G" (Uric acid std solution)

Uric acid	60 mg
Lithium carbonate	36 mg
Sodium azide	1.5 g
Distilled water up to 1 litre	

Preparation is the same than Reagent "D".

Reagent "H"

Same as Reagent "G", but instead of sodium azide the solution contains 0.1% thiomersal. Preparation is the same, than Reagent "G".

Reagent "I"

Uricase	60 U/l
Peroxidase	600 U/l
4-aminophenazone	1 mmol/l

Dissolve 60 U uricase, 600 U peroxidase and 0.231 g 4-aminophenasone in 200 ml distilled water, and after perfect dissolution the volume is completed to 250 ml.

After filtration 5-5 mls are filled into 10 ml glass vials and lyophilized.

Temperature: -35 -40°C

Time: after reaching -30°C (1 hour)

-30 -25°C	1-8 h
-25 - 0°C	8-18 h
0°C- +40°C	18-26 h

Reagent "J"

Phosphate buffer (pH=7.5)		30	mmol/l
4-aminophenasone		1	mmol/l
Uricase		60	U/l
Peroxidase		600	U/l
Phenol		0.523	g/l
Glicerin		500	g/l
Distilled water	ad	1	litre

Dissolve 0.231 g 4-aminiphenasone and 0.523 g phenol in 200 ml 0.15 M phosphate buffer (pH=7.5). Add 600 U peroxidase and dissolve it. Simultaneously dissolve 60 U uricase in 300 ml 50% glicerin solution and add to the phosphate buffer. Add 350 ml glicerin to the solution and after homogenization fill up to 1 litre with distilled water.

Reagent "K" (Reconstituted solution)

Dissolve 1 vial Reagent"I" in 20 ml Reagent "E"

Reagent "L" (Reconstituted solution)

Dissolve 1 vial Reagent "J" in 20 ml Reagent "F".

Measurement

Waveleugth: 500 nm

	I.	II.	III.	IV.	V. (Blank)
Reagent "J"	1 ml	-	-	-	-
Reagent "K"	-	1 ml	-	-	1 ml
Reagent "L"	-	-	1 ml	1 ml	-
Reagent "G"	25 µl	25 µl	25 µl	-	-
Reagent "H"	-	-	-	25 µl	-

Incubate at 37°C for 5 min or at 25°C for 15 min. Determine the absorbance against blank at 600 nm.

Results

The method with the tried reagents (II and III) works well. To exclude the use of lyophilized reagent the suitability of I. variant can be proved by stability testing. The applicability of thiomersal instead of sodium azide is evident , but the reagent stability can be proved.

Laboratory prescription (in Spanish): See Annex III.

3. Determination of detection limit and lowest detectable quantity in control sera

(a) Direct method

Two types of experiments are ferformed, one with control sera, another with only standard.

	ADDED AMOUNT OF STD	
	TEST WITH CONTROL SERA	TEST WITHOUT CONTROL SERA
(i)	NO	X
(ii)	X/2	3X/2
(iii)	X	2X
(iv)	2X	3X

where X = the measured uric acid content in control sera.

Reactions can be made both with Finlay and BM uricase.

(b) Uricase/peroxidase method

The same test as in (a) should be made.

(c) Evaluation

Plot the added uric acid concentration against the absorbance measured, calculate the equations and interpolate to the zero added uric acid.

Determine the baseline noise by measuring control sera without enzyme.

Detection limit: 3 X NOISE

Lowest detectable quantity: 10 X NOISE

4. Other validation prameters

Linearity and range: calculated from experiments No. 3.

Accurancy: $\frac{b_2}{b_1}$ from $Y = mx + b$ equation

b_2 = experiments with control sera

Precision: calculated for 20 X NOISE.Quantity from 7 independent experiments.

5. Stability test

(a) Uricase stab. in 50% glicerin solution

Type: accelarated

Treatments: $-20^{o}C$, $+4^{o}C$, RT, $37^{o}C$

Testing periods: zero, 2 weeks, 1,2,3 months

Analytical methods:

(i) Specific enzyme assay

(ii) Uric acid reaction UV-test

(b) Freeze-dried products

(i) Uricase only

After dissolution in 50% glicerin the same test is performed as (a)

(ii) Peroxidase only

Same as (i)

(iii) Uricase + peroxidase + 4-aminophenasone

Treatments, testing times same as in (a)

Methods: uric acid colour test

(c) Reagent "J" stability

Same as (a), method uric acid colour reaction.

(d) Reconstituted solution stability

The lyophylized powder is dissolved in the reagents.

Treatment: $4^{o}C$, RT, reflected light

Testing periods: zero, 6 hours, 10 hours, 1,2,3,5,7,10,14 days

Method: uric acid UV or colour tests.

(e) Reagents stability - STD stability

Type: long-term

Same as Bilirubin-sulfanilic acid test.

Anal. methods:

Phenol (dichlorophenol)	UV-spectrophotometry
Uric acid	UV-soectrophotometry
Visual inspection	
pH	

6. Quality control requirements

(a) Quality of raw materials and other ingredients

(i) For inorganic materials: analytical grade according to the standards

(ii) For uric acid: UV test

(spectrum + assay at the maximum)

(iii) For enzymes: specific enzyme tests

(iv) For 4-aminophenozone: Pharmacopoeia Quality

(investigation according to the Pharmacopoeia)

(b) Quality of of packaging materials

(i) Reagents (including std sol): ambered glass bottles with 2nd class surface treated quality (insert can be used)

(ii) For lyophylized products: 1st class glass vial good, quality rubber stoppers.

(c) Quality of finished products

a./ Direct method: visual inspection (Appearence)

(i) Reagent: pH

refractive index

(ii) Std. sol: uric acid content - UV test

visual inspection

pH

(iii) Uricase sol: specific enzyme test

b./ Uricase-peroxidase methods

(i) Reagents: visual inspection

pH

phenol (dichlorophenol) content

refractive index

(ii) Standard: same as at direct method

(iii) Enzymes - solution

colour reaction with uric acid

4-aminophenason content - UV test

Appeareance

pH

(iv) Enzymes - freeze dried

Appearance

Reconstitution

Colour reaction with uric acid or

direct method

filling weight

pH of reconstituted solution.

7. Future experiments

After arrival of DHBS, the same investigations should be made than with phenol/dichlorophenol.

C. Preparation of lithium standard solution

The uric acid standard solution icludes precisely weighed amount of lithium salt. This solution can be advantageously used as lithium standard solution.

D. Preparation of reagents and standard for iron determination

(Reaction based on CAB-method)

1. Preparation of reagents

Reagent "A"	Chromazurol "B" (CAB) (acidic)	0.2	mmol/l
	Ethanol	100	ml /l
	CTMA-bromide	2.0	mmol/l
	Guanidine HCl	3.0	mmol/l
	Acetate buffer (pH=5)	45	mmol/l
	Sodium azide	0.1	g/l

Dissolve 2.6 g sodium acetate xH_2O or 4.31 g sodium acetate $x\ 3H_2O$ in 700 ml distilled water. Add 0.77 ml acetic acid and homogenize. Control the pH of solution (5.0 ± 0.1) and adjust, if necessary.

Dissolve 616 mg CTMA bromide in the solution with gentle shaking, than dissolve 337.5 mg guanidin HCl and 0.1 g sodium azide,

Dissolve 107.9 mg Chromarusol B in 100 ml ethanol, than add under continuous stirring the buffer solution to the ethanolic solution.

Homogenize and control the pH of solution again and adjust if necessary (5.0 ± 0.1).

Fill up to 1 litre volume with distilled water.

Filter!

Reagent "B" (standard stock solution)

Prepare a 100 mg iron (III) 1 litre solution from iron (III) chloride with 0.1N hydrochloric acid.

Reagent "C"

Prepare a 0.5 mmol/l EDTA solution with distilled water.

Reagent "D" (standard solution)

Measure 10 ml Reagent "B" 1 litre volumetric flask. Add 100 ml Reagent "C" and fill up to volume with acetate buffer, pH=5.0 prepared as described at Reagent "A".

Measurement

Waveleught: 630 nm

	Blank	Std
Reagent "A"	1000 µl	1000 µl
Distilled water	50 µl	-
Reagent "D"	-	50 µl

After mixing wait 15 min and read the absorbance.

Results

Chromazurol "B" and guanidine HCl were not available during my stay. After arrival of the materials, the reagents will be produced. Necessary instructions have been given.

Laboratory prescription (in Spanish): see Annex IV.

2. Stability test

The same stability tests (long-term) will be performed as described at Bilirubin-Reagents stability.

3. Quality control requirements

(a) Quality control of raw materials and other ingredients

Chromazurol "B" according to the vendor specification (UV assay)

Other ingredients: analytical grade, investigated according to the standards.

Water: very important that would be iron-free!!!

(b) Quality control of packaging materials

Ambered glass 2nd (preferably 1st) class with surface treatment.

(c) Quality control of finished products

Reagent "A"

Chromazurol "B" assay by UV-test

visual inspection

pH

refractive index

Reagent "D"

visual inspection

pH

iron (III) content - colour reaction

E. Total protein - Biuret reaction

Finlay Institute recently has developed two different formulations in glass bottle. They were interested in finding the optimum composition which can be filled into plastic containers and in the preparation of protein standard.

1. Preparation of reagent and standard

Reagent "A"

Potassium iodide	30	mmol/l
Potassium sodium tartrate	100	mmol/l
Copper sulfate	30	mmol/l
Sodium hydroxide	38	mmol/l

Prepare a 0.038 N sodium hydroxide solution (1.520 mg in 1000 ml water).

Dissolve 28.22 g potassium sodium tartrate $x4H_2O$, in 400 ml 0.038 N sodium hydroxide. Add 5.0 g potassium iodide, then 7.5 g copper sulfate $x5H_2O$ into the solution. After perfect dissolution, fill up to 1.0 litre with 0.038 N sodium hydroxide. Homogenize and filter!

Reagent "B" (standard solution)

Bovine serum albumin (BSA)	60 g/l
Sodium azide	1 g/l

Dissolve 6.3 g BSA in 50 ml isotonic distilled water. Determine the protein content of this solution by Kjeldahl method . Convert the protein nitrogen assay to gm% protein by using a conversion factor: 1 mg protein nitrogen/ml is equivalent to 6.25 mg/ml protein.

Add 0.1 g sodium azide to the solution and add the quantity of isotonic distilled water to achive a protein content of 6 mg% by using the following formula:

$$\text{Dilution (ml)} = \frac{\text{BSA calculated}}{\text{BSA weighed}} \times 100-(50+S)$$

where S = ml of sample used for protein assay

Reagent "C" (isotonic sodium chloride solution)

Dissolve 7.9 g sodium chloride in 1000 ml distilled water.

Measurement

Waveleugth: 550 nm

	Blank	Std
Reagent "A"	3 ml	3 ml
Reagent "C"	50 µl	-
Reagent "B"	-	50 µl

Mix the reagents and measure the absorbance after 10 min.

Results

The reagents satisfactorily work. Finlay Institute will perform comparative stability test (see later) to select the optimum composition.

Laboratory prescription (in Spanish): see Annex IV.

2. Stability test (Reagent "A" and "B")

Type: comparative continued as long-term

Samples: (a-1) Reagents prepared in this composition filled in glass bottles

(a-2) as A-1 filled into plastic containers

(b-1) Finlay-1 composition filled into glass bottles

(b-2) same as b-1 filled into plastic containers

(c-1) Finlay-2 composition filled into glass bottles

(c-2) as c-1 filled into plastic containers.

Treatments and testing times

Treatment	Testing time (in month)									
	0,	1,	2,	3,	6,	9,	12,	18,	24,	36
$4^{o}C$	+	+	+	+	+	+	+	+	+	+
RT	+	+	+	+	+	+	+	+	+	+
$37^{o}C$	+	+	+	+	+	+	+	+	+	+
$60^{o}C$	+	+	+	+						
Reflected light	+	+	+	+						

Analytical methods

Reagent "A"	visual inspection
	pH
	Colour of solution
	Colour reaction
Reagent "B"	visual inspection
	pH
	Colour of solution
	Colour reaction

3. Quality control of raw materials

Inorganic materials: analytical grade, investigated according to the standards

BSA: protein content

water content

4. Quality control of packaging material

For Reagent "A" Ambered glass, 2nd class quality with surface treatment .

For Reagent "B" 1st class glass bottle.

5. Quality control of finished products

Reagent "A" visual inspection

pH

colour of solution

Cu^{2+} ion content (titration)

Reagent "B" visual inspection

pH

protein content (Kjeldahl)

F. Iron binding capacity measurement (TIBC)

Instead of albumin kit preparation (Finlay has good quality marketed product) the determination of TIBC (total iron binding capacity) was discussed.

1. Preparation of reagents

Reagent "A"

iron (III) chloride	5.03 mg/l

Reagent "B"

alumina	0.35 g

Measurement

Reagent "A" 1 ml

Sample 0.5 ml

Mix well, and after 3-5 min add Reagent "B". Cap and place on a rotator or roller mixer for 10 min. Remove the tubes and allow to stand for 3 min or centrifuge for 1 min at 5000 rpm.

(Sample supernatant is used = SS)

	Blank	Sample	Std
SS	-	50/ul	-
STD	-	-	50/ul
Water	50/ul	-	-
CAB reagent	1000/ul	1000/ul	1000/ul

After mixing wait for 15 min and read the absorbance at 630 nm.

Laboratory prescription (in Spanish): see Annex V.

Results

Due to the lack of appropariate aluminium oxide quality, Finlay will try this method after its arrival, because it is much faster than the present method.

2. Quality of raw materials and other ingredients

Same as in CAB method (D.3)

Aluminium oxide: Brockmann II. quality

active before use

Distilled water: iron-free!!!

3. Quality of packaging materials

Reagent "A": Sames as in CAB method (D.4.)

For aluminium oxide: conical plastic container with plastic caps, in which the reaction can be performed.

4. Quality of finished products

No any difference from CAB method (D.5) and F.2.

5. Stability test

No needed.

II. ASSISTANCE IN THE PREPARATION BUFFER SOLUTION

The composition of following buffer solution have been given.

A. TRIS-buffer (0.1 M)

Reagent "A": Dissolve 24.2 g TRIS in 1000 ml distilled water (0.2 M)

Reagent "B": 0.2 M agneous hydrochloric acid

Mix 500 ml A and "X ml B and dilute to total volume of 1 litre with distilled water

X	pH
50	9.0
81	8.8
122	8.6
165	8.4
219	8.2
268	8.0
325	7.8
384	7.6
414	7.4
442	7.2

B. Glycine-HCl buffer

Reagent "A": 15.01 g glycine is dissolved in 1000 ml distilled water

Reagent "B": same as Reagent "B" at "A"

Dilution, mixing same as "A"

X	pH	X	pH
50	3.6	168	2.8
64	3.4	242	2.6
82	3.2	324	2.4
114	3.0	440	2.2

C. Phthalate - HCl - NaOH buffer

Reagent "A": Dissolve 40.84 g potassium hydrogen phthalate in 1000 ml distilled water

Reagent "B": 0.2 N hydrochloric acid

Reagent "C": 0.2 N sodium hydroxide

Mix 500 ml Reagent "A" and X ml Reagent "B" or Y ml Reagent "C" and complete the volume to 1000 ml.

X	pH	X	pH
467	2.2	37	4.2
396	2.4	75	4.4
330	2.6	122	4.6
264	2.8	177	4.8
203	3.0	239	5.0
147	3.2	300	5.2
99	3.4	355	5.4
60	3.6	398	5.6
26.3	3.8	430	5.8
		455	6.0

D. Citrate buffer

Reagent "A": Dissolve 21.01 g citric acid in 1000 ml distilled water

Reagent "B": Dissolve 29.41 g sodium citrate x $2H_2O$ in 1000 ml distilled water.

Mix "X" ml Reagent "A" and "Y" ml Reagent "B" and dilute to 1000 ml.

X	Y	pH
465	35	3.0
437	63	3.2
400	100	3.4
370	130	3.6
350	150	3.8
330	170	4.0
315	185	4.2
280	220	4.4
255	245	4.6
230	270	4.8
205	295	5.0
180	320	5.2
160	340	5.4
137	363	5.6
118	382	5.8
95	405	6.0
72	428	6.2

E. Acetate buffer

Reagent "A": Dilute 11.55 ml glacial acetic acid to 1000 ml

Reagent "B": Dissolve 16.4 g sodium acetate xH_2O or 27.2 g sodium acetate $x3H_2O$ on 1000 ml distilled water.

Composition (mixing-dilution) as in "D"

X	Y	pH
463	37	3.6
440	60	3.8
410	90	4.0
368	132	4.2
305	195	4.4
255	245	4.6
200	300	4.8

X	Y	pH
148	352	5.0
105	395	5.2
88	412	5.4
48	452	5.6

F. Succinate buffer

Reagent "A": Dissolve 23.6 g succinic acid in 1000 ml distilled water

Reagent "B": 0.2 M sodium hydroxide

Mix 250 ml Reagent "A" and X ml reagent "B" and dilute to 1000 ml

X	pH	X	pH
75	3.8	267	5.0
100	4.0	303	5.2
133	4.2	342	5.4
167	4.4	375	5.6
200	4.6	407	5.8
235	4.8	435	6.0

G. Maleate - buffer

Reagent "A": Dissolve 23.2 g maleic acid and 8 g sodium hydroxide in 1000 ml distilled water

Reagent "B": 0.2 M sodium hydroxide

Mix 500 ml Reagent "A" and "X" ml Reagent "B" and fill up to 1000 ml with distilled water.

X	pH	X	pH
72	5.2	330	6.2
105	5.4	380	6.4
153	5.6	416	6.6
208	5.8	444	6.8
269	6.0		

H. Borax - boric acid - NaOH buffer

Reagent "A": Dissolve 19.05 borax in 1000 ml distilled water

Reagent "B": 0.2 M sodium hydroxide

Reagent "C": Dissolve 12.4 g boric acid in 1000 ml distilled water

Reagent	A	B	C	pH	up to
	20	-	500	7.6	2000 ml
	31	-	500	7.8	
	49	-	500	8.0	
	73	-	500	8.2	
	115	-	500	8.4	
	175	-	500	8.6	
	300	-	500	8.8	
	590	-	500	9.0	
	1150	-	500	9.2	
	500	-	500	9.3	
	500	70	-	9.35	
	500	110	-	9.4	
	500	230	-	9.6	
	500	340	-	9.8	
	500	430	-	10.0	

I. Phosphate - buffer

Reagent "A": Dissolve 27.8 g sodium dihydrogen phosphate in 1000 ml distilled water

Reagent "B": Dissolve 53.6 g dinatriumhydrogen phosphate x $7H_2O$ in 1000 ml distilled water

Mix X ml Reagent "A" and Y ml Reagent "B" and dilute to 2000 ml with distilled water

X	Y	pH	X	Y	pH
935	65	5.7	330	670	7.1
900	100	5.9	230	770	7.3
850	150	6.1	160	840	7.5
775	225	6.3	130	870	7.6
685	315	6.5	105	895	7.7
565	435	6.7	85	915	7.8
450	550	6.9	70	930	7.9
			53	947	8.0

J. PIPES -buffer (pH=7.5)

PIPES Na	13.86	g
Sodium hydroxide	0.711	g
Distilled water ad	1000	ml

K. Triethanolamine - EDTA buffer

(pH=8.0)

Dissolve 12 g triethanolamine HCl, 1.2 g EDTA Na_2H_2 x $2H_2O$ in 800 ml distilled water .

Control the pH and adjust if necessary with 1 M HCl or 1 M NaOH to pH=8.0$\pm$0.1.

Complete the volume to 1000 ml

Quality control of raw materials

All ingredients are analytical grade quality and can be investigated according to the Standards.

III. ASSISTANCE IN THE PREPARATION OF ENZYME DIAGNOSTIC REAGENTS

This was the point which was perfectly revised for the request of Finlay Institute, because they have no synthetic laboratories where the substrates can be produced. We discussed which topic would be discussed instead of them and for their suggestion we started the experiments with the preparation of enzyme diagnostic reagents. Although some of the materials were not abailable at Finlay Institute, I consider that they can prepare these reagents after arrival of the materials to Finlay based on the intensive discussion and written materials given to them.

A. Determination of glucose

Two methods have been discussed in details.

1. Glucose - hexocinase method

Reagent "A" (Buffer)

Phosphate buffer (pH=7.7)	100 mmol/l
ATP	4 mmol/l
NAD	3 mmol/l
Mg-ions	10 mmol/l
Sodium azide	0.5 g/l

Dissolve 1.4595 g sodium dihydrogen phosphate and 23.986 g disodium hydrogen phosphate x $7H_2O$ in 800 ml distilled water. Control the pH of solution (7.7$\pm$ 0.1) and adjust, if necessary.

Add 2.47 g manesium sulphate x $7H_2O$, 2.210 g $ATPNa_2$x H_2O to the solution.

Finally add 0.5 g sodium azide and control the pH again. Fill up to 1000 ml with distilled water , homogenize and filter.

Reagent "B" (Enzyme reagent)

Hexokinase	100 KU/l
G-6P-dehydrogenase	3000 KU/l
Sodium azide	1 g/l

Dissolve 100 KU hexokinase and 300 KU glucose-6-phosphate-dehydrogenaze G-6P-DH) in 800 ml distilled water. Add 1 g sodium azide and fill up to 1000 ml with distilled water.

Store the reagent at $4^{o}C$, protected from light.

Reagent "C" (Glucose STD solution)

Glucose	1 g/l
Trichloracetic acid	0.5 mmol/l

Accurately weigh 1 g glucose and dissolve it in 600 ml distilled water. Add 81.7 g trichloracetic acid and complete the volume to 1000 ml with distilled water.

Glucose should be stored at $50^{o}C$ for 48 hours!!

Reagent "D" (deproteinizing reagent)

Dissolve 1.6 g uranyl acetate in 1000 ml freslely prepared 0.9% sodium chloride solution.

Measurement

Wavelength: 365 nm

(a) Deproteinization

Reagent "D"	500 µl
Sample	50 µl

Mix well, centrifuge with high speed for 5 to 10 min.

(b) Pipetting scheme

Supernatant 100/ul

Reagent"A" 1000/ul

Mix and measure the absorbance (A_1)

Add Reagent "B" 10/ul

Mix and measure the absorbance (A_2) after 3-30 min.

(c) Determination in hemolysate

Reagent "A", "B" and "C" are the same

Reagent "E"

Dissolve 2 g CTMA bromide and 1 g sodium azide in 1000 ml distilled water.

Reagent"E" 1000/ul

Sample (blood) 20/ul

Mix well for fast hemolysis.

Hemolysate 200/ul

Reagent "A" 1000/ul

Mix well and measure the absorbance at 365 nm (A_1)

Reagent "B" 10/ul

Mix well and measure the absorbance 3-30 min (A_2)

Make a standard dilution from Reagent "C" (10 times dilution with distilled water and handle as a sample).

Calculation

$C(mg/dl) = 1365 \times \Delta A$ $\Delta A = A_2 - A_1$ STD sol.

$$C\ (sample) = \frac{C_{std} \times A_{sample}}{\Delta A_{std} + A_{sample}}$$

Results

The reagent preparation has been started, will be finished soon according to the written instructions.

Laboratory prescription (in Spanish): see Annex V.

(a) Stability testing

(i) Reagent "A" and "B"

Type: accelerated

Treatments: -20°C, 4°C, RT, RL, 37°C

Testing times: zero, 2 weeks, 1,2,3 months

Analytical methods: visual inspection

pH

colour reaction with

glucose standard

(ii) Glucose std solution

Type: long term

Treatments: -20°C, 4°C, RT, RL, 37°C

Testing times: zero, 3,6,9,18,24 and 36 months

Analytical methods: visual inspection

glucose - HPLC

colour of solution

(iii) Other reagents (Reagent "D" and "E")

Same as (a)

Method: visual inspection

Applicability

(b) Quality of raw materials, other ingredients

Inorganic materials: analytical grade and investigated according to standards.

Enzyme: according to vendor specification, sepcific enzyme test

ATP, NAD: same as enzyme.

(c) Quality of packaging materials

Ambered 1st class glass bottles

(d) Quality of finished product

Reagent "A":	visual inspection
	pH
	UV-spectrum
	Magnesium content
	(complexometry)
	Refractive index
	NAD - UV test
Reagent "B":	Colour reaction with
	glucose
	visual inspection
	pH
Reagent "C":	glucose content - HPLC
	colour of solution
	visual inspection
Reagent "D" and "E":	visual inspection
	refractive index

2. Glucose - peroxidase method

(a) Preparation of reagents

Reagent "A"	phosphate buffer (pH=7.4)	100 mmol/l
	phenol	24 mmol/l
	sodium azide	0.05 g/l

Dissolve 13.6 g natriumdihydrogen phosphate and 3.3 g sodium hydroxide in 800 ml distilled water. Control the pH of solution (7.4 ± 0.05).

Dissolve 0.941 g phenol and 0.05 g sodium azide and complete the volume to 1000 ml.

Homogenize and filter!

Store at room temperature protected from light.

Reagent "B-1" (enzyme reagent)

glucose oxidase	12000 U/l
peroxidase	700 U/l
4-aminoantypyrin	0.4 mmol/l

Dissolve 12000 U glucose oxidase, 700 U peroxidase and 0.0924 g 4-aminophenazone in 1000 ml cool (5-10°C) distilled water.

Fill in 300 ml glass bottle and lyophylize as described under uric acid.

Reagent "B-2" (enzyme solution)

Phosphate buffer (pH=7.4)	100	mmol/l
4-aminophenazone	0.25	mmol/l
phenol	0.75	mmol/l
glucose oxidase	15000	U/l
peroxidase	1500	U/l
distilled water up	1000	ml

In 800 ml phosphate buffer (pH=7.4) prepared as described in Reagent "A" dissolve 57.75 mg 4-aminophenazone, 29.4 mg phenol, 15000 U glucose oxidase, 1500 U peroxidase. Control the pH of solution (7.4 $\pm$ 0.05) adjust if necessary, then fill up to 1000 ml with distilled water. Store at 4^{o}C protected from light.

Reagent "C" (glucose STD solution)

Dissolve 1 g benzoic acid in 800 ml distilled water at 90^{o}C. Cool down the solution to room temperature and dissolve 1 g accurately weighed (previously dried at 45^{o}C for 48 hours) glucose. Complete the volume to 1000 ml with distilled water.

Reagent "D" (Reconstituted solution)

Dissolve the content of one vial Reagent "B-1" in 250 ml Reagent "A".

(b) Measurement

Wavelength: 500 nm

(i) With Reagent "D"

	Blank	STD	Sample
Reagent "D"	1000 µl	1000 µl	1000 µl
Reagent "C"	-	10 µl	-
Sample	-	-	10 µl

After mixing incubate the mixtures at 37^{o}C for 5 min or at 20-25^{o}C for 15 min. Read the absorbance of standard solution (A_{std}) and sample solution (A_{sam}) against reagent blank.

Calculation: $C\ (mg/dl) = \frac{A_{sam}}{A_{std}} \times 100$

$$C\ (mmol/l) = \frac{A_{sam}}{A_{std}} \times 5.58$$

(ii) With reagent "B-2"

	Blank	STD	Sample
Reagent "D" (from method-1)	1000/ul	1000/ul	1000/ul
Sample	-	-	100/ul
STD	-	100/ul	-
water	100/ul	-	-

Mix, centrifuge the sample with high speed for 5-10 min.

	Blank	STD	Sample
Supernatant	100/ul	100/ul	100/ul
Reagent "B-2"	2000/ul	2000/ul	2000/ul

The method and coleulation are the same as described above.

Laboratory prescription (in Spanish): see Annex VI.

(c) Results

The kit has not been completed due to the shortage of time. After that the glucose-hexokinase method will be tried, the kit will be ready, Finlay will start the experiments. Necessary instructions have been given.

(d) Stability test

(i) Reagent "A" and Reagent "B-2" are the same as in Method-1 (glucose-hexokinase method)

(ii) Reagent "B-1"

X Reconstituted solution

The same as described at uric acid under 5.d.

X Freeze-dried product

Same as (ii) after dissolution the product is examined

(iii) Reagent "C"

Same as Method-1 under (a)ii

(e) Quality control of raw materials

Same as Method-1

(f) Quality coantrol of packaging materials

Same as Method-1

(g) Quality control of finished product

(i)	Reagent "A"	visual inspection
		pH
		phenol content (titrimetry)
		refractive index
(ii)	Reagent "B-1"	solubility
		colour of solution
		colour reaction
(iii)	Reagent "B-2"	visual inspection
		pH
		refractive index
		colour of solution
		colour reaction
(iv)	Reagent "C"	visual inspection
		colour of solution
		glucose content

B. Creatine kinase

1. Preparation of reagent solutions

Reagent "A"	Imidazol buffer (pH=7.3)	100 mmol/l
	glucose	20 mmol/l
	magnesium acetate	10 mmol/l
	EDTA	2 mmol/l
	Sodium azide	1 g/l

Dissolve 2.83 g imidazol, 2.47 g magnesium acetate x$4H_2O$, 0.85 g EDTA Na_2H_2x $2H_2O$ and 3.604 g glucose (previously dried) in 800 ml distilled water. Add 1 M acetic acid (60 g in 1000 ml distilled water) until the pH of solution will be $7.3^{+}_{-}0.1$. Add 1 g sodium azide and complete the volume to 1000 ml with distilled water.

Mix, homogenize and filter!

Reagent "B"	N-acetyl cysteine	20 mmol/l
	ADP	2 mmol/l
	AMP	5 mmol/l
	NADP	2 mmol/l
	diadenosinepentaphosphate	10 mmol/l
	hexokinase	3500 U/l
	G6P-DH	2000 U/l
	creatinine phosphate	30 mmol/l

Dissolve 2.9 $AMPNa_2$, 1.153 g ADP-Kx$2H_2O$, 1.81 g $NADPNa_2$ and 11 mg A_{p5}-A-trilithium (p_1-$p_5$diadenosine-5-pentaphosphate)in 800 ml distilled water.

Cool down the solution to $+5^{o}C$. Add 2.562 g N-acetyl-cysteine,and 11.3 g creatinine phosphate-Na_2x $4H_2O$ to the solution and dissolve them.

Dissolve 3500 U hexokinase and 2000 U glucose-6-phosphate dehydrogenase, then complete the volume to 1000 ml with distilled water. Fill the solution into 10-10 mls vial and lyophilize as was recently given.

Measurement

Waveleugth: 340 nm

Dissolve 1 vial Reagent "B" in 10 ml Reagent "A"

(Reagent "C")

Reagent "C"	1000 µl
Sample	20 µl

Mix and incubate the samples for 25°C or 30°C and 37°C.

Read the absorbance after 3 min at 25°C, 2 min at 30°C and 1 min at 37°C.

Start the stop watch and read the absorbance again after exactly 1,2 and 3 min. Calculate the main absorbance change per minute (ΔA/min).

Calculate the activity of the sample using the following equation.

IU/l = ΔA/min (340 nm) x 8199

Results

N-acetyl-cystein was not available at the Institute during my stay. Finlay starts with the experiments after arrival of the material. Necessary instructions have been given.

Laboratory prescription (in Spanish): see Annex VII.

2. Stability tests

(a) For Reagent "A"

Same as glucose-hexokinase method (Method-1) (1.a.i)

(b) For Reagent "B"

Same as glucose-peroxidase method (Method-2) (2.d.ii)

Analytical methods

colour reaction for

control sera containing creatinine kinase

visual inspection (directly or after dissolution)

pH

3. Quality control requirements

(a) QC requirements for raw materials and other ingredients

For inorganic materials: analytical grade, investigated according to the Standards

For N-acetyl-cystein, ADP, AMP, diadenosin pentaphosphate, creatinine phosphate, imidazol, EDTA according to the vendor specification.

For NADP: according to the vendor specification and specific test for NAD impurities.

For glucose: pharmacopoeial quality investigated according to the pharmacopoeial prescription.

For enzymes: according to the vendor specification, specific enzyme test.

(b) Quality control criteria for packaging material

1st class glass bottles (for Reagent "A") and vials (Reagent "B") are required.

(c) Quality control requirements for finished product

Reagent "A" visual inspection

pH

colour of solution

glucose content

magnesium ion content

Reagent "B" visual inspection after dissolution

colour of reconstituted solution

colour reaction for control sera containing known amount of creatinine kinase

C. Cholinesterase

1. Preparation of reagents

Reagent "A" Phosphate buffer (pH=7.7) 80 mmol/l

dithio-bis-nitrobenzoate 400 mmol/l

(DTNB)

Dissolve 1.5856 g DTNB in 800 ml phosphate buffer (0.08 M) and fill up to 1000 ml with 0.08 M phosphate buffer. Fill 30-30 mls in 50 ml glass bottles and lyophylize as described at uric acid.

Reagent "B-1"

Each vial contains 2.0 mg butirylthiocholine iodide (B-BCJ).

Reagent "B-2" (solid powder)

20 mg butiryl thiocholin is grounded with 1000 mg sodium chloride. Determine the B-BCJ content by UV spectrometry prepaning a solution containing 10 ug/ml B-BCJ.

Prior to use dissolve Reagent "A" in 30 ml (Reagent "C") and Reagent "B" (Reagent "D") in i ml distilled water.

Measurement

Wavelength: 405 nm

Reagent "D"	1500 /ul
Sample	10/ul
Reagent "B"	50/ul

Mix, measure the absorbance at 405 nm, immediately after completition of the reacting mixture, then after 30, 60 and 90 sec.

Calculate ΔA/min.

at 25°C U/l = ΔA/min x 11 730

2. Results

The DTNB an 8-BCJ were not available in the time of my stay. After arrival of these materials the kit will be prepared. Necessary instructions have been given.

Laboratory prescription (in Spanish): see Annex VIII.

3. Stability tests

For Reagent "A": same as bilirubin reagent test (I)
For Reagent "B-1": same as described for uric acid (II.5 (b) (i)
For Reagent "B-2": same as described for uric acid (II.5 (b) (i)
For Reagent "C" and "D": same as uric acid (II.5 (d)

Methods

8-BCJ test (Reagent "B-1", "B-2")

DTNB test (Reagent "A")

4. Quality control requirements

Will be established after the KIT is prepared.

D. Lactatedehydrogenase

1. Preparation of reagent solutions

Reagent "A":

TRIS buffer (pH=7.2)	100 mmol/l
Sodium chloride	200 mmol/l
Sodium pyruvate	1.6 mmol/l
Ethyleneglycol	18.3 g/l
Sodium azide	1 g/l

Dissolve 12.2 g TRIS base in 300 ml distilled water. Add 44,2 ml 1 N hydrochloric acid. Mix and control the pH of the solution (7.2+0.1), and adjust, if necessary. Dissolve 11.5 g sodium chloride, 176.2 mg sodium pyruvate, 18.3 g ethylene glycol and 1 g sodium azide into the solution. Control the pH again, then complete the volume to 1000 ml with distilled water. Mix and filter!

Reagent "B":

Each vial contains 0.01 mmole (6.634 mg) NADH.
Prepare a solid dilution with sodium chloride (234 mg sodium chloride and 6.64 mg NADH) and fill it into glass vials.

Reagent "C":

Prior to use dissolve the content of one vial Reagent "B" in 20 ml Reagent "A".

Measurement:

Wavelength: 340 nm

Reagent "C" 1000 ul

STD or Sample 20 ul

Mix and after 1 min follow the procedure described at Creatinine kinaze ("B")

Calculation:

IU/l (340 nm)= A/min x 8095

Laboratory prescription (in Spanish): see Annex IX.

Results:

The kit has been prepared and it works. Stability testing has been started.

2. Stability testing program:

(a) Stability tests for Reagent "A":

Type: Accelerated

Treatments: $-20^{o}C$,$+4^{o}C$,$+20^{o}C$(RT),$37^{o}C$,$60^{o}C$.

Testing intervals: zero, 2 weeks, 1, 2, 3 months

Analytical methods: visual inspection

pH

sodium pyruvate content

(b) Stability tests for Reagent "B":

Type: Accelerated

Treatments: $-20^{o}C$,$+4^{o}C$,$+20^{o}C$(RT),$37^{o}C$,$60^{o}C$.

Testing intervals: zero, 2 weeks, 1, 2, 3 months

Analytical methods: visual inspection (after dissolution)

pH

NAD impurity content (UV)

colour reaction with control sera

(c) Stability tests for Reagent "C" (Reconstituted solution):

Type: Preliminary

Treatments: $-20^{o}C$, $+4^{o}C$, $+20^{o}C$(RT), reflected light (RL), $37^{o}C$

Testing intervals: zero, 5 hours, 1, 2, 5, 7, 10 and 14 days (after reconstitution)

Analytical methods: visual inspection
pH
colour reaction with control sera
NAD content (UV)

3. Quality control requirements:

(a) For the raw materials and other ingredients

For buffer component, sodium chloride, ethylene glycol: analytical grade, investigated according to the Standards.

For sodium pyruvate: according to the vendor specification

For NADH: limited NAD content (less than 0.1%), others according to the vendor specification.

(b) For the packaging materials:

For Reagent "A": Ambered 2nd class glass bottles with surface treatment

For Reagent "B": 2nd class glass vials with surface treatment

(c) For the finished products:

Reagent "A": visual inspection
pH
colour of solution
refractive index
sodium pyruvate content

Reagent "B": dissolution characteristic

NADH content

NAD impurity

E. Acid phosphatase

Two methods have been advised for kit preparation.

1. Nitro-phenyl-phosphate method (Method-1)

(a) Preparation of reagent solutions

Reagent "A":

Dissolve 13.5 g trisodium citratex$2H_2O$ in 700 ml distilled water, adjust the pH of solution to 4.9+0.05 with 1 N hydrochloric acid. Fill up to 1000 ml with distilled water. Mix and filter!

Reagent "B": (substrate without tartric acid)

Dissolve 288 mg 4-nitrophenyl-phosphate-Na_2 in 1000 ml Reagent "A".

Reagent "C": (substrate with tartric acid)

Dissolve 720 mg 4-nitrophenyl-phosphate-Na_2 in 240 ml Reagent "A". Add 5 ml Reagent "D".

Reagent "D":

Dissolve 150 g tartric acid in 700 ml distilled water. Adjust the pH of solution with 1 N sodium hydroxide to 4.9+0.05, and complete the volume to 1000 ml.

Reagent "E":

Dissolve 4 g sodium hydroxide in 1000 ml distilled water (0.1 N)

Measurement:

Wavelength: 405 nm

	Total activity	Tartric acid resisitence	Blank
Reagent "B"	500 ul	-	-
Reagent "C"	-	500 ul	500 ul
Incubate at 37°C for 3 min			
Sample	100 ul	100 ul	-
Incubation at 37°C for 30 min			
Reagent "E"	2000 ul	2000 ul	2000 ul
Sample	-	-	100 ul
Read the absorbance	A_1	A_2	A_0

Calculation:

I IU/l (Total)= $(A_1 - A_0)/30min \times 1405$

II IU/l (Tart.res.) = $(A_2 - A_0)/30min \times 1405$

III IU/l (Tart.inhib.) = I - II

Laboratory prescription (in Spanish): see Annex X.

Results:

The kit has not yet been prepared, due to the shortage of time. Finley will prepare the reagents after the alkaline phosphatase kit will be completed. Necessary instructions have been given.

2. FAST-RED-T method (Method-2)

(a) Preparation of reagent solutions

Reagent "A":

Dissolve 14 7 g trisodium citratex$2H_2O$ in 700 ml distilled water, adjust the pH of solution to 5.6+0.05 with 1 N hydrochloric acid. Add 1 ml Triton-X-100

and fill up to 1000 ml with distilled water. Mix and filter!

Reagent "B": (Tartrate solution)

Dissolve 14.7 g trisodium citratex$2H_2O$ and 15 g tartric acid in 700 ml distilled water, adjust the pH of solution to 5.6+0.05 with 1 N sodium hydroxide. Add 1 ml Triton-X-100 and fill up to 1000 ml with distilled water. Mix and filter!

Reagent "C": (substrate)

Dissolve 2 681 g disodium napthyl-phosphate and 400 mg Fast-Red-T in 600 ml Reagent "A". Fill up to volume (1000 ml) and fill in 5 ml glass vials. Lyophilize as described at uric acid.

Reagent "D": (stabilizer)

Add 0.15 ml glacial acetic acid to 1000 ml distilled water.
Add 1 drop to the sample to stabilize the enzyme activity.

Reagent "E":

Prior to use dissolve the content of 1 vial of reagent "C" in 3 ml Reagent "A".

Reagent "F":

Prior to use dissolve the content of 1 vial of reagent "C" in 3 ml Reagent "B".

Measurement:

Wavelength: 405 nm

	Total activity	Tartric acid resisitence	Blank
Reagent "E"	1000 ul	-	-
Reagent "F"	-	1000 ul	1000 ul

Incubate at 37°C for 3 min

Sample	100 ul	100 ul

Incubation at 37°C for 5 min, then read the absorbance change for 3 min.

Calculation:

I IU/l (Total)= $(A_1 - A_0)$/min x 743

II IU/l (Prostatic) = $(A_1 - A_0)$/min (For Reagent "E") - $(A_1 - A_0)$/min (For Reagent "F") x 743

Laboratory prescription (in Spanish): see Annex XI.

Results:

The kit has not yet been prepared, due to the lack of naphthyl-phosphate and Fast-Red-T. Finley will prepare the reagents after the arrival of the materials. Necessary instructions have been given.

2. Stability testing program:

(a) Stability tests for Reagent "B" (Method -1), and Reagent "C" (Method-1):

Type: Accelerated

Treatments: -20°C,+4°C,+20°C(RT),37°C,60°C.

Testing intervals: zero, 2 weeks, 1, 2, 3 months

Analytical methods: visual inspection

pH

4-nitro-phenyl-phosphate content (with colour reaction performed for control sera)

4-nitrophenol impurity (UV test)

(b) Stability tests for Reagent "C" (Method-2):

Type: Accelerated

Treatments: $-20^{o}C$,$+4^{o}C$,$+20^{o}C$(RT),$37^{o}C$,$60^{o}C$.

Testing intervals: zero, 2 weeks, 1, 2, 3 months

Analytical methods: visual inspection (after dissolution)

pH

colour reaction with control sera

napthol content (UV)

(c) Stability tests for Reagent "E" and Reagent "F" (Reconstituted solution - Method-2):

Type: Preliminary

Treatments: $-20^{o}C$,$+4^{o}C$,$+20^{o}C$(RT), reflected light (RL),$37^{o}C$

Testing intervals: zero, 5 hours, 1, 2, 5, 7, 10 and 14 days (after reconstitution)

Analytical methods: visual inspection

pH

colour reaction with control sera

naphthol content (UV)

3. Quality control requirements for Method-1:

(a) For the raw materials and other ingredients

For buffer component, tartric acid: analytical grade, investigated according to the Standards.

For 4-nitrophenyl-phosphate: low nitrophenol content (less than 0.1%), others according to the vendor specification

(b) For the packaging materials:

For Reagent "B" and "C": Ambered 1st class glass bottles

For Reagent "E": 2nd class glass bottles with surface treatment

(c) For the finished products:

Reagent "B" and C": visual inspection
pH
colour of solution
refractive index
nitrophenol impurity (UV)
nitrophenyl-phosphate content (UV)

Reagent "E": visual inspection
sodium hydroxide content

4. Quality control requirements for Method-2:

(a) For the raw materials and other ingredients

For buffer component, tartric acid: analytical grade, investigated according to the Standards.

For Naphthyl-phosphate: low naphthol content (less than 0.1%), others according to the vendor specification

For Fast-RED-T: according to the vendor specification

(b) For the packaging materials:

For Reagent "A", "B" and "D": Ambered 2nd class glass bottles with surface treatment

For Reagent "C": 2nd class glass vials with surface treatment

(c) For the finished products:

Reagent "A": visual inspection
pH
colour of solution
refractive index

Reagent "B": visual inspection

pH

tartric acid identification

colour of solution

refractive index

Reagent "C": visual inspection of reconstituted solution

colour of solution

colour reaction with control sera

F. Alkaline phosphatase

Two methods differing in the buffers used and evaluation methods have been advised for kit preparation.

1. Using diethanolamine buffer (Method-1)

(a) Preparation of reagent solutions

Reagent "A":

Diethanolamine buffer (pH=9.8)	1 mmol/l
Magnesium chloride	0.5 mmol/l
Sodium azide	1 g/l

Dissolve 105.1 mg diethanolamine in 300 ml distilled water. Adjust the pH of solution to 9.8±0.1 with 1 N hydrocholoric acid. Dissolve 151.7 mg magnesium chloride$\times 6H_2O$ and 1 g sodium azide and fill up to volume with distilled water. Homogenize, control the pH of solution, and adjust, if necessary. Filter!

Reagent "B": (substrate)

Each vial contains 200 umol 4-nitrophenyl-phosphate-Na_2 (52.61 mg).

Reagent "C":

Prior to use dissolve the content of 1 vial Reagent "B" in 20 ml Reagent "A".

Measurement:

Wavelength: 405 nm

Reagent "C"	1000 ul
Sample	20 ul

Mix and after 1 min at 25°C read the absorbance at 405 nm against water. Measure the absorbance change within 3 min.

Calculation:

IU/l (405nm)= A/min x 2750

Laboratory prescription (in Spanish): see Annex XII.

Results:

The kit has been successfully prepared and it works without problems. Stability tests have been started. Neccessary instructions have been given.

2. Using 2-amino-2-methyl-1-propanol (2A2M1P) buffer (Method-2)

(a) Preparation of reagent solutions

Reagent "A":

Dissolve 86 g 2A2M1P in 500 ml distilled water which is previously warmed up to 40°C. Add 120 ml 1 N hydrochloric acid. Separately dissolve 264 mg magnesium sulfatex7H_2O under continuous stirring. Add to this solution the previous one and adjust the pH of solution to 10,5+0.02 under continuous stirring. Fill up to volume (1000 ml) with distilled water. Homogenize and filter!

Reagent "B": (substrate)

Each vial contains 1 mmol 4-nitrophenyl-phosphate-Na_2 (205 mg).

Reagent "C":

Prior to use dissolve the content of 1 vial Reagent "B" in 50 ml Reagent "A".

Measurement-1:

Wavelength: 405 nm

Reagent "A"	1400 ul
Sample	50 ul

Incubate 3 min at 37°C.

Reagent "C"	50 ul

Mix and after 1 min at 37°C read the absorbance at 405 nm against water.

Measure the absorbance change within 3 min.

Calculation:

IU/l (405nm)= A/min x 1590

Measurement-2:

Wavelergth: 405 nm

Reagent "A"	1400 ul
Sample	50 ul

Incubate 3 min at 37°C.

Reagent "C"	50 ul

Incubate for 30 min+10 sec, then add 5.0 ml 0 5 N sodium hydroxide.

Read the absorbance at 405 nm against water.

Calculation:

umol/l (405nm)= A x 6.67/A'

Results:

The kit has been successfully prepared and it works without problems.

Stability tests have been started. Neccessary instructions have been given.

3. Stability testing program:

(a) Stability tests for Reagent "A" (Method -1), and Reagent "A" (Method-2):

Type: Accelerated

Treatments: $-20^{o}C, +4^{o}C, +20^{o}C(RT), 37^{o}C, 60^{o}C$.

Testing intervals: zero, 2 weeks, 1, 2, 3 months

Analytical methods: visual inspection

pH

Magnesium ions (titrimetry)

Colour of solution

(b) Stability tests for Reagent "B" (Method-1 and Method-2):

Type: Accelerated

Treatments: $-20^{o}C, +4^{o}C, +20^{o}C(RT), 37^{o}C, 60^{o}C$.

Testing intervals: zero, 2 weeks, 1, 2, 3 months

Analytical methods: visual inspection (after dissolution)

pH

colour reaction with control sera

4-nitrophenol impurity content (UV)

4-nitro-phenyl-phosphate content (with colour reaction performed for control sera)

(c) Stability tests for Reagent "C" (Reconstituted solution - Method-1 and -2):

Type: Preliminary

Treatments: $-20^{o}C, +4^{o}C, +20^{o}C(RT)$, reflected

light (RL), 37°C

Testing intervals: zero, 5 hours, 1, 2, 5, 7, 10 and 14 days (after reconstitution)

Analytical methods: visual inspection

pH

4-nitrophenol impurity content (UV)

4-nitro-phenyl-phosphate content (with colour reaction performed for control sera)

4. Quality control requirements for Method-1 and Method-2:

(a) For the raw materials and other ingredients

For buffer component, magnesium salts: analytical grade, investigated according to the Standards.

For 4-nitrophenyl-phosphate: low nitrophenol content (less than 0.1%), others according to the vendor specification

(b) For the packaging materials:

For Reagent "A" (Method-1 and -2): Ambered 2nd class glass bottles with surface treatments

For Reagent "B" (Method-1 and -2): 1st class glass vials

(c) For the finished products:

Reagent "A" (Method-1 and -2): visual inspection

pH

colour of solution

refractive index

magnesium ion content

Reagent "B" (Method-1 and -2): visual inspection

nitrophenol impurity (UV)

nitrophenyl-phosphate content (UV)

IV. ASSISTANCE IN THE PREPARATION OF SERA FOR THE QUALITY CONTROL OF LIPIDS, BILIRUBIN AND ENZYMES

Presently Finley Institute produces control sera with the exception of the above mentioned ones, and these are marketed and used in the clinical laboratory. The quality and stability of these sera in my opinion are good. Inspite of this fact I felt it is advisable to discuss all types of control sera. The main reason is that they can utilize the experiences obtained with normal sera, and the control sera base is different, in our practice we use bovine sera, while Finley applies horse sera.

During my stay we could only discuss the topic of preparation of control sera, however, I am sure they can utilize the conclusions of our discussion. We have not separately talked about the preparation of bilirubin control sera, because these experiments are included into the bilirubin program.

A. Preparation of serum base

Bovine, ship (possible horse) blood is taken without anticoagulant and transfered into a volumetric vessel and placed for 1 hour at 37^{o}C (during this process a glass stick is used "going round" the glass wall to avoid precipitation at the wall). After that the blood is placed into refrigerator for 24 hours. The sera is decanted first, then centrifuged the blood (1000 rpm, 30 min). Aerosil is added to the sera (0.5 g/100 ml), after rigorous shaking at 37^{o}C for 24 hours, the sera centrifuged again to remove proteins. 0,01% sodium azide is given to the solution. The sera base is placed into deep freezer (-30-35^{o}C) until its use, or filled into ampoules (vials) and lyophilized as described at uric acid.

9. Preparation of control sera for normal range

Raw material: IV/"A" (stored in deep freezer)

Melt the serum base and prepare a 23 litre pool from that. Correct the concentration by adding the following materials (it is valid for bovine sera, in other cases the sera base can be controlled for each element, and the necessary correction can be made in the knowledge of the results):

glucose	5.5 mmol/l
urea	2.0 mmol/l
albumin (BSA or human)	5.0 g/l

Filter through a Seitz K5 and EK5 filters then a Pall-filter (SLK7002NRP) Fill 5.3 ml into 10 mls ampoules (or vials) and lyophilize as described at uric acid.

C. Preparation of control sera for low range

Raw material: IV/"A" (stored in deep freezer)

Melt the serum base and prepare a 9.2 litre pool from that. Add 13,8 l "dilution solution" and homogenize.

"Dilution solution":		
	Sodium chloride	48.67 g
	Sodium nitrate	71.37 g
	Potassium chloride	1.76 g
	Calcium chloride$\times 2H_2O$	2.32 g
	Distilled water up to	14000 ml

Prior to filtering add 10.35 g glucose and follow the procedure descibed under "B".

D. Preparation of control sera for high range

Raw material: IV/"A" (stored in deep freezer)

Melt the serum base and prepare a 20.7 litre pool from that. Add 2.3 l "adjusting solution" and homogenize.

"Adjusting solution":		
	Sodium chloride	47.03 g
	Potassium chloride	2.57 g
	Calcium chloride$\times 2H_2O$	2.30 g
	Magnesium sulfate$\times 7H_2O$	1.10 g
	Urea	27.6 g
	Potassium hydrogenphosphate	4.28 g
	Creatinine	0.92 g
	Iron(III) chloride$\times 6H_2O$	17,25 g
	Uric acid	3.00 g
	(Previously dissolved in 50 ml 0.5 N lithium hydroxide)	
	Distilled water up to	2300 ml

Prior to filtering add 56.9 g glucose and 460 g human albumin. Follow the procedure descibed under "B".

E. Preparation of control protein sera

Prepare from human blood as described at IV/A.

F. Preparation of enzyme containing sera

Raw materials: frozen serum base

frozen enzyme concentrate

Melt the serum base yielding 23.0 l pool. Add the calculated amount of enzyme concentrate. Mix and follow the procedure as described at IV/B.

The enzyme concentrate is prepared from white (albino) rats. After

decapitulation the tissues of liver (GOT, gamma GT, GPT), intestine (alkaline phosphatase), heart (GOT,CK), kidney (gamma-GT) and pancrease (alpha-amilaze) are homogenized with 10 volumes of TRIS buffer (0.05 M, pH=7.2), cooled with ice-distilled water which contains 0.1% Triton-X-100. The mixture is centrifuged at $4^{O}C$ (15 min, 7000 g), then it is allowed to stand at $4^{O}C$ for 24 hours, and centifuged again as above. Store at $-20^{O}C$.

G. Preparation of lipide containing sera

1. Raw material production with high lipid content

The pH of lypemic human plasma is adjusted to 5.4 with 0.025 mole/l acetic acid solution. Cool down to $+2^{O}C$, then add abs. ethanol by drop-to-drop until the ethanol content reaches 15.5%. Allow to stand for 1 hour and centrifuge.

2. Preparation of the control sera

The precipitate produced as above is mixed with human serum (preparation see IV/E) to achieve the required lipid concentration. Fill into 5.0 ml vials and lyophilize as described at uric acid.

H. Stability testing program:

My experiences with the stability test performed by Finley Institute can be summarized as follows:

Basically the organisation of long-term stability tests used for control-sera is good. The statistical methods used for the evaluation of stability test results are also good.

I made some recommendations to improve the quality of the work, such as:

(a) the number of samples analysed in the same time can be standardized,

(b) the number of laboratories involved into the stability tests can be

standardized,

(c) stability protocol can be established prior to the start of any stability test (its format has been discussed),

(d) criteria can be formulated to accept or exclude:

x the results of a test in anytime of the investigation,

x the results of the stability test at the end of the investigation period,

(e) analytical methods can be standardized, that each investigation period the same method would be used in each laboratory for the determination of each element.

I. Quality control requirements for control sera

1. For the raw materials and other ingredients

For organic and inorganic materials: analytical grade, investigated according to the Standards.

For sera base: in-house standard

For enzyme: specific enzyme methods

2. For the packaging materials:

Ambered 1st class glass ampoules (vials) or at least 2nd class glass with surface treatment.

3. For the finished products:

For Finley Institute it is recommended to find outer laboratories (at least three), who analyse the prepared control sera and perform the in-process control, too.

V. ASSISTANCE IN THE PREPARATION OF COLORANTS FOR MICROBIOLOGICAL AND HERMATOLOGICAL TINCTURES

Finley Institute produces colorants for microbiological and hermatological tinctures. They have had problems on two fields:

(a) Stability of methylene blue solution associated with changed colour of the solution during the storage.

(b) Stability of methylene blue solution regarding precipitation in the solution during the storage.

Because Finley has more formulations containing methylene blue, the problem is important and can be urgently solved.

Another problem is that Finley wants to change the packaging materials turning from glass to plastic bottles, and requesting recommendations about the suitable stability tests should be performed.

A. Stability problems associated with changed colour of solution

Colour change in the solution can be led back to three possible reason:

(a) reversible change in colour depending on the pH of solution,

(b) irreversible change in colour as a results of oxidation of the azo-dye,

(c) reversible or irreversible change in colour as a results of metal-chelate complex formation.

To investigate thee problem the following experiments were advised:

(a) To change the pH of solution using acetic acid to lowering the pH. If the change is caused reversible by the change in pH, the original colour will appear again. If the colour on the effect of acetic acid will change, but not the original colour appears again, the possible reason of colour change is metal complexation. In that case the application of EDTA can give

back the original colour of the solution. If the colour will not change the possible reason is oxidation.

(b) In case of oxidation, antioxidant (sodium bisulphite, pyrosulphite) can be used in the solution.

B. Stability problems associated with precipitation in the solution

Precipitation can be led also back to three possible reasons:

(a) Microbiological instability caused by fungi.

Conservants, such as phenol can be used.

(b) Salt formation of the azo-dyes with metals disolved from the glass surface. The salt is insoluble in water.

Better glass as packaging material can be used.

(c) The degradation product of the azo-dye is insoluble in water.

Ethanol can be used in the solution.

C. Recommendations.

(a) Changing the pH of solution using 5% glacial acetic acid.

(b) Adding 20% ethanol into the solution.

(c) Combination of (a) and (b).

(d) Using only 100% ethanol (it cannot be done with the presently used packaging materials due to the ease of evaporation, which can cause serious problems during the storage).

(e) To perform comparative stability tests by using the following containers:

- x 3rd class glass bottles (present formulation)
- x 2nd class glass bottles with surface treatment
- x 1st class glass bottles
- x Polypropylene bottles (Lupolene-BASF) or equivalent available

x Plastic container available in Cuba.

Compositions: (a) to (c) as above.

Testing intervals: zero, 1, 2, 3 months

Treatments: +4°C, 20°C, 37°C, 60°C, reflected light

(e) To perform long-term stability tests with the best two compositions, filled into the best glass and plastic containers, respectively, based on the results of comparative stability tests.

Testing intervals: zero, 3, 6, 9, 12, 18, 24, 36 months

Treatments: +4°C, 20°C, 40°C

(f) Analytical performance parameters for stability testing:

x visual inspection,

x pH,

x UV-spectrum,

x Methylene blue assay based on UV-spectrophotometry.

(g) Evaluation of the stability test results:

x Accelerated test:

If stable at 37°C and 60°C: 6 months expiry time at 25°C

If stable at 37°C, but not at 60°C: six month expiry time at +4°C.

x Long-term stability test:

If stable at 37°C after 1 year, expiry time: two years at 25°C.

If stable at 25°C after 1 year, but not at 37°C, expiry time: two years at +4°C.

If stable at 37°C after 2 years, expiry time: three years at 25°C.

If stable at 25°C after 2 years, but not at 37°C, expiry time: three years at +4°C.

Detailed description of the stability tests including number of samples, etc. can be found (in Spanish) in Annex XIII.

Results:

During my stay the application of 5% acetic acid has been successfully tried. The colour of a previously colour-changed solution on the effect of acetic acid turned back to be blue again. Last minute discussion: to clarify the amount of acetic acid resulting a pH-change from 7-8 to 4. After selection of the necessary amount of acetic acid the stability tests can start.

VI. RECOMMENDATIONS FOR THE QUALITY OF RAW MATERIALS AND PACKAGING MATERIALS

In case of each products in connection with which we had detailed discussions I gave all recommendations for the quality of raw materials and packaging materials, and these can be found as a part of laboratory descriptions. My experiences on this field were not so positive as in other cases. Raw materials are used without indication that these were eexamined according to a specified Standards and released. The packaging materials used for the product are not satisfactory, do not give suffecient protection for the products during the storage.

Therefore, I highly recommend to send the QC manager for a longer GMP training, as well as to support CUBA by UNIDO to develop the glass industry producing the glass bottles (vials) not only for diagnostics, but also for pharmaceuticals. Presently this is one of the most important discrepancy from the international standard.

VII. RECOMMENDATIONS FOR THE DESIGN OF THE TECHNOLOGICAL PROCESSES AND STABILITY TESTING

In case of each product in connection with which we had detailed discussions (and we performed experiments) recommendations have been given to the technological processes and stability testing (partly included into the spanish version of the technological description. See Annex II to XIII). The basic GMP requirements (process and method validation, stability testing) were selected as main subjects of the three lectures presented during my stay at Finley Institute. (Brief summaries can be found in Annex XIV to XVI .)

VIII. RECOMMENDATIONS FOR THE REQUIRED EQUIPMENTS

Finley Institute just is now being built a new diagnostic production plant, which will be finished soon. I could see the basic equipment, and according to my opinion these are basically good for the production. However, the size of the equipments are too small (two 300 l and two 150 l total volumes). If Finley really wants to realize a larger production scale, equipments with larger working capacity (600 to 1000 l) is required and I think it would be useful if this project would be supported by UNIDO. I have a similar opinion about the filling equipment, which is in my opinion too small for a large scale production.

Regarding the new plant I visited in each floor (three times half day), and I found this building will be very good for diagnostic production.

Especially I like the working and packaging areas, the place of analytical laboratories. I made some recommendations to satisfy the most important GMP regulations, such as process validation, cleaning instructions, batch record system, etc. In my sincere opinion that Finley Institute will satisfy the

requirements, and good quality products can be produced there.

IX. RECOMMENDATIONS FOR THE PROCESS AND SUBSTANCES TO UTILIZE IN ORDER TO ASSURE THE STABILITY OF THE PRODUCTION

This topic is included into the design of newly introduced diagnostic kit (XIII.).

X. RECOMMENDATIONS FOR THE MOST APPROPRIATE FORMULATION TO PRESENT THE PRODUCT

This topic is also included into the design of newly introduced diagnostic kit (XIII.).

XI. RECOMMENDATIONS FOR THE APPLICATION OF THE GMP AND QUALITY CONTROL TECHNIQUES FOR THE RAW MATERIALS AND FINAL PRODUCTS. RECOMMENDATIONS FOR IN-PROCESS CONTROL PROCEDURE

This topic is also included into the design of newly introduced diagnostic kit (XIII.), and concrete recommendations have been given in the recent subsections for the products to be discussed.

XII. TO ADVISE ON THE ESTABLISHMENT OF PARAMETERS OF ACCEPTANCE FOR EACH PRODUCT

Recommendations for acceptance parameters of the formulated products (named as "Quality control requirements for the finished product") have been given in the case of each developed product.

XIII. TO GIVE RECOMMENDATIONS FOR THE ESTABLISHMENT OF EVALUATION SYSTEMS FOR THE NEWLY INTRODUCED DIAGNOSTIC REAGENTS

One of the most important aspects when a reasarch laborato., wants to deal with the development of a new diagnostic reagent is, how the experiments can be organised, which logical approaches should be followed during the various steps of the experiments. Because the colleagues at Finlay Institute were very interested in this problem I organised a one week seminar (3-4 hours/day) for some selected researcher (5 persons including the head of the department). Depending on the selected topics some other persons (from the QC department) have also been participated on the seminar.

During the seminar the following topics were discussed following the logical way of a product development:

(A) Design of the chemical reaction.

(B) Validation of the chemical reaction and reaction conditions.

(C) Optimization of KIT composition.

(D) Re-validation.

(E) QC control criteria for raw materials and other ingredients.

(F) Selection of KIT design.

(G) Stages of various stebility tests.

(H) Evaluation system - Standard Operating Conditions (SOPs).

(I) Standardization.

A shortened version of the seminar is given below.

A. Design of the chemical reaction

1. General principles for the reaction.

(a) Chemical reaction.

Only known reaction can be used. Reactions would be unidirectional without significant formation of by-products. Reaction would be well-reproducible.

(b) Reaction conditions.

Reactions must be nearly quantitative, with known and reproducible reaction kinetics. Reaction conditions can be freely selected (excess of reagent, solvent and temperature), and the reactions would be as fast as possible.

(c) Formed derivatives.

From chemically different compounds different derivatives must be formed. The formed derivatives should differ in the detection characteristics from the parent compounds which enables their selective detection. Derivatives must be stable within a reasonable time.

2. General principles for method validation.

The following validation steps are required for method validation of chemical reaction:

(a) Validation of sample preparation.

(b) Validation of chemical reaction.

(c) Investigation of the effect of instrumental and environmental conditions.

The following analytical performance parameters (APP) are used for method validation:

- x accuracy (recovery),
- x precision (method and system precision),

x reproducibility (in-day, day-to-day, interlaboratory),

x limit of detection,

x limit of quantitation,

x linearity and range,

x specificity (selectivity),

x sample stability,

x variation in reaction conditions:

- reagent batch to batch,
- temperature,
- elapsed time,
- wavelength,

x ruggedness testing.

3. Chemical reaction.

The effect of change in the following parameters on the chemical reaction can be studied:

(a) Variation of the reagent concentration (in volume).

(b) The stability of the reagent in solution (reconstitution).

(c) Stability of the formed derivatives:

x during the chemical reaction,

x temperature,

x in time.

(d) Variation of reaction conditions:

x reaction temperature,

x time,

x combination of the above two parameters.

(e) Variation of the standing time and conditions after completition of the reaction.

(f) Batch-to-batch variation of the reagents.

4. Ruggedness testing.

(a) General principles.

Ruggedness is a measure of reproducibility of the individual test results, when the procedure is used repeatedly to determine the same homogeneous sample in a variety of specifieed experimental conditions.

(b) Type-A investigations:

Tests are based on the variation in different environmental conditions, sucha as: instrument-to-instrument, laboratories-to-laboratories, analyst-to-analyst.

(c) Type-B investigations:

Type-B-1: ruggedness testing of sample preparation.

Type-B-2: reggedness testing of the operational conditions.

Prior to start with any ruggedness testing:

(i) the factors (experimental parameters, such as: solvent, pH, temperature, reaction time) which have significant influence on the analytical results can be selected and listed;

(ii) minimum acceptable deviations from the specified experimental conditions can be selected:

x -1 (lowest acceptable value)

x 0 (nominal value),

x +1 (highest acceptable value);

(iii) The APP to be sensitive to the changes in the experimental conditions (precision, accuracy, LDQ, etc.) **can be selected and listed;**

(iv) The acceptable deviations from the values of the preselected APP can be defined:

x maximum value;

x minimum value.

Some practical advises:

(i) not every step included into the procedure is checked, only those ones which are expected to highly influence on the value of APP;

(ii) not every APP is included in the test, only the critical ones;

(iii) one part of analytical performance parameters (precision, recovery) can be controlled by other methods, which are more precise and accurate compared to the method in question.

5. Evaluation of ruggedness testing

Two methods have been discussed in details (not discussed here):

(a) single criterion - single parameter system

(b) multicriteria - multiparameter system.

9 Optimisation of KIT composition

1. The knowledge of the effect of the constituents on the chemical reaction.

Example: Creatinine kinaze KIT

Chemical reaction:

creatinine kinaze

I. creatinine phosphate + ADP ----------▶ creatinine + ATP

hexokinaze

II. ATP + glucose ---------▶ Glucose-6-phosphate + ADP

glucose-6-P-dehydrogenaze

III. G-6-P + NAD ----------▶ NAOH + H+ + gluconate-6-P

(a) Compounds needed for the reactions - Variables

x ADP	Concentration
x NAD	Concentration
x Creatinine phosphate	Concentration
x Glucose	Concentration
x Hexokinaze	Concentration
x Glucose-6-P-dehydrogenaze	Concentration

The application of these compounds are clear and evident

(b) Other compounds needed for reaction conditions:

Compounds	Effect	Variables
AMP	Inhibition of 2ADP ⇌ AMP + ATP reaction catalysed by adenyl kinaze	Concentration
Diadenosyl-pentaphosphate	Inhibition of 2ADP = AMP + ATP reaction through deactivation of adenyl kinaze	No
N-acetyl-cysteine	Reactivation of Creatinine kinaze by activating SH-groups	Concentration
Magnesium acetate	Reactivation of Creatinine kinaze	Concentration
EDTA	Metal complexation avoiding oxidation	NO

(c) Other conditions needed for the reaction:

Conditions	Effect	Variables
Buffer	Optimum conditions for enzyme reaction	pH
		Buffer conc.
		Buffer type
Temperature	Optimum conditions for enzyme reaction	

2. Optimization of KIT composition by factorial design.

(The principle of factorial design is not discussed here, it was discussed during the seminar in details)

Compounds used in the KIT are divided into two groups:

(a) Primary compounds (these basically determine the reaction)

Compounds listed in point (a) are belonging into this group.

In the first run (first 15 experiments) the concentration of these compounds are changed, concentration of others are constant.

(b) Secondary compounds (these have secondary importance regarding the reaction).

Compounds listed in point (b) are belonging into this group.

In the second run (second 15 experiments) the concentration of these compounds are changed, while the concentration of the primary components are constant (optimum).

(c) Important conditions.

These are listed at point (c) and optimized in the second run.

3. Evaluation of results of optimization of KIT composition by factorial design.

The evaluation system is not discussed here, it was discussed in detail during the seminar. Only a brief summary is given.

The purpose is to find and clearly identify the main effect(s). Evaluation is based on the selection of the best three experimental runs using sensitive APPs for the comparisons. After the columns are compared and selected the optimum values of the various factors.

Basic rules:

(i) If the best three experiments are comparable (there is no great difference in the APP-values):

x select that value where more the same data,

x if both three values are different, select +1 value,

x if both three values are the same, select this one.

(ii) If two experiments are comparable, the third one not:

x and this third one extremely good, select this one,

x this third one is significantly wronger, the other two experiments are duplicated at the comparison,

x if two values are the same, select this one,

x if two are different, the third experiment will determine the optimum value (if the third value differs from the other two, select the more positive extreme value).

4. Optimization of the chemical reaction by factorial design.

(The principle of factorial design is not discussed here, it was discussed during the seminar in details)

The principle of optimization and the evaluation of the results are the same as discussed above.

Factors to be varied:

x reagent volume

x sample volume

x volume ratio of reagent and sample

x incubation temperature using constant time

x incubation time using constant temperature

x wavelength for absorbance reading

5. Optimization of KIT composition by using the "walking method"

Basic principle: change first and optimize the step which is closest to the measurement (last step) and go in reversed direction.

In case of Creatinine kinaze this step is step III.

(a) Change the concentration of G-6-P-DH (the concentration of others are constant) and select the optimum concentration of G-6-P-DH

Examples were given to evaluate three different types of correlation.

(b) Change the other parameters regarding the reaction conditions (optimization of reaction conditions) using optimum G-6-P-DH concentration, others are the same as (a). Optimization of pH, buffer concentration and type, temperature, reaction time, wavelength are performed.

Examples were given to evaluate the different types of correlation.

(c) Change the concentration of NAD using optimum concentration of G-6-P-DH, and optimum reaction conditions. Others are used in constant concentration.

Select the optimum NAD concentration.

Examples were given to evaluate the different types of correlation.

(d) Change the concentration of hexokinaze using optimum concentration of G-6-P-DH and NAD, and optimum reaction conditions. Others are used in constant concentration.

Select the optimum hexokinaze concentration.

Examples were given to evaluate the different types of correlation.

(e) Change the concentration of glucose using optimum concentration of G-6-P-DH, NAD, and hexokinaze and optimum reaction conditions. Others are used in constant concentration.

Select the optimum glucose concentration.

Examples were given to evaluate the different types of correlation.

(f) Change the concentration of ADP using optimum concentration of G-6-P-DH, NAD, hexokinaze and glucose and optimum reaction conditions. Others are used

in constant concentration.

Select the optimum ADP concentration.

Examples were given to evaluate the different types of correlation.

(g) Change the concentration of creatinine phosphate using optimum concentration of G-6-P-DH, NAD, hexokinaze, glucose and ADP and optimum reaction conditions. Others are used in constant concentration.

Select the optimum creatinine phosphate concentration.

Examples were given to evaluate the different types of correlation.

(h) Change systhemathically the concentration of secondary components as described above using optimum concentration of primary components and optimum reaction concditions.

Examples were given to evaluate the different types of correlation.

C. Selection of detergents, conservants and stabilizers

1. Selection of detergents.

Detergents are used to avoid specific interactions (i) between polypeptides and proteins, and the substarte and enzymes, respectively, or with the glass surface; (ii) to liberate the compounds from peptide (protein) absorption bound; (iii) to avoid precipitation of the formed derivatives in aqueous solution.

Mostly non-ionic detergent, such as: Triton-X-100, Tween 80 and Tween 20 are used, but ionic detergents (SDS or CTMA) cab also be applied.

2. Selection of conservants.

Conservants are used to protect the product from microbial contamination.

Most frequently used materials: sodium azide, phenol, thymol.

Most important requirements towards a good conservants are as follows:

x Can be effectively used in low concentration.

x No reaction should be observed with the substrate, enzyme or with the formed derivative.

x Would be stable at the pH of reagent solution.

x Does not adsorb to the glass or plastic surface of the container.

Microbiol stability is also an important question. It is performed by exposing a high amount of selected microorganisms (10^{10} concentration) and the decrease of germ count in time is investigated.
(Requirements: within one week max. $5x10^5$ is the international requirement, our in-house requirement is max. 10/ml within 1 day.)

3. Selection of stabilizers.

Stabilizers are used to protect the formed derivatives or the compound (enzyme) in blood, as well as to stabilize the colour of solution which is measured spectrophotometrically. Mostly antioxidants and alcohols (ethanol, isopropanol) are used.

D. Design of packaging materials

For the production of diagnostic reagents good quality packaging materials (bottles, vials, stoppers, cups, etc.) can be used. Special stability test is required to clarify the suitability of a packaging materials, performed by using stress conditions.

(a) Glass containers

The use of 1st class glass bottles, vials is highly recommmended to avoid the interaction with the glass surface. If it is not available 2nd class glass with surface treatment would the second choice. In our practice the application of 3rd class glass bottles and vials is not a good choice.

(b) Plastic containers.

The resistance (water vapour penetration, resistency against chemicals, etc.) can be previously specified. Methods can be found in each Pharmacopoeia. The application of polypropylene is highly recommended.

E. KIT design.

Appropriate size of teh reagents has a great importance. KIT should contain all the materials (accessoires) necessitated for the work. Good application sheet (leaflet) containing the all instructions, warnings connected to the use and storage of the reagents are also advisable.

F. Stability testing of the product during development.

1. Preliminary stability tests

It provides information about the estimated stability of the product in the early stage of development.

Treatments: -20^{O}C, +4^{O}C, 25^{O}C (RT), 37^{O}C, 60^{O}C

Testing period: zero, 1,2,3,6,10 and 14 days

Sample: first laboratory batch

Analytical method: Assay

2. Accelerated stability tests

It is performed by the optimised composition.

Treatments: -20^{O}C, +4^{O}C, 25^{O}C (RT), 37^{O}C, 60^{O}C

Testing period: zero, 1,2 and 3 months

Sample: three laboratory batches

Analytical method: Assay + visual inspection + pH

3. Comparative stability tests

When the composition of the product is changed, or we want to compare our product with a previously marketed one we perform this test.

Treatments: -20°C, +4°C, 25°C (RT), 37°C, 60°C

Testing period: zero, 1,2,3 months

Sample: pilot plant batch and production batch (composition is changed) or production batches (our one compared with the marketed one)

Analytical method: same as 2.

4. Long-term stability tests

Scale up experiments, to establish the expiry date of our product.

Treatments: (-20°C), +4°C, 25°C (RT), 37°C

Testing period: zero, 3,6,9,12,18,24,36 and (60) months

Sample: 1 laboratory, 1 pilot, and 1 production batches

Analytical method: same as 2.

5. Stability protocol

Containing the following information:

Type of stability test

(a) Standard Operating Procedure (SOP) for the Analytical Methods:

- x Objective
- x Materials used for the experiments
- x Equipments
- x Sample
- x Procedure
- x Calculation
- x Validation data
  - Specificity

- Accuracy
- Precision
- Ruggedness testing
- Linearity and Range
- Lowest detectable quantity

(b) Samples used for stability tests:

x Composition

x Brief technological description

x Packaging materials

(c) Sample amount needed for the test

(d) Sample treatments

(e) Duration of the test and testing intervals

(f) Evaluation of the results

G. Standardization.

1. Standardization of the quality of each incoming materials

(raw materials, other ingredients)

(a) At least: IR spectrum

UV spectrum

Loss on drying

Assay

Solubility

(b) Specific test for impurity may participate in the reactions:

x metal ions

x decomposition products

(c) Specific assay for enzymes

2. Standardization of the quality of the formulated products

(a) General tests: Appearance
pH (if solution)
pH (after dissolution, if solid)
substrate concentration
weight variability (if solid)
solubility (if solid)

(b) Applicability test: chemical reaction according to the use against standard or contol sera

3. Standardization of the quality of packaging materials

As it was recently discussed.

4. Standardization of the manufacturing procedure

- x technological prescription
- x batch records
- x in-process controls

Each point of this topic has been discussed in details.

5 External Quality Control Program (EQCP)

- x To give the product to exteranal laboratories as much as possible.
- x Report requested about all measured data,
- x Statistical evaluation of the results is prepared:
  - in-side the laboratory
  - within laboratories

6. Internal Quality Control Program (IQCP)

x All validation data (validation package) are collected.

x All stability test data (stability protocols) are collected.

x Comparative test with a competitive product is performed, and the equivalency is established.

7. Construction of leaflet

It is a very important part of standardization to construct the well-applicable leaflet.

It advantageously contains:

x Chemical reaction

x Composition of the reagents

x Detailed instructions for the test performance

x Calculation of the results

x Storage of the product

x Shelf life of the product

x Warnings and percautions regarding the use, applicability and storage of the product after its use.

XIV. TO ADVISE ON THE APPLICATION OF THE AUTOMATIC ANALYTICAL SYSTEMS FOR THE ELABORATED DIAGNOSTIC REAGENTS

It is very difficult to advise on the applicability of a reagent for automatic analysis, because the conditions are highly dependent on the instrument construction. Practically no two instruments for which the same conditions can be applied. The researcher who developed the product can advise on the following areas:

(a) Volume ratio

This can be the same as in case of manual work.

(b) Reaction temperature

This can be the same as in case of manual work.

(c) Detection wavelength

This can be the same as in case of manual work.

(d) Blank and standard composition

This can be similar to the case of manual work.

(e) Reaction time

It can give a good estimation for the use of automatic instrument.

To give more information I gave copies of determination of GOT, GPT, gamma GT, acidic and alkaline phosphatase, uric acid, cholesterol, triglicerides, CK, glucose, urea, proteinase, LDH, amylase in three different automatic instruments (HitachiH704, Abbott VP and Centriphichem) to Finlay.

XV. TO ORGANIZE LECTURES OR SEMINARS ACCORDING TO THE DEVELOPED ACTIVITIES

During my stay I presented three lectures and one seminar (it was previously mentioned at point XIII). On the lectures 60-70 people have been participated partly from the Instuiitute, and partly from other companies. My lectures were presented in English with simultaneous spanish translation. Duration was 1,5 hour, and several questions were asked at the end. I am personally very satisfied with these lectures.

Topics:

(1) Basic GMP and GLP rules (brief summary is given in Annex XIV)

(2) Stability testing (brief summary is given in Annex XV).

(3) Process and method validation (brief summary is given in Annex XVI)

ANNEX 1

UNITED NATIONS INDUSTRIAL DEVELOPMENT ORGANIZATION Sanchez/sk
20 November 1990

JOB DESCRIPTION
DG/CUB/86/015/11-51

Post title	Consultant in stability and production of clinical reagents
Duration	1 month
Date required	January 1991
Duty station	Havana, Cuba
Purpose of project	To assist the technical staff of the 'Finlay Institute' in the production and quality control of clinical reagents.
Duties	The expert is expected to carry out the following activities at laboratory and pilot plant scale:

1. Assistance in the preparation of reference solutions of proteins, uric acid, ferrum lithium bilirubin, albumin, etc.
2. Assistance in the preparation of buffers of triethanolamin, EDTA, TRIS, potassium phosphate, etc.
3. Assistance in the preparation of substats of G-6P-Na, tiocalin ruteril iodide, benzoilarginin-pnitoranilid, etc.
4. Assistance in the preparation of sera for the quality control of lipids, bilirubin, enzymes, etc.
5. Assistance in the preparation of Colorants for microbiological and hermatological tinctures.
6. To give recommendations on the quality of raw materials and packaging materials to be utilized.

..../..

7. To advise on the design of the technological processes and stability studies.

8. To give recommendations on the required equipment.

9. To advise on the type of processes and substances to utilize in order to assure the stability of the production.

10. To advise on the most appropriate formulation to present the product.

11. To give recommendations for the application of the G.M P. and quality control techniques for raw materials and final products. Recommendations for in-process control procedures.

12. To advise on the establishment of parameters of acceptance for each product.

13. To give recommendations for the establishment of evaluation systems for the newly introduced diagnostic reagents.

14. To advise on the application of the automatic analytical systems for the elaborated diagnostic reagents.

15. To organize lectures or seminars according to the developed activities.

16. To present a typed, detailed mission report.

Qualifications	Pharmacist, Chemical Engineer or Medical Doctor with experience in the production and quality control of clinical reagents.
Language	English, preferably with knowledge of Spanish.

Background information

The 'Finlay Institute' is working on the development of technologies for biological products as vaccines, blood derivatives, diagnostic media and others. Recently the Institute started the research and development activities in the field of clinical reagents, which started with the basic qualification of the personnel. The available infrastructure and equipment in the 'Finlay Institute' are not sufficient in order to guarantee the appropriate technology development as well as the quality of the produced products.

UNIDO's assistance was requested to advise on the production, quality control, G.M.P. application as well as for recommendations on the requirements for production and quality control equipment.

Annex II.

REACTIVOS PARA DETERMINACION DE BILIRRUBINA

REACTIVO A

Acido sulfanílico	32,2 mmol/L
Acido clorhídrico	165 mmol/L
Dimetil sulfóxido	7 mmol/L

Preparación:

Pesar exactamente 5,57 g ácido sulfanílico en frascos volumétricos de 1 L, disuelvalo en 600 mL de 0,165 N ácido clorhídrico con agitación suave.

Después de completa disolución, añada 0,5 mL de dimetil sulfóxido a la solución entase al volumen con 0,165 N HCl. Agite bien hasta homegenización.

Filtre a través de un filtro (0,8 - 0,2)u o (0,4 - 0,2)u

REACTIVO B

Acido sulfanílico	32,2 mmol/L
Acido clorhídrico	165 mmol/L

Preparación: igual al A

REACTIVO C

Nitrito de sodio	29 mmol/L

Preparación:

Disolver 2,5 g de nitrito de sodio en agua destilada o desionizada y complete el volumen a 1 L, filtre igual que en el caso A.

Almacene en frasco ámbar a 4°C. (dura 18 meses)

REACTIVO D

Acido sulfanílico	32,2 mmol/L
Acido clorhídrico	165 mmol/L
CTMA - cloride	30 g/L (aproximadamente 0,1 mol/L)

Preparación:

Disuelva 5,57 g ácido sulfanílico en 300 mL de ácido clorhídrico (165 mmol/L), paradamente disuelva 30 g de CTMA en 500 uL de HCl (165 mmol/L) transferir la solución a la solución de ácido sulfanílico (este orden es muy importante, agitar muy suavemente), completar el volumen con HCl, con mucho cuidado, agitar con movimientos suaves, filtrar en igual forma que en los casos anteriores.

REACTIVO E (Reactivo de trabajo)

2,4 dicloroanilina	1,5 mmol/L
Acido clorhídrico	40 mmol/L
CTMA	30 g/L

Preparación: (Para 500 mL)

Disuelva 2,43 g de 2,4 dicloroanilina en 200 mL de solución 0,08 N HCl, separadamente disuelva 30 g en 200 mL de ácido clorhídrico 0,08 N.

Mantener iguales precauciones que en el reactivo D.

Enrase a 500 mL con HCl 0,08 N. Filtrar en igual forma que en las anteriores.

REACTIVO F

2,4 dicloroanilina	1,5 mmol/L
Acido clorhídrico	40 mmol/L
Dimetilsulfóxido	7 mmol/L

Preparación:

Disolver 2,43 g de 2,4 dicloroanilina en 300 mL de ácido clorhídrico 0,08 N. Añada 0,5 mL de dimetilsulfóxido a la solución, enrase a 500 mL con ácido clorhídrico 0,08 N, agitar para homogenizar y filtre.

REACTIVO G

Nitrito de sodio 1,5 mmol/L

Preparación:

Disuelva 0,1275 g de nitrito de sodio en 500 mL de agua destilada, filtre y guarde en frasco ámbar a 4°C.

Antes de usar mezcle Reactivo E o Reactivo F con el Reactivo G en proporción 1:1.

REACTIVO H

Reactivo blanco muestra

Preparación:

Mezcle Reactivo E o Reactivo F con agua destilada en proporción 1:1.

(Reactivo EH o FH)

REACTIVO J

2,4 dicloroanilina	1,5 mmol/L
Acido chorhídrico	40 mmol/L

Preparación: igual al caso E, pero sin CTMA.

REACTIVO K (preparar antes de usar)

Mezcle en proporción 1:1 Reactivo J con Reactivo G, deje reposar al menos 15 minutos antes de usar.

REACTIVO L

Preparación: (Antes de usar)

Diluya Reactivo J en proporción 1:1 con agua destilada. Dejar reposar 15 minutos - antes de usar.

9- Solución Calibradora de bilirrubina

A. Sin albúmina

mg/L	Total	Directa
Nivel 1	10	7
Nivel 2	50	30
Nivel 3	170	85

9A- Solución Stock de bilirrubina (BSTS)

Disuelva 155,5 mg pesados exactamente y previamente secado sobre sílica gel 24 hrs ($E^{1\%}_{1cm}$ = 60500 ± 800, calculada en cloroformo y medida a 455 nm en cubeta de 1 cm), en 700 ml 0,1 M Carbonato de sodio, eliminar el O_2 de la solución con N_2 o Argón 3 o 4 hrs. PROTEJA DE LA LUZ. Enrase al volumen.

Use 5 mL de la solución stock en cada caso.

	BSTS 5 mL	H_2O 10,3	Solvente 4,5 mL	Acido acético 0,2
9A1 -			ETANOL	
9A2 -			ISOPROPANOL	
9A3 -			ETILENGLICOL	
9A4 -			PROPILENGLICOL	
9A5 -			ACETONITRILO	
9A6 -			DIMETIL SULFOXIDO	
9A7 -			WATER	

Se prepararán alrededor de

9C- Prueba de estabilidad.

A- Condiciones de almacenamiento

I - 2 - 8°C

II - T. ambiente (20°C)

III - 40°C

IV - 60°C

B- Periodo de Investigación

T - 0

- 5 hr

- 1 - 2 - 3 - 4 - 6 - 10 - 14 días

C- Curva

Características visuales

pH

Método de Análisis.

Temperatura: 20 - 25°C

Longitud de onda: 550 nm (a 546 si se usa fotómetro de filtro)

Cubeta: 1 cm

a) Con ácido sulfanílico

Bilirrubina Total

	Blanco	STD	Blanco	STD
Reactivo A	1,5 mL	1,5 mL	-	-
" D	-	-	1,5 mL	1,5 mL
" C	-	50 µL	-	50 µL
Calibrador	100 uL	100 µL	100 µL	100 µL

Bilirrubina Directa

Reactivo B	1,5 mL	1,5 mL
" C	-	50 µL
Calibrador	100 µL	100 µL

Leer a los 5 minutos a 20 - 25°C.

B_1 - Con 2,4 dinitroanilina

Antes de usar, mezcle:

I Reactivo E con Reactivo G en proporción 1:1 (Reactivo EG)

II " F " " G " 1:1 (Reactivo FG)

Déjela reposar al menos 15 minutos a temperatura ambiente, protegido de la luz.

BILIRRUBINA TOTAL.

	I		II	
	Blanco	STD	Blanco	STD
Reactivo EG	-	1000 µL	-	-
" FG	-	-	-	1000 µL
" EH	1000 µL	-	-	-
" FH	-	-	1000 µL	-
Calibrador	100 µL	100 µL	100 µL	100 µL

BILIRRUBINA DIRECTA

Reactivo K	-	1000 µL
" L	1000 µL	-
Calibrador	100 µL	100 µL

PRECAUCION: TODO EL TRABAJO SE DEBE REALIZAR PROTEGIDO DE LA LUZ

TECNICA DE PREPARACION DE JUEGO DE REACTIVOS DE ACIDO URICO

II/A. Método Uricasa/POD

REACTIVO A.- Solución buffer

NaH_2PO_4	20,4 g
NaOH	4,95 g
EDTA 2Na	1,86 g
Azida Sódica	0,1 g
(diclorofenol sulfonic acid)Na	0,523 g
Tritón X - 100	1 mL
H_2O destilada	p/ 1 L

Preparación:

20,4 g NaH_2PO_4 y 4,95 g NaOH se disuelven en 800 mL H_2O destilada -pH se ajusta - 7,5 $\pm$ 0,1. El resto de los productos se disuelve en este orden:

EDTA
NaH_2
DHBS
Tritón X - 100

se completa el volumen a 1 L, el pH se controla nuevamente a 7,5 $\pm$ 0,1 (18-20°C)

Filtrar la solución a través de 0,8 - 0,2 u

REACTIVO B.- Solución Standard

Acido úrico	60 mg
Carbonato de litio	36 mg
Azida	1,5 g
Agua destilada	p/ 1 L

Preparación:

Primeramente 36 mg de Carbonato de litio se disuelven en agua destilada a 90°C, después se disuelve o mezclada con 5 mL de agua, se añade la solución de carbonato de litio (aproximadamente a 60°C) sobre el ácido úrico, agitar vigorosamente y disolver perfectamente, llevar a temperatura ambiente y añadir la ázida sódica y completar el volumen. Mantener a T 2-8°C protegida de la luz.

REACTIVO C.- Solución reactivo

Uricasa	60 U/L		
POD	600 U/L	4-aminofenazona	1 mmol/L

Preparación:

C,231 g de 4-aminofenazona, se disuelven en 100 mL de H_2O destilada a 90°C, después de disuelta enfriar a temperatura ambiente, disolver las enzimas y completar el volumen a 250 mL. Filtrar a través de 0,4 - 0,2 u (si es posible en condiciones asépticas) y dispensar en viales 5 mL; liofilizar

T-	-35°C	-40°C	
después de	-30°C	1 hr	
	-30	-25°C	1-8 hrs
	-25°C	-0°C	8-18hrs
	0 -	+40°C	18-26hrs

N_2 como protección

REACTIVO D.- Reactivo reconstituido

1 fc del reactivo C, se disuelven en 20 mL del reactivo A (buffer)

Método de Análisis

	Blanco	Std
Reactivo D	1 mL	1 mL
Reactivo B	-	25 uL

Incubar la solución 5 min. a 37°C o 15 min de 20 - 25°C. Determinar la absorbancia de la solución Std contra blanco a 500 nm (492 - 550 nm) en cubeta de 1 cm.

Método Directo

C- Uricase

Reactivo A - Igual al IIA pero sin DHBS

Reactivo B - Acido Bórico 12,5 g
Na_2CO_3 20 g
H_2O dest. 800 mL

Disolver el ácido bórico aproximadamente a 90°C 800 mL, después de enfriar, disolver el carbonato, medir el pH 9,5 ± 0,1, depués de ajustado llevar a 1 L.

Reactivo C - Uricase 1 U/mL
50% glicerol

Reactivo D - Standard

Acido úrico	180 mg
Carbonato de litio	108 mg
Azida	1,5 g
Agua destilada	1 L

Preparar igual que en el caso anterior.

REACTIVOS PARA DETERMINACION DE HIERRO

Método CAB.

Reactivo A

Chromoazurol "B" (acida)	0,2 mmol/L
$CTMA^+$-(cloruro o bromuro)	2,0 mmol/L
Guanidine -HCl	3,0 mmol/L
Buffer acetate pH 5	45,0 mmol/L
Azida	0,1 g/L

Preparación:

Disuelva 2,6 g acetato de sodio.H_2O 4,31 g acetato de sodio. $3H_2O$ en 800 mL - agua destilada.

Añada 0,77 mL ácido acético a la solución. Homogenizar, pH = 5,0 $\pm$ 0,1, ajuste si es necesario (NaOH o AcH).

Disuelva primero 6/6 mg CTMA.Br en el buffer, después 337,5 mg guanidine HCl y 0,1 g azida sódica. Disuelva 107.9 mg cromoazurol B separadamente en aproximadamente - 100 mL de agua si no se disuelve, caliente ligeramente y añada a la solución de buffer, arrastrando con agua la cantidad total. Completar el volumen y controlar el pH.

Filtrar a través 0,8 - 0,2 u filtro.

* Cetil Trimetil Amonio

Precauciones: El agua debe estar libre de Fe. Chequear.

HIERRO.-

Stock - 100 mg/L en HCl 0,1 N

Standard

A- 1 mg Fe/L + HCl

100 mL 0,5 mmol/L EDTA

Llevar a 1 L con buffer acetato pH 5,0
(45 mmol/L)

a- 10 mL Stock solución en HCl 0,1 N /1 L

Solución EDTA 0,5 mmol/L EDTA

Método λ - 620 nm (620 - 640 nm)

	Blank	STD
Reactivo A (CAB)	1 mL	1 mL
H_2O	50 uL	-
Std	-	50 uL

Mezclar, esperar 15 min leer absorbancia

VII. CAPACIDAD FIJACION DE HIERRO (TIBC)

Reactivo A

1)	Clorure hierro (III)	0,09 mmol/L
	equivalente a	5,03 mg/L
2)	Oxide aluminie	0,35g
	(en fe plástico con tapa)	
3)	Sample	

Mezole bien, después de 3-5 minutos, añada aproximadamente (0,35 g) óxide de aluminie colocar en retor por 10 minutos, esperar 3 minutos. Tomar el sobrenadante 50 uL.

	Blanco	Muestra
Muestra	-	50 uL
Iron free water	50 uL	-
Reactivo	1000 uL	1000 uL

Oxido de aluminie 120° (4-6 hrs)
(Grade II)

Annex V.

GLUCOSA - HEXOQUINASA

1. Reactivo A - Buffer

Buffer Fosfate pH 7.7	100 mmol/L
ATP	4 mmol/L
NAD	3 mmol/L
Magnesium sulfate	10 mmol/L
Azida	0,5 g/L

Preparación:
Disuelva 1,4595 g NaH_2PO_4, 23,986 g $Na2HPO_4.7H_2O$ en 800 ml de H_2O destilada, controle el pH de la solución 7,7 ± 0,1 ajuste si necesario c| HaOh ó PO_4H_3, disuelva 2,47 g $Mg\ SO_4.7H_2O$, 2,210 g ATP $Na_2.H_2O$ y 2,30 g NADP.Na_2·H_2O a la solución controle el pH y ajuste si es necesario; finalmente añada 0,5 g de NaN_3 y lleve a volumen con agua destilada.

Homogenizar y filtrar por vidrio (0,8 - 0,2 u)

2. Reactivo B

Hexoquinasa	100 U/ml	-	100 KU/L
G6P-DH	300 U/ml	-	300 KU/L
Azida Sódica	0,01 g/ml	-	1 g/L

Preparación:

Disuelva 100 KU hexoquinasa y 300 KU G6P-DH en 800 ml de 1:1 agua destilada y glicerina, añada 0,1 g azida y complete a 1 L con H_2O/glic. 1:1

NO FILTRAR

Almacena a 4°C protegido de la luz.

3. Reactivo C - Standard de glucosa

Glucosa

La Glucosa se debe secar 48 hrs a 50°C antes de usar.

Pesar exactamente 1 g de glucosa en 600 ml de agua. Añada 81,7 g de acido-tricloroacético a la solución y lleve al volumen con agua destilada a 1 L.

Puede prepararse con ácido benzoico.

Precauciones con el tricloroacético.

4. Reactivo D

Uranil Acetato	1,6 g
NaCl	

Disuelva 1,6 g Uranil acetato en 1 L de NaCl 0,9% recién preparado.

Método: Desproteinización

Reactivo D	500 uL
Muestra	50 uL

Mezole bien, centrifugue la muestra a alta velocidad (10000 - 12000 rpm), de 5 - 10 minutos.

Longitud de onda λ	- 365 nm
Sobrenadante	100 uL
Reactivo A	1000 uL

Mezole y mida la absorbancia a 365 nm. A_1

Add 10 uL Reactivo B

Mezole y mida la absorbancia después 3 - 30 min. A_2

Si no se desproteiniza

Preparación del hemolisado

Reactivos A - B - C iguales

Reactivo E CTMA-bromuro 0,2% y 0,1% azida en H_2O destilada.

Método

Reactivo E	1000 uL
Sangre	20 µL

Mezcle bien y tome 200 uL

Hemolisado	200 µL
Reactivo A	1000 µL

Mezcle bien y mida la absorbancia A_1

Reactivo B	10 µL

Continúa igual al método de desproteinización

$$\frac{20\ uL \cdot 200\ uL}{1000\ uL \cdot 1000} = \frac{\cancel{20} \cdot \cancel{200}}{\cancel{1000} \cdot \cancel{1000}} = \frac{1}{100}$$

Lectura en Hemolisado:

Reactivos A -B - C - iguales

Reactivo B - 0,2% CTMA-bromuro y 0,1% NaN_3 en agua destilada.

Método Reactivo B 1000 µL
Muestra(sangre) 20 µL

Mezcle bien para rápida hemólisis

Longitud de onda 365 nm

Tomar: Hemolisado 200 µL

Reactivo A 1000 µL

Mezcle y mida la absorbancia A_1

Reactivo B 10 µL

Continuar igual al métodoA (desproteinización)

Cálculo:	365 nm	354	340
C(mg/dl) =	1635 x ΔA	899 x ΔA	882 x ΔA
C(mmol/L)=	90,7 x ΔA	49,9 x ΔA	49,0 x ΔA

MÉTODO B - GLUCOSA. GOD-POD

Reactivo A

Buffer pH 7,4 fosfato	- 100 mmol/L
Fenol	- 24 mmol/L
Azida	- 0,05 g/L

Preparación:

Disuelva 13,6 g de NaH_2PO_4 y 3,3 g NaOH en 800 mL H_2O destilada, controle el pH de la solución 7,4 $\pm$ 0,05, disuelva 0,941 g fenol y 0,05 g de azida sódica y - lleve la solución a 1L y controle el pH, filtre a través de filtro de vidrio y almacene en frasco ámbar a temperatura ambiente.

Reactivo B_1 - (liofilizado)

Preparación:

Disuelva 12000 U GOD, 700 U POD y 0,0924 g 4-aminofenazona en 100 mL de agua - destilada FRIA (5-15°C). Liofilizar según las condiciones de URICASA. Calcular la cantidad para obtener concentración del diluido.

Reactivo B_2 - (líquido)

Buffer PO_4 pH 7,4	- 100 mmol/L
4-aminofenazona	- 0,25 mmol/L
GOD	- 15000 U/L
POD	- 1500 U/L
Fenol	- 0,75 mmol/L
H_2O destilada	- 1 L

Preparación:

Preparar buffer con iguales cantidades de sales, disolver en 800 mL de H_2O y - añadir a la solución, 57,75 mg 4-aminofenazona, 29,4 mg fenol, 15000 U GOD y - 1500 U POD y completar a 1 L con H_2O destilada. Almacenar a temperaturas bajas alrededor de -20°C.

Reactivo C - Standard de Glucosa.

1 g ácido benzoico/800 mL H_2O destilada . 90°C.

Después de dilución completa enfriar y disolver 1 g de glucosa EXACTAMENTE PESADO y que ha sido secado previamente a 50°C 48 hrs.

Complete la dolución a 1 L.

Medida λ = 500 nm

Reactivo B_1

Disolver el contenido de 1 frasco en 250 mL reactivo A (Reactivo E)

Reactivo	Blanco	Stand	Muestra
React. E	1 mL	1 mL	1 mL
Std	-	10 uL	-
Sample	-	-	10 uL

Después de mezclar incube 5' a 37°C y lea la absorbancia a 500 nm blanco

Cálcule - C(mg/dl) = Asam/Astd X 100

C(mmol/L) = Am/Astd X 5,58

Reactivo B_2

	Blanco	Muestra	Std
Sample	-	100 uL	-
Stand	-	-	100 uL
Sen.desprot.	-	1000 uL	-
H_2O destilada	-	-	1000 uL

Después mezcle y centrifugue a alta velocidad 5 - 10 min y del sobrenadante - tomar 100 uL.

	Std	Sample	Blank
Sobrenadante	100 uL	100 uL	-
H_2O	-	-	100 uL
Reactivo B_2	2000 uL	2000 uL	2000 uL

Mezcle bien, incube 10 min - 20 - 25

5 min - 37°C

Mida la absorbancia a 500 nm contra Bl. y calcule.

Annex VII.

CREATIN QUINASA

Preparación buffer Reactivo A

Buffer Imidazol pH 7,3	100 mmol/L
Glucosa	20 mmol/L
Acetato magnesio	10 mmol/L
EDTA	2 mmol/L
Azida	1 g/L

Preparación:

Disolver 2,83 g de imidazol, 2,47 g acetato $Mg.4H_2O$ y 0,85 g EDTA $Na_2.2H_2O$, - 3,604 g glucosa (tratada como standard), disuelva en 800 mL H_2O destilada después añada solución AcH 1 mol (60 g/1000 mL) hasta que el pH sea 7,3 ± 0,1. Añada 1 g azida y complete el volumen a 1 L con H_2O destilada.

Mezcle para homogenizar y filtrar 0,8 - 0,2 u.

Reactivo B - Enzimas

N-acetilcisteína	20 mmol/L
ADP	2 mmol/L
AMP	5 mmol/L
NADP	2 mmol/L
Diadenosin 5P	10 mmol/L
Hexoquinasa	3500 U/L
G6PDH	2000 U/L
Fosfato Creatina	30 mmol/L

Preparación:

Disuelva 2,9 g AMP Na_2, 1,153 g ADPK.$2H_2O$, 1,81g; NADPNa_2 y 11 mg AP_5-A3h en - 800 mL H_2O destilada. Enfríe la solución a +5°C y añada 2,562 g NAC y 11,3 g CP. 2Na $4H_2O$ a la solución después de completa disolución, añada 3500 U/L HK y 2000 U G6PDH y complete a 1 L con H_2O destilada.

Distribuya en viales 10 mL de la solución y liofilize de acuerdo a lo descrito p/ ácido úrico.

Medidas

Disuelva 1 bb reactivo B en 10 mL Reactivo A.

- REACTIVO C -

λ = 340 nm

React. C	1 mL
mta	20 µL

Mezcle e incube la muestra 25°C ó 37 - 30°C.

Después de 3 min de incubación a 25°C
2 " " 30°C
1 " " 37°C

Lea la absorbancia y lea nuevamente después de 1 - 2 - 3 min y calcule $\Delta A/min$.

Calcule la actividad

1 U/L = $\Delta A/min$(340 nm) x 8199

Annex VIII.

COLINESTERASA

Reacción:

Butiriltiocolina + H_2O —colinesterasa→ tiocolina + butirato

tiocolina + DTNB ——→ nitro - 2 - mercapto - 5 benzoato

Reactivo A - Buffer

Buffer Fosfato	pH 7,7	80 mmol/L
DTNB (ditio-bis-nitrobenzoato)		400 mmol/L

Disuelva en 800 mL buffer fosfato (80,08 M), 1,5856g DTNB lleve al volumen (1 L) con buffer. Dispensar en viales 30 mL y liofilizar con iguales condiciones que - las anteriores.

Reactivo B

Cada vial contiene 2 mg de 8-butiriltiocolina ioduro.

Disolver en NaCl (1:100) o (1:50) y dispensar (en mezcla sólida) la cantidad necesaria.

Antes de usar disolver Reactivo A en 30 mL y Reactivo B en 1 mL agua destilada.

Medida λ - 405 nm

Reactivo A	1,5 mL
Muestra	10 µL
Reactivo B	50 µL

Mezcle, mida la absorbancia a 405 nm inmediatamente después de completar la mezcla de reacción y después de 30, 60 y 90 seg. de la reacción.

Calcule:

$\Delta A/min$

$25^{\circ}C$ $\quad U/L = \Delta A/min \times 11730$

Preparación:

Pesar 20 mg butiriltiocolina (BCI) + 1000 mg NaCl, pulverizar en un mòrtero, a esta mezcla se le determina la concentración 8BCI en UV espectro, preparando una dilución 10 µg/mL.

Se pesa la cantidad de la mezcla necesaria para tener 2 mg/vial de 8 BCI.

Annex IX.

LACTATO DESHIDROGENASA (LDH)

Reacción:

$$\text{Piruvato} + \text{NADH} + H^+ \underset{\text{pH 8,5-9,0}}{\overset{\text{pH 7,8-8}}{\rightleftharpoons}} \text{Lactato} + \text{NAD}$$

Reactivo A - Buffer

Buffer Tris pH 7,2	- 100 mmol/L
NaCl	- 200 mmol/L
Na Piruvato	- 1,6 mmol/L
Etilenglicol	- 18,3 g/L
Azida	- 1 g/L

Disolve 12,2 g TRIS (base) en 300 mL de H_2O destilada y 44,2 mL HCl 0,1 N, mezcle y controle el pH a 7,2 ± 0,1, disuelva 11,7 g NaCl y 176,16 mg Na piruvato en la solución, añada 18,3 etilenglicol y 1 g de azida sódica, controle el pH nuevamente y complete a 1 L con H_2O destilada, mezcle y filtre (0,8 - 0,2 µ).

Reactivo B

Cada vial contiene 0,01 mmol (6,634 mg) NADH se puede hacer igual mezcla que en el caso de colinesterasa con NaCl y quitar el NaCl del buffer.

Antes de usar, disolver el contenido de 1 vial en 20 mL de buffer Reactivo C

Medida

Longitud de onda = 340 nm

Reactivo C	1000 µL
Std o muestra	20 µL

Mezcle y después de 1 min lea la absorbancia según lo descrito en CK.

IU/L(340nm) = A/min X 8095

Annex X.

FOSFATASA ACIDA

Método p-nitrofenil fosfato

Reacción:

p-nitrofenil fosfato + H_2O $\xrightarrow[\text{pH 4,9}]{\text{Fósf. acida}}$ p-nitrofenol + fosfato

p-nitrofenol $\xrightarrow{\text{pH 11,0}}$ p-nitrofenolato

Reactivo A

Disuelva 13,5 g Citrato de Na $2H_2O$ en 700 mL de H_2O destilada ajuste el pH - 4,9 $\pm$ 0,05 con HCl 1N lleve a 1 L con agua destilada.

Reactivo B - Sustrato sin ácido tartárico

Disuelva 288 mg p-nitrofenil fosfato Na_2 $6H_2O$ en 1 L de Reactivo A.

Reactivo C - Sustrato con ácido tartárico

Disuelva 720 mg p-nitrofenil fosfato en 245 mL de Reactivo A y añada 5 mL - Reactivo D.

Reactivo D

Disuelva 150 gL-ácido tartárico en 700 mL H_2O destilada. Ajuste el pH de la - solución a 4,9 $\pm$ 0,05 con NaDH y lleve el volumen a 1 L.

Reactivo E

4 g NaOH en 1 L H_2O destilada

Método

Longitud de onda = 405 nm

	Total act.	Tartárico acid resist	React. Blanco
React. B	0,5 mL	-	-
" C	-	0,5 mL	0,5 mL
	Incube a 37°C por 3 min		
Muestra	0,1 mL	0,1 mL	-
	Incube 30 min a 37°C		
React. E	2,0 mL	2,0 mL	2,0 mL
Muestra	-	-	0,1
	A_1	A_2	A_o

Cálculo:

I U/L total : 1405 X d A_1/30 min $A_1 - A_o$

II Tart-resist : 1405 d A_2/30 min $A_2 - A_o$

III Inhib. (I - II)

Annex XI.

FOSFATASA ACIDA
Método FAST - RED - T:

Reacción:

1-naftil fosfato + H_2O —Fosf. Acida→ 1-naftol + fosfato

1-naftol + FAST-RED T ——————→ Azocompuesto

Reactivo A - Buffer

14,7 g Citrato de Na.$2H_2O$ en 700 mL H_2O destilada. Ajuste el pH 5,6 $\pm$ 0,1 con HCl 1N controle el pH y añada 1 mL de TRITON X-100 y lleve a 1 L.

Reactivo B - Solución tartárica

Disuelva 14,7 g Citrato Na.$2H_2O$ y 15 g de ácido tartárico en 700 mL de H_2O destilada. Ajuste el pH con NaOH 1N a 5,6, añada 1 mL Tritón X-100 y lleve a 1 L con H_2O destilada.

Reactivo C - Sustrato

Disuelva 2,681 g naftilfosfato de Na y 100 mg Fast Red-T en 600 mL react. A, - lleve a 1 L y liofiliza en porciones de 3 ml.

Reactivo - Estabilizador

Lleve 0,15 mL AcH a 1 L H_2O, añada 1 gota de este reactivo a la muestra.

Reactivo D

Disuelva antes de usar 1 vial react. C en 3 mL React. A.

Reactivo E

Antes de usar añada 3 mL React. B a 1 vial del reactivo C.

Método

Longitud de onda: 405 nm

	Total	Tartar. resist.
React. D	1 mL	-
React. E	-	1 mL
Muestra	100 uL	100 uL

Mezcle y después de 5 min incubación a 37°C, lea la variación de la absorbancia c/min hasta 3 min.

Total: A/min X 743 Prostatic: A(Dreact) - A/min (Ereact) X 743

FOSFATASA ALCALINA

Reacción:

p-nitrofenilfosfato $\xrightarrow{FALC}$ p-nitrofenol + fosfato

Reactivo A Buffer

Dietanolamina buffer	1 mmol/L
Cloruro magnesio	0,5 mmol/L
Azida sódica	1 g/L

Pese 105,1 mg dietanolamina en volumétrico de 1 L, añada 300 mL H_2O destilada, ajuste pH 9,8 ± 0,1 con HCl 1N, disuelva 151,7 mg cloruro $Mg.6H_2O$ y 1 g azida lleve 1 L con H_2O destilada. Controle el pH.

Reactivo B Sustrato

Viales conteniendo 52,61 mg p-nitrofenilfosfato (200 µmol - 0,2 mmol)

Reactivo C

Antes de usar disuelva el contenido de 1 vial en 20 mL Reactivo A.

Método

Longitud de onda: 405 nm

Reactivo C	1 mL
Muestra	20 µL

Mezcle y después de 1 min a 25°C lea la absorbancia a 405 nm contra H_2O.

Mida la reacción de absorbancia en 3 min.

U/L (405 nm) = ΔA/min X 2750

COLORANTES Annex XIII.

1.- Azul de Metileno 0.1% en agua

Problemas:

A) Cambios de color

a) Cambiod de acuerdo con cambios de pH.

b) Cambios en la intensidad del color.

B) Precipitación

a) Producida por contaminación microbiológica.

b) Formación de sales del colorante que precipitan.

1) Ver el rango de cambio de pH.

2) Medir el pH a las soluciones.

- Tomar 10 mL de muestra y añadir 0,5 mL de HAc glacial. El pH debe ser aproximadamente 3.
- Añadir aproximadamente 5 mL de HAc glacial a 120 mL de Azul de Metileno si se agita el precipitado debe redisolverse.

Antioxidante Bilsulfitó de Na.

También se usa 2% de Azul de Metileno en etanol; es mucho mejor, sólo se usan 2 gotas.

Preparar pequeñas cantidades + concentrados y diluir antes de usar.

Y preparar las soluciones en etanol.

El precipitado debesser por cambios de pH y reacción con el vidrio.

Recomendación general.

1) Estabilidad (Envases)

Tres tipos de envase: I, II, III, IV

Clase I

Clase II con tratamiento en la superficie

Clase III

Polipiopileno (Lipolen R BAS F alemana)

Polietileno (Que sean resistentes al calor y a los solventes)

Lavar con agua destilada y filtrar las soluciones pues el etanol es un buen - conservante.

2) Composición:

1- Formulación original

2- " " + 5% Hác glacial

3- " al 2% en etanol (Diluir antes de usar con H_2O destilada)

4- Formulación original + 20% etanol

3) Temperatura

$4^{\circ}C$ (temperatura ambiente, luz reflejada)

$40^{\circ}C$ y $60^{\circ}C$

Medir 0, 1, 2, 3 meses

Para $4^{\circ}C$ temperatura y $40^{\circ}C$ además 6, 9, 12, 18, 24, y 36 meses.

$4^{\circ}C$
Temp. amb } 9 x 3 = 27 muestras
$40^{\circ}C$

Luz reflejada
$60^{\circ}C$ } 3 x 2 = 6

27
6
33
x 3 - 3 focos en cada temp.
99 = 100 muestras

100 muestras 1 comp. 1 envase

3 formulaciones 4 envases - 1200 muestras

Composición	1	2	3
No. de muestras	100	100	100
Envases	100 100 100 100	100 100 100 100	100 100 100 1000

400 muestras de cada componente

1) Estudio Acelerado

Tiempo 0, 1, 2, 3 meses

Temp. 4°, Temp. A, luz reflejada, $40^{\circ}C$, 60°

Composición	(1)	(2)	(3)
Azul de Metileno	-	-	-
Hac		-	
Etanol			-
H_2O destilada	-	-	-

Envases I
II
III
IV

No. de muestras. (Tabla igual a la anterior). Pero como es acelerado sólo se necesitan 50 muestras cada vez en lugar de 100.

2) Tiempo Largo

Tiempo: 0, 3, 6, 9, 12, 18, 24, 36. (7 x 3 = 21)

Temp 40C, temp. A, 40°C. 21 x 3 = 63 aproximadamente 7 70 muestras

No. de muestras

Formulación	1		2	
Envase	A	B	A	B
	70	70	70	70

A = original

B = mejor envase obtenido en el estudio acelerado.

1 = Original

2 = a B

Analizan

Visual

pH

UV-Espectro

(conc.)

Cada vez preparar un standard para el ensayo que sirva como referencia. Standard fresco.

Evaluación de los test de estabilidad

(A) Acelerado

Si se mantiene a 37° ó 40°C estable por 3 meses, se puede decir: Fecha de vencimiento 6 meses a 25°C

Fecha de vencimiento 1 año a

$$CV = \frac{SD}{\bar{X}} \times 100$$

Después de 2 años a 37°C es estable 3 años a temp. ambiente

si a 37°C no es estable y a 25°C estable 3 años a 4°C

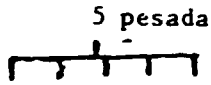
Cálculos

Contenido a tiempo cero = 100%

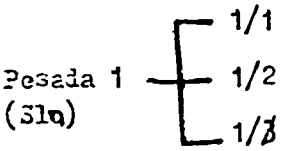
Se considera estable cuando la concentración está entre 98% y 102%

Para evaluar la precisión se hace:

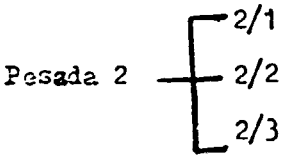
Se preparan 5 soluciones = 5 con 5 pesadas independientes



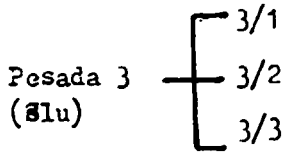
3 variantes de cada caso (3 diluciones)



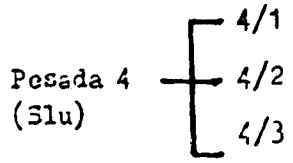
3 diluciones de la Slu 1



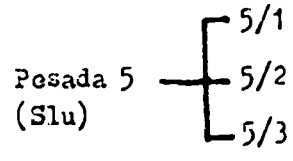
3 diluciones de la Slu 2



3 diluciones de la Slu 3



3 diluciones de la Slu 4



3 diluciones de la Slu 5

Se realiza el ensayo a cada dilución y se calcula la $\bar{X}$ en cada grupo.

Absorbancia: $A = \frac{(A_{1/1} + A_{1/2} + A_{1/3}) \times 10}{3 \times W}$

W = pesada de la Slu

Luego se calcula la media de las 5 medias

$$\frac{\bar{1} + \bar{2} + \bar{3} + \bar{4} + \bar{5}}{5} = \bar{X} \text{ y la SD y el CV}$$

El intervalo en que se deben mover los valores hallados en el tiempo es:

$\bar{X} \pm CV$ CV debe ser menor que 1%

Mientras eso suceda no hay diferencias significativas en las concentraciones.

Si se quiere trabajar con % se prepara un standard que contenga 500 ug en 100 mL, se realiza una dilución tomando 2 mL y llevando a 100 mL y de aquí 10 mL y se lleva a 100 mL para que la Slu final quede 10 ug/mL. Se evalúa la concentración del standard y de las muestras.

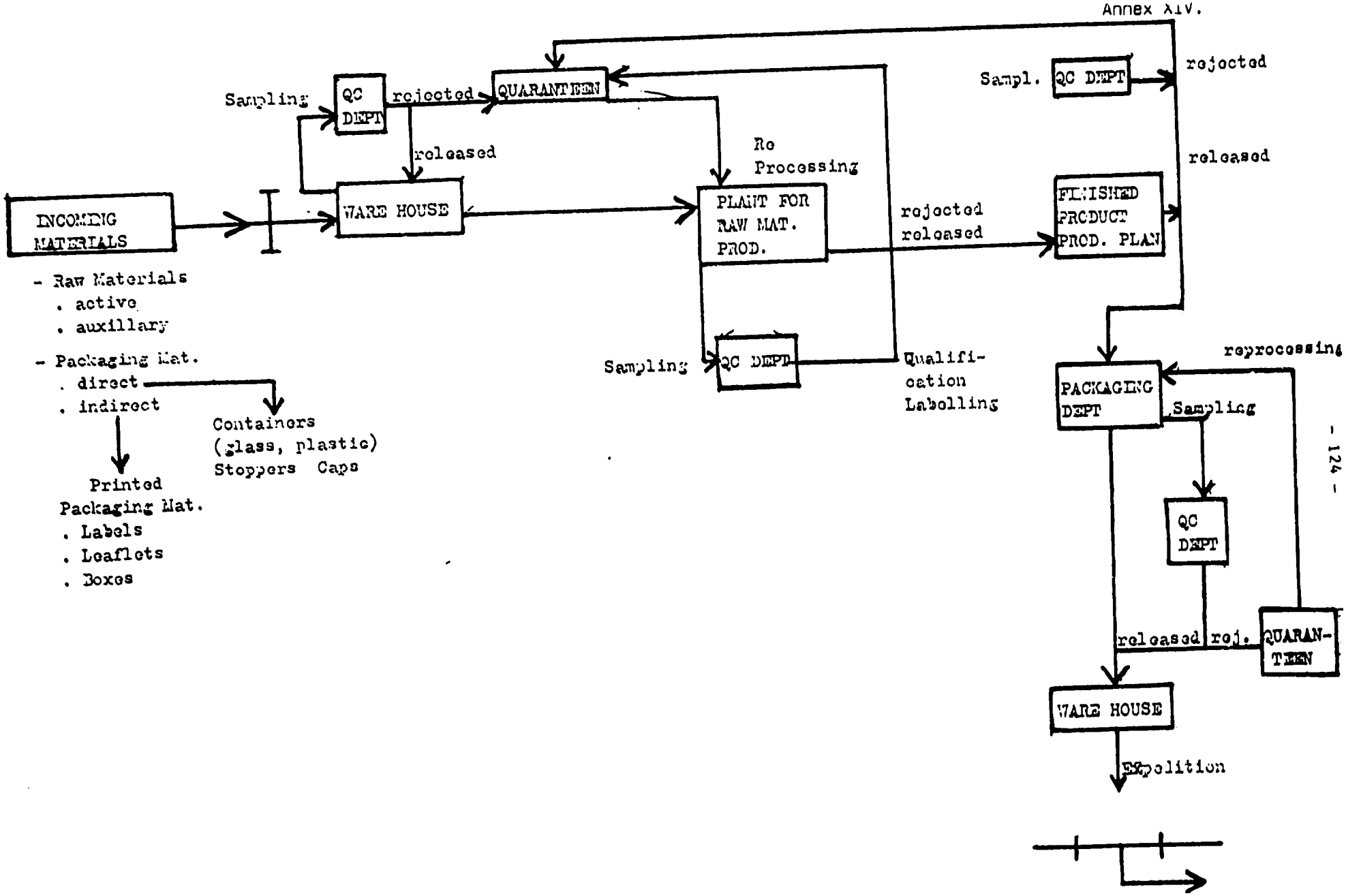
Cálculos:

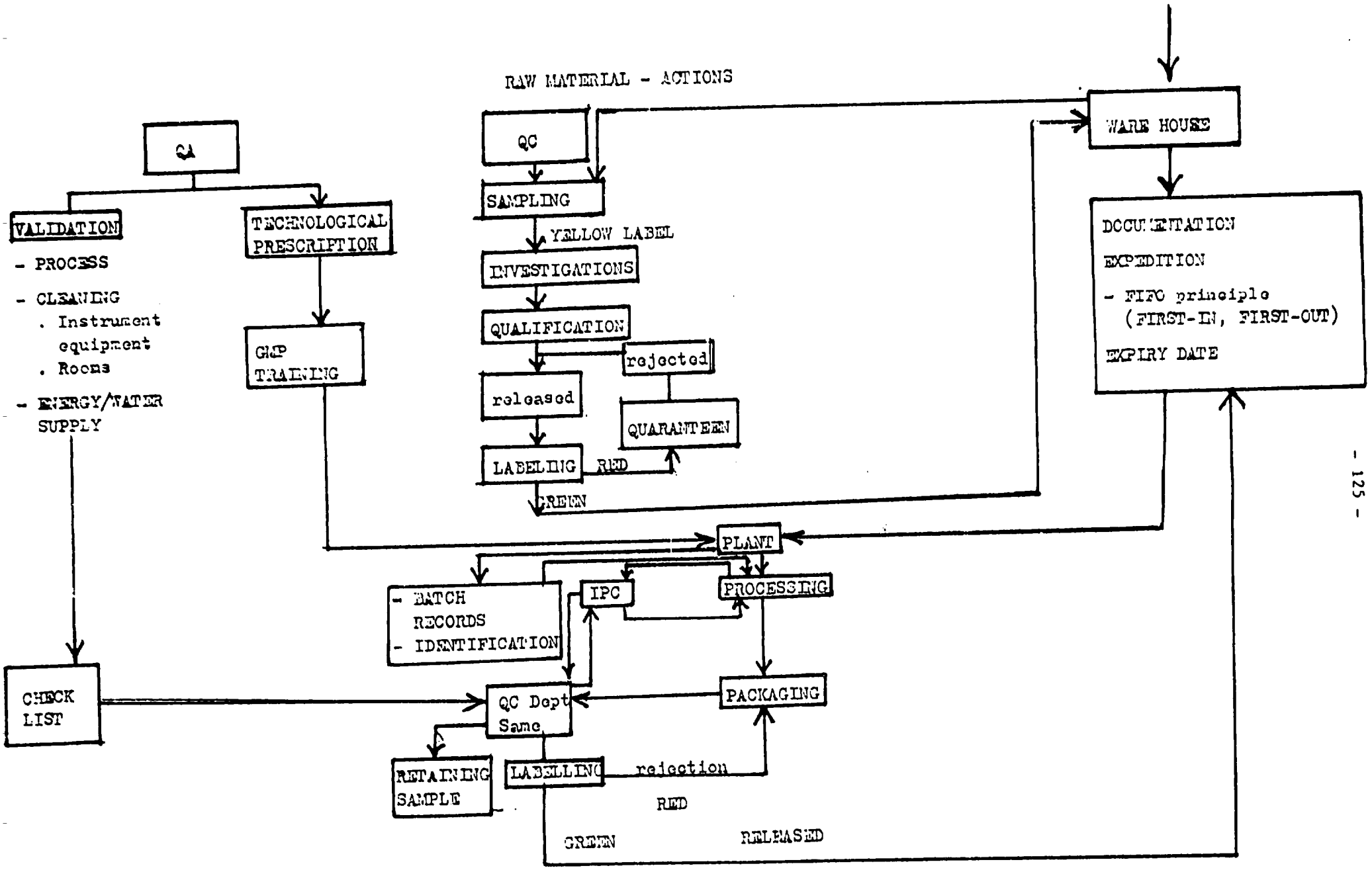
$$\% = \frac{\text{Amuestra X Wstandard}}{\text{Astandard X 100 X 10}} \times 50$$

50 = dilución
10 = peso standard

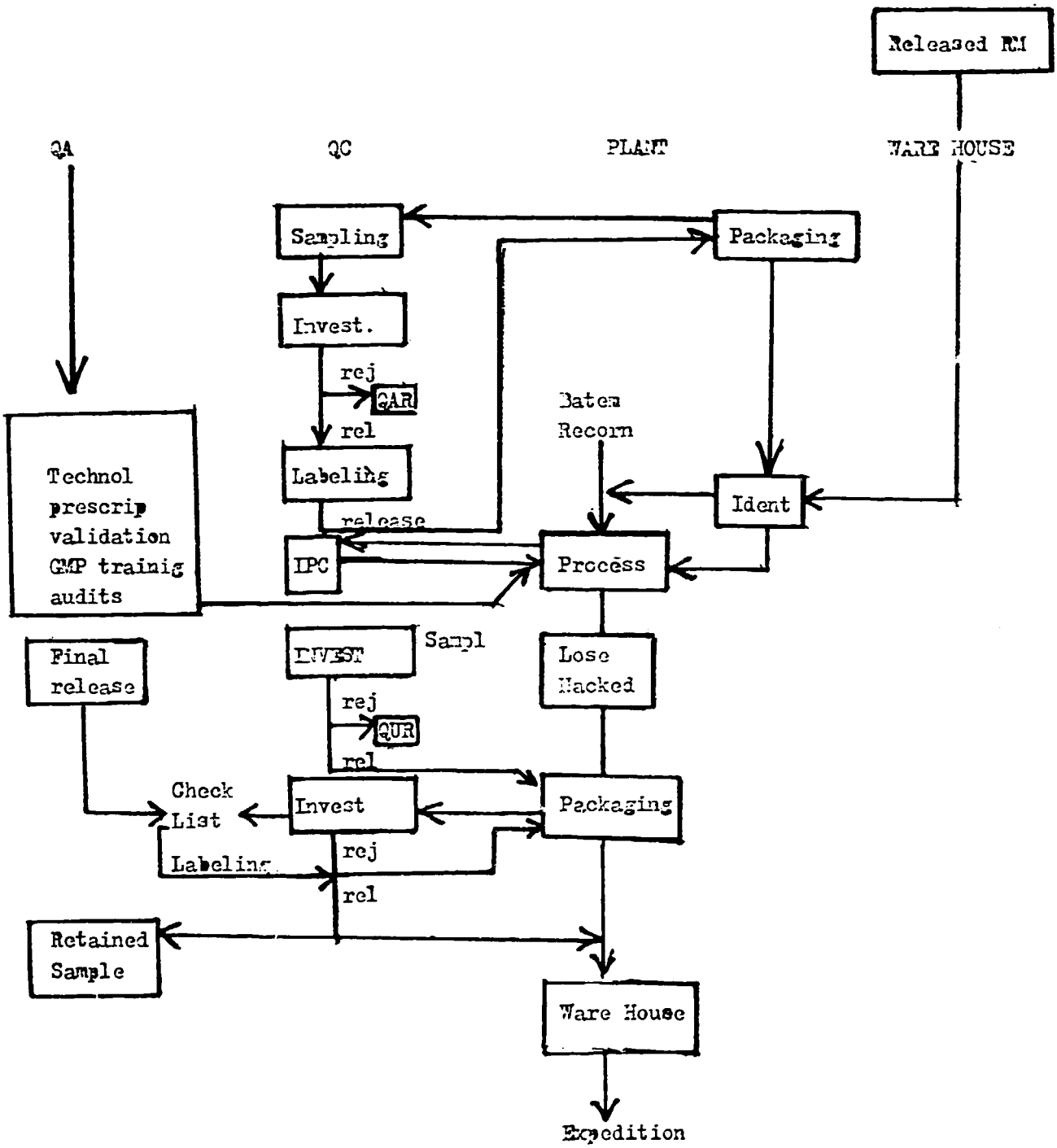
$$\% = \frac{\text{Amuestra}}{\text{Astandard}} \times W_{std} \times 0{,}05$$

Nota: Se puede usar la Regresión Lineal para calcular la fecha de vencimiento hasta el tiempo exacto en que se realizó el estudio; por ejemplo hasta 1 año pero en base a esto no se puede estimar el futuro.

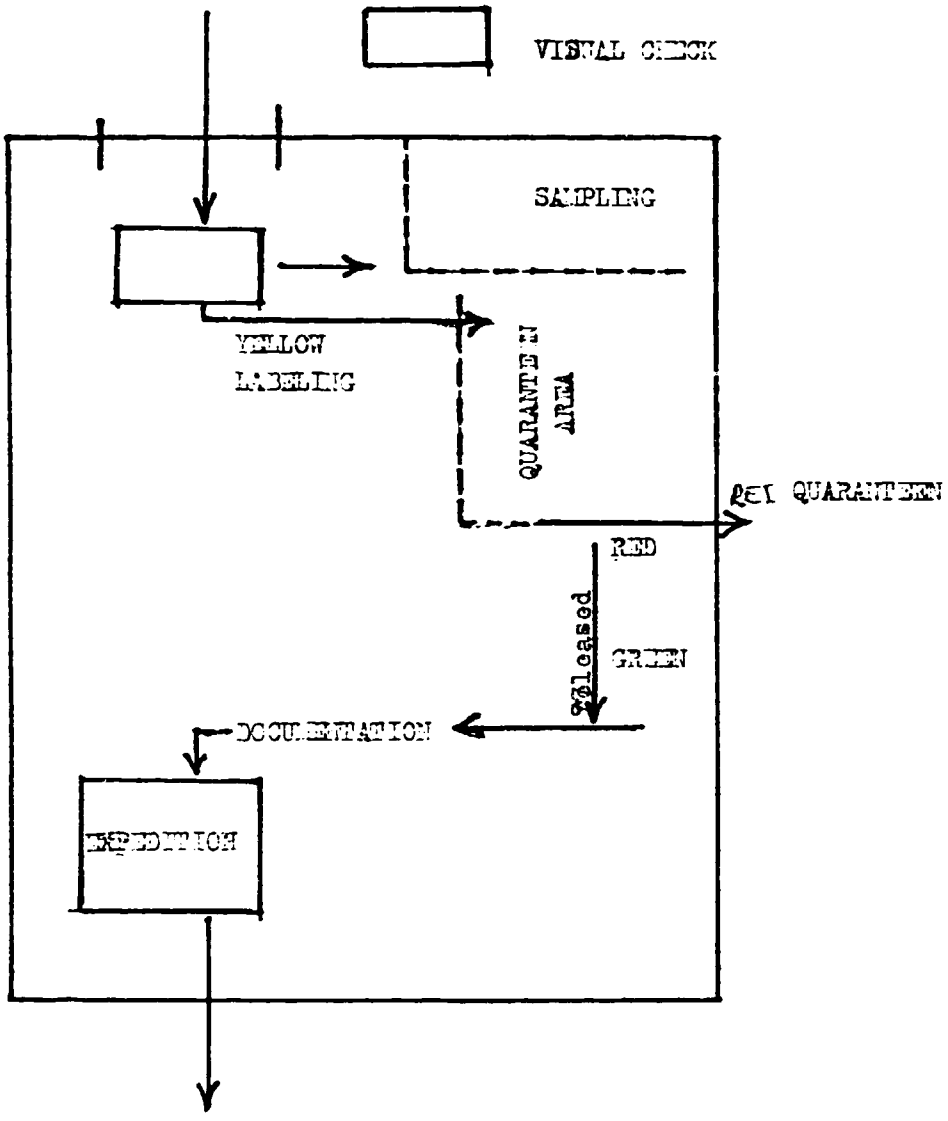

Sampling
QC DEPT
rejected
QUARANTEEN
released
WARE HOUSE
INCOMING MATERIALS
- Raw Materials
. active
. auxillary
- Packaging Mat.
. direct
. indirect
Containers (glass, plastic) Stoppers Caps
Printed Packaging Mat.
. Labels
. Leaflets
. Boxes
Re Processing
PLANT FOR RAW MAT. PROD.
Sampling
QC DEPT
Qualification Labelling
rejected released
Sampl.
QC DEPT
rejected
released
FINISHED PRODUCT PROD. PLAN
reprocessing
PACKAGING DEPT
Sampling
QC DEPT
released
rej.
QUARANTEEN
WARE HOUSE
Expedition


RAW MATERIAL - ACTIONS
QA
VALIDATION
- PROCESS
- CLEANING
. Instrument equipment
. Rooms
- ENERGY/WATER SUPPLY
TECHNOLOGICAL PRESCRIPTION
GMP TRAINING
QC
SAMPLING
YELLOW LABEL
INVESTIGATIONS
QUALIFICATION
rejected
released
QUARANTEEN
LABELLING
RED
GREEN
WARE HOUSE
DOCUMENTATION
EXPEDITION
- FIFO principle (FIRST-IN, FIRST-OUT)
EXPIRY DATE
PLANT
- BATCH RECORDS
- IDENTIFICATION
IPC
PROCESSING
CHECK LIST
QC Dept Same
PACKAGING
RETAINING SAMPLE
LABELLING
rejection
RED
GREEN
RELEASED

FORMULATION - ACTIONS
DEPARTMENT



WAREHOUSE ACTIONS



DOCUMENTATION

(1) Organization Charts

(2) Personnels - Responsibilities

(3) QC (Most Important)

- Sampling Plan: Raw material / Packaging / Formulated PR

- Sops (all activities)

- Retaining Samples

- Release Systems

- Labeling Instructions (YELLOW GREEN RED)

- Quality Controller instructions

- Prevention of mixing active RM and formulated products - C R O S S CONTAMINA T I O N PROBLEM

- Re-call System

- Trend Analysis

- Performing method validation and stability testing

- Instruction for expiry date determination

  - in house
  - out house

(4) QA (most important)

- Validation

  * Energy supply system (water, steam, air)

  * Process

  * Cleaning

  * Equipment

  * Rooms - Plants

- Batch numbering system

- Technological Prescriptions

- GMP Audits
- GMP Trainings
- Archivation
- Trend Analysis

(5) Plant (Most important)

- Process Prescriptions (in details)
- Clothing Rules
- Maintenance Rules
- In Process Control
- Identification of all incoming material

(6) Warehouse (most importan)

- Proper rules for storage
- Fife principles
- Expiry date
- Quarantees rules
- Documentation
- Expedition Rules

Annex XV.

STABILITY TESTING

Purpose:

(1) Determination of optimum formulation

(2) Establishment of the stability of a product which quarantee the full activity up to the end of shelf life.

Questions:

- How precise the methods used?
- How specific ?
- How sensitive ?
- How extensive are the changes that occur ?
- How far can changes be tolerated ?
- To what extent can changes be tolerated ?
- What is the cause of the changes ?
- After what time may binding statements about stability be made ?
- To what extent after may stability-related changes be tolerated ?

Considerations:

(1) Analytical method

A) - only active ingfedient
- only decomposition product
- active ingredients + decomposition product

B) Requirements:
- high specificity
- high sensitivity
- Ruggedness !

(2) Testing criteria

(See A, + visual insp, pH, etc.)

(3) Storage conditions
- Effect of heat
- Efect of light
- Effect of moisture (humidity)
- Effect of metal ions, pH, oxygen (electrochemical effects)
- Experimental effects of combination

Study of appropriate storage conditions:

- Confirm the suitability and validity of the analytical methods.
- Help to identify potential degradation products.
- May identify the weak points of formulations.
- Identify the stability limiting parameters.
- Identify the potential problems during transport and storage.
- Help in establishment of shelf life.
- Help in selection suitable packaging materials.
- Cover the climatic factors.

TESTING INTERVALS AND DURATION

- Preliminary (only 1 testing time, short duration) Well-defined stress conditions
- Accelerated (3 month duration with three or four testing intervals) Storage conditions are based on the date of preliminary test.
- Comparative (duration and intervals same as above) Extreme storage conditions are used.
- Long-term (duration: the entire shelf life + 1 year) Testing intervals: 0, 3, 6, 9, 12, 18, 24, 36, 60 months. Storage: conditions which maybe encountered
- Follow up (on-going): Entire shelf life with one testing time recommended - storage cond.

EVALUATION AND EXPIRY DATE !!

(1) From preliminary:

Only estimation with max. shelf life 6 months

(2) From accelerated:

Provisional shelf life up to 1 year

(3) From comparative:

Shelf life of the reference product

(4) Long-term:

Continuously extended up to 5 years

(5) Follow up.

Confirmation of (4)

Testing Plans for the Individual Phases of Stability Investigations

	Phases of stability testing				
	Preliminary	Accelerated	Comparative	Long term	Follow-up
Samples	Active substance or various compositions	1 selected batch	1-1 batch from developed and marketed formulations	1-1 laboratory pilot and production batches	1-2 batches per year
Duration of test	2 - 6 weeks	12 weeks	12 weeks	3 - 6 years	6 months or 2-5 years
Testing intervals including zero time	At the end of storage	6, 12 weeks or 1, 2, 3 months	6 - 12 weeks	3, 6, (9), 12, (18) 24, 36, (48), (60)	At the end of storage
Storage conditions					
100°C	(+)				
75°C	(+)		+		
50°C	+	+			(+)
40°C				+	
40°C/80% R.H.	+	+	+	+	+
25°C		+		+	+
5 - 15°C		+		(+)	(+)
0 - 5°C	+	+	+	(+)	(+)
Reflected light	+	+	+		
UV-254/10 h	+	+	+		
At pH 2 (in solution)	(+)				
At pH 12 (in solution)	(+)				
Evaluation	Construction of stability profile or verification of peak purity	Identification of stability-limiting parameters	Proof of stability equivalency	Derivation of real shelf life	Confirmation lives

\+ - recommended

(+) - only for an active substance and special cases

Annex XVI

WHAT IS VALIDATION?

Validation of a method or a process is the planning, performance and interpretation of series of experiments designed to reveal the operational characteristics of the method and/or process, which allows it's use for a relatively long period of time with acceptable accuracy and reliability.

Do not mix with:

- Standardization
- Verification
- Qualification

(1) STANDARDIZATION:

Refers to constancy

- Quality of the product

- Performance of the process, etc.

Validation is necessary for standardization, to establish the weakest points.

(2) VALIDATION is the most important tool for standardization and refers to the suitability of the method or process for the intended application.

(3) VERIFICATION is a statement that something meets with the standard, the re fore it refers to the applicability of an instrument, equipment, etc. basic tool for qualification.

(4) Qualification refers to the ability of an instrument, and equipment to perform the intended application.

For example:

Each sterilization autoclave contains thermometers. Thermometer is checked, that the temperature measured is valid with a standard thermometer placed into the autoclave. This is verification.

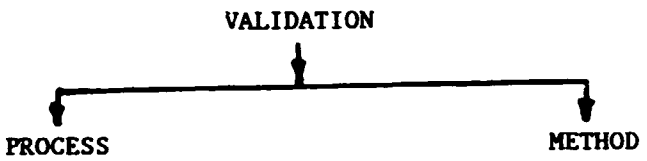
The autoclave has several sterilization programme possibilities stated in the operational characteristics to control it, it is qualification.

If we place microorganism with various thermal stability and investigate for the microbial count this is high degree of qualification but not validation.

Validation is if we put the materials into the autoclave in an amount which we want to sterilize and inside we contaminate the solution with thermal-sensitive bacteria.

Validation process should be made for each product which differs size and materials of container, size of lot placed into or the type of solution regarding viscosity, suspension emulsion or clear liquid, or the sterilization programme regarding heat sensitivity of the material.

If we have many results and the process is validated, without any problem, the process is standardized.



PROCESS VALIDATION

(1) Validation of processes connected to the many factor (extraction, drying, milling, filtering, centrifugation, chemical reactions, etc.) Product is controlled. Results: in-process control tests.

(2) Validation of equipment used for the process: results, daily qualification tests.

(3) Validation of cleaning procedure of the equipment very different:

- From process to process
- From product to product
- From equipment to equipment
  * used for the same process
  * used for different process

Results:

(a) Qualification after each product (in-process)

(b) Prescription valid for the process until it is constant (no change in the procedure).

METHOD VALIDATION:

- Accuracy
- Precision
- Reproductibility
  * in day
  * day to day
  * ruggedness
- Specificity

- Linearity and range
- Limit of detection
- Limit of quantitation
- Sample stability

RUGGEDNESS

Is a measure of reproductibility of the individual test results, when the procedure is repeatedly used to determine the same homogeneous sample in a variety of specified experimental conditions.

Sensitivity of the method to the change in environmental conditions.

PRIOR TO THE TES

(1) Selection of factors

(2) Minimum acceptable criteria for the factors

(3) Selection of APP

(4) Acceptable deviations of APP

RE-VALIDATION

(1) Method-procedure is found to be deficient in some areas

(2) Method-process has been changed

(3) Environmental conditions has been changed

- Different laboratory plans
- Different instrument - equipment
- Composition of the product has been changed

Annex XVII

Senior counterpart staff and list of peoples with whom I met during my stay

1. Finlay Institute

Dr. Martha Carralero	Director of Finlay Institute
Dr. Lillian Valdes Diez	Head of Reasearch Department

Approximately 30 other colleagues dealing with this project.

2. Other persons

Ms. Maria Julia Guesta	Head of Cooperation Department Pharmaceutical Union
Mr. Rosty Batista Arnedo	Responsible for Cooperation Pharmaceutical Union
Mr. Sixto Montano	Protocal Attache Pharmaceutical Union
Mr. Ordonez	Vice Representative UNDP Office - Havanna
Ms. Ana Maria Gudz Rabak	Project Officer UNDP Office - Havanna
Mr. Jesus Garcia Valdes	Natural Product Specialist Emp.Lab. "Mario Munoz"
Dr. W.N. Walker	Consultant Chemist - Scotland UNDP expert at "Mario Munoz"

Annex XVIII

UNIDO comments on Dr. Szepesi's technical report DG/CUB/86/015/11-51

During Dr. Szepesi's mission to Havana, the staff of the Institute Finlay received direct assistance for the daily production and research activities. The expert also advised on the performance of construction works for the new reagents plant.

Special emphasis was done to the proper application of the G.M.P. regulations to the production processes, and also the performance of validation procedures in the plant. In order to guarantee the quality and durability of the produced reagents, it is necessary to improve the quality of the locally produced packaging materials. Special UNIDO's assistance in this field would be advisable.

The expert assisted in the preparation of reference solutions, buffers, control sera and colorants for microbiological and haematological tinctures, application of G.M.P., G.L.P., etc.

During the mission, the expert assisted on the preparation of reference solutions of uric acid, protein, iron, bilirubin, albumin, etc.. The descriptions for the preparation of the substances are given in the report, as well as the requirements of packaging materials and methods for quality determination.

Upon request of the personnel of the Institute, the expert assisted on the preparation of enzyme diagnostic reagents. It is advisable that the Institute establish collaboration with some national groups working in the development of synthetic products for the development of their own synthetic reagents.

The expert's assistance for the preparation of specific sera for the quality control of lipids, bilirubin and enzymes was highly evaluated by national technicians, as guidelines for introduction of non produced in the Institute control sera were given, also assistance for the programming and performance of stability tests for the production of the Institute were given.

Some minor typing mistakes are found in the report, but it was satisfactorily evaluated by UNIDO substantive area.