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EXPERIMENTAL PRODUCTION OF PHARMACEUTICAL RAW MATERIALS FROM ANIMAL BY-PRODUCTS OBTAINED FROM SLAUGHTER HOUSES

DP/VIE/86/016

SOCIALIST REPUBLIC OF VIET NAM

Technical report: Findings and recommendations*

Prepared for the Government
of the Socialist Republic of Viet Nam
by the United Nations Industrial Development Organization,
acting as executing agency for the United Nations Development Programme

Based on the work of Ms. Blazenka Pavelic,
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* This document has not been edited.

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a. Prof. Oleg Ščedrov: CHYMOTRYPSIN AND TRYPSIN
b: Prof. Oleg Ščedrov: DRY BILE

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I N T R O D U C T I O N

Blaženka Pavelić spent 23 days (in the frame of 1 month-mission, 20 working days) at the Project site (Ho Chi Minh City, Vietnam, Project DP/VIE/86/016) as a quality control expert (QCE). According to the Job Description received from B. S. Officer, UNIDO, Vienna, QCE was expected to carry out the following duties:

- organize and deliver training courses at the Project site in the quality control of bioactive substances in bulk as well as that of the final formulations,
- introduce modern quality control techniques for bioactive substances as starting raw materials and final products, and assist in the implementation and the start-up of laboratory facilities,
- prepare quality control protocols for each product, and participate jointly with the CTA, PTE and DICI in the preparation of the maintenance and operation manual,
- prepare a technical report on the modern quality control techniques for bioactive substances,
- prepare a detailed report of her findings, achievements and recommendations for future action.

Except for this Job Description, QCE received the Work Programme of the CTA, agreed upon by the NPD. Both documents are enclosed.

According to the mentioned above, the QCE tried to realize the programme as much as possible in the frame of the time given and the working possibilities at the Project site.

The QCE would like to stress that many, very different types of examinations are embraced in the quality control of pharmaceutical enzymes. As it can be seen in the pharmacopoeia's monographs enclosed, there are chemical, biological, microbiological and biochemical examinations included. Because of the lack of the needed equipment and the chemicals at the Project site, and some technological problems in the production as well, it is clear that a big part of the work could not be realized.

At the same time, it must be said that the time envisaged for the realization of such a work was absolute ly unrealistically set up. Even under ideal working conditions it couldn't be fully realized within 20 working days.

All the essential details and facts, connected in any way with the realization of the QCE duties, are explained in this report.

1. PROPOSED METHODS FOR THE QUALITY CONTROL OF THE PROJECT PRODUCTS

1.1. PANCREATIN
1.2. CHYMOTRYPSIN AND TRYPSIN

The Project products are intended to be pharmaceutical raw materials, and so they should be analysed and evaluated according to the pharmacopoeia's prescription. The monograph "Pancreatis pulvis", (1984), Ph. Eur. 2nd edition, (Annex 2.a.), is the proposed quality-control procedure for Pancreatin. "Chymotrypsin and Trypsin" is an crude mixture of chymotrypsinogen and trypsinogen, which as such can not be considered as a pharmaceutical substance at all. Having in mind the inclusion of additional technological steps, which will enable that separated and sufficiently pure enzymes be obtained, the quality control procedures for Chymotrypsin and Trypsin are chosen. The monograph "Chymotrypsinum" (1986), Ph. Eur. 2nd, (Annex 2.b.), is the proposed analytical procedure for the quality control of Chymotrypsin. Also, for Trypsin (pure and successfully separated from Chymotrypsin), the monograph "Trypsinum" (1990), Ph. Eur. 2nd, (Annex 2.c.), is proposed as the quality control procedure. In the cited monographs there are analytical procedures for enzyme activity determination, which are selected by FIP (Federation Internationale Pharmacetique) International Commission for standardization of pharmaceutical enzymes, and proposed to be internationally accepted as standard assay methods. The enzyme analytical methods and the enzyme-activity definitions proposed by FIP are adopted by all the European national Pharmacopoeias, recently issued. The proposed monographs are enclosed.

1.3. DRY BILE

"Dry bile" is not introduced in any pharmacopoeia as a pharmaceutical substance. In the pharmacopoeias issued many years ago, fresh ox bile and extract of the ox bile can be found. The French pharmacopoeia 10th edition is the only modern pharmacopoeia which contains the monoghraph "Extract of ox bile" (1986). There is no technological data given in this monograph, but regarding the analytical procedures included, and the quality requirements to be met, it seems to be applicable to the dry bile. This monograph is proposed as the analytical prescription for the quality control of dry bile.

In accordance to the mentioned above it should be emphasized that all the prescriptions and the quality requirements cited in all pharmacopoeias are related only to ox bile, not to pig bile.

In "Hagers Handbuch der Pharmazeutischen Praxis", 4th edition, Springer-Verlag, Berlin/Heidelberg/New York, 1973, Part IV (Cl-G), p. 920-922, it is mentioned that in recent time a pig bile is also used in therapy but in L. Goodman and Gilman`s "The Pharmacological Basis of Therapeutics", 5th edition, MacMillan Publishing Co., Inc. New York, 1975, p. 971-973, is cited: the composition of an ox bile closely resembles that of humans, with respect to the bile salts, but hog bile contains hyocholic and hyodeoxycholic acids of undetermined toxicity, so it is advisable to avoid using hog bile preparations in which these foreign bile acids are found. In the last edition of the same book, L. Goodman and Gilman: "The Pharmacological Basis of Therapeutics", 8th edition, Pergamon Press Inc.,New York, 1991, p. 930-931, hyocholic acid and hyodeoxycholic acid are not mentioned among bile acids having curative properties.

2. WORKING CONDITIONS IN THE QC LABORATORY related to carrying out the quality control of the Project products

2.1. LACKING EQUIPMENT

- high speed mixer (>8000 rpm),
- graduated quick delivery pipettes,
- vacuum thermostat dryer,
- aspiration or vacuum pumps operated under the influence of water pressure,
- microburette,
- porous thimble (inner tube) for Soxhlet apparatus,
- volumetric flasks of 10 ml and 25 ml,
- exchange units for dosimat (piston burette a 1 ml and 10 ml),
- microscope eye piece equipped with a calibrated micrometer,
- microscope cover glasses and slides.

2.2. LACKING CHEMICALS

- petrol ether,
- toluen,
- tris (hydroxymethyl) aminomethane,
- disodium hydrogen phosphate x $2H_2O$,
- L-tyrosine,
- chromotropic acid,
- furfural,
- ammonium molybdate,
- copper,
- cupric sulphate.

It should be emphasized that in the quality control laboratory there are only a few pipettes and funnels, only one mortar with a pestle ...

As it was absolutely impossible to start the training in the quality control laboratory with the existing glassware, prof. Huyen, the Dean of the Faculty of Biology in Ho Chi Minh City, was asked to borrow some of the needed items. Two days later some glass stoppered erlenmayer-flasks, pipettes, funnels, and volumetric flasks were borrowed from the Faculty.

3. WORKING PROGRAMME

01	NOV93		CTA and QCE arrival - Arrangements for first meetings with counterparts and UNIDO Hanoi No 1 PRIORITY
week	Month		

18 Thursday	PM:	CTA and QCE arrival at TSN airport	
19 Friday	AM: PM:	Meeting with NPD and project staff Checking all the works performed so far at the Project site At UNPD office QCE working at QC Lab	
20 Saturday	AM: PM:	CTA meeting DICI, BASON QCE working at QC Lab Arrangements for next week meetings	NPD, NPC, HOU,NTA,HOL
21 Sunday		Free	
22 Monday		CTA: Checking the test run of equipment Discussing work programme with NPC, HOU Drafting work programme QCE training QC personnel at QC labs: Pancreatic amylase - theoretical considerations, preparation of reagents solutions, assays in different samples of pancreatin, discussion (22-23/11).	
23 Tuesday	AM: PM:	CTA meeting at project-site A.P.Nguyen van Quan, vice-director of Subinstitute for Scientific and Technical Research on Occupational Safety in HCM CTA, QCE visiting Drugs Control Subinstitute and meeting the Director Nguyen van Thi At UNIPHA office: Preben Hjortlund (UCD), Nguyen khac Tiep from UNIDO Hanoi meeting CTA about mission timing and project's subcontractors about contracts and expenditures QCE training QC personnel at QC labs	DICI, HOU, NPC Hieu (QC Lab) DICI, Bason, NPD, NPC

02	NOV93		CTA and QCE arrival - Arrangements for first meetings with counterparts and UNIDO Hanoi No 1 PRIORITY
week	Month		

24 Wednesday		CTA: Test run of cooling room, refrigerated truck, cooling water system. Temperature did not meet requirements To be tested again next day QCE training QC personnel at QC labs: Pancreatic protease - theoretical considerations, preparations of reagents solutions, determination of protease activity in different samples of pancreatin discussion (24-25/11).	
25 Thursday		CTA: Test run of cooling room, refrigerated truck, cooling water system. Redrafting workplan QCE training QC personnel at QC labs	
26 Friday		CTA: Cooling water system and refrigerated truck OK. Meeting NPD about work program QCE training QC personnel at QC labs: Assay of fat, loss on drying in different samples of pancreatin.	NPC, HOU
27 Saturday	AM: PM:	CTA: QCE training QC personnel at QC labs: Enzyme analytics: Questions - answers	
28 Sunday		Free	
29 Monday	AM: PM:	CTA: QCE training QC personnel at QC labs: Pancreatic lipase - theoretical considerations, preparation of reagents solutions; determination of lipase activity in different samples of pancreatin, discussion (29/11-01/12) Meeting project team to discuss preparations for the test run of dry bile and chymotrypsin.	QCE,HOU,NPC,QC and Biochem lab, DICI
30 Tuesday	AM: PM:	CTA: QCE training QC personnel at QC labs	
01 Wednesday	AM: PM:	CTA: QCE training QC personnel at QC labs	

03	NOV93 DEC93		No 1 PRIORITY
week	Month		

02 Thursday	AM: PM:	CTA weekly meeting QCE training QC personnel at QC labs: Chymotrypsin - theoretical considerations, preparation of reagents solutions; assay in different samples, discussion (02-03/12)	NPD,NPC,HOU (Head of Unit), DICI, BASON, CEC2 (Civ.Eng. Co. No.2), Civeng, Interpret
03 Friday			
04 Saturday	AM: PM:	CTA: QCE training QC personnel at QC labs: Enzyme analytics: Questions - answers	
05 Sunday		Free	
06 Monday	AM: PM:	CTA: QCE training QC personnel at QC labs: Dry bile - theoretical considerations, preparation of reagents solutions, assay in different samples, discussion. Microbiological purity test, histamin substances - short briefing (06-07/12)	
07 Tuesday			
08 Wednesday	AM: PM:	CTA: QCE training QC personnel at QC labs: Stability of products, in process control - GMP, protocols (08-10/12)	

NOTE: The determination of microbial contamination and histaminic substances is not included in the working progaramme, since it was absolutely impossible to perform this kind of examination at the Project site. The determination of microbial impurities should be carried out in especially equipped microbiological laboratory, and the histaminic substances can be assayed in a biological lab, especially equipped for such type of analysis.

Lipase activity assay, and dry bile examination were included in the working programme, because at the time of drawing up this Programme there were some possibilities of a purchase or a loan of a mixer and the needed chemicals to the Project site. In spite of the QCE's requests repeated every day, nothing of that was realized.

3.1. WORK PERFORMED AT THE QC LAB

Realization in QC labs:

Pancreatic amylase assay - theoretical consideration and practically carrying out the examination of the Pancreatin-Project product, according to the proposed monograph "Pancreatis pulvis".

Pancreatic protease assay - theoretical considerations and practically carrying out the examination of the Pancreatin-Project product according to the proposed monograph "Pancreatis pulvis".

Pancreatic lipase assay - only theoretical considerations of the assay method cited in the monograph "Pancreatis pulvis". All details of analysis were discussed with the stress on the substrate (the quality of the olive oil, the way of preparing the emulsion, control of the size of the droplets by the microscope), and the quality of sodium taurocholate. PH-stat potentiometric titration was discussed as the best way of following of the reaction rate.

Loss on drying - practically carried out the examination of the Pancreatin-Project product according to the prescription in the proposed monograph "Pancreatis pulvis".

Fat in pancreatin - practically carried out the examination of the Pancreatin-Project product according to the prescription in the monograph "Pancreatis pulvis".

The results of analysis:
Sample being examined: Pancreatin, Project product batch 040993.
Analytical procedure: "Pancreatis pulvis", Ph. Eur. 2nd (Annex 2.a.).
- Amylase activity found: 36 FIP U/mg, (requirement: not less than 12 FIP U/mg),
- Protease (total) activity found: 1.9 FIP U/mg, (requirement: not less than 1 FIP U/mg),
- Loss on drying found: 6.5%, (requirement: not more than 5%),
- Fat content found: 1.54%, (requirement: not more than 5%).

The cited results of amylase activity and total protease activity are the average values of the three analysis. Enzyme activities are determined in comparison of the sample examined with Pancreatin "Merck" used as the house standard. It should be emphasized that enzyme activities of Pancreatin "Merck" are not checked in comparison with the official FIP standards.

The cited result of the loss on drying is the average value of two determinations.

Chymotrypsin assay - theoretical consideration and practically carrying out the assay according to the proposed monograph "Chymotrypsinum", Ph. Eur. 2nd. The examination was performed on Chymotrypsin substance "Collectorgane" France, because there was no possibility of examining the Project product (semi product in zymogene form).

The results of analysis:

1. Sample being examined: Chymotrypsin "Collectorgane".
Analytical procedure: "Chymotrypsinum", Ph. Eur. 2nd (Annex 2.b.).
Chymotrypsin activity found: 4.8 μkat/mg, (requirement: not less than 5 μkat/mg).

Enzyme activity is determined in comparison of the sample examined with Chymotrypsin "Merck" used as the house standard. The enzyme activity of the house standard is not checked in comparison with the official FIP standard.

2. Sample being examined: Chymotrypsin tablets (declaration: 21 μkat/1 tablet)
Analytical procedure: written by QCE (for training the QC-Personnel)
Chymotrypsin activity found: about 15 μkat/1 tablet which makes about 70 per cent of the declared amount (requirement: 90-110 per cent).

The cited result is the average value of two analysis. The result is too low. Activity is determined in comparison with the house standard Chymotrypsin "Merck" whose activity is not checked in comparison with the official FIP standard. Here, we should have in mind that the sample examined are tablets (not enteric coated), packed in blister and stored under room conditions (it means at 28° to 40°C and very high relative humidity), so, the loss of enzyme activity in tablets is expected.

Dry bile - theoretical consideration only. Because of the lack of the needed chemicals there was no possibility to practically perform the examination of the Project product of Dry bile. (The results of analysis performed later in Zagreb are cited on page 18).

Stability - an establishment of expiry date and shelf life on the basis of the stability tests related to storage conditions was discussed. It was emphasized that the respective environment conditions have an essential influence on the stability of the products. In the case of insufficient microbial purity, the stability of the products can be impaired by growth of microorganisms. Unsuitable packaging shortens the stability of the products as well. It was stressed that the following GMP rules proposed by WHO in all the steps of the production is of great importance for improving stability of the products. It was suggested to monitor the stability of the Project products (on 3 batches) stored under the storage conditions.

Suggested storage conditions:
- Pancreatin - store in an airtight container, at a temperature below 15°C (Ph. Eur. 2nd),
- Chymotrypsin - store in an airtight container, at 2°C to 8°C, protected from light (Ph. Eur. 2nd),
- Dry bile - store in an airtight container, equipped with a suitable desiccant, protected from light (Österreichisches Arzneibuch, Amtliche Ausgabe, 1981).

For the reason of checking the stability of the products, the relevant parameters should be examined after 14 days, 1 month, 2 months, 4 months, 6 months, 12 months, ... of storage.

The relevant parameters, which have to be checked are: characteristics of the substances (visually checked), microbial purity, loss on drying, enzyme activities, content of the active ingredients.

Microbiological purity tests - theoretical considerations with the stress on the microbial norms for medicaments and Ph. Eur. 2nd prescriptions.

The significance of microorganisms in pharmaceutical products was discused with the emphasis being placed especially on raw materials of animal origin, which may contain microorganisms not destroyed during subsequent processing. Microbial limits for certain categories of products incorporated in specific monographs are considered. It was suggested that the Project products Pancreatin, Chymotrypsin, Trypsin and Dry bile be tested for the total viable count of microorganisms, and for some specified microbial contaminants, at least for Escherichia coli and Salmonella. The examination should be performed according to the procedures Ph. Eur. 2nd (V.2.1.8.) using culture media cited in Ph. Eur. 2nd (VIII.10.), (Annex 3) or equivalent.

Requirements: the Project products Pancreatin, Chymotrypsin and Dry bile should comply with a limit for the total viable aerobic count of 10^4 microorganisms per gram, determined by a plate count. The products are required to comply with the tests for Escherichia coli and Salmonella (it means that they should be free of E. coli and Salmonella).

The proposed schemes of the microbial purity examinations:

TOTAL VIABLE AEROBIC COUNT

Dissolve 10g of the sample being examined in a buffered sodium chloride-peptone solution pH 7.0 (Ph.Eur. 2nd, VIII.10), adjust the volume to 100 ml with the same liquid, and prepare the following dilutions using the same liquid according to the scheme:

10g → 100ml ..... (sample dilution 1 in 10)
↓
1ml → 10ml ..... (sample dilution 1 in 100)
↓
1ml → 10ml ..... (sample dilution 1 in 1000)
↓
1ml → 10ml ..... (sample dilution 1 in 10000)

Using Petri dishes 9cm to 10 cm in diameter, add to each dish a mixture of 1ml of the prepared sample dilution and about 15ml of the liquified medium B (Casein soya bean digest agar) heated to no more than 45°C. Prepare at least two such Petri dishes using the same dilution and incubate at 30°C to 35°C for 5 days, unless a more reliable count is obtained in a shorter time. Count the number of the colonies which develop. Calculate the results using the plates with the greatest number of colonies, but regarding 300 colonies per plate as the maximum consistent with good evaluation.

ENTEROBACTERIACEAE, ESCHERICHIA COLI, SALMONELLA

10g sample → 100ml medium D (Lactose Broth) and incubate the mixture at 35°C to 37°C for 2 to 5 hours

ENTEROBACTERIACEAE

10ml → 100ml of medium E (Broth acc, to Mossel) and incubate at 35°C to 37°C for 18 to 48 hours

Subculture on the plates of agar medium F (VRBD AGAR acc. to Mossel) and incubate at 35°C to 37°C for 18 to 24 hours
The product passes the test if there is no growth of colonies of gram-negative bacteria on any plate.

ESCHERICHIA COLI

10ml → 100ml medium G (mac CONKEY broth) and incubate at 43°C to 45°C for 18 to 24 hours
Subculture on agar medium H (mac CONKEY AGAR), and incubate at 43°C to 45°C for 18 to 24 hours.
Growth of red, generally non-mucoid colonies of gram-negative rods, sometimes surrounded by a reddish precipitation zone, indicates the possible presence of Escherichia coli. This has to be confirmed by the formation of indol at 44±0.5°C (appearance of red colour as a result of the reaction of E. coli colonies with indol-reagent, KOVACS-INDOLREAGENZ, MERCK,art. nr.9293; Merck Catalogue 1992/93, or by other biochemical reactions).

SALMONELLA

10ml → 100ml medium I (Tetrathionate Brilliant-green Bile Enrichment Broth) incubate at 42°C to 43°C for 18 to 24 hours.

Subculture on agar medium J (Leifson Agar = Deoxycholate citrate Agar) and incubate at 35°C to 37°C for 24 to 48 hours
The probable presence of salmonellae is indicated by well-developed, colourless colonies.

Subculture on agar medium K (XLD Agar) and incubate at 35°C to 37°C for 24 to 48 hours.
The probable presence of salmonellae is indicated by well-developed red colonies with or without black centres.

The provisionally confirmation of salmonellae: transfer separately a few suspect colonies to agar medium M (Triple sugar iron Agar) in tubes, using surface and deep inoculation. The presence of salmonellae is provisionally confirmed if in the deep inoculation, but not in the surface culture, there is a change of colour from red to yellow, and usually a formation of gas, with or without production of hydrogen-sulphide in the agar.
The precise confirmation of salmonellae may be carried out by the appropriate biochemical and serological tests (SALMOSYST BASISBOUILLON, art. nr.10153; SALMOSYST SELECTIV-SUPPLEMENT, art. nr.10141, MERCK CATALOGUE 1992/93).

IN-PROCESS CONTROL

In process control embraces all the checks performed during production in order to monitor and, if necessary, to adjust the process, to ensure that the product conforms to its specifications. The control of the environment or equipment may also be regarded as a part of in-process control.

In-process control suggested bel ow is directed primarily to ensure that the products be consistently produced, and to diminish the risks of cross-contamination by unexpected contaminants, and mix-ups caused by false labels being put on containers of chemicals.

The efficacy of the separations of proteins on the basis of different solubility, that means the efficacy of the isolation of enzymes as well, depends on the efficiency in adjusting the essential parameters: pH, temperature, and salt concentration. So, monitoring of these parameters is the most important part of the in-process control in the production of enzymes.

CHYMOTRYPSIN production (in-process control)

- check of glands (checked by a veterinarian),

- check of sulphuric acid 0.25 N

  Identification tests:

  1) solution yields with barium chloride (5% in water) a white precipitate, which is insoluble in hydrochloric acid or nitric acid.

  2)hydrochloric acid produces no precipitate when added to solution of the sulphate.

  Assay: titration with 1N-NaOH using methyl-red as the indicator.

  Each ml of 1N-NaOH is equivalent to 0.04904g of H_2SO_4.

- check of water: (purified water should be used)

  - water should conform to the standard for microbial purity required for purified or drinking water.

  - pH of water should be 5.0-7.0 (procedure: 0.3ml of saturated aqueous solution of potassium chloride is added to 100ml of examined water and check the pH value, using pH-meter)

  - water must be free of heavy metals (procedure: 1ml of acetic acid (30%) is added to 40ml of water heated to 50°C, and then 10ml of freshly prepared hydrogen sulphide saturated solution should be added, and the mixture be allowed to stand for 10 minutes. The colour of the liquid should be no darker than 50ml of the same water.

  - water should comply with the test for oxydisable substance (procedure: 5ml of sulphuric acid (16%) is added to 100ml of water, and then 0.1ml of 01N-$KMnO_4$ solution should be added and the mixture boiled 5 minutes. The solution must remain faintly pink.).

-check of ammonium sulphate:

  Identification:

  1) by the addition of an excess of sodium-hydroxide (15%), the salt is decomposed, with the evolution of ammonia, recognizable by its odour, and by its alkaline effect upon moistened red litmus paper exposed to the gas. Warming of the solution,accelerates the decomposition

2) aqueous solution of ammonium sulphate (3%) yields with barium chloride (5%) a white precipitate, which is insoluble in hydrochloric acid or nitric acid.

- check pH, temperature, and ammonium-sulphate concentration in each production stage, closely follow step by step the written instructions cited in the technological procedure.

- the control of the moisture content in the product, should be done at the final step of the production (determination of loss on drying at 60°C in vacuum during 2 hours). Requirement: not more than 5% of moisture.

- check of the yield.

- control of the cleanliness of the equipment used in the production which should be washed according to the instructions given in the technological procedure.

PANCREATIN production (in-process control)

- check of pancreas (checked by a veterinarian),

- check of sodium bicarbonate:

Identification:

1) aqueous solution (3%) effervesces by the addition of hydrochloric acid.

2) the colour of freshly prepared aqueous solution (3%) by the addition of phenolphtalein solution remains unchanged, or is only slightly coloured.

3) sodium imparts an intensive yellow colour in a nonluminous flame.

- check of acetone:

Identification: warm mixture of 1ml of an aqueous dilution of acetone (1:200) and 1ml of sodium hydroxide yields by addition a few ml of iodine solution, a yellow precipitate of iodoform.

- check pH, temperature and time of the duration of each single procedure-stage, closely follow step by step the written instructions cited in the technological procedure.

- control of the moisture content in the product at the final step of the production (determination of loss on drying at 60°C in vacuum, 4 hours). Requirement: not more than 5% of moisture.

- check of the yield.

- control of cleanliness of the equipment used in the production and washed according to the written instructions given in the technological procedure.

DRY BILE production (in-process control)

- check of the bile collected (checked by a veterinarian),

- check of the formalin (=formaldehyde):

  Identification:

  - dilute 2 ml of formaldehyde solution with 10ml of water in a test tube, and add 1ml of silver-ammonium-nitrate solution. Metallic silver is produced either in the form of gray precipitate, or as a bright, metallic mirror on the sides of the test tube.

- check of the time and temperature of the production stages, following strictly the instructions given in the technological procedure.

- control of the moisture content in the product at the final step of the production (determination of loss on drying at 100°C to the constant weight). Requirement: not more than 6% of moisture.

- check of the yield.

- control of cleanliness of the equipment used in the production and washed according to the written instructions given in the technological procedure.

Each in-process control should be recorded, and the records maintained as a part of the batch records.

GMP, PROTOCOLS

Basic principles of GMP were discussed. Quality assurance-problems in connection with the control-laboratory equipment and instruments, washing and cleaning of the equipment, validation and calibration of the analytical apparatus, reagent-chemicals-quality and reference-standards were discussed. Performed analytical tests should be recorded.

Analysis records should include the following data:
- the name of the sample being examined,
- the animal origin of the sample being examined,
- the batch number and the date of the production,
- the relevant specification, and testing procedure,
- the test results:

a) description: characteristics - odour, colour ..., observations and requirements.
b) identification: the tests performed, observations and requirements.
c) purity tests: the tests performed, observations and requirements.
d) assay: the method performed, analytical data, calculations and the limits required.
e) remarks
f) the date of testing
g) conclusions: clear statement whether the sample being examined meets or does not meet the requirements cited in the testing procedure mentioned above.
h) the name of the responsible analyst.

OTHER QCE ACTIVITIES at the Project site

1) ANALITYCAL PROCEDURES for pharmaceutical formulations.
QCE was asked by the analyst in QC lab to prepare an analytical procedure for the quality control of chymotrypsin tablets at the Project site. QCE explained that for the analytical procedure to be correctly prepared it is necessary that the responsible analyst knows the exact composition of the drug, not only related to the active ingredients, but also to all the inactive excipients. Some relevant technological data have to be known as well. In some cases, because of the excipients contained, and the technology used, certain modifications in the analytical procedure may be needed. Each analytical procedure should be verified through the experimentally obtained analytical results of certain exactly known pharmaceutical preparation. These results should be compared with the analytical results obtained for the same preparation, using some other analytical procedure.
QCE prepared a provisional analytical procedure for chymotrypsin tablets (not enteric coated) just for the training of the analysts.

2) PARTICIPATION AT THE MEETINGS WITH CTA, NPD, DICI and the other experts at the Project site

Taking part at the meetings in discussions related to the arrangements for the beginning of the production of chymotrypsin and dry bile, QCE called attention to:

- **inadequate quality of tape water** used in the production, and suggested the use of demineralized or/and distilled water. An appropriate microbial purity of water was required.

- **washing and cleaning procedure** for the production equipment. It should be incorporated in the written technological procedure.

- applicability of the **infected animal-start materials** for the production. Parasites contained, as Eurytrema pancreaticum, Fasciola gigantica and Fasciola haepatica are dangerous for humans. According to prof. Wickerhouser, former Head of the tropic parasitology Department, the Faculty of Veterinary Medicine at the University of Zagreb, pancreas infected by flukes can not be used for processing of drugs as enzymes unless it is carefully cleaned from the flukes. Fasciola heapatica and Fasciola gigantica are contained in bile. In the opinion of the Project veterinarian, these flukes can be dangerous for the workers. The Project veterinarian was asked to find out the method of identification of the flukes. QCE promised to consult some experts at the Faculty of Veterinary Medicine at the University in Zagreb.

- lack of fire-extinguishers,
an emergency telephone,
warning signs,
first-aid kits and first-aid written instructions.

QCE ACTIVITIES in Zagreb, upon the arrival from the mission in Vietnam

- **Infected animal raw material** by Eurytrema pancreaticum, Fasciola haepatica and Fasciola gigantica can be used for the production.
QCE consulted Dr. Nikola Džakula, the professor at the Department for parasitology and invasive diseases at the Faculty of Veterinary Medicine in Zagreb. According to Dr. Džakula, infected animal raw material is not directly a danger to humans. The reason for such a statement is the life cyclus of these species: the landsnails serve as the first intermediate hosts. Two generations of sporocytes occur in the snails, the second producing cercariae about five months after the infection. Cercariae are extruded onto herbage and are eaten by grasshoppers and crickets, which serve as secondary intermediate hosts. Here metacercariae occur in the haemocele, becoming infective three weeks after the infection. Humans, just like cattle, can be infected while eating herbage with metacercariae.

- **Dry bile**
Dry bile (laboratory scale Project product) was not analysed during QCE's stay at the Project site, for the reason of the lack of the chemicals needed.
A small sample of this product QCE has brought with her, and she tried to analyse it in Zagreb:

Analysis

sample being examined: Dry bile Project-product.
comparative sample: Fel bovis siccum "Kali Chemie"
analytical procedure: monograph "Extractum fellis bovis", 1986, Ph. Franc. 10th edition.

- Identification (TLC): seven separated spots appeared on the chromatogram of the sample being examined, (requirement: at least six separated spots coloured from yellow to blue should appear on the chromatogram of the sample being examined).

Note: On chromatogram plate parallel with the Project-sample being examined, cholic acid and comparable sample - Fel bovis siccum "Kali Chemie" were run. The differences between the sample being examined and the comparable sample can be seen regarding the Rf-values and colour of the separated spots. There was no spot of cholic acid on the chromatogram of the Project-sample being examined.

- Bile acids (expressed as cholic acid) content found: less than 5 per cent, (requirement: 45 to 55 per cent).

Note: Bile acids are determined spectrophotometrically measuring the colour produced in reaction of bile acids with furfural. Hyodeoxycholic acid, lithocholic acid and different keto-acids and 7-ketosteroids as well,do not give the colour reaction with furfural (cited in B. Kakač: Handbuch der photometrischen Analyse organischer Verbindungen. Verlag Chemie GmBH, Weinheim/Bergstr. 1974, Volume 2, p. 1061)

- Loss on drying found: 19%, (requirement: not more than 6%),
- Sulphated ash found: 11.5%, (requirement: not more than 35%),
- Indol, scatol, tryptophane: meets the requirement, (requirement: negative reaction with xanthydrol),
- Substances insoluble in 80%-alcohol found: 4%, (requirement: not more than 2%),
- Microbial purity: meets the requirements, (requirements: not more than 10^4 total viable aerobic microorganisms per gram; free of Escherichia coli and Salmonella).
- Formaldehyde: meets the requirement, (requirement: less than 0.2%).

All the examinations were performed twice.
The examinations of cholesterol content, and reducing sugars after hydrolyse, are not performed because there is no sample examined left.

On the basis of the obtained results, the following comments can be made:
- the examined sample had not been dried enough, and it had not been stored properly, so the bile acids might be decomposed, or
- the examined sample was prepared from pig bile, and not from ox bile, and so the possibility is that the examined product can not be analysed according to the analytical procedure prescribed for the control of ox bile, and can not meet the quality control requirements of ox bile as well.
It should be emphasized here that ox bile (Fel bovis) is the only official bile in pharmacopoeias, and so all the analytical procedures and quality control requirements are set for ox bile. Taking into consideration all these facts, the reexamination is needed. Only from the examination of both dry bile samples (one prepared from ox bile, and one prepared from pig bile), it can be seen where the problem is.

FINDINGS, ACHIEVEMENTS, RECOMMENDATIONS FOR THE FUTURE ACTION

1 FINDINGS

- scanty and inadequate equipment of the QC labs,
- lack of the essential apparatus and glassware,
- lack of chemicals and the official standard-substances (FIP controlled standards, and preparations),
- lack of GLP in QC lab (in using and maintaining of laboratory equipment).

> **Note**:
> In such operational conditions, it was not possible to achieve the required accuracy and reproducibility of the analytical results, despite of the efforts made by pharmacists included in the QC-training.

- Some parts of the production-equipment were partly corroded before being used in the production.
- Capacity of the distil-water apparatus was too small, for all the needs at the Project site to be settled.

2 ACHIEVEMENTS

- introduction of the quality control procedures for the assay of alpha amylase, total protease, loss on drying and fat in pancreatin were accepted.
- the quality control procedure for the assay of the activity of chymotrypsin was accepted.
- the ways of monitoring the stability and proposed analysis-recording were accepted,
- the following relevant literature was given:
  - R. Ruyssen: "Pharmaceutical enzymes", E. Story-Scientia, Gent, Belgium, 1978, 256 pages.
  - B. Deasy: "The Quality control of medicines", Elsevier/North-Holland Biomedical Press, Amsterdam, 1976, 398 pages.
  - WHO REPORT thirty-second, Geneva 1992: "WHO expert committee on specifications for pharmaceutical preparations", 133 pages.
  - WHO REPORT forty-second, Geneva 1992: "WHO expert committee on biological standardization", 85 pages.

3 RECOMMENDATIONS FOR FUTURE ACTION

QCE proposes the following actions to be taken:

1) completing the equipment and the chemicals in QC labs, so that the reliability of analytical results can be achieved,

2) introducing the GLP in QC labs - cleaning and maintaining the equipment, evaluation of the analytical procedures,

3) purchase of FIP controlled standards:
lipase (pancreatic),
protease (pancreatic),
amylase (pancreatic),
chymotrypsin
trypsin,
purchase of FIP controlled preparations:
amylum solubile,
casein,
enterokinase,
sodium taurocholate,

4) checking the reliability of the analytical procedures (checking the precision and the accuracy of the employed methods under QC-lab conditions).
- assaying the exact activity of, so called, "house" standard substances to be used in every day work.

5) introducing the quality control procedures for the assaying lipase and trypsin.

6) performing the examinations of the Project products - chymotrypsin and pancreatin (pilot--plant produced) and evaluating them as pharmaceutical raw materials.

7) performing the examinations of the dry bile, checking the proposed analytical procedure and the quality requirements; making corrections if necessary. Evaluating dry bile as a pharmaceutical raw material.

8) introducing the quality control procedures for certain pharmaceutical formulations, including methods for the determination of laboratory equivalence of parallel products.

9) furnishing the microbiology-lab with the needed equipment and introducing the methods for the determination of microbial purity of products.

SUMMARY

In the frame of the Project DP/VIE/86/016 the manufacture of Dry bile, Chymotrypsin and Trypsin, and Pancreatin has to start on a pilot plant scale, and the adequate quality-control has to be established.

The Project products are intended to be pharmaceutical substances used in drug-production, and they should meet the pharmacopoeia's quality requirements.

Conformably to this fact, the choice of suitable analytical procedures for the quality control of these substances is made. All the needed analytical procedures, and respective quality requirements for the up to date quality-control of Pancreatin are included in the monograph "Pancreatis pulvis", Ph. Eur. 2nd edition, and it is proposed to be accepted as its quality control procedure. The Pancreatin, Project-product, batch 040993 is examined according to that procedure, and it is found that it meets the requirements set up for amylase-activity, total protease-activity, and fat-content in pancreatin, but loss on drying found, is somewhat higher than allowed. The found, total protease activity is about twice, and amylase activity is three times stronger than are the respective required minimums. The other prescribed examinations (lipase activity, microbial purity) can not be performed at the Project site because of the lack of the equipment and chemicals needed.

The Project-product "Chymotrypsin and trypsin" produced according to the existing technological procedure is a crude mixture of chymotrypsin and trypsin, in zymogene form. As such, a semi product, it can not be considered as a pharmaceutical substance. Having in mind the inclusion of additional technological steps, which would enable that separated and sufficiently pure enzymes chymotrypsin and trypsin be obtained, the quality-control procedures are chosen. So, the monograph "Chymotrypsinum", Ph. Eur. 2nd edition, is proposed for chymotrypsin. The method is included in the training of the Project personnel, carrying out at the Project site, using the Chymotrypsin substance "Collectorgane" France, as a sample to be examined. By the results obtained (4.8 μkat/mg), the chymotrypsin-activity of the sample examined meets the requirement. The chymotrypsin tablets taken from the local market were examined as well, according to the analytical procedure written by QCE. The found enzyme activity is about 70 per cent of the declared activity. The tablets are packed in blister, and stored at room temperature - it means at temperature from 28°C to 40°C and very high per cent of relative humidity, so the loss of activity is probable.

According to the technological procedure for Dry bile, pig bile can be used instead of cattle bile as raw material. The monograph "Extractum fellis bovis", Ph. Franc. (1986), is proposed as an appropriate quality-control procedure for ox Dry bile. Examining the Project-product "Dry bile" manufactured from pig bile (laboratory scale), the results obtained do not meet the quality requirements set up in the monograph cited above. The analysis is performed using Fel bovis siccum "Kali Chemie" as a comparative sample. On TLC plate, there were seven spots separated on the chromatogram of the sample examined, but their Rf-values and colours differ from those of the spots of the comparative sample. There is no spot of cholic acid on the chromatogram of the Project sample examined. The result of the assay of bile acids (free and conjugated) in Dry bile (Project-product) is less than 5%. Beside the possible decomposition of bile acids in the Project-product caused by moisture, the analytical results point at the differences between pig bile and cattle bile. Hyodeoxycholic acid contained in pig bile does not give a positive colour reaction with furfural (Pettenkofer reaction), while bile acids contained in ox bile react with furfural producing blue colour. Namely, bile acids are determined spectrophotometrically measuring the colour produced in reaction of bile acids with furfural. Besides, there are no data in technical papers about curative properties of pig bile acids in human use, but a warning can be found saying that it is advisable to avoid the use of pig bile because of the contained foreign bile acids of undetermined toxicity.

Raw material eventually infected with Eurytrema pancreaticum, Fasciola hepatica and Fasciola gigantica, is not directly dangerous for humans because the life cyclus of the parasites concerned makes that way of human infection impossible.

UNITED NATIONS INDUSTRIAL DEVELOPMENT ORGANIZATION
UNIDO

Project in the Government of Vietnam
Job Description

DP/VIE/86/016/11-03

Post Title: Quality control Expert

Duration: 1 man/month

Date required: asap

Duty Station: Ho Chi Minh City, Vietnam

Purpose of Project: Establishment of a production unit for manufacture of enzymes, hormones and other bioactive substances in UNIPHA from by-products obtained from slaughterhouses of Ho Chi Minh City.

Duties: The Quality control expert will be expected to carry out the following duties:

1. Organize and deliver training courses at the project in quality control of bioactive substances in bulk as well as that of the final formulations.

2. Introduce modern quality control techniques for bioactive substances (enzymes, hormones, etc.) as starting raw materials and final products, and assist in the implementation and the start-up of laboratory facilities.

3. Prepare quality control protocols for each product, and participate jointly with the CTA, PTE and DICI, in the preparation of maintenance and operation manual.

4. Prepare a technical report on the modern quality control techniques for bioactive substances.

5. Prepare a detailed report on his findings, achievements and recommendations for future action.

28 June 1993

Quality Control Expert Work Programme for Four Weeks

First week: Introduction of the up-to-date methods of the quality control of the pancreatin enzymes, protease, emylase and with stress to lipase, then chymotrypsin and trypsin, and dry bile. Training course for the local personnel using standard enzyme preparations with different dilutions.

Second week: Quality control tests of enzymes in raw materials from the "Vissan" slaughterhouse, pancreas of pigs and cattle, and cholic acid and dry matter in bile of pigs and cattle. Training .. . of the local personnel.

Third and fourth week: Quality control of the final products received by processing of pancreatin, dry bile, chymotrypsin and trypsin on a laboratory and pilot plant scale. Quality control of semi products and raw products are included to reach the quality assurance.

Fourth week: Preparation of everyday quality control protocols for each one of raw materials, semi products and final productschecked. Microbiological tests of final products, pancreatin, dry bile, chymotrypsin and trypsin performed at the Government Drug Quality Control Institute in HoChiMing City.

Agreed Mr. Tran Tuu, NPD

Prof.O.Scedrov, CTA

EUROPEAN PHARMACOPOEIA

SECOND EDITION

Printed and published by
MAISONNEUVE S.A.
57 - Sainte-Ruffine - France
1980

PANCREATIS PULVIS

Pancreas Powder

Pancreas powder is prepared from the fresh or frozen pancreases of mammals. It contains various enzymes having proteolytic, lipolytic and amylolytic activities.

1 milligram of pancreas powder contains not less than 1.0 European Pharmacopoeia Unit (Ph. Eur. U.) of total proteolytic activity, 15 European Pharmacopoeia Units of lipolytic activity and 12 European Pharmacopoeia Units of amylolytic activity.

Pancreas powder is prepared in conditions designed to minimise the degree of microbial contamination.

CHARACTERS

A slightly brown, amorphous powder with a faint characteristic odour, partly soluble in water, practically insoluble in alcohol and in ether.

IDENTIFICATION

A. Triturate 0.5 g with 10 ml of water and adjust to pH 8 by the addition of 0.1N sodium hydroxide, using 0.1 ml of cresol red solution R as indicator. Divide the suspension into two equal parts (suspension (a) and suspension (b)). Boil suspension (a). To each suspension add a few particles of fibrin congo red R, heat to 38 °C to 40 °C and maintain at this temperature for 1 h. Suspension (a) is colourless or slightly pink and suspension (b) is distinctly more red.

B. Triturate 0.25 g with 10 ml of water and adjust to pH 8 by the addition of 0.1N sodium hydroxide, using 0.1 ml of cresol red solution R as indicator. Divide the suspension into two equal parts (suspension (a) and suspension (b)). Boil suspension (a). Dissolve 0.1 g of soluble starch R in 100 ml of boiling water, boil for 2 min, cool and dilute to 150 ml with water. To 75 ml of the starch solution add suspension (a) and to the remaining 75 ml add suspension (b). Heat each mixture to 38 °C to 40 °C and maintain at this temperature for 5 min.

To 1 ml of each mixture add 10 ml of iodine solution R2. The mixture obtained with suspension (a) has an intense blue-violet colour; the mixture obtained with suspension (b) has the colour of the iodine solution.

TESTS[1]

Fat content In an extraction apparatus, treat 1.0 g with light petroleum R1 for 3 h. Evaporate the solvent and dry the residue at 100 °C to 105 °C for 2 h. The residue weighs not more than 50 mg (5.0 per cent).

Loss on drying (V.6.22) Not more than 5.0 per cent, determined on 0 50 g by drying at 60 °C at a pressure not exceeding 670 Pa (5 Torr) for 4 h.

ASSAY

Total proteolytic activity The total proteolytic activity of pancreas powder is determined by comparing the quantity of peptides non-precipitable by a 5 per cent *m/V* solution of trichloroacetic acid R released per minute from a substrate of casein solution[2] with the quantity of such peptides released by pancreas powder (protease) BRP from the same substrate in the same conditions.

For the test suspension and the reference suspension, prepare the suspension and carry out the dilution at 0 °C to 4 °C.

Test suspension Triturate 0.100 g of the substance to be examined for 5 min adding gradually 25 ml of 0.02M calcium chloride R. Transfer completely to a volumetric flask and dilute to 100.0 ml with 0.02M calcium chloride R. To 10.0 ml of this suspension add 10.0 ml of enterokinase solution[3] and heat in a water-bath at 35 ± 0.5 °C for 15 min. Cool and dilute with borate buffer solution pH 7.5 R at 5 ± 3 °C to a final concentration of about 0.065 Ph. Eur. U. of total proteolytic activity per millilitre calculated on the basis of the stated activity.

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(1) National authorities may require that pancreas powder comply with a limit for total viable count of 10^4 micro-organisms per gram, determined by plate count (V.2.1.8.1). National authorities may require that pancreas powder comply with the tests for *Escherichia coli* and *Salmonella* (V.2.1.8.2).

(2) *Casein solution* Suspend a quantity of casein BRP equivalent to 1.25 g of dried substance in 5 ml of water, add 10 ml of 0.1N sodium hydroxide and stir for 1 min. (Determine the water content of casein BRP prior to the test by heating at 60 °C *in vacuo* for 4 h.) Add 60 ml of water and stir with a magnetic stirrer until the solution is practically clear. Adjust to pH 8.0 with 0.1N sodium hydroxide or 0.1N hydrochloric acid. Dilute to 100.0 ml with water. Use the solution on the day of preparation.

(3) *Enterokinase solution* Dissolve 50 mg of enterokinase BRP in 0.02M calcium chloride R and dilute to 50.0 ml with the same solvent. Use the solution on the day of preparation.

Reference suspension Prepare a suspension of pancreas powder (protease) BRP as described for the test suspension but without addition of enterokinase so as to obtain a known final concentration of about 0.065 Ph. Eur. U. per millilitre calculated on the basis of the stated activity.

Designate tubes in duplicate T, T_b, S_1, S_{1b}, S_2, S_{2b}, S_3, S_{3b}; designate a tube B.

Add borate buffer solution pH 7.5 R to the tubes as follows:

B: 3.0 ml,

S_1 and S_{1b}: 2.0 ml,

S_2, S_{2b}, T and T_b: 1.0 ml.

Add the reference suspension to the tubes as follows:

S_1 and S_{1b}: 1.0 ml,

S_2 and S_{2b}: 2.0 ml,

S_3 and S_{3b}: 3.0 ml.

Add 2.0 ml of the test suspension to tubes T and T_b.

Add 5.0 ml of a 5.0 per cent *m/V* solution of trichloroacetic acid R to tubes B, S_{1b}, S_{2b}, S_{3b} and T_b. Mix by shaking.

Place the tubes and the casein solution in a water-bath at 35 ± 0.5 °C. Place a glass rod in each tube. When temperature equilibrium is reached, add 2.0 ml of the casein solution to tubes B, S_{1b}, S_{2b}, S_{3b} and T_b. Mix. At time zero, add 2.0 ml of casein solution successively and at intervals of 30 s to tubes S_1, S_2, S_3 and T. Mix immediately after each addition. Exactly 30 min after addition of the casein solution, taking into account the regular interval adopted, add 5.0 ml of a 5.0 per cent *m/V* solution of trichloroacetic acid R to tubes S_1, S_2, S_3 and T. Mix. Withdraw the tubes from the water-bath and allow to stand at room temperature for 20 min.

Filter the contents of each tube twice through the same suitable filter paper(1) previously washed with a 5.0 per cent *m/V* solution of trichloroacetic acid R, then with water and dried.

A schematic presentation of the above operations is shown in the Table below.

(1) A suitable filter paper complies with the following test: filter 5 ml of a 5.0 per cent *m/V* solution of trichloroacetic acid R on a 7 cm disc of white filter paper; the absorbance (V.6.19) of the filtrate, measured at 275 nm using unfiltered trichloroacetic acid solution as the compensation liquid, is less than 0.04.

	Tubes								
	S_1	S_{1b}	S_2	S_{2b}	S_3	S_{3b}	T	T_b	B
Buffer solution	2	2	1	1			1	1	3
Reference suspension	1	1	2	2	3	3			
Test suspension							2	2	
Trichloroacetic acid solution		5		5		5		5	5
Mix		+		+		+		+	+
Water-bath, 35 °C	+	+	+	+	+	+	+	+	+
Casein solution		2		2		2		2	2
Mix		+		+		+		+	+
Casein solution	2		2		2		2		
Mix	+		+		+		+		
Water-bath, 35 °C, 30 min	+	+	+	+	+	+	+	+	+
Trichloroacetic acid solution	5		5		5		5		
Mix	+		+		+		+		
Room temperature, 20 min	+	+	+	+	+	+	+	+	+
Filter	+	+	+	+	+	+	+	+	+

Measure the absorbance (V.6.19) of the filtrates at 275 nm using the filtrate obtained from tube B as the compensation liquid.

Correct the average absorbance values for the filtrates obtained from tubes S_1, S_2 and S_3 by subtracting the average values obtained for the filtrates from tubes S_{1b}, S_{2b} and S_{3b} respectively. Draw a calibration curve of the corrected values against volume of reference suspension used.

Determine the activity of the substance to be examined using the corrected absorbance for the test suspension ($T - T_b$) and the calibration curve and taking into account the dilution factors.

The test is not valid unless the corrected absorbance values are between 0.15 and 0.60.

Lipolytic activity The lipolytic activity is determined by comparing the rate at which a suspension of pancreas powder hydrolyses a substrate of olive oil emulsion (1) with the rate at which a suspension of pancreas powder (amylase and lipase) BRP hydrolyses the same substrate under the same conditions. *The test is carried out under nitrogen.*

Apparatus Use a reaction vessel of about 50 ml capacity provided with:

— a device that will maintain a temperature of 37 ± 0.5 °C,

— a magnetic stirrer,

— a lid with holes for the insertion of electrodes, the tip of a burette, a tube for the admission of nitrogen and the introduction of reagents.

An automatic or manual titration apparatus may be used. In the latter case, the burette is graduated in 0.005 ml and the pH-meter is provided with a wide reading scale and glass-calomel electrodes. After each test the reaction vessel is evacuated by suction and washed several times with water, the washings being removed each time by suction.

Test suspension In a small mortar cooled to 0 °C to 4 °C, triturate carefully a quantity of the substance to be examined equivalent to about 2500 Ph. Eur. U. of lipolytic activity with 1 ml of cooled maleate buffer solution pH 7.0 R (lipase solvent) until a very fine suspension is obtained. Dilute the suspension with cold maleate buffer solution pH 7.0 R, transfer quantitatively to a volumetric flask and dilute to 100.0 ml with the cold buffer solution. Keep the flask containing the test suspension in iced water during the titration.

Reference suspension. To avoid absorption of water formed by condensation, allow the reference preparation to reach room temperature before opening

(1) *Olive oil emulsion*

Stock emulsion In an 800 ml beaker 9 cm in diameter, place 40 ml of olive oil R, 330 ml of acacia solution R and 30 ml of water. Place an electric mixer at the bottom of the beaker. Place the beaker in a vessel containing alcohol R and a sufficient quantity of ice as a cooling mixture. Emulsify using the mixer at an average speed of 1000 r/min to 2000 r/min. Cool to 5 °C to 10 °C. Increase the mixing speed to 8000 r/min. Mix for 30 min, keeping the temperature below 25 °C by the continuous addition of crushed ice into the cooling mixture. (A mixture of calcium chloride and crushed ice is also suitable). Store the stock emulsion in a refrigerator and use within 14 days. The emulsion must not separate into two distinct layers. Check the diameter of the globules of the emulsion under a microscope. At least 90 per cent have a diameter below 3 µm and none has a diameter greater than 10 µm. Shake the emulsion thoroughly before preparing the emulsion substrate.

Olive oil emulsion For ten determinations, mix the following solutions in the order indicated: 100 ml of stock emulsion, 80 ml of tris(hydroxymethyl)aminomethane solution R1, 20 ml of a freshly prepared 8 per cent *m/V* solution of sodium taurocholate BRP and 95 ml of water. Use on the day of preparation.

the container. Prepare a suspension of pancreas powder (amylase and lipase) BRP as described for the test suspension using a quantity equivalent to about 2500 Ph. Eur. U.

Carry out the titrations immediately after preparation of the test suspension and the reference suspension. Place 29.5 ml of olive oil emulsion in the reaction vessel equilibrated at 37 ± 0.5 °C. Fit the vessel with the electrodes, a stirrer and the burette (the tip being immersed in the olive oil emulsion).

Put the lid in place and switch on the apparatus. Carefully add 0.1N sodium hydroxide with stirring to adjust to pH 9.2. Using a rapid-flow graduated pipette transfer about 0.5 ml of the previously homogenised reference suspension, start the chronometer and add continuously 0.1N sodium hydroxide to maintain the pH at 9.0. After exactly 1 min, note the volume of 0.1N sodium hydroxide used. Carry out the measurement a further four times. Discard the first reading and determine the average of the four others (S_1). Make two further determinations (S_2 and S_3). Calculate the average of the values S_1, S_2 and S_3. The average volume of 0.1N sodium hydroxide used should be about 0.12 ml per minute with limits of 0.08 ml to 0.16 ml.

Carry out three determinations in the same manner for the test suspension (T_1, T_2 and T_3). If the quantity of 0.1N sodium hydroxide used is outside the limits 0.08 ml to 0.16 ml per minute, the assay should be started again with a quantity of test suspension which is more suitable but situated between 0.4 ml and 0.6 ml. Otherwise the quantity of the substance to be examined should be adjusted to comply with the conditions of the test. Calculate the average of the values T_1, T_2 and T_3.

Calculate the activity in Ph. Eur. Units per milligram from the expression:

$$\frac{n \times m_1}{n_1 \times m} \times A$$

n = average volume of 0.1N sodium hydroxide used per minute during the titration of the test suspension,

n_1 = average volume of 0.1N sodium hydroxide used per minute during the titration of the reference suspension,

m = mass in milligrams of the substance to be examined,

m_1 = mass in milligrams of the reference preparation,

A = activity of pancreas powder (amylase and lipase) BRP in Ph. Eur. Units per milligram.

Amylolytic activity The amylolytic activity is determined by comparing the rate at which a suspension of pancreas powder hydrolyses a substrate of starch solution (1) with the rate at which a suspension of pancreas powder (amylase and lipase) BRP hydrolyses the same substrate under the same conditions.

Test suspension Triturate a quantity of the substance to be examined equivalent to about 1500 Ph. Eur. U. of amylolytic activity with 60 ml of phosphate buffer solution pH 6.8 R1 for 15 min. Transfer quantitatively to a volumetric flask and dilute to 100.0 ml with phosphate buffer solution pH 6.8 R1.

Reference suspension Prepare a suspension of pancreas powder (amylase and lipase) BRP as described for the test suspension, using a quantity equivalent to about 1500 Ph. Eur. U.

In a test tube 200 mm long and 22 mm in diameter, fitted with a ground-glass stopper, place 25.0 ml of starch solution, 10.0 ml of phosphate buffer solution pH 6.8 R1 and 1.0 ml of a 1.17 per cent *m/V* solution of sodium chloride R. Close the tube, shake and place in a water-bath at 25.0 ± 0.1 °C. When the temperature equilibrium has been reached, add 1.0 ml of the test suspension and start the chronometer. Mix and place the tube in the water-bath. After exactly 10 min, add 2 ml of 1N hydrochloric acid. Transfer the mixture quantitatively to a 300 ml conical flask fitted with a ground-glass stopper. Whilst shaking continuously, add 10.0 ml of 0.1N iodine and, immediately, 45 ml of 0.1N sodium hydroxide. Allow to stand in the dark at a temperature between 15 °C and 25 °C for 15 min. Add 4 ml of a mixture of four volumes of water and one volume of sulphuric acid R. Titrate the excess of iodine with 0.1N sodium thiosulphate using a microburette. Carry out a blank titration adding the 2 ml of 1N hydrochloric acid before introducing the test suspension. Carry out the titration of the reference suspension in the same manner.

Calculate the amylolytic activity in Ph. Eur. Units per milligram from the expression:

$$\frac{(n' - n)m_1}{(n'_1 - n_1)m} \times A$$

n = number of millilitres of 0.1N sodium thiosulphate used in the titration of the test suspension,

n_1 = number of millilitres of 0.1N sodium thiosulphate used in the titration of the reference suspension,

(1) *Starch solution* To a quantity of starch BRP equivalent to 2.0 g of the dried substance add 10 ml of water and mix. (Determine the water content of starch BRP prior to the test by heating at 120 °C for 4 h). Add this suspension, whilst stirring continuously, to 160 ml of boiling water. Wash the container several times with successive quantities, each of 10 ml, of water and add the washings to the hot starch solution. Heat to boiling, stirring continuously. Cool to room temperature and dilute to 200 ml with water. Use the solution on the day of preparation.

n' = number of millilitres of 0.1N sodium thiosulphate used in the blank titration of the test suspension,

n'_1 = number of millilitres of 0.1N sodium thiosulphate used in the blank titration of the reference suspension,

m = mass in milligrams of the substance to be examined,

m_1 = mass in milligrams of the reference preparation,

A = activity of pancreas powder (amylase and lipase) BRP in Ph. Eur. Units per milligram.

STORAGE

Store in an airtight container, at a temperature below 15 °C.

VII.1.1. REAGENTS

Acacia Complies with the requirements prescribed in the monograph on Acaciae Gummi.

Acacia solution Dissolve 100 g of acacia R in 1000 ml of water. Stir with a mechanical stirrer for 2 h. Centrifuge at about 2000 g_n for 30 min to obtain a clear solution.

Store in plastic containers of about 250 ml capacity at 0 °C to - 20 °C.

Calcium chloride

Calcium chloride solution 0.02M Dissolve 2.94 g of calcium chloride R in 900 ml of water, adjust to pH 6.0 to 6.2 (V.6.3.1) and dilute to 1000.0 ml with water.

Store at 5 ± 3 °C.

Fibrin congo red Cut fibrin into small pieces and leave overnight in a 2 per cent m/V solution of congo red R in alcohol (90 per cent V/V). Filter, rinse the fibrin with water and store under ether R.

Maleic anhydride. — $C_4H_2O_3$ (M_r 98.1). 2,5-Furandione.

White crystals, soluble in water forming maleic acid, very soluble in acetone and in ethyl acetate, freely soluble in chloroform and in toluene, soluble in alcohol with ester formation, very slightly soluble in light petroleum.

mp: about 52 °C.

Any residue insoluble in toluene does not exceed 5 per cent (maleic acid).

Tris(hydroxymethyl)aminomethane

Tris(hydroxymethyl)aminomethane solution R1 Dissolve 60.6 mg of tris(hydroxymethyl)aminomethane R and 0.234 g of sodium chloride R in water and dilute to 100 ml with the same solvent.

Store at 5 ± 3 °C and use within 3 days.

VII.1.3. BUFFER SOLUTIONS

Buffer (phosphate) solution pH 6.8 R1 To 51.0 ml of a 2.72 per cent *m/V* solution of potassium dihydrogen phosphate R add 49.0 ml of a 7.16 per cent *m/V* solution of disodium hydrogen phosphate R. Adjust the pH (V.6.3.1) if necessary.

Store at 5 ± 3 °C.

Buffer (maleate) solution pH 7.0 Dissolve 10 g of sodium chloride R, 6.06 g of tris-(hydroxymethyi)aminomethane R and 4.90 g of maleic anhydride R in 900 ml of water. Adjust to pH 7.0 (V.6.3.1) using a 17 per cent *m/V* solution of sodium hydroxide R. Dilute to 1000 ml with water.

Store at 5 ± 3 °C and use within 3 days.

Buffer (borate) solution pH 7.5 Dissolve 2.5 g of sodium chloride R, 2.85 g of disodium tetraborate R and 10.5 g of boric acid R in water and dilute to 1000.0 ml with the same solvent. Adjust the pH (V.6.3.1) if necessary.

Store at 5 ± 3 °C.

CHYMOTRYPSINUM

Chymotrypsin

Chymotrypsin is a proteolytic enzyme obtained by the activation of chrymotrypsinogen extracted from the pancreas of beef (*Bos taurus* L.). It has an activity not less than 5.0 microkatals per milligram. In solution it has maximal enzymic activity at about pH 8 ; the activity is reversibly inhibited at pH 3, at which pH it is most stable.

Chymotrypsin is prepared in conditions designed to minimise microbial contamination.

CHARACTERS

A white, crystalline or amorphous powder, sparingly soluble in water ; the amorphous form is hygroscopic.

IDENTIFICATION

A. Dilute 1 ml of solution S (see Tests) to 10 ml with water. In a depression in a white spot plate, mix 0.05 ml of this solution with 0.2 ml of substrate solution(1). A purple colour develops.

B. Dilute 0.5 ml of solution S to 5 ml with water. Add 0.10 ml of a 2.0 per cent *m/V* solution of tosylphenylalanylchloromethane R in alcohol R. Adjust to pH 7.0 and shake for 2 h. In a depression in a white spot plate, mix 0.05 ml of this solution with 0.2 ml of substrate solution(1). No colour develops within 3 min of mixing.

TESTS

Solution S Dissolve 0.10 g in carbon dioxide-free water R and dilute to 10.0 ml with the same solvent.

Appearance of solution Solution S is not more opalescent than reference suspension II (V.6.1).

---

(1) *Substrate solution for the identification.* To 24.0 mg of acetyltyrosine ethyl ester R add 0.2 ml of alcohol R, and swirl until solution is effected. Add 2.0 ml of 0.067M phosphate buffer solution pH 7.0 R and 1 ml of methyl red mixed solution R and dilute to 10.0 ml with water.

pH (V.6.3.1) The pH of solution S is 3.0 to 5.0.

Absorbance (V.6.19) Dissolve 30.0 mg in 0.001N hydrochloric acid and dilute to 100.0 ml with the same acid. The solution shows an absorption maximum at 281 nm and a minimum at 250 nm. The specific absorbance at the maximum is 18.5 to 22.5 and that at the minimum is not greater than 8.

Trypsin Transfer to a depression in a white spot plate 0.05 ml of tris(hydroxymethyl)aminomethane buffer solution pH 8.1 R and 0.1 ml of solution S. Add 0.2 ml of substrate solution[1] (test solution). Prepare at the same time a control solution using the substance to be examined to which not more than 1 per cent *m/m* of trypsin BRP has been added. Start a timer. No colour appears in the test solution within 3 min to 5 min after the addition of the substrate solution. A purple colour is produced in the control solution.

Histamine (V.2.1.6) Not more than 1 µg (calculated as histamine base) per 5 microkatals of chymotrypsin activity. Before carrying out the test, heat the solution of the substance to be examined on a water-bath for 30 min.

Loss on drying (V.6.22) Not more than 5.0 per cent, determined on 0.100 g by drying at 60 °C at a pressure not exceeding 0.7 kPa (5 Torr) for 2 h.

ASSAY

The activity of chymotrypsin is determined by comparing the rate at which it hydrolyses acetyltyrosine ethyl ester R with the rate at which chymotrypsin BRP hydrolyses the same substrate under the same conditions.

Apparatus. — Use a reaction vessel of about 30 ml capacity provided with :

- a device that will maintain a temperature of 25.0 ± 0.1 °C,
- a stirring device, for example a magnetic stirrer,
- a lid with holes for the insertion of electrodes, the tip of a burette, a tube for the admission of nitrogen and the introduction of reagents.

An automatic or manual titration apparatus may be used. For the latter the burette is graduated in 0.005 ml and the pH meter is provided with a wide scale and glass-calomel electrodes.

(1) *Substrate solution for the test for trypsin* To 98.5 mg of tosylarginine methyl ester hydrochloride R, suitable for assaying trypsin, add 5 ml of tris(hydroxymethyl)aminomethane buffer solution pH 8.1 R and swirl to dissolve. Add 2.5 ml of methyl red mixed solution R and dilute to 25.0 ml with water.

Test solution Dissolve 25.0 mg of the substance to be examined in 0.001N hydrochloric acid and dilute to 250.0 ml with the same acid.

Reference solution Dissolve 25.0 mg of chymotrypsin BRP in 0.001N hydrochloric acid and dilute to 250.0 ml with same acid.

Store the solutions at 0 °C to 5 °C. Warm 1 ml of each solution to about 25 °C over 15 min and use 50 µl of each solution (corresponding to about 25 nanokatals) for each titration. Carry out the titration in an atmosphere of nitrogen. Transfer 10.0 ml of 0.01M calcium chloride solution R to the reaction vessel and, while stirring, add 0.35 ml of 0.2M acetyltyrosine ethyl ester solution R. When the temperature is steady at 25.0 ± 0.1 °C (after about 5 min) adjust the pH to exactly 8.0 with 0.02N sodium hydroxide. Add 50 µl of the test solution (equivalent to about 5 µg of the substance to be examined) and start a timer. Maintain the pH at 8.0 by the addition of 0.02N sodium hydroxide, noting the volume added every 30 s. Calculate the volume of 0.02N sodium hydroxide used per second between 30 s and 210 s. Carry out a titration in the same manner using the reference solution and calculate the volume of 0.02N sodium hydroxide used per second.

Calculate the activity in microkatals per milligram using the expression :

$$\frac{m' \times V}{m \times V'} \times A$$

m = mass in milligrams of the substance to be examined,

m' = mass in milligrams of chymotrypsin BRP,

V = volume of 0.02N sodium hydroxide used per second by the test solution,

V' = volume of 0.02N sodium hydroxide used per second by the reference solution,

A = activity of chymotrypsin BRP in microkatals per milligram.

STORAGE

Store in an airtight container at 2 °C to 8 °C, protected from light.

LABELLING

The label on the *container* or the label on the *package* states :

— the quantity of chymotrypsin and the total activity in microkatals per container,

— for the amorphous substance, that it is hygroscopic.

VII.1.1. REAGENTS

Acetyltyrosine ethyl ester. — $C_{13}H_{17}NO_4,H_2O$ (M_r 269.3). Ethyl *N*-acetyl-L-tyrosinate monohydrate.

A white, crystalline powder suitable for the assay of chymotrypsin.

α_D^{20} : +21° to +25°, determined on a 1.0 per cent *m/V* solution in alcohol R.

$A_{1\,cm}^{1\ per\ cent}$: 60 to 68, determined at 278 nm using a solution in alcohol R and calculated with reference to the dried substance.

Acetyltyrosine ethyl ester solution 0.2M Dissolve 0.539 g of acetyltyrosine ethyl ester R in alcohol R and dilute to 10.0 ml with the same solvent.

TRYPSINUM

Trypsin

Trypsin is a proteolytic enzyme obtained by the activation of trypsinogen extracted from the pancreas of healthy mammals. It has an activity not less than 0.5 microkatals per milligram, calculated with reference to the dried substance. In solution, it has maximum enzymic activity at pH 8; the activity is reversibly inhibited at pH 3, at which pH it is most stable.

Trypsin is prepared in conditions designed to minimise the degree of microbial contamination.

CHARACTERS

A white or almost white, crystalline or amorphous powder, sparingly soluble in water. The amorphous form is hygroscopic.

IDENTIFICATION

A. Dilute 1 ml of solution S (see Tests) to 100 ml with water. In a depression in a white spot-plate, mix 0.1 ml of this solution with 0.2 ml of tosylarginine methyl ester hydrochloride solution R. A reddish-violet colour develops within 3 min.

B. Dilute 0.5 ml of solution S to 5 ml with water. Add 0.1 ml of a 2.0 per cent *m/V* solution of tosyl-lysyl-chloromethane hydrochloride R. Adjust to pH 7.0, shake for 2 h and dilute to 50 ml with water. In one of the depressions of a white spot-plate, mix 0.1 ml of this solution with 0.2 ml of tosylarginine methyl ester hydrochloride solution R. No colour develops within 3 min.

TESTS(1)

Solution S Dissolve 0.10 g in carbon dioxide-free water R and dilute to 10.0 ml with the same solvent.

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(1) The national authority may require that trypsin comply with a limit for total viable count of 10^3 micro-organisms per gram, determined by plate count (V.2.1.8.1). The national authority may require that trypsin comply with the test for *Escherichia coli* and *Salmonella* (V.2.1.8.2).

Appearance of solution Solution S is not more opalescent than reference suspension II (V.6.1).

pH (V.6.3.1) The pH of solution S is 3.0 to 5.5.

Absorbance (V.6.19) Dissolve 30.0 mg in 0.001N hydrochloric acid and dilute to 100.0 ml with the same acid. The solution shows an absorption maximum at 280 nm and a minimum at 250 nm. The specific absorbance at the maximum is 13.5 to 16.5 and that at the minimum is not greater than 6.5.

Chymotrypsin To 1.8 ml of buffer solution pH 8.0 R add 7.4 ml of water and 0.5 ml of 0.2M acetyltyrosine ethyl ester R. While shaking the solution, add 0.3 ml of solution S and start a stop-watch. After exactly 5 min, measure the pH (V.6.3.1) (test solution). Prepare a reference solution in the same manner, replacing solution S by 0.3 ml of a 0.05 per cent *m/V* solution of chymotrypsin BRP and measure the pH (V.6.3.1) exactly 5 min after adding the chymotrypsin. The pH of the test solution is higher than that of the reference solution.

Histamine (V.2.1.6) Not more than 1 µg of histamine base per 0.2 microkatal of trypsin activity. Use a 1 per cent *m/V* solution of the substance to be examined in borate buffer solution pH 8.0 (0.0015M) R inactivated by heating in a water-bath for 30 min. Carry out dilutions with a 0.9 per cent *m/V* solution of sodium chloride R.

Loss on drying (V.6.22) Not more than 5.0 per cent, determined on 0.500 g by drying at 60 °C, at a pressure not exceeding 670 Pa (5 Torr) for 2 h.

ASSAY

The activity of trypsin is determined by comparing the rate at which it hydrolyses benzoylarginine ethyl ester hydrochloride R with the rate at which trypsin BRP hydrolyses the same substrate in the same conditions.

Apparatus. — Use a reaction vessel of about 30 ml capacity provided with:

— a device that will maintain a temperature of 25.0 ± 0.1 °C,

— a stirring device (for example, a magnetic stirrer),

— a lid with holes for the insertion of electrodes, the tip of a burette, a tube for the admission of nitrogen and the introduction of reagents.

An automatic or manual titration device may be used. For the latter, the burette is graduated in 0.005 ml and the pH meter is provided with a wide-range scale and glass-calomel electrodes.

Test solution Dissolve sufficient of the substance to be examined in 0.001N hydrochloric acid and dilute to 25.0 ml with the same acid in order to obtain a solution containing approximately 700 nanokatals per millilitre.

Reference solution Dissolve 25.0 mg of trypsin BRP in 0.001N hydrochloric acid and dilute to 25.0 ml with the same acid.

Store the solutions at 0 °C to 5 °C. Warm 1 ml of each solution to about 25 °C over 15 min and use 50 µl of each solution for each titration. Carry out the titration in an atmosphere of nitrogen. Transfer 10.0 ml of borate buffer solution pH 8.0 (0.0015M) R to the reaction vessel and, while stirring, add 1.0 ml of a freshly prepared 0.686 per cent *m/V* solution of benzoylarginine ethyl ester hydrochloride R. When the temperature is steady at 25.0 ± 0.1 °C (after about 5 min) adjust the pH to exactly 8.0 with 0.1N sodium hydroxide. Add 50 µl of the test solution and start a timer. Maintain the pH at 8.0 by the addition of 0.1N sodium hydroxide the tip of the microburette being immersed in the solution ; note the volume added every 30 s. Follow the reaction for 8 min. Calculate the volume of 0.1N sodium hydroxide used per second. Carry out a titration in the same manner using the reference solution and calculate the volume of 0.1N sodium hydroxide used per second.

Calculate the activity in microkatals per milligram using the expression:

$$\frac{m' \times V}{m \times V''} \times A$$

m = mass in milligrams of the substance to be examined.

m' = mass in milligrams of trypsin BRP.

V = volume of 0.1N sodium hydroxide used per second by the test solution.

V' = volume of 0.1N sodium hydroxide used per second by the reference solution.

A = activity of trypsin BRP in microkatals per milligram.

STORAGE

Store in an airtight container, protected from light, at a temperature of 2 °C to 8 °C.

LABELLING

The label on the *container* states, in particular:

— the activity in microkatals per milligram.

VII.1.1. REAGENTS

Tosylarginine methyl ester hydrochloride

Tosylarginine methyl ester hydrochloride solution To 98.5 mg of tosylarginine methyl ester hydrochloride R add 5 ml of tris(hydroxymethyl)aminomethane buffer solution pH 8.1 R and shake to dissolve. Add 2.5 ml of methyl red mixed solution R and dilute to 25.0 ml with water.

Tosyl-lysyl-chloromethane hydrochloride. — $C_{14}H_{22}Cl_2N_2O_3S$ (M_r 369.3). *N*-Tosyl-L-lysyl-chloromethane hydrochloride. (3*S*)-7-Amino-1-chloro-3-(4-methylbenzenesulphonamido)heptan-2-one hydrochloride.

$[\alpha]_D^{20}$: – 7° to – 9°, determined on a 2 per cent *m/V* solution.

mp: about 155 °C, with decomposition.

$A_{1\,cm}^{1\,per\,cent}$: 310 to 340, determined at 230 nm using a solution in water.

PHARMACOPÉE FRANÇAISE

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EXTRAIT DE BILE DE BOEUF

Extractum fellis bovis

L'extrait de bile de boeuf contient au minimum 45,0 pour cent et au maximum 55,0 pour cent d'acides biliaires libres et conjugués exprimés en acide cholique ($C_{24}H_{40}O_5$ M_r 408,6), calculé par rapport à la substance desséchée.

CARACTÈRES

Poudre hygroscopique de couleur plus ou moins foncée, beige à verdâtre, facilement soluble dans l'eau. La solution aqueuse à 10 pour cent, complète, peut présenter une opalescence.

IDENTIFICATION

Opérez par chromatographie sur couche mince (V.6.20.2) en utilisant une plaque recouverte de gel de silice G R.

Solution à examiner. Dissolvez 0,100 g d'extrait de bile de boeuf dans du méthanol R et complétez à 10 ml avec le même solvant.

Déposez sur la plaque 10 µl de la solution à examiner. Développez sur un parcours de 15 cm avec un mélange de 5 volumes d'eau, de 50 volumes de toluène R et de 55 volumes d'acide acétique R. Séchez la plaque à l'étuve à 100-105 °C pendant 10 min. Laissez refroidir. Pulvérisez une solution préparée de la manière suivante: dissolvez 0,50 g de molybdate d'ammonium R dans 4 ml d'acide sulfurique R à 50 pour cent *V/V*; ajoutez un copeau de cuivre R et laissez 2 h en contact, en agitant de temps de temps; retirez le copeau, ajoutez 80 ml d'eau et 6,5 ml d'acide sulfurique R. Après pulvérisation, séchez la plaque à l'étuve à 100-105 °C pendant 10 min. Le chromatogramme obtenu présente au moins 6 taches distinctes, de couleur variant du jaune au bleu violacé (acides biliaires libres et conjugués).

ESSAI

Détermination du pH (V.6.3.1). Dissolvez 0,200 g d'extrait de bile de boeuf dans de l'eau exempte de dioxyde de carbone R et complétez à 20 ml avec le même solvant. Le pH de la solution est de 5,0 à 7,0.

Substances insolubles dans l'alcool à 80 pour cent *V/V*. Dissolvez 5,00 g d'extrait de bile de boeuf dans de l'alcool R à 80 pour cent *V/V* et complétez à 100 ml avec le même solvant. Chauffez à reflux pendant 10 min et filtrez à chaud sur un filtre en verre fritté (16) préalablement taré. Lavez le résidu avec 3 fois 20 ml d'alcool R à 80 pour cent *V/V*. Séchez le filtre à l'étuve à 100-105 °C pendant 1 h et pesez. La masse du résidu, calculée par rapport à la substance desséchée, n'est pas supérieure à 0,100 g (2,0 pour cent).

Sucres réducteurs après hydrolyse. Dissolvez 50 mg d'extrait de bile de boeuf dans de l'eau et complétez à 5 ml avec le même solvant. Ajoutez 0,10 ml d'acide sulfurique R et chauffez au bain-marie pendant 15 min. Alcalinisez avec une solution d'hydroxyde de sodium R à 10 pour cent *V/V* jusqu'à obtention d'une solution limpide. Ajoutez 2 ml en excès puis 2 ml de solution de sulfate de cuivre R à 10 pour cent *m/V*. Chauffez à l'ébullition pendant 1 min. La solution ne se décolore pas et ne donne pas de précipité rouge d'oxyde cuivreux.

Cholestérol.

Solution à examiner. Introduisez dans une ampoule à décantation 0,350 g d'extrait de bile de boeuf. Ajoutez 5 ml d'eau puis 40 ml de solution d'hydroxyde de sodium R à 0,5 pour cent *m/V* dans de l'alcool R à 60 pour cent *V/V* et 50 ml d'éther R. Agitez pendant 1 min, laissez reposer et éliminez la couche inférieure. Ajoutez 50 ml d'éther de pétrole R et agitez énergiquement. Soutirez soigneusement la couche inférieure en ayant soin de bien détacher les gouttelettes adhérant aux parois. Recueillez la couche éthérée dans une capsule; rincez l'ampoule avec 10 ml d'éther R et éliminez le solvant par chauffage au bain-marie. Reprenez le résidu par du chloroforme R et complétez à 20,0 ml avec le même solvant. A 5,0 ml de solution chloroformique, ajoutez 2 ml d'un mélange de 2,5 volumes d'acide sulfurique R et de 20 volumes d'anhydride acétique R.

Solution témoin. Dissolvez 0,6 mg de cholestérol R dans du chloroforme R et complétez à 5,0 ml avec le même solvant.

Laissez reposer à l'obscurité pendant 25 min. Mesurez l'absorbance (V.6.19) des deux solutions à 630 nm en utilisant comme liquide de compensation le chloroforme R. En tenant compte des absorbances mesurées et de la concentration des solutions, calculez la teneur en cholestérol, qui n'est pas supérieure à 0,7 pour cent, calculée par rapport à la substance desséchée.

Formaldéhyde.

Solution à examiner. Introduisez 0,50 g d'extrait de bile de boeuf et 100 ml d'eau dans un ballon rodé de 1 000 ml possédant une tubulure latérale.

Montez sur ce ballon une colonne de distillation munie d'un réfrigérant descendant terminé par un tube dont l'extrémité plonge dans une fiole jaugée de 200 ml contenant 50 ml d'eau, le corps de la fiole étant placé dans un bain de glace. Ajoutez 10 ml d'acide sulfurique 1N et laissez en contact pendant 30 min. Faites barboter lentement dans le ballon par la tubulure latérale un courant de dioxyde de carbone R et portez à l'ébullition. Distillez environ 50 ml. Complétez à 200,0 ml avec de l'eau. Prélevez 10,0 ml de cette solution et complétez à 50,0 ml avec de l'eau.

Solution témoin. A 5,0 ml de formaldéhyde R, ajoutez de l'eau et complétez à 1 000,0 ml avec le même solvant (solution (a)). Déterminez la concentration en formaldéhyde, en milligramme par millilitre, de la solution (a) en opérant selon la technique décrite au réactif formaldéhyde (VII.1.1) sur 10,0 ml de solution (a). Diluez dans de l'eau une prise d'essai appropriée de solution (a) pour obtenir une solution contenant 2 µg par millilitre de formaldéhyde.

Dans 2 tubes jaugés de 20 ml, introduisez respectivement 2 ml de solution à examiner et 1 ml de solution témoin additionné de 1 ml d'eau. Dans chaque tube, ajoutez 5 ml d'une solution obtenue en dissolvant 0,2 g de sel sodique d'acide chromotropique R dans 5 ml d'eau et en complétant à 100 ml avec de l'acide sulfurique R. Agitez et portez au bain-marie pendant 30 min. Refroidissez. Complétez à 20 ml avec de l'eau. Observez dans l'axe des tubes sur fond blanc. Si le tube contenant la solution à examiner présente une coloration, celle-ci n'est pas plus prononcée que celle du tube contenant la solution témoin (0,2 pour cent en formaldéhyde).

Indole, scatole, tryptophane. Dissolvez 10 mg d'extrait de bile de boeuf dans 1 ml d'acide acétique R. Ajoutez 5 ml de solution de xanthydrol R à 0,2 pour mille *m/V* dans un mélange de 1 volume d'acide chlorhydrique R et de 99 volumes d'acide acétique R. Chauffez au bain-marie pendant 10 min. Il ne se développe pas de coloration rose violacé.

Perte à la dessiccation (V.6.22). Déterminée à l'étuve à 100-105 °C, sur 1,00 g d'extrait de bile de boeuf, la perte à la dessiccation n'est pas supérieure à 6,0 pour cent.

Cendres sulfuriques (V.3.2.14). Déterminé sur 1,0 g d'extrait de bile de boeuf, le taux des cendres sulfuriques n'est pas supérieur à 35,0 pour cent.

Contamination microbienne (V.2.1.8). L'extrait de bile de boeuf satisfait à l'essai du dénombrement des germes aérobies viables totaux (V.2.1.8.1) avec un maximum de 10^4 microorganismes par gramme, déterminé par filtration sur membrane. L'extrait de bile de boeuf satisfait à l'essai d'*Escherichia coli* (V.2.1.8.2) et à l'essai des salmonelles (V.2.1.8.2).

DOSAGE

Solution à examiner. Dissolvez 0,100 g d'extrait de bile de boeuf dans de l'acide acétique R à 60 pour cent *V/V* et complétez à 100,0 ml avec le même acide. Agitez et filtrez si nécessaire.

Solution témoin. Dissolvez 50,0 mg d'acide cholique R dans de l'acide acétique R à 60 pour cent *V/V* et complétez à 100,0 ml avec le même solvant. Agitez.

Dans 3 tubes à essai, introduisez respectivement 1 ml de solution à examiner, 1 ml de solution témoin et 1 ml d'acide acétique R à 60 pour cent *V/V*. Ajoutez 1 ml de solution de furfural R à 1 pour cent *V/V*. Agitez et placez les tubes à 0 ± 1 °C pendant 5 min. Ajoutez 13 ml d'un mélange de 50 volumes d'acide sulfurique R et de 65 volumes d'eau. Portez les tubes au bain-marie à 70 ± 1 °C pendant 10 min, puis refroidissez à 0 ± 1 °C pendant 2 min. Mesurez immédiatement l'absorbance (V.6.19) des deux solutions à 680 nm en utilisant comme liquide de compensation le contenu du troisième tube à essai.

En tenant compte des absorbances mesurées et de la concentration des solutions, calculez la teneur en $C_{24}H_{40}O_5$.

ANNEX 2.1

ÖSTERREICHISCHES ARZNEIBUCH

(PHARMACOPOEA AUSTRIACA)

BAND II

AMTLICHE AUSGABE

WIEN 1981

VERLAG DER ÖSTERREICHISCHEN STAATSDRUCKEREI

Trockenhefe zur Pillenbereitung

Trockenhefe, die zur Pillenbereitung dienen soll, muß durch mindestens 2 Stunden im Trockenschrank auf 120° erhitzt werden. Sie ist unter der Bezeichnung »Trockenhefe zur Pillenbereitung« vorrätig zu halten.

P r ü f u n g : 1,0 g Trockenhefe zur Pillenbereitung darf bei der *Bestimmung der Gärkraft* in der oben angegebenen Weise innerhalb von 3 Stunden keine Gärung hervorrufen.

Zubereitungen: Extractum Faecis. Pilulae.

Fel Bovis depuratum
Gereinigte Rindergalle

Synonyme: Fel Tauri depuratum siccum. Extractum Fellis Bovis.

1 Teil Gereinigte Rindergalle entspricht 10 Teilen frischer Rindergalle.

Bereitung:

Frische Rindergalle ..... 100 Teile
Äthylalkohol ..... 250 Teile
Milchzucker ..... nach Bedarf

Die Rindergalle muß aus frischen, sinnfällig nicht veränderten Gallenblasen von Tieren stammen, die vor und nach der Schlachtung tierärztlich untersucht und im Sinne der Fleischbeschauvorschriften tauglich befunden wurden. Die Galle muß frisch und darf sinnfällig nicht verändert sein. Die frische Rindergalle wird unter stetem Rühren allmählich mit 200 Teilen Äthylalkohol versetzt. Nach zwei- bis dreitägigem Stehen wird die Flüssigkeit vorsichtig vom Bodensatz abgegossen; der Rückstand wird auf ein Filter gebracht und nach dem Abtropfen mit 50 Teilen Äthyalkohol in kleinen Portionen nachgewaschen. Die vereinigten alkoholischen Lösungen läßt man 12 Stunden lang bei höchstens 5° absetzen und filtriert dann. Im Filtrat bestimmt man den Trockenrückstand, destilliert sodann den Äthylalkohol im Wasserbad größtenteils ab und löst im Rückstand so viel Milchzucker, daß nach dem Eindampfen unter vermindertem Druck unterhalb 50° ein Gesamtgewicht von 10 Teilen gereinigter Rindergalle erhalten wird. Die Masse wird mittelfein gepulvert (V).

Beschreibung: Hygroskopisches, hellgelbes bis grünlichbraunes Pulver, das fast geruchlos ist und stark bitter schmeckt.

Löslichkeit: Gereinigte Rindergalle ist in Wasser oder verdünntem Alkohol sehr leicht löslich; in Alkohol löst sie sich nur teilweise.

Prüfung:

Identität:

1. 1 g Gereinigte Rindergalle muß sich in 2 ml Wasser fast klar lösen; die Lösung schäumt beim Schütteln sehr stark.

2. Etwa 0,01 g Gereinigte Rindergalle wird in 10 ml einer Mischung von 6 ml konzentrierter Essigsäure (R) und 4 ml Wasser gelöst. Zu 1 ml dieser Lösung fügt man 1 ml einer frisch bereiteten Lösung von 0,1 ml Furfurol (R) in 10 ml Wasser hinzu. Versetzt man diese Lösung mit 10 ml einer Mischung von 50 ml konzentrierter Schwefelsäure (R) und 65 ml Wasser und erhitzt 10 Minuten lang im Wasserbad bei 70°, so tritt eine blauviolette Färbung auf.

Reinheit:

Alkoholunlösliche Anteile 5,00 g Gereinigte Rindergalle werden mit 100 ml 80 vol.%igem Alkohol (R) 15 Minuten lang unter Rückflußkühlung auf dem Wasserbad extrahiert; die Flüssigkeit wird durch einen tarierten Filtertiegel filtriert. Der Rückstand wird dreimal mit je 10 ml 80 vol.%igem Alkohol (R) nachgewaschen und dann 1 Stunde lang getrocknet. Sein Gewicht darf nicht mehr als 0,0100 g betragen.

Trocknungsverlust: Höchstens 4,0% (S. 23).

Asche: 10,0—14,0% (S. 23).

Aufbewahrung: Vor Licht geschützt, in dicht schließenden Gefäßen, mit einem geeigneten Trocknungsmittel.

Dosierung:

Gebräuchliche Einzeldosis: 0,2—0,5 g.

Ferrum chloratum
Eisen-III-chlorid

$FeCl_3 . 6 H_2O$ Mol.-Gew. 270,3

Synonyme: Ferrum sesquichloratum crystallisatum. Ferri Chloridum.

Beschreibung: Gelbbraune, zerfließliche, geruchlose, kristalline Stücke, die in verdünnter Lösung herb metallisch schmecken. Konzentrierte Lösungen wirken ätzend auf Schleimhäute.

EUROPEAN PHARMACOPOEIA

SECOND EDITION

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VIII.10. MICROBIAL CONTAMINATION OF PRODUCTS NOT REQUIRED TO COMPLY WITH THE TEST FOR STERILITY - RECOMMENDED SOLUTION AND CULTURE MEDIA

The following solution and culture media have been found satisfactory for the purposes for which they are prescribed in the test for microbial contamination in the Pharmacopoeia. Other media may be used if they have similar nutritive and selective properties for the micro-organisms to be tested for.

Buffered sodium chloride-peptone solution pH 7.0

Potassium dihydrogen phosphate	3.56 g	equivalent to 0.067M
Disodium hydrogen phosphate dihydrate	7.23 g	
Sodium chloride	4.30 g	
Peptone (meat or casein)	1.0 g	
Purified water	1000 ml	

0.1 per cent *m/V* to 1.0 per cent *m/V* of polysorbate 20 or 80 may be added. Sterilise by heating in an autoclave at 121 °C for 15 min.

Broth medium A (Casein soya bean digest broth)

Pancreatic digest of casein	17.0 g
Papaic digest of soya bean	3.0 g
Sodium chloride	5.0 g
Dipotassium hydrogen phosphate	2.5 g
Dextrose monohydrate	2.5 g
Purified water	1000 ml

Adjust the pH so that after sterilisation it is 7.3 ± 0.2. Sterilise by heating in an autoclave at 121 °C for 15 min.

Agar medium B (Casein soya bean digest agar)

Pancreatic digest of casein	15.0 g
Papaic digest of soya bean	5.0 g
Sodium chloride	5.0 g
Agar	15.0 g
Purified water	1000 ml

Adjust the pH so that after sterilisation it is 7.3 ± 0.2. Sterilise by heating in an autoclave at 121 °C for 15 min.

Agar medium C (Sabouraud-dextrose agar with antibiotics)

Peptones (meat and casein)	10.0 g
Dextrose monohydrate	40.0 g
Agar	15.0 g
Purified water	1000 ml

Adjust the pH so that after sterilisation it is 5.6 ± 0.2. Sterilise by heating in an autoclave at 121 °C for 15 min. Immediately before use, add 0.10 g of benzylpenicillin sodium and 0.10 g of tetracycline per litre of medium as sterile solutions or, alternatively, add 50 mg of chloramphenicol per litre of medium before sterilisation.

Broth medium D (Lactose broth)

Beef extract	3.0 g
Pancreatic digest of gelatin	5.0 g
Lactose	5.0 g
Purified water	1000 ml

Adjust the pH so that after sterilisation it is 6.9 ± 0.2. Sterilise by heating in an autoclave at 121 °C for 15 min and cool immediately.

Enrichment broth medium E (Enterobacteria enrichment broth-Mossel)

Pancreatic digest of gelatin	10.0 g
Dextrose monohydrate	5.0 g
Dehydrated ox bile	20.0 g
Potassium dihydrogen phosphate	2.0 g
Disodium hydrogen phosphate dihydrate	8.0 g
Brilliant green	15 mg
Purified water	1000 ml

Adjust the pH so that after heating it is 7.2 ± 0.2. Heat at 100 °C for 30 min and cool immediately.

Agar medium F (Crystal violet, neutral red, bile agar with dextrose)

Yeast extract	3.0 g
Pancreatic digest of gelatin	7.0 g
Bile salts	1.5 g
Lactose	10.0 g
Sodium chloride	5.0 g
Dextrose monohydrate	10.0 g
Agar	15.0 g
Neutral red	30 mg
Crystal violet	2 mg
Purified water	1000 ml

Adjust the pH so that after heating it is 7.4 ± 0.2. Heat to boiling: do not heat in an autoclave.

Broth medium G (MacConkey broth)

Pancreatic digest of gelatin	20.0 g
Lactose	10.0 g
Dehydrated ox bile	5.0 g
Bromocresol purple	10 mg
Purified water	1000 ml

Adjust the pH so that after sterilisation it is 7.3 ± 0.2. Sterilise by heating in an autoclave at 121 °C for 15 min.

Agar medium H (MacConkey agar)

Pancreatic digest of gelatin	17.0 g
Peptones (meat and casein)	3.0 g
Lactose	10.0 g
Sodium chloride	5.0 g
Bile salts	1.5 g
Agar	13.5 g
Neutral red	30 mg
Crystal violet	1 mg
Purified water	1000 ml

Adjust the pH so that after sterilisation it is 7.1 ± 0.2. Boil for 1 min with constant shaking then sterilise by heating in an autoclave at 121 °C for 15 min.

Broth medium I (Tetrathionate bile brilliant green broth)

Peptone	8.6 g
Ox bile, dried	8.0 g
Sodium chloride	6.4 g
Calcium carbonate	20.0 g
Potassium tetrathionate	20.0 g
Brilliant green	70 mg
Purified water	1000 ml

Adjust the pH so that after heating it is 7.0 ± 0.2. Heat just to boiling. Do not re-heat.

Agar medium J (Deoxycholate citrate agar)

Beef extract	10.0 g
Meat peptone	10.0 g
Lactose	10.0 g
Sodium citrate	20.0 g
Ferric citrate	1.0 g
Sodium deoxycholate	5.0 g
Agar	13.5 g
Neutral red	20 mg
Purified water	1000 ml

Adjust the pH so that after heating it is 7.3 ± 0.2. Heat gently to boiling and boil for 1 min, cool to 50 °C and pour into Petri dishes. Do not heat in an autoclave.

Agar medium K (Xylose, lysine, deoxycholate agar)

Xylose	3.5 g
L-Lysine	5.0 g
Lactose	7.5 g
Sucrose	7.5 g
Sodium chloride	5.0 g
Yeast extract	3.0 g
Phenol red	80 mg
Agar	13.5 g
Sodium deoxycholate	2.5 g
Sodium thiosulphate	6.8 g
Ferric ammonium citrate	0.8 g
Purified water	1000 ml

Adjust the pH so that after heating it is 7.4 ± 0.2. Heat just to boiling, cool to 50 °C and pour into Petri dishes. Do not heat in an autoclave.

Agar medium L (Brilliant green-phenol red-lactose-sucrose agar)

Peptones (meat and casein)	10.0 g
Yeast extract	3.0 g
Sodium chloride	5.0 g
Lactose	10.0 g
Sucrose	10.0 g
Agar	20.0 g
Phenol red	80 mg
Brilliant green	12.5 mg
Purified water	1000 ml

Heat to boiling for 1 min. Adjust the pH so that after sterilisation it is 6.9 ± 0.2. Immediately before use, sterilise by heating in an autoclave at 121 °C for 15 min, cool to 50 °C and pour into Petri dishes.

Agar medium M (Triple sugar iron agar)

Beef extract	3.0	g
Yeast extract	3.0	g
Peptones (casein and beef)	20.0	g
Sodium chloride	5.0	g
Lactose	10.0	g
Sucrose	10.0	g
Dextrose monohydrate	1.0	g
Ferric ammonium citrate	0.3	g
Sodium thiosulphate	0.3	g
Phenol red	25	mg
Agar	12.0	g
Purified water	1000	ml

Heat to boiling for 1 min with shaking. Adjust the pH so that after sterilisation it is 7.4 ± 0.2. Fill into tubes to one-third of their height, sterilise by heating in an autoclave at 121 °C for 15 min and allow to cool in a position that gives a deep portion and a sloping surface.

Agar medium N (Cetrimide agar)

Pancreatic digest of gelatin	20.0	g
Magnesium chloride	1.4	g
Dipotassium sulphate	10.0	g
Cetrimide	0.3	g
Agar	13.6	g
Purified water	1000	ml
Glycerol	10.0	ml

Heat to boiling for 1 min with shaking. Adjust the pH so that after sterilisation it is 7.2 ± 0.2. Sterilise by heating in an autoclave at 121 °C for 15 min.

Agar medium O (Baird-Parker agar)

Pancreatic digest of casein	10.0	g
Beef extract	5.0	g
Yeast extract	1.0	g
Lithium chloride	5.0	g
Agar	20.0	g
Glycine	12.0	g
Sodium pyruvate	10.0	g
Purified water	950	ml

Heat to boiling for 1 min, shaking frequently. Adjust the pH so that after sterilisation it is 6.8 ± 0.2. Sterilise by heating in an autoclave at 121 °C for 15 min, cool to 45 °C to 50 °C and add 10 ml of a sterile 1 per cent *m/V* solution of potassium tellurite and 50 ml of egg-yolk emulsion.

Medium P (Reinforced medium for clostridia)

Beef extract	10.0	g
Peptone	10.0	g
Yeast extract	3.0	g
Soluble starch	1.0	g
Dextrose monohydrate	5.0	g
Cysteine hydrochloride	0.5	g
Sodium chloride	5.0	g
Sodium acetate	3.0	g
Agar	0.5	g
Purified water	1000	ml

Hydrate the agar, dissolve by heating to boiling with continuous stirring. If necessary, adjust the pH so that after sterilisation it is about 6.8. Sterilise by heating in an autoclave at 121 °C for 15 min.

Medium Q (Columbia Agar)

Pancreatic digest of casein	10.0	g
Meat peptic digest	5.0	g
Heart pancreatic digest	3.0	g
Yeast extract	5.0	g
Maize starch	1.0	g
Sodium chloride	5.0	g
Agar, according to gelling power	10.0 to 15.0	g
Purified water	1000	ml

Hydrate the agar, dissolve by heating to boiling with continuous stirring. If necessary, adjust the pH so that after sterilisation it is 7.3 ± 0.2. Sterilise by heating in an autoclave at 121 °C for 15 min. Allow to cool to 45°C to 50 °C, add, where necessary, gentamicin sulphate corresponding to 20 mg of gentamicin base and pour into Petri dishes.

Medium R (Lactose sulphite medium)

Pancreatic digest of casein	5.0	g
Yeast extract	2.5	g
Sodium chloride	2.5	g
Lactose	10.0	g
Cysteine hydrochloride	0.3	g
Purified water	1000	ml

Dissolve, adjust to pH 7.1 ± 0.1 and fill to 8 ml in 16 mm by 160 mm tubes containing a small Durham tube. Sterilise by heating in the autoclave at 121 °C for 15 min and store at 4 °C.

Before use, heat the medium for 5 min in a water-bath and cool. Add to each tube 0. 5 ml of a 1.2 per cent *m/V* solution of sodium metabisulphite R and 0.5 ml of a 1.0 per cent *m/V* solution of ferric ammonium citrate, both solutions being freshly prepared and filtered through membranes (pore size: 0.45 µm).

ANNEX 4.a

2 Dec. 1993

C H Y M O T R Y P S I N A N D T R Y P S I N

Raw material ,pancreas,is collected at a slaughterhouse of international standard, as the Viasan, not later than half an hour after the slaughter.
Cattle pancreas is preferable because of the rate of enzymes, and less fat than at the pig pancreas.
Cattle pancreas is collected by knife and pig pancreas by hand. Right after the collection, the pancreas must be cleaned from fat and connective tissues. It must be frozen, not later than one hour after slaughter, at -50°C in a single layer on a tray, using contact freezer. After a half to one hour, the pancreas is moved to the freezing store at -20°C, and put in plastic bags with indicated weight and date. Pancreas can be kept at -20°C no longer than six months because the autolysis starts after that time.
Frozen pancreas is transported to the pilot plant by the refrigerated truck at -20°C (to -16°C at least) . About 100 to 200 kilos of frozen pancreas can be kept in Westinghouse chest freezers of the pilot plant.
On the pilot plant scale, 35kg is chosen as one batch. For that quantity of pancreas, it is required 250 heads of cattle (in average 150gr of pancreas per cattle head) or 500 pigs (in average 70gr of pancreas per pig).
Frozen pancreas is weighted on a balance of 100kg and the weight of pancreas from the slaughterhouse is taken into consideration. Pancreas is transported by a lift from the ground floor to the second floor where it is minced in frozen state in the mincing machine 01.1 . The minced pancreas is as soon as possible put into the jacketed vessel of 250 lit with stirrer, item 03.1 . In this vessel (03.1), it is already placed 90 lit of 0.25 N sulphuric acid prepared with demineralized water and cooled in advance to +10°C . The 0.25 N H_2SO_4 is prepared from the concentrated H2SO4 pure 13.5gr (or 7.5ml) up to 1000ml of solution, using demineralized water, and 1,215 gr (or 675 ml) of concentrated H2SO4 pure up to 90 lit of the ready solution for the extraction already cooled at +10°C. The extraction lasts 8 hours, using cooled water of +6.5°C to +8°C in the jacket of the vessel 03.1, to keep the extraction temperature at +10°C. After that the mixture is discharged to the vacuum Nutch filter of 1,200 mm diameter, with a filter cloth, item 03.2 . Only the experiments can solve how tick has to be the filter cloth for an efficient and successful sucking.
The remained pancreas at the filter cloth is moved to the

pressure vessel of 100 lit with perforated basket and a lifting device for the basket, item 01.9 , for sterilization of waste pancreas, using live steam. After that, the sterilized waste pancreas of about 42 kg have to be kept at +4°C (in the cold room) and the next day, it is sent to the Vissan for the processing together with the slaughterhouse solid waste in their pressure drier. The dry waste pancreas is used as animal feed.

The filtered extract from item 03.2 of about 80 Kg is collected into the vessel of 250 lit with stirrer, item 03.3 , at the first floor. Solid ammonium sulphate, technical pure, 17.3 kg (21.5 % by weight of the filtered extract), is added by stirring. After one hour of continuous stirring, the salted out and precipitated impurities are removed using the filter centrifuge ' Rina ' of 1,200 to 1,400 r.p.m. with a filter cloth, item 03.4 . The obtained centrifugate (filtrate) is collected in the vessel of 200 lit, item 03.8 , at the ground floor. The precipitated impurities, mainly proteins, are discharged into the sewage.

The filtrate from item 03.8 is pumped by the pump, item 03.9, for an additional clarification to the vacuum Nutch filter with a filter cloth, item 03.5 , at the first floor. The clear filtrate of about 95 kg is collected in the vessel 03.8 (at the ground floor) and pumped again by item 03.9 to the 200lit vessel with stirrer, item 03.6, at the first floor. Crude proenzymes are salted out from the filtrate by adding 31.5 kg of ammonium sulphate technical pure (33% of ammonium sulphate by weight of the filtered extract) and stirring at the same time. After one hour of continuous stirring, it is left till next morning for sedimentation in the item 03.6 .

After that the clear solution is decanted by a hose and a decantation pipe and is discharged to the sewage. The product is filtered through the vacuum Nutch filter, item 03.5, using the tick filter cloth. Approximately 1.4kg of wet cake of crude proenzymes is obtained. It is moved to the big laboratory refrigerator at +4°C for the further purification on the laboratory scale.

The filtrate is disregarded and discharged into the sewage.

The washing with warm water has to be done right after completion of the batch, item by item, including pipes and the premises. The washing lasts about four hours.

Three shifts of 8 hours or 24 hours and 4 hours the next day or one half shift, in total three and half shifts, or 28 hours run are needed for the completion of one batch at the pilot plant scale for obtaining crude proenzymes.

Personnel :

- Slaughterhouse (collection of pancreas):
  . One veterinarian, 8 hours ,
  . Three skilled workers, 8 hours per man (8 x 3 = 24 hours).
+ Production (pilot plant scale):
  . One pharmacist or biochemist to supervise the process in the first shift, three hours.
  . One technician to supervise the process in the second shift, four hours.
  . One skilled worker to operate all items of the process (8x3=24) 24+4= 28 hours or one man in three and half shifts, or three and half mans of 8 hours.
  . One labourer for transportation of frozen pancreas from the ground floor to the second floor and for mincing the pancreas in item 01.1 , assisting the skilled worker in the production process, and washing all equipment, pipes, and premises after completion of a batch, 8x3=24 hours, or one man in three shifts or 3 mans of 8 hours.
  . Two skilled workers to operate the water demineralisation and water distilling units.

D R Y B I L E

Fresh bile is collected at a slaughterhouse of international standard, as the Vissan is, right after and not later than one hour after the slaughter.
Cattle bile is preferable.
The gall bladder is open by a knife and filtered through a double gauze to a 20 lit plastic can. Formalin pure, 0.1 % of bile is added and the quantity of formalin is calculated according to the quantity of bile collected. The collected bile is to be kept at +4°C for a week and no more than ten days.
- For one batch at the pilot plant scale, 35kg of fresh bile is used. For that amount of bile, 175 heads of cattle are required, expecting 200 gr per cattle head in average or 530 pigs, in average 65gr per pig.
To 35kg of fresh bile, about 33.7 lit, 0.035 kg of formalin is added .
The fresh bile in cans is transported to the Project site by the Project car (but not by the refrigerated truck) and must be kept in the cold room at +4°C.
In the morning at 8:00 a.m. one batch (35kg) has to be weighted on a balance of 100 kg and put in the vessel of 100 lit, item 04.1 . It is continuously filled by vacuum into the glass rotary vacuum evaporator 04.2 . The evaporator has a rotating flask submerged into water bath . Evaporated water is condensed in the close connected cooling section and the condensed water flows to the second flask. The evaporator works under vacuum. After completion of the evaporation, the container with water bath is dropped down by an hydraulic device. The flask with the concentrated bile is disconnected and the content is gathered in the vessel 04.3 of 20 lit .

The bile is concentrated (in item 04.2) to 20 % of his starting volume, to approximately 7 lit. About four hours is estimated for the evaporation. It depends on the vacuum and the temperature of the water in the water bath and of the cooling water. Only the experiments can give the right time consumption needed for the evaporation. It seems that the concentrated bile have to be collected and removed from item 04.2 two to three times during a batch run. The concentrated and thickened bile of about 50 % of dry substance is in the vessel 04.3 . Evaporated and condensed water is discharged to the sewage.

The concentrated bile is pumped by a dosing pump to the laboratory size spray drier 04.4 . Dry bile is obtained after approximately one hour run of the spray drier and 2.8 kg to 3.0kg of dry bile is expected.
In the final product, it must be less than 6 % of moisture.
The quality control is to be done following the Q.C. consultant's prescription.
One batch can be completed in 8 hours of continuous work.
The washing of all equipment and room must be performed right after the completion of the batch and can be done in the next two to three hours.

Personnel :

- Slaughterhouse (collection of bile)
  - One veterinarian , 6 hours.
  - Two skilled workers , 6 hours per man.
- Production (pilot plant)
  - One pharmacist or biochemist to supervise the process, one and half hour.
  - One technician to run the process and to operate the rotary evaporator and the spray drier, eight hours.
  - One labourer for the transportation of bile from the cold room to the first floor to the item 04.1 , one hour, and to wash all equipment and room after completion of the batch, three hours.
  - Two skilled workers to operate the water cooling unit and the vacuum pump (not only for the Dry Bile but also for other processes) .

    Two shifts are required to complete one batch and all washings after that.

29 November 1993

Mr.Tran Tuu,

NPD of the Project VIE/86/016,
Animal By-Products,

Dear Sir,

I would like to draw your attention to some facts connected with the quality control work in the Q.C. laboratory. There is no helping personnel in the quality control laboratory and the pharmacists have to loose a lot of working time, so, instead of focussing at the training quality control procedures, they are occupied by washing the glasswares.

I suggest you to solve this problem as soon as possible. So many things have to be done in a very short period of time. It is not possible to fulfil my work programme in such conditions. For the reasons mentioned above, I must stress on my expectation that the pharmacists are to be completely engaged in the training during the full working time.

Expecting your help and kindest regards,

Yours sincerely,

Mrs. Pavelic,
Q.C. Consultant

GIUN SÁN KÝ SINH Ở LOÀI NHAI LẠI

(List of Parasites)

Những kết quả điều tra nghiên cứu cho đến nay đã phát hiện những loài giun sán sau đây ở súc vật nhai lại nước ta :

	Loài giun sán	Trâu Buffalo	Bò Ox	Dê Goat	Cừu Sheep
1	2	3	4	5	6
	Lớp Trematoda (sán lá)				
1	*Ogmocotyle indica* (có khi ở ống mật) Bile)		+		
2	*Eurytrema pancreaticum* (ở tụy) (pancreas)	+	+	+	
3	*E. coelomaticum*		+		
4	*E. dajii*		+		
5	*Fasciola hepatica* (ống mật) lấy mũ (bile)	+	+	+	
6	*F. gigantica* (ống mật) lấy mũ (bile)	+	+	+	
7	*Schistosoma spindale*		+		
8	*Ornithobilharzia sp.*		+		
9	*Paramphistomam cervi*	+	+	+	
10	*P. epiclitum*		+		
11	*P. liorchis*	+	+	+	
12	*P. gotoi*		+		
13	*P. explanatum* (lại ống mật) (bile)	+	+		
14	P. skrjabini		+		
15	P. orthocoelium	+			
16	Gigantocotyle explanatum	+			
17	G.bathycotyle	+			
18	G. formosanum	+			
19	G. anisocotylea	+			
20	Calicophoron ijinai		+		
21	C. calicophorum		+		
22	C. cauliorchia			+	
23	Cotylophoron cotylophorum		+		
24	C. indicum		+		
25	Ceylonocotyle scoliocoelium			+	
26	C. dieranocoelium	+	+		

1	2	3	4	5	6
27	*C. streptocoelium*	+			
28	*C. orthocoelium*		+		
29	*Gastrothylax crumenifer*	+	+	+	+
30	*G. glandiformis*	+		+	
31	*G. minutus*	+			
32	*Fischoederius elongatus*	+	+		
33	*F. cobboldi*	+	+	+	
34	*F. japonicus*	+			
35	*Carmyerius gregarius*		+		
36	*C. spatiosus*	+			
37	*Homalogaster paloniae* (sống mắt) (Cile)	+	+	+	
	Lớp Cestoidea (sán dây)				
38	*Moniezia expansa*	+	+	+	+
39	*M. benedeni*	+	+	+	+
40	*Avitellina centripunctata*	+	+	+	+
41	*Taenia hydatigena* (larvae)	+	+	+	+
42	*Taenia multiceps* (larvae)				+
43	*Taeniarhynchus saginatus* (larvae) ngoài (Anen)	+	+		
44	*Echinococcus granulosus* (larvae)	+		+	
	Lớp Nematoda giun tròn				
45	*Ascaris ovis*				+
46	*Neoascaris vitulorum*	+	+		
47	*Skrjabinema ovis*				+
48	*Strongyloides papillosus*		+		
49	*Oesophagostomum radiatum*	−	+	+	
50	*Oe. venulosam*			+	+
51	*Oe. columbianum*			+	+
52	*Oe. asperum*			+	
53	*Agriostomum wryburgi*		+		
54	*Bunostomum phlebotomum*	+	+		
55	*B. wryburgi*		+		
56	*B. trigonocephalum*			+	+
57	*Syngamus (Mammomonogamus) laryngeus* (Lợn)	+		−	
58	*Trichostrongylus axei*	+	+	+	+
59	*T. colubriformis*		+	+	
60	*T. probolurus*				+
61	*Sarwaria (Gruhneria) bubalis*	+	+		
62	*Cooperia erchovi*		+		
63	*C. laterouniformis*	+	+		
64	*C. pectinata*	+	+		
65	*C. punctata*	+	+		
66	*Ostertagia ostertagi*		+		
67	*Haemonchus contortus* (dạ dày ngoài)	+	+		+
68	*H. similis*		+		
69	*H. placei*				+
70	*Mecistocirrus digitatus* (dạ dày ngoài)	+	+	+	

ANNEX 7

COMMENTS OF THE SUBSTANTIVE BACKSTOPPING OFFICER

The quality control expert's report gives a very good and correct review of the project. It covers, after a brief introduction, the suggested quality control techniques of the enzyme products to be manufactured, an assessment of the working conditions in the quality control laboratory, a detailed log-book of the assignment, an account of the expert's activities not directly related to the quality control and her recommendations for future actions. In addition to these, the quality control expert reports on her activities related to the project upon her arrival to Zagreb, Croatia from Viet Nam. IN the addendum the pharmacopoeial monographs of the enzyme products are given.

The structure of the report is very clear and the information given is very relevant. The descriptive parts are action oriented, they are very concise but easy to follow such as from a recipe book.

One of the particular values of the report, according to the substantive backstopping officer, that the suggested methods for quality control tests both for the starting raw materials and the finished products are relatively simple techniques. They would be easy to implement. The quality control expert has clearly expressed the importance of the quality of the starting raw materials, that is the quality of the animal organs, glands and tissues. As the pharmacopoeial requirements will become more and more strict, more and more emphasis should be given on the microbiological purity of the raw materials of animal origin. The development of a microbiological laboratory in BIOPHA should, therefore, be one of the priority objectives for the forthcoming phases of the project. Even if the product-mix of the pilot plant would be changed by introducing products of plant and marine life origin, a well functioning microbiological laboratory is a must for the company.

It is a unique feature of the technical report that the quality control expert gives a full account of her activities in a log-book format. In this presentation it is very transparent that her assignment was fully utilized.

The quality control expert has, implicitly, made some comments on the acceptability and marketability of the products selected by the national counterpart to be produced. She, in agreement with the Chief Technical Adviser and the substantive backstopping officer feels that the selected enzyme products have only a modest market potential, and some of them have become old. One can hardly find some of the selected products in the internationally accepted pharmacopoeias any more. New products of high market potential and of high added value, both at the domestic and the export market, should be identified and developed to be produced in the pilot plant.