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IMPROVED PRODUCTION OF PENICILLIN

DP/CPR/89/021

THE PEOPLE'S REPUBLIC OF CHINA

Technical report: First visit to the Guangzhou pharmaceutical factory and research institute, July 1992*

Prepared for the Government
of the People's Republic of China
by the United Nations Industrial Development Organization,
acting as executing agency for the United Nations Development Programme

Based on the work of A. E. Bird,
expert in instrumental analysis

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* This document has not been edited.

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INTRODUCTION

This is a report on the first mission of the Technical Advisor in instrumental analysis to the Guangzhou Pharmaceutical Factory. The job description is given in Annex 1. In preparation for the visit I read all the reports on the project written by Dr Batchelor, Dr Cole, Mr Barley and Dr Jong, and met with Drs Batchelor and Cole to discuss analytical requirements for the project. Also I read relevant scientific literature to prepare my seminar presentations and to familiarise myself with the analytical chemistry of Penicillin V.

The mission lasted for 9 days, from Sunday 5 July to Tuesday 14 July.

The objectives of the mission were to review the analytical work associated with the penicillin V project, to provide advice about this work and to assist with the initial commissioning of the HPLC system. These objectives were achieved.

The analytical procedures being used were:

- The iodometric method for pen V in fermentation broth, process extraction samples and isolated pen V (acid and potassium salt)
- The iodometric method for isolated 6APA
- The PDAB method for acylase activity for conversion of Pen V to 6APA

The iodometric method measures all penicillins and 6APA. The fermentation broth almost certainly contains a significant amount of p-OH Pen V and probably contains other penicillins as well. Some of the p-OH Pen V will come through the isolation procedure and be present as an impurity in the Pen V. The Project requires specific information on the content of Pen V in fermentation broth and other samples. The only reliable method to obtain this is HPLC. GPF staff have not been able to obtain this information because of the lack of HPLC equipment and have had to rely on the total penicillin results from the iodometric assay.

I. IODOMETRIC ASSAY

Work on this method was discussed with Mr Cheng Houghui and Mr Chou Zhengleun, who was concerned about the validity of results on his MIBK and acetone solutions of Pen V.

The iodometric assay was being used for both 6APA and Pen V. The method was that of the US Pharmacopoeia 1985, which is a reliable and satisfactory procedure.

I was asked to carry out the assay, to demonstrate it to Mr Cheng and others. I explained that my role is to give advice, not to carry out practical work of this type which I have not done routinely for well over 20 years. We agreed that Mr Cheng would carry out the assay and that I would watch and comment.

Mr Cheng asked for advice about reproducibility of the method. He was concerned that duplicate results were sometimes not in good agreement. However, in the results I was shown most duplicates were within 1%. Only a few were worse than this, and the worst pair were only about 4% apart. I explained that this was an acceptable pattern and that occasionally random errors will produce duplicates further apart than usual. When this happens the assay should be repeated to establish which is the more reliable result.

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Explanatory Notes:

6APA	6-aminopenicillanic acid
HPLC	High Performance Liquid Chromatography
Pen V	Penicillin V
PDAB	p-Dimethylaminobenzaldehyde
GPF	Guangzhou Pharmaceutical Factory
p-OH Pen V.	p-Hydroxy Penicillin V

ABSTRACT

Technical Support to Guangzhou Pharmaceutical Factory, China. DP/CPR/89/021. Mission of the TA for instrumental analysis 5-14 July 1992, to review analytical work for the Pen V project and make recommendations.

Some of the Pen V and 6APA results which had been obtained by the iodometric method are likely to be incorrect because of errors in the way the method was applied. Correct procedures were explained.

HPLC methods for Pen V and 6APA were provided and applied satisfactorily. The HPLC operator requires more training on use of this complex system. Some additional items need to be obtained for efficient operation of the HPLC.

Improved procedures for storage and use of reference standards are needed and were recommended.

Seminars were given on Penicillin Assay Methods and on Validation of Analytical Methods.

Mr Chou and Mr Cheng queried whether MIBK and acetone interfere in the method so that incorrect results are obtained for solutions of Pen V in these solvents. I suggested a very simple experiment (carry out the assay on MIBK and on acetone alone, with no penicillin) which would take about 45 mins. to find out whether interference occurs. The results with MIBK showed that it does respond in the assay, so that the iodometric method will not give correct results for penicillin solutions in this solvent. HPLC would be the best way to assay these solutions, but the hydroxylamine method would probably also be suitable, although it would be necessary to check that MIBK does not interfere. I provided experimental details for the hydroxylamine method, which could be used prior to the HPLC system being operational.

My observations of the assay technique, discussions arising from my scrutiny of the laboratory notebook and the results of the MIBK experiment led to the following list of likely causes of error in the way the iodometric assay was being carried out.

1. The factor from the reference standard has been measured only when a new $Na_2S_2O_3$ solution has been prepared. This is about every 2 weeks. This factor MUST be measured each day when iodometric assays are done. This is necessary because there are many causes of variability in the amount of iodine that reacts. These include reaction time, temperature and pH. Possible errors due to these effects can be prevented only by measuring the reference standard factor at the same time as the sample assays.

2. The same reference standard factor has been used for assays of Pen V and 6APA. The factor was determined with 6APA. The factor for Pen V will almost certainly not be the same as that for 6APA so that assays of Pen V that are calculated with the 6APA factor will be incorrect.

3. The reaction time with iodine was not measured accurately. The amount of iodine that reacts can vary with the reaction time and it is essential that this time is measured accurately and kept the same for each sample.

4. Pipettes were being used incorrectly in such a way that they would not deliver the required volume accurately.

5. On some occasions the burette was held at an angle while the volume was read. Burettes must be held vertically while being read.

6. Fermentation broth samples, which contain a high concentration of Pen V, were assayed by pipetting 0.1ml. Even when pipettes are used correctly a volume as low as 0.1ml will not be measured very accurately. It would be more accurate to pipette 1ml (or more), dilute it to a known volume in a volumetric flask and pipette 1ml (or more) of this diluted

solution for analysis.

7. MIBK was shown to interfere in the assay. Consequently assays of Pen V in MIBK solution gave results which were too high. The iodometric method must not be used for MIBK solutions. HPLC (when operational) or the hydroxylamine method should be suitable, although possible interference by MIBK must be checked.

8. It is possible that acetone will interfere in the same way as MIBK. This must be checked. If interference is found the iodometric method must not be used for acetone solutions.

I gave a handwritten copy of this list to Mr Liu Guangtao (the Director of the Research Institute) and Mr Cheng. I went through the list with Mr Cheng to ensure that he understood the points. On the issue of incorrect use of pipettes, I demonstrated the correct technique in the laboratory and provided brief written notes on correct technique. Mr Cheng agreed to explain the correct technique to other staff carrying out this assay.

The most serious of the likely causes of error is the use of the 6APA reference standard factor for assays of Pen V. [This had certainly been done for recent assays. I did not try to establish whether it had been done throughout the programme]. 6APA and Pen V react with different amounts of iodine per mole and have very different molecular weights so that Pen V assays carried out using the 6APA factor will almost certainly be incorrect. No clear explanation was given for use of the 6APA factor in Pen V assays, although a comment was made that not enough Pen V reference standard was available.

During the discussions on the iodometric assay the absence of a complete written procedure became apparent. I explained the importance of writing out the experimental procedure and the calculation, so that the analyst can follow it and to provide a permanent record of how the assays have been done.

II. p-DIMETHYLAMINOBENZALDEHYDE (PDAB) METHOD FOR 6APA

I discussed this method with Mr Peng Zhaohui and Mr Cheng. Isolated 6APA is assayed by the iodometric method but the PDAB method is also used for 6APA, apparently to measure penicillin acylase activity. I explained the limitations of the PDAB method, that it does not differentiate between 6APA and the penicilloic acid of 6APA and that other compounds with a primary amino group might interfere. However, if the method is used only to measure penicillin acylase activity the lack of specificity for 6APA relative to its penicilloic acid probably does not matter because acylase activity must take place for either compound to be produced.

No written procedure describing how the method was being applied was available and I was given two conflicting accounts of the PDAB reaction step. The procedure was said to be based on a paper by Balasingham (Biochem.Biophys.Acta 276 250 (1972)) but no copy was available. I agreed to obtain one and send it to Mr Peng after my return to the UK. (This has been done). As with the iodometric method, the 6APA reference standard was not being used each time the assay was done. I explained that it is essential with methods like this that the reference standard is used each time to correct for variations in response due to differences in experimental conditions.

Mr Peng expressed concern about variation of colour intensity with temperature. However, the results I was shown had no clear trend of colour with temperature. The variability appeared to me to be random scatter.

III. HPLC

A. Installation

At the start of the visit the HPLC system had not been unpacked or installed. GPF management intended to wait for completion of the analytical laboratory in the new building before installation. I requested that it be installed immediately elsewhere and subsequently be moved to the new building when that is ready. The system (Waters model Baseline 810) was installed by engineers from the manufacturer's Guangzhou office on 8 and 9 July. No paper had been ordered for the printer so the only way to view chromatograms and other output was on the computer screen.

B. Training

Mr Xiao Zhong Lin had previously attended a 6 day Waters training course in Hong Kong. Consequently I expected him to be able to operate the system, but I told him to get the installation engineer to go through the operation with him, as a reminder. On 10 July Mr Xiao told me that he did not know how to operate the system and that the engineer had been unable to help. He said that on the Waters training course he had not been allowed to use a system himself, just to watch a demonstrator using it.

I had no previous experience with the Baseline 810, which is a complex and sophisticated computer based system, operation of which is difficult to learn from the instruction manual. I did not know before going to Guangzhou what system had been bought and had no opportunity to familiarise myself with it. Nevertheless, between Mr Xiao and myself we were able to obtain chromatograms of 6APA, but we could not get the system to integrate, i.e. to measure the peak area. I phoned Waters in Hong Kong, complained strongly about the inadequate training given to Mr Xiao and arranged for a local engineer to come to the factory to provide basic training. This was done on the evening of 10 July, 5pm to 11pm. As a result Mr Xiao became much more confident in use of

the system and was able to integrate peaks. Nevertheless he clearly needed more help. On 13 July I again complained to Waters and asked for help. In response to specific queries I was told that both their Guangzhou and Beijing offices will provide advice and help by phone and that there are several other users of the Baseline 810 in the Guangzhou area, a list of which would be made available by their Guangzhou office.

I told the Director of the Research Institute and Mr Xiao about this and emphasised to both of them that Mr Xiao should be allowed to learn to use the system for at least 2 to 3 weeks, with whatever help could be arranged from other users in Guangzhou, before being expected to analyse real samples.

C. Methods

I provided written methods for HPLC assay of 6APA (Annex 2) and Pen V (Annex 3). Satisfactory chromatograms were obtained from standard samples of both compounds but it was not possible to run any investigative samples because of the difficulties with use of the system referred to above. An authentic sample of p-OH Pen V was also run in the Pen V system and shown to be very well separated from Pen V (about 6 and 14 mins. respectively).

The Pen V method I provided was taken from the US Pharmacopoeia, XXII, (1990) for assay of Pen V in drug substance and formulated products. Subsequently the Director of the Research Institute asked me to comment on a method he had obtained, apparently from a US pharmaceutical company, which included assay of p-OH Pen V, Pen V and phenoxyacetic acid in fermentation broth. The chromatographic conditions were identical to those I had taken from the USP and the retention times for p-OH Pen V and Pen V were close to those obtained by Mr Xiao. This method specifies the use of a p-OH Pen V reference standard each time that compound is to be assayed. This will not be possible at GPF because they have only a very small amount of authentic p-OH Pen V. Consequently I wrote instructions (Annex 4) on measurement of a response factor so that Pen V can be used as the reference standard for assays of p-OH Pen V. I gave this to the Director of the Research Institute on 14 July, to be passed on to the analysts. Also I pointed out that GPF fermentation broth will be different from the American company's broth and could contain compounds which interfere in the HPLC method even though that method was suitable for the American company's samples.

Because measurement of p-OH Pen V is important for this project I gave details of a very simple but less specific method (Annex 5) to Mr Cheng and to Mr Zheng (who is responsible for Pen V extraction work). This method could be useful for approximate assays of p-OH Pen V before the HPLC is fully operational, although the method is unlikely to be suitable for assay of fermentation broth.

D. Practical Aspects

I gave a list of twelve points (Annex 6) on basic practical aspects of HPLC to Mr Xiao and discussed them with him and Mr Cheng. These are all things which should have been covered in the Waters training course. However, I noticed that some essential things were not being done, e.g. wash the syringe after each injection and rinse it with the new sample solution before each injection.

E. Additional Items Required for HPLC

I discussed a list of eight additional items (Annex 7) with the Director of the Research Institute and Mr Li Jian on 13 July. The most expensive of these is a water purification system. The need for this was based on the facts that high purity water is required for HPLC and the water used in the GPF laboratory was obtained from a old copper still, kept outside on a balcony in very dirty surroundings. I was concerned about the quality of this water; (a) because of the unsatisfactory facility which produced it, (b) because chromatograms of 6APA run on 11 July showed two negative peaks, which can arise from poor quality water and (c) because it is very likely to contain copper ions, which can catalyse degradation of penicillins. When I explained this to the Director of the Research Institute on 13 July I was told that water doubly distilled from glass is available from the factory, which uses it for the manufacture of Injection products. The availability of this water had not been mentioned in a previous discussion with GPF managers about the need for high purity water for the HPLC. I told Mr Xiao to obtain and use this water in future. In view of this, purchase of a water purification system can be delayed until more experience has been obtained with the HPLC and I can assess whether it is really necessary (see FAX of 17 July to Li Jian, Annex 8).

The Director of the Research Institute and Mr Li Jian agreed to progress local acquisition of items 5 to 8 on the list, although the Director thought that re-allocation of existing equipment rather than new purchases should be possible for items 5 to 7.

In this discussion I tried to emphasise that GPF managemen. must realise that use of HPLC involves a continuing commitment to local purchase of expensive items such as high purity solvents, replacement columns, filters etc. It is essential for successful use of HPLC that they understand that it is not only expensive to buy, it is also expensive to operate and involves a high ongoing revenue cost.

IV. REFERENCE STANDARDS

Most quantitative analytical methods for penicillins and 6APA require use of a reference standard of the compound that is being analysed. The reference standard should be a very pure sample of the compound which has been thoroughly analysed so that the purity is known as accurately as possible. The purity that is assigned to the reference standard is used to calculate

results for the samples which are assayed by comparison with it. Consequently the accuracy of the results is dependent on the validity of the purity assigned to the reference standard. If the reference standard has been stored under conditions which allow it to degrade or to absorb water and its purity assignment has not been changed to allow for this, then assay results obtained with that reference standard will be incorrect.

Penicillins and 6APA are inherently unstable compounds, especially if the water content is high. Reference standards of them should be stored at 0 to 5°C in a desiccator to prevent water uptake. I was astonished to find the GPF 6APA reference standard stored at room temperature in a desiccator the silica gel in which was completely white, indicating that it had taken up its full water load some time before. I insisted on prompt replacement of the silica gel. The refrigerator in the analytical laboratory had no space for reference standards and was operating at about 15°C, not 0 to 5°C. The need for a refrigerator for storage of reference standards was made verbally to the National Project Director (Mr Liang) and the Director of the Research Institute and in the list of additional items for HPLC, Annex 7.

I provided notes (Annex 9) on the storage and use of reference standards to the Director of the Research Institute, Mr Cheng and Mr Xiao. Also I gave the Director samples of SmithKline Beecham reference standards of 6APA (0.5g) and Pen V (1.0g) and notes (Annex 10) on their use in assay of GFF in house standards.

V. GOOD LABORATORY PRACTICE

Basic aspects of GLP which were, or appeared to be, absent or inadequate were:

1. Written procedures for standard analytical methods.

2. Labelling of sample solutions, reagents etc. used during analysis.

3. Sample identification and record keeping to make it obvious which analytical result belongs to which sample.

I gave preliminary guidance on items 1 and 2 but GLP is an area that needs more detailed attention.

The importance and value of labelling solutions in use was vividly demonstrated on my last day. I had left instructions the previous day for the mobile phase for HPLC of Pen V to be prepared. This had already been done when I arrived and, because of my previous insistence that things be labelled, it was labelled. I saw immediately that one of the constituents was wrong; CH_3OH had been used instead of CH_3CN [My written instruction was perfectly clear, it had been

read carelessly!. If it had not been labelled I would not have known about the mistake and the whole day's work would almost certainly have been wasted.

VI. SEMINARS

I presented two seminars to 20 to 30 people, one on Penicillin Assay Methods and one on Validation of Analytical Methods. They were very ably translated by Daniel Cai. The overheads are attached as Annexes 11 and 12 respectively. A copy of text and overheads was given to the National Project Director for distribution to interested staff.

The talk on Penicillin Assay Methods concentrated on methods that are, or are likely to be, used at GPF (iodometric, hydroxylamine and PDAB for 6APA) and on the use of paper chromatography, TLC and HPLC for penicillin analysis.

The validation talk put considerable emphasis on accuracy of analysis, with discussion of basic aspects such as correct pipetting which enable accurate results to be obtained. Also the relationship between accuracy and specificity was emphasised. This is particularly important for non-chromatographic methods of penicillin analysis because these methods generally measure all penicillins and do not give results specific to a single penicillin.

VII. DOCUMENTS

I gave Mr Xiao a copy of High Performance Liquid Chromatography by Lindsay from John Wiley's Analytical Chemistry by Open Learning series, which I had bought at a cost of £20. This is a useful text for beginners in HPLC. I told Mr Xiao that he is to make it available to any GPF staff who wish to study it.

I gave Mr Xiao and Mr Cheng a copy of a chapter by D.H. Sieh on Penicillin V Potassium from Analytical Profiles of Drug Substances, (Ed. K. Florey) Vol. 17 (1988), pp. 677-748. This summarises an extensive literature survey of analytical work on Pen V and includes details of many chromatographic methods.

VIII. MAJOR FINDING

Some (at least) of the Pen V and 6APA assay results are likely to be incorrect because of errors in the way the iodometric assay has been carried out. This applies particularly to Pen V results where the 6APA reference standard factor has been used.

RECOMMENDATIONS

- Staff carrying out the iodometric assay need to make the changes detailed in points 1 to 8 on p.4 of this report.

- The HPLC operator needs a further period (at least 2-3 weeks) of training and familiarisation with use of this complex system before being expected to use it to analyse real samples.

- Assays for Pen V and p-OH Pen V should be carried out by HPLC as soon as is practicable.

- GPF management needs to provide the additional items nos. 5-8 for HPLC listed in Annex 7.

- GPF management and UNIDO need to provide additional items nos. 2-4 for HPLC listed in Annex 7.

- The need for a water purification system (additional item no. 1 in Annex 7) needs to be assessed during the TA's next visit.

- GPF management and analytical staff need to establish adequate supplies of a Pen V reference standard and to make arrangements for the storage and use of reference standards as outlined in Annex 9.

- GPF management need to make financial provision for the regular purchase of consumable items (replacement columns, high purity solvents etc.) for the HPLC system.

- Analytical staff need to adopt basic aspects of Good Laboratory Practice such as documentation of analytical methods and labelling of samples during analysis.

All of these recommendations were made either verbally or in writing to the Director of the Research Institute during the mission. Also, some of the recommendations were made to the appropriate analytical staff during the mission.

A E Bird
26/8/92

ANNEX 1

UNITED NATIONS INDUSTRIAL DEVELOPMENT ORGANIZATION

JOB DESCRIPTION

DP/CPR.89/021/11-04

Post Title: Expert in Instrumental Analysis

Duration: 2 months (split mission) - first mission: 0.5 m/m

Date Required: ASAP - April 1992

Duty Station: Guangzhou, China

Purpose of the Project

To strengthen the technical capabilities and know-how for the production of the main raw material (penicillin V) used for the production of semi-synthetic derivatives in the Guangzhou Pharmaceutical Factory.

Duties:

Assist in the commissioning of and demonstrate the operation and use of modern analytical techniques.

Advice on analytical procedures for the control of the fermentation processes, raw materials and finished products.

Recommend and devise simple and readily available mehtods of analysis to supplement the use of expensive equipment which will be limited in availability.

Liaise with other technical experts and introduce analytical techniques required by them.

Make all personnel - both analytical and process staff fully aware of the specificity and limitations of all assay methods related to the products and processes and recommend the most appropriate methods for each purpose.

Train local technicians.

Evolve methods for the improvement of good laboratory practice and record keeping.

Qualifications:

Chemist with extensive experience in analytical methods preferably related to the fermentation industry and -lactam antibiotics.

Language English

Background Information Whereas the total production of antibiotics in China, is 15,000 tons/year, the consumption of penicillin semi-synthetic derivatives is 13% of this. Only 150 tons of semi-synthetic derivatives are produced annually in China. This means that approximately 1,850 tons of semi-synthetic derivatives must be imported each year in order to meet the demand. In addition, the demand for semi-synthetic derivatives is rapidly growing as antibiotics are being replaced, for therapeutic reasons, by more modern less toxic semi-synthetic penicillins.

Out of the twelve different antibiotics derived from penicillin, only ampicillin is locally produced at full scale. In 1988 total production of ampicillin amounted to only 150 tons, a quantity well below the requirements of the health sector.

The country's manufacturers are faced with two main difficulties, namely:

-Insufficient production of Penicillin V and, consequently,
-Insufficient production of 6APA and 7ADCA.

These products persently have to be imported at high cost in order to ensure a minimum output of easily applicable antibiotics.

In the Guangdong province, advanced chemical industry is highly developed among the pharmaceutical companies. Two of them, the Guangzhou Pharmaceutical Factory (GPF) and the Guangdong Pharmaceutical Factory have well established production units which are manufacturing a large amount of pharmaceutical products by fermentation and chemical synthesis with a combined annual production capacity of 4,000 tons. (GPF has been the first company in China to manufacture Penicillin V.)

In the field of antibiotics the Government and the State Pharmaceutical Administration of China (SPAC) are aiming at improving self-reliance capacities by an increase of the local production of semi-synthetic penicillin derivatives from the present 2% to 30% of the total production of antibiotics. To achieve this target, the SPAC has the following strategies:

-Local production of 6APA and 7ADCA which are the intermediates for production of semi-synthetic derivatives.
-Local production of Penicillin V which is the starting material for the production of 6APA and 7ADCA.

The Bio-engineering Research and Development Centre of the State Commission for Science and Technology have included this project as one of the main priorities of the 7th Five Year Programme. Accordingly, Guangzhou Bio-engineering is sponsoring a total of 530,000 RMB Yuan and the State Commission for Science and Technology 80,000 RMB Yuan for the establishment of a 1,000 M^2 fermentation pilot plant.

ANNEX 2

HPLC Assay of 6APA

Column:	Waters Microbondapak C18
Mobile Phase:	Mix 50ml of methanol with 950ml of 0.05M sodium acetate pH 4.0 [Weigh 6.8g sodium acetate trihydrate or 4.1g sodium acetate anhydrate, dissolve in about 600ml water, adjust the pH to 4.0 with glacial acetic acid and dilute to 1 litre with water].
Flow rate:	1 ml/min
Detection:	254nm
Injection volume:	20µl
Retention time:	4-5 mins.

Reference Standard Solution

Dissolve an accurately weighed amount of 6APA reference standard in 0.1M sodium acetate (13.6g sodium acetate trihydrate or 8.2g sodium acetate anhydrate dissolved in water to make 1 litre of solution) and dilute to a known volume to give a concentration of about 1.0mg/ml.

Sample Solutions

(a) Solid samples
Prepare as for the reference standard

(b) Solution samples
Dilute if necessary with 0.1M sodium acetate to give a 6APA concentration of about 1mg/ml. Filter if necessary through a 0.45µ membrane before injection.

Calculation

Solid samples

$$\text{Purity} = \frac{A_1 \times C_2 \times P}{A_2 \times C_1} \%$$

where A_1 is the area of the 6APA peak in the sample chromatogram

A_2 is the area of the 6APA peak in the reference standard chromatogram

C_1 is the concentration of the sample solution in mg/ml

C_2 is the concentration of the reference standard solution, in mg/ml

P is the % 6APA content of the reference standard

Solution Samples

$$\text{6APA content} = \frac{A_1 \times C_2 \times P \times D}{A_2 \times 100} \text{ mg/ml}$$

of solution

where A_1, A_2, C_2 and P are as for solid samples

D is the dilution factor for the sample [E.g. if 1.0ml of sample was diluted to 10ml before injection, D = 10].

ANNEX 3

HPLC Assay of Pen V

Column:	Waters Microbondapak C18
Mobile phase:	Mix 650ml water, 350ml acetonitrile and 5.75ml glacial acetic acid
Flow rate:	1 ml/min
Detection:	254nm
Injection volume:	10µl

Reference Standard Solution:

Dissolve an accurately weighed amount of Pen V reference standard in mobile phase and dilute to a known volume to give a concentration of about 2.5mg/ml. [E.g. about 60mg to 25ml]

Sample Solutions:

(a) Solid samples
Prepare as for the reference standard

(b) Fermentation broth
Dilute with mobile phase to give a concentration of about 2.5mg/ml and filter through a 0.45µ membrane before injection

(c) Other aqueous solution samples

Dilute with mobile phase to give a concentration of about 2.5mg/ml. Filter if necessary before injection.

(d) Organic solution samples (e.g. MIBK or acetone)

Dilute with mobile phase to give a concentration of about 2.5mg/ml. Make a similar dilution of the MIBK or acetone ALONE and inject to find out where the MIBK or acetone elutes.

CALCULATION

Purity of solid samples

$$\text{Pen V content} = \frac{A_1 \times C_2 \times P}{A_2 \times C_1} \% \text{ or } \frac{A_1 \times C_2 \times P \times 10}{A_2 \times C_1} \text{ ug/mg}$$

where: A_1 is the area of the Pen V peak in the sample chromatogram
A_2 is the area of the Pen V peak in the reference chromatogram
C_1 is the concentration of the sample solution in mg/ml
C_2 is the concentration of the reference solution in mg/ml
P is the % Pen V content of the reference standard

Solution Samples

$$\text{Pen V content} = \frac{A_1 \times C_2 \times P \times D}{A_2 \times 100} \text{ mg/ml or}$$

$$= \frac{A_1 \times C_2 \times P \times D \times 10}{A_2} \text{ ug/ml}$$

where A_1, A_2, C_2 and P are as for solid samples
D is the dilution factor for the sample solution [E.g. If 1.0ml of fermentation broth was diluted to 10.0ml before injection, D = 10].

ANNEX 4

Reference Standard for HPLC Assays of p-hydroxy Pen V

The method from an American company requires use of a reference standard of p-OH Pen V each time assays are done. You will not have enough material to do this. What you must do is to use some of the material you have to measure the response relative to Pen V. Then for assay of samples you use Pen V as the reference standard and include the response factor in the calculation for p-OH Pen V.

1. Prepare two solutions, both containing accurately known concentrations of both p-OH Pen V and Pen V reference standards, both about 1.5mg/ml. [Because you have such a small amount of p-OH Pen V you will have to weigh only a few mgs. and dissolve in a small volume. If possible use a 5 or 6 place balance for these weighings, so that the weights are as accurate as possible].

NOTE

You must have both the p-OH Pen V and Pen V in the same solution.

2. Inject the solutions, twice each. [Make the first injection immediately after the solution has been prepared and the second one as soon as the first chromatogram has finished].

3. Measure the area of the p-OH Pen V and Pen V peaks in each chromatogram.

4. Calculate the response factor (F) from

$$F = \frac{A_1 \times C_2 \times P_2}{A_2 \times C_1 \times P_1}$$

A_1 = area of p-OH Pen V peak

A_2 = area of Pen V peak

C_1 = concentration of p-OH Pen V, in mg/ml

C_2 = concentration of Pen V, in mg/ml

P_1 = % purity of p-OH Pen V (Assume 100% if you have no figure)

P_2 = % purity of Pen V reference standard

Average the value of F from all 4 chromatograms. F will probably be quite close to 1, but I am not sure about this.

To measure p-OH Pen V in samples, chromatograph the sample solution (as described in

the American method) and a solution of Pen V reference standard of accurately known concentration.

Calculation:

$$\text{p - OH Pen V content} = \frac{A_3 \times C_4 \times P_2}{A_4 \times F \times C_3}\%$$

where F and P_2 are as above.

A_3 is the area of the p-OH Pen V peak in the sample chromatogram

A_4 is the area of the Pen V peak in reference chromatogram

C_3 is the sample concentration

C_4 is the Pen V reference standard concentration

Note that the p-OH Pen V retention time might vary from day to day and that broth samples might have other peaks close to p-OH Pen V. You will need to chromatograph a dilute solution (~0.5mg/ml or less if you can detect it) of p-OH Pen V to check the retention time occasionally to make sure you are measuring the correct peak in sample chromatograms.

Remember that your broth is different from the American one and so might give interfering peaks which theirs did not have.

ANNEX 5

Approximate Method for p-OH Pen V in Pen V

Dissolve 0.1g sample in 50ml 0.1M NaOH. Measure the absorbance (optical density) at 306nm.

$$\text{p-OH Pen V content} = \frac{A \times 100}{74.3 \times W \times 2} \%$$

where A is the absorbance (in a 1cm cell)

74.3 is the E_1^1 value for p-OH Pen V

W is the weight of sample taken, in g.

This method is based on the absorbance of the phenoxide ion. Pen V does not interfere but other phenols (e.g. p-OH phenoxyacetic acid; p-OH Pen V penicilloic acid) do interfere so the method is NOT specific for p-OH Pen V.

The method would not be useful for fermentation broths because many interfering substances will be present. It would be useful for isolated samples of Pen V and perhaps for samples from the extraction steps BUT THE LIMITATION MUST BE CLEARLY UNDERSTOOD IN INTERPRETING RESULTS; that is that the result could be higher than the true p-OH Pen V content because of interference by other phenols etc.

ANNEX 6

HPLC - Practical Hints

1. Use clean glassware and equipment.

2. Use high purity solvents and chemicals.

3. Clean the syringe with water immediately after each sample injection.

4. Rinse the syringe 3 times with each sample solution before injection.

5. At the end of the day, or if you change from one mobile phase to another, wash the column with water for 5-10 mins.

6. At the end of the day, after the water wash, pump 50/50, CH_3OH/H_2O for about 10 mins. and leave this in the column overnight. [You can use a higher concentration of CH_3OH but 50/50 should be OK and will save the cost of expensive CH_3OH]. **NEVER** leave the column with only H_2O in it. Bacteria will grow and ruin it.

7. **NEVER** pump a high concentration of organic solvent (e.g. 50/50 CH_3CN/H_2O) immediately after a mobile phase which contains a buffer. **ALWAYS** wash with water first to remove the buffer. Some buffers (especially phosphate) precipitate with organic solvents and will block the column etc.

8. After a set of dirty samples has been analysed (e.g. fermentation broths), wash with 50/50 CH_3CN/H_2O (after the H_2O wash if a buffer was used), to clean the column. Observe whether any peaks elute and if necessary wash with even stronger CH_3CN, up to 100%, to clean the system.

9. Filter dirty samples (e.g. fermentation broths) through a 0.45µm filter before injection.

10. Keep a daily note of system use and performance. This should include the column no., date, assay (e.g. 6APA, Pen V), sample type (e.g. solid, fermentation broth etc.), flow rate, pump pressure and comments. This is best done as a table, with these items as headings for columns.

11. Change the guard column insert:

a) If the pump pressure increases significantly day to day for the same mobile phase and flow rate

b) After analysis of a set of dirty samples (e.g. fermentation broth), before washing the column.

c) As otherwise indicated in the Waters instructions with the guard column Kit.

12. For quantitative analysis:

a) Use 2 separate weighings (or separate dilutions for liquid samples). Make 2 injections from each as soon as possible after the solution has been prepared. DO NOT leave solutions of penicillins for quantitative analysis standing before the first injection. If the area from the second injection is much below that from the first injection the compound is probably unstable in that solvent. Check this by making more injections as the solution ages. If this confirms that the compound is unstable make only one injection of each solution, immediately after preparation. [Alternatively, try a different solvent and check for stability again].

b) Use at least 2 separate weighings of reference standard.

c) First inject one reference standard solution (but see point g) below), (2 injections if it is stable), then the sample solution (or some of the solutions if more than one is to be assayed), then another reference standard solution, then more sample solutions etc.

d) The reference standard MUST always be done in the same set of chromatograms as the samples. You cannot use a reference standard response from one day to calculate results for samples done another day.

e) If results from the different reference standard solutions are close together (e.g. within about 2.0%), average them and use the average to calculate the results for the samples. Calculate and report a separate result for each sample weighing. If these are not in good agreement, do a third weighing if possible.

f) If results from different reference standard solutions are NOT close together, try to find out why. This could be an error in weighing, or in transfer of the standard into the volumetric flask or a problem with the HPLC system, or it might be a drift due to temperature change during the day. If results from the first 2 reference solutions are not close together you MUST inject a third solution, then decide what to do. Generally this will be either to average all 3 of them or to average only 2 of them, if 2 are close together and the third is very different.

g) Whenever you have enough time you should inject 2 reference standard solutions and get results close together from them BEFORE injecting any samples. Then inject at least one more reference solution during the series of chromatograms if several samples are being assayed.

ANNEX 7

Additional Items Required for HPLC

1. Water purification system (about US $ 6000)

2. Sample filtration kit (to filter fermentation broth samples) (about US $ 300)

3. Spare column (about US $ 400)

4. More guard column inserts (about US $ 200)

5. Magnetic stirrer for preparation and filtration/de-gassing of mobile phases. [The magnetic stirrer that is already in the lab. is kept in a fume cupboard for other work. An additional one is needed].

6. Refrigerator for storing reference standards and samples awaiting analysis. [This is needed for all analytical work, not just HPLC]

7. Additional laboratory glassware. Efficient use of the HPLC requires more volumetric flasks, beakers and conical flasks in a range of sizes so that reagents and sample solutions can be prepared promptly. Also a 1 litre flask for use with the mobile phase filtration and degassing unit is URGENTLY needed.

8. Paper for the HPLC system printer is URGENTLY needed.

COMMENTS

1. Dr Batchelor will ask UNIDO if funds are available for items 1 to 4. The others must be purchased locally by GPF. In view of the need to obtain item 2 as soon as possible it might be best if that also is purchased locally.

2. GPF management must realise that use of HPLC will require continuing purchases of expensive items such as replacement columns, high purity chemicals and solvents, filtration membranes, guard column inserts etc., which will not be funded by UNIDO.

ANNEX 8

To: Li Jian

Guangzhou Pharmaceutical Factory 17 July 1992

ADDITIONAL ITEMS FOR HPLC

Dr Batchelor obtained UNIDO approval for purchase of:
One additional Microbondapak column, C18.
One pack of 10 guard column inserts
One pack of 100 sample clarification filters (Millex HV 13mm, Waters catalogue number SJHV 013 NS). You will also need some glass hypodermic syringes for use with these filters. The syringes must have Luer lok fitting. I think five 2ml syringes would be enough.
Please order these items locally and charge to the UNIDO budget. You told me that you can order items costing less than US$2000 on the UNIDO budget without going through Vienna. Dr Batchelor does not know about this system. If you have any difficulty, or need any confirmation from us that these items can be bought on the UNIDO budget please FAX us on (44) 293 863006.

I have decided not to go ahead with purchase of the water purification system at present. The water that Mr. Liu told us about on Monday 13th, that is double distilled from glass and used in the factory for Injection products, should be adequate for HPLC. After Mr. Liu told us about it I asked Mr. Xiao to start using this water. He will need to collect it in a glass container fresh 3 times a week, Monday, Wednesday and Friday. By the time I come again you will have enough experience for me to decide whether there is any problem with this water. Also I expect to bring a procedure with me for testing the water for suitability for HPLC.

Please tell Mr. Liu, Mr. Xiao and Mr. Cheng about this FAX.

A E BIRD

ANNEX 9

Storage and Use of Reference Standards of Penicillins and 6APA

STORAGE

Reference standards must be stored in a desiccator with silica gel in a refrigerator at 0-5°C.

The standards must be in a glass container with a screw cap or other tight closure to prevent entry of water vapour.

Most of the material should be kept in a single container but this MUST NOT be opened regularly for day to day use of the standard. Several small amounts (about 1g) should be kept in small containers for day to day use. These small containers can be re-filled from the large container when necessary. This will minimise the number of occasions on which the large container is opened and so help to avoid entry of water vapour into the large container.

The silica gel in the desiccator must be replaced as soon as the blue colour starts to fade.

USE

The reference standard **MUST** be allowed to warm to room temperature without deliberate heating on each occasion **BEFORE** the container is opened. This is **ESSENTIAL** to prevent condensation of water on the cold material which would lead to some decomposition. If there is a large amount of standard in a single container it will take several hours for it all to warm to room temperature. The container must be kept open for as short a time as possible, be re-closed immediately after use and returned to the refrigerator.

ANNEX 10

Purity Assignment of Reference Standards of 6APA and Pen V

Determine the purity of your own batch of reference standard by analysis relative to the SmithKline Beecham standards which have been given to Mr Liu. These are

6APA:	LRS 16; Assigned purity 99.0%
Pen V (potassium salt);	LRS 2. Assigned purity 88.0% as Pen V acid. [This is equivalent to 97.6% as Pen V potassium salt]

Assay your standard relative to the SmithKline Beecham standards by both HPLC and the Iodometric method. Do 6 replicate assays by both methods, 3 on one day and 3 on another day. If results do not show good agreement carry out 3 more on another day. FAX the results to Dr Batchelor who will pass them to me for comment.

ANNEX 11

PENICILLIN ASSAY METHODS

Introduction

Titrimetric Methods

Spectrophotometric Methods

Chromatographic Methods

INTRODUCTION

Many methods in literature

Concentrate on widely used methods

Important to:

- Understand the chemistry
- Know what the method measures
- Know what else will respond
- Carry out validation tests

IODOMETRIC

Penicillin does not react with I_2

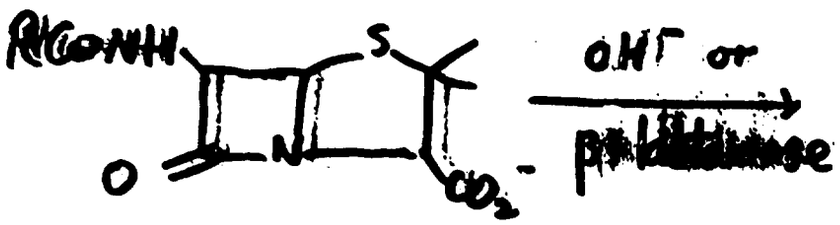
Penicilloic acid does react with I_2

Need sample blank

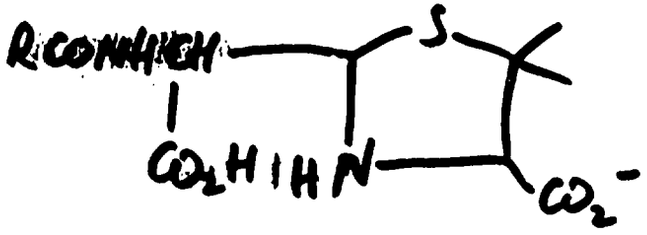
Amount of I_2 varies with conditions

Need reference standard

Difficult to use with fermentation samples



Penicillin



Penicilloic Acid

HYDROXYLAMINE

React with NH_2OH at pH7, then with Fe^{3+}

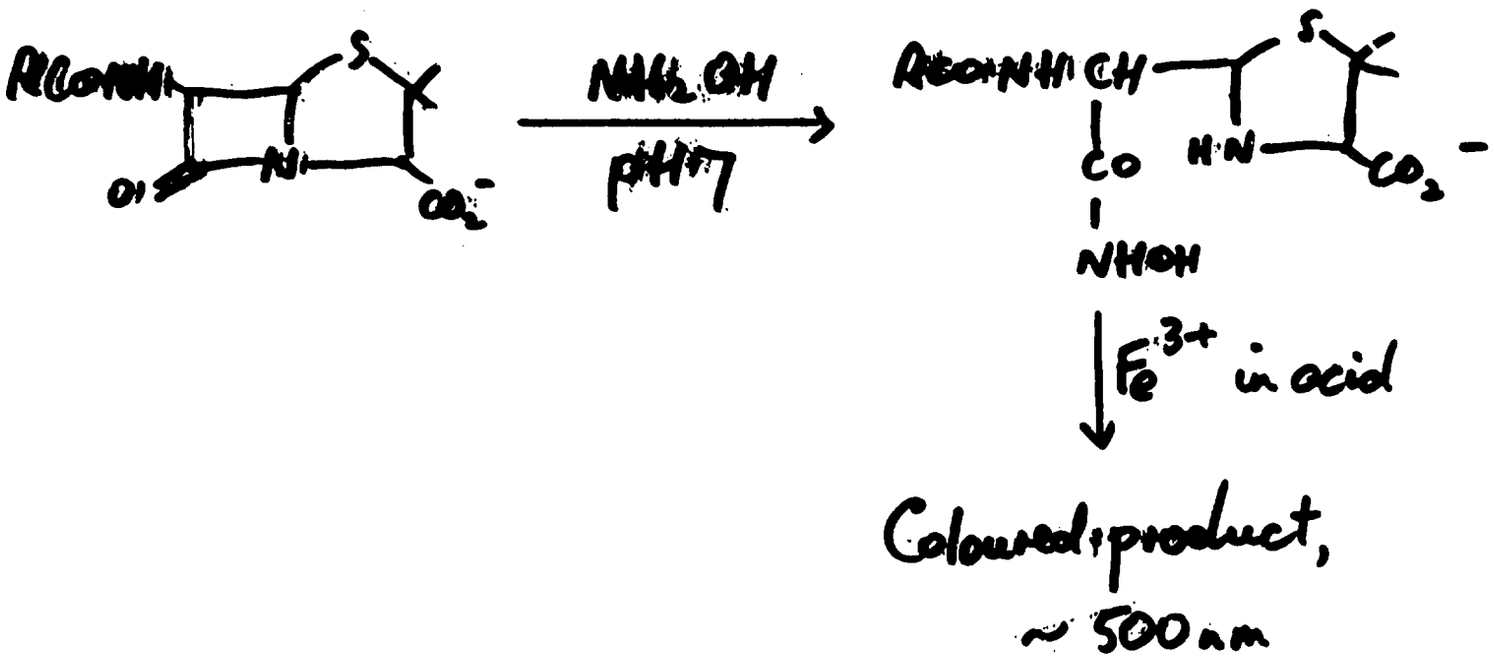
Coloured product, 500nm

Need reference standard

Need sample blank (hydrolysed penicillin)

Phenolic esters interfere

Can be used for fermentation samples


NH2OH
pH 7
Fe3+ in acid
Coloured product,
~ 500 nm

p-DIMETHYLAMINOBENZALDEHYDE (PDAB) FOR 6-APA

Either

React with PDAB at pH2.5, colour at 415nm

Or

React with 2,4-pentanedione,then PDAB, colour at 538nm

Need a reference standard

Penicilloic acid of 6-APA interferes

PAPER CHROMATOGRAPHY

Simple equipment

Detect by antibacterial activity or spray

Slow

Poor resolution (cf. TLC and HPLC)

Difficult to use quantitatively

THIN LAYER CHROMATOGRAPHY

Fairly simple equipment

Reasonably fast

Reasonable resolution

Methods available for many penicillins

Major use for identification tests

Can be used for quantitative limit tests (eg. POAA in Pen V)

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Expensive equipment

Fast

Good resolution

Detection of penicillins usually by UV

Pre- or post- column derivatisation can be used

Methods available for many penicillins

Reverse phase methods preferred

Major uses; Purity assay and impurity content

Penicillin content in complex samples

ANNEX 12

VALIDATION OF ANALYTICAL METHODS

Introduction

Validation test parameters

INTRODUCTION

Validation is a process which establishes that a method is adequate for the intended use

Validation should be done:

-as part of development of a new method

-when an existing method is applied to a new matrix

-when an existing method is first applied in a lab.

VALIDATION TEST PARAMETERS

Precision

Accuracy

Linearity and range

Specificity (selectivity)

Limit of detection and quantification

Ruggedness

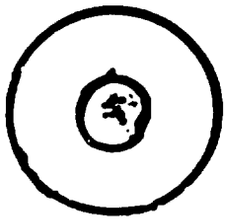
Stability in solution

Acceptance criteria

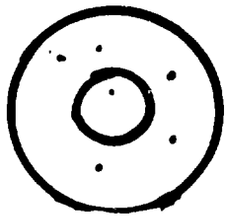
PRECISION AND ACCURACY- DEFINITION

Precision is the degree of agreement between individual results.

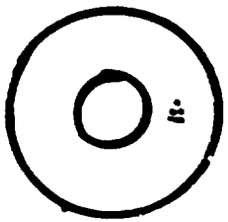
Accuracy is the closeness of results to the true value



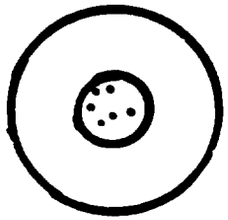
Precise and Accurate



Not precise and Not accurate



Precise but not accurate



Accurate but not precise

PRECISION - TEST

Assay 6 or more portions of sample

Calculate coeff. of variation (CV)

For purity assay, acceptable CV normally 1.5% max.

For other assays acceptable CV depends on analyte concentration and matrix. For low conc. CV may be high; perhaps 5% or more.

ACCURACY - TEST

Either:

Apply method to samples to which known amounts of analyte have been added. The % found normally should be 95-105%, but may depend on analyte content.

Or:

Apply both the test method and a different validated method to several samples. Results should not be statistically significantly different.

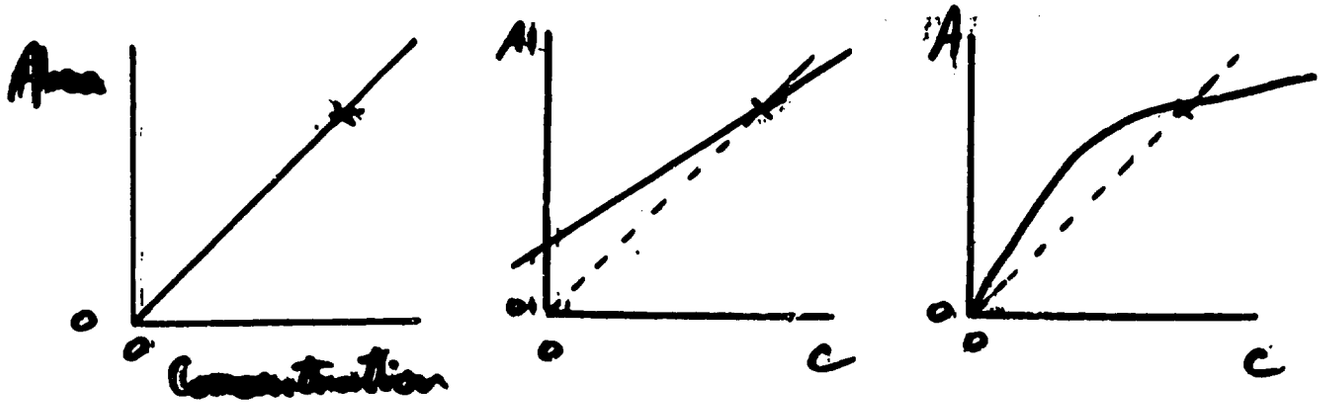
Not possible for some assays

LINEARITY AND RANGE - DEFINITION

Linearity is a test of the relationship between analyte concentration and analytical response.

Range is the difference between the lowest and highest analyte concentrations for which satisfactory accuracy and precision have been demonstrated.

Linearity essential for methods that use Single Point Calibration


A
O
O
Concentration
A
O
C
A
O
C

LINEARITY - TEST

Assay at least 5 solutions with analyte concentration between 20 and 150% of the nominal concentration for the assay. Plot response vs. concentration.
The plot must appear linear with little scatter and negligible intercept. Statistical calculations can be used.

SPECIFICITY - DEFINITION

The ability of the method to measure only the analyte(s) of interest, without interference by other components of the sample.

SPECIFICITY - TEST

For chromatographic methods:

Check that other possible components of the sample are resolved from the analyte.

For other methods:

Carry out the assay with other possible components deliberately added to assess their effect.

LOD AND LOQ - DEFINITION

The lowest concentration of analyte that can be reliably detected or quantified under the standard conditions of the method.

LOD AND LOQ - TEST

Apply the method to known low levels of the analyte and estimate LOD and/or LOQ from the results. A statistical calculation from the noise level of blank samples can be used for some methods.

RUGGEDNESS - DEFINITION

The effect of normal variations in operating conditions, eg. different analysts, different equipment, different environmental conditions.

RUGGEDNESS - TEST

Select critical variables.

Carry out assays of the same sample(s) using the selected conditions.

Results should not be statistically significantly different.

STABILITY IN SOLUTION

DEFINITION:

The stability of the analyte in the solution used for the assay

TEST:

Repeatedly assay a solution held for longer than the time it will be held in normal use of the method.

VALIDATION REQUIRED FOR VARIOUS ASSAYS

Validation is time consuming

Do not need all tests for all methods

Use judgement to decide the minimum that is necessary

USP list

B.S.O. Technical Comments on the Reports of Dr. Batchelor (11-01), Dr. Cole (11-02) and Dr. Bird (11-04)

In their reports, the experts present extensive information and sound recommendations for the continuation of the experimental work in GPF.

The initial breakthrough for installation and commissioning of the HPLC and the clarification of the characteristics and problems of every analytical method used or available to be used (potentially useful), proved to be essential for the whole advance of the research and final results of the project.

Dr. Bird's clear recommendations for analytical working procedures, GLP, record-keeping and additional laboratory equipment/items required, are most valuable and should be closely followed.

The supply of reference standards and literature is highly appreciated.

The methods recommended by Dr. Cole for strain improvement and for the conduction of fermentation have to be tried from now on. As strongly recommended, it is essential to maintain a good level of communication between the project team to keep detailed records of the experiments' conditions and results, and prepare summaries of the work done, in order to get the best advantage of the experts! advice and to facilitate all the necessary analysis for the work progress. The measures suggested for the laboratory building finishing/design, especially those related to the avoidance of microbial contamination, should be faithfully followed.

The installation of pending equipment/instruments should be also finished before the next experts' visit.

Work on the Pen V recovery aspects has to be stressed after the installatio is finished and fermentation conditions settled, following Dr. Batchelor's recommendations.

The possibility of purchasing the Westphalia centrifuge as pointed out by Dr. Batchelor, will require financial arrangements in the project budget which could be discussed/approved during the next experts' mission in October 1992.

The individual and team work of the experts is regarded as highly qualified and efficient; their personal interest and contribution to the project's success is also recognized.