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THE PEOPLE'S REPUBLIC OF CHINA

Technical report: Second visit to the Guangzhou pharmaceutical
factory and research institute, October 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

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Explanatory Notes:

6APA	6-aminopenicillanic acid
GLP	Good Laboratory Practice
GPF	Guangzhou Pharmaceutical Factory
HPLC	High Performance Liquid Chromatography
Pen V	Penicillin V
p-OH Pen V	p-Hydroxy Penicillin V

ABSTRACT

Technical Support to Guangzhou Pharmaceutical Factory, China. DP/CPR/89/021. Visit of the TA for instrumental analysis, 5-17 October 1992, to review analytical work on the Pen V project and to make recommendations.

No use had been made of the HPLC system for work related to the Pen V project since my first visit in July.

HPLC assay of fermentation and extraction samples shows that the true Pen V content is considerably lower than previously estimated by the iodometric method.

Some components of the fermentation medium interfere in the iodometric assay, leading to over-estimation of the Pen V content. Possible ways to avoid this were proposed.

Basic training in operation of the HPLC was provided and an operating procedure for use of the system was written and supplied.

The compliance with basic aspects of GLP such as unambiguous labelling of samples and adequate record keeping still requires improvement.

INTRODUCTION

This is a report on the second visit of the Technical Advisor in instrumental analysis to the Guangzhou Pharmaceutical Factory. The objectives of the visit were to review progress in the analytical work for the Penicillin V project, to introduce procedures for the HPLC analysis of fermentation broth and extraction samples and to provide training for some GPF staff in use of the HPLC. These objectives were achieved.

In preparation for the visit I spent 2 to 3 days in SmithKline Beecham laboratories in the UK, and in discussions with Waters staff, to familiarise myself with the operation of computer controlled Waters HPLC systems. Although the software in the SmithKline Beecham systems was not absolutely identical to that in the GPF system, this preparation proved to be invaluable in enabling me to understand the operation of the system and to train GPF staff in its use.

The visit lasted for 13 days, from Monday 5th October to Saturday 17th October. Arrival in Guangzhou had been scheduled for Sunday 4th October but was delayed because the Hong Kong-Guangzhou rail ticket had not been purchased by the Guangdong Pharmaceutical Committee office in Hong Kong and all trains were fully booked.

I. HPLC

A. General Issues

During my visit in July 1992 the HPLC system had been installed and commissioned and the basic methodology for assay of Pen V and 6APA had been demonstrated. Also the need to purchase some additional items for use with the system had been identified, the most important of these being sample clarification filters for filtration of fermentation broth samples. On arrival for this second visit I was disappointed to find that the HPLC system had not been used for any Pen V work and that the sample clarification filters had not been ordered until the week before my visit. They had still not been delivered at the end of my visit. Fortunately I had taken a few filters with me, so analysis of fermentation broth and related samples was possible.

Re-location of the HPLC into a laboratory in the new building to be occupied by the GPF Research Institute was arranged during my visit. At the start of the visit I provided advice on the facilities required in the laboratory, which was just an empty room with no benching etc. Benching and a sink were provided and the HPLC was moved on Friday 16th October. The waste pipe for the sink, and a water supply to it, had still to be installed, as had window blinds and a wall mounted temperature control unit. Also, ancillary equipment such as a pH meter, a magnetic stirrer and an adequate supply of glassware was not yet available.

In the report on my first visit I expressed concern about the suitability of the distilled water for HPLC work. The results obtained on this visit gave no evidence of any problem arising from water quality, although I did not have time to carry out the objective test which I had planned.

B. Training and Operation of the System

During my first visit the HPLC had been operated by Mr Qiu Zhong Lin (incorrectly named as Mr Xiao Zhong Lin in my report on that visit). Mr Qiu is now working for the GPF Quality Control Department, not for the Research Institute, but apparently retains an overview role for care of the HPLC.

Most of my time was devoted to training GPF research institute staff in use of the HPLC system. This was done in parallel with analysis of Pen V fermentation broth and extraction samples. The staff being trained were Miss Cheng Meiling and Miss Qu Junhua, although Miss Qu was available for only about half of the time. Translation was very ably carried out by one of the non technical staff of the institute, Miss Nie Qin. Because of the complexity of this computer based system the learning process was slow, but by the end of the visit Miss Cheng and particularly Miss Nie were familiar with basic operation of the system and should be able to continue to use it for Pen V samples, although assistance from Mr Qiu Zhong Lin will be required if any problems occur with the equipment.

The instruction manuals provided by Waters with the HPLC system are extremely detailed but are set out in such a way that they are virtually useless to a beginner in HPLC. For example, they contain no clear step by step instructions on what to do to obtain a chromatogram. I wrote simple instructions (Annex 1) for the main functions of the system and gave a copy to the Director of the Research Institute (Mr Liu Guangtao) and to the staff for use in the laboratory.

C. HPLC Methods

Procedures were developed for preparation and HPLC assay of fermentation whole broth and of samples taken at various stages during the Pen V extraction process. Annex 2 gives the procedures for assay of Pen V content and Annex 3 gives those for assay of p-OH Pen V and phenoxyacetic acid. A copy of these procedures was given to the Director and to the laboratory staff.

D. Results

Two main objectives were addressed; comparison of HPLC and iodometric assay results for Pen V content and assay at various stages of extraction to estimate yields and identify stages at which significant losses occur. Also, some Pen V potassium samples isolated from earlier fermentations were analysed.

HPLC comparison with iodometric

	Sample	Treatment	Pen V (units/ml)		HPLC as % of I ₂
			HPLC	I ₂	
1.	Whole broth 8, harvest	Filtered, filtrate 1 to 10	32400	37128	87%
2.	Whole broth 8, in tank	Filtered, filtrate 1 to 10	30916	36816	84%
3.	Whole broth 8, harvest , (after 1 day in fridge)	Diluted 1 to 10, filtered for HPLC. Filtered, 0.1ml filtrate for I ₂	28444	33852	84%
4.	Whole broth 7, harvest	Diluted, 1 to 10, filtered for HPLC. Filtered, 0.1ml filtrate for I ₂	22434	32916	68%
5.	Whole broth 7, in tank	Diluted 1 to 10, filtered for HPLC. Filtered, 0.1ml filtrate for I ₂	22183	34320	65%
6.	Broth 7 at pH 4.5	Diluted 1 to 10, filtered for HPLC. Filtered, 0.1ml filtrate for I ₂	19203	32448	59%
7.	pH 4.5 filtrate, start	Diluted 1 to 10, filtered	20526	30888	66%
8.	pH 4.5 filtrate, end	Diluted 1 to 10, filtered	20818	29640	70%
9.	pH 4.5 filtrate plus wash	Diluted 1 to 10, filtered	20137	28392	71%
10.	Shaker flask A	Diluted 1 to 10, filtered	20337	30340	67%
11.	Shaker flask B	Diluted 1 to 10, filtered	25626	40300	64%
12.	Pen V (K) 7-65 A		1438*	1450*	99%
13.	Pen V (K) 7-65 B		1455*	1399*	104%
14.	Pen V (K) 7-65 Mixed		1458*	1438*	101%

* Units/mg

The HPLC and I₂ results for samples 3 to 6 are not directly comparable because the sample treatment was different. In these cases the iodometric assay was carried out in the way it has always been done at GPF, by filtering whole broth and assaying 0.1ml of filtrate. This gives a result in units/ml of filtrate which is bound to be higher than the content in whole broth, because the solids will contain little or no Pen V. A direct comparison by HPLC in 3 broth samples showed the content in whole broth to be 5-8% lower than that in filtrate.

The results given above show acceptable agreement between HPLC and I₂ for the solid Pen V (K) samples, but the iodometric method greatly over-estimates the Pen V content in the broth and pH 4.5 filtrate samples, although the over-estimate is less for broth 8 than for broth 7 and the two shaker flask samples.

Stage yields and losses

HPLC assays were carried out on samples taken at various stages during the extraction of Pen V from fermentation 7-65. The yields and losses are detailed in Dr Batchelor's report.

Assay of Pen V (K) samples

Pen V (K salt) samples dated 18/7/92, 21/8/92, 24/8/92, 12/9/92 and 16/9/92 were assayed with results (from a single injection of one solution) between 1244 and 1422 units/mg. Most of the samples showed several small impurity peaks and two of them (18/7/92 and 12/9/92) showed one or two large impurities, the identity of which is not known.

p-OH Pen V and phenoxyacetic acid

The chromatograms of the fermentation samples showed no detectable p-OH Pen V. Time was not available for an estimate to be made of the detection limit.

The chromatograms showed a peak at the retention time of phenoxyacetic acid, corresponding to about 4mg/ml broth in whole broth 8.

II. IODOMETRIC ASSAY

During my first visit I identified several likely causes of error in the way the iodometric assay was being carried out. The most important of these were that results for Pen V were calculated using a factor from a 6APA reference standard and that the factor was measured only when a new $\text{Na}_2\text{S}_2\text{O}_3$ titrant solution was prepared, instead of being measured each day the assay is used. On this visit I found that Pen V results are now calculated with a factor from a Pen V reference standard, but the factor is still being measured only once when the $\text{Na}_2\text{S}_2\text{O}_3$ solution is first prepared. I emphasised again the importance of measuring the factor every day and this was done during my visit. I did not have time to check whether the other, less important, potential causes of error identified in my first visit have been corrected.

The comparison of results by HPLC and the iodometric assay given above shows that the iodometric method greatly over-estimates the amount of Pen V in fermentation broth and extraction samples. Some of this over-estimate could be due to other naturally occurring penicillins, but it is very unlikely that such large amounts of these will be present. Consequently the possibility of interference by other components of the fermentation broth was considered and an iodometric assay was carried out on sterilised whole medium before addition of the inoculum, so that no penicillin could be present. This gave a result equivalent to about 6000 units/ml of Pen V. Two possible

approaches to overcoming this interference were proposed; the use of β -lactamase instead of alkali to hydrolyse the penicillin in the assay and identification and replacement of the component of the medium which causes the interference. The availability of β -lactamase will be investigated and measurements will be carried out on the individual components of the medium.

Because of this interference in the iodometric assay, HPLC should be used whenever possible. However, the sample throughput by the iodometric method is considerably higher than by HPLC, so continuing use of the iodometric method is likely to be required.

III. GOOD LABORATORY PRACTICE

The staff working with me on HPLC soon learned and enthusiastically complied with the need to label everything. Regrettably this is still not the case in other areas. Samples for analysis from the fermentation and extraction staff were delivered either unlabelled or inadequately labelled. The labelling of sample solutions during analysis by the iodometric method was either haphazard or non-existent. On two occasions two different fermentation samples were being filtered into unlabelled tubes, side by side in a rack! On another occasion fermentation samples numbered 8, 11 and 12 were being filtered into tubes labelled 6, 7 and 8 respectively! Dr Batchelor and I strongly emphasised to the National Project Director and the Director of the Research Institute that complete and correct labelling is essential. There was a noticeable improvement during the course of the visit, but I think this is a subject that will require frequent re-emphasis from GPF management to change the casual attitude of many of the staff.

The language problem makes it difficult to check whether adequate written records are kept to relate unambiguously the analytical results to individual samples. However, I did find out that no written record had been made of the sample treatment (1 to 10 dilution, filtration and use of 1ml for assay) used for the iodometric assay of two shaker flask samples, even though this was different from the standard procedure previously used for such samples. The need to record such information was emphasised.

IV. SEMINAR

I presented a seminar on HPLC to about 30 staff, which was very ably translated by Mr Cai Shichao. The overheads are attached as Annex 4.

V. DOCUMENTS ETC.

I provided a mouse mat for use with the HPLC computer mouse and documents on HPLC fittings and on trouble shooting in HPLC to laboratory staff. A copy of documents on GLP and on an introduction to Good Manufacturing Practice was supplied to Mr Cai Shichao.

I supplied 3g of p-OH Pen V, a gift from SmithKline Beecham, for use as a reference standard.

VI. MAJOR FINDING

The true Pen V content of the fermentation broth, as measured by HPLC, is much lower than was previously thought from the results by iodometric assay.

RECOMMENDATIONS

The Pen V content of fermentation and extraction samples has to be determined by HPLC rather than the iodometric method.

Attempts should be made to overcome the interference in the iodometric assay by fermentation broth components.

GPF Research Institute staff need continuing on the job practice to ensure their full familiarisation with use of the HPLC. The notes provided in Annex 1 should be used, instead of the manufacturer's operating manual, as the basis for training.

Ancillary equipment for HPLC (pH meter, magnetic stirrer, glassware) needs to be provided in its new location.

The sink and water supply, window blinds and a cooling unit need to be installed in the HPLC laboratory.

GPF Research Institute management need to continue to ensure that basic aspects of GLP, particularly the unambiguous labelling of samples and record keeping, are complied with.

ANNEX 1

Baseline 810 HPLC system - Operating Procedure

When the computer is turned 2 or 3 pages of text are displayed. Wait for this to end with one line showing C:. Type BASE and press enter. The question OK to delete log and scratch files? appears. Type Y and enter. Another question appears. Type Y, enter. The BASELINE 810 logo screen appears. Press Enter. The main menu screen appears.

Prepare the mobile phase and prime the pump.

F6 gives the Monitor Control Events screen. Set %A, %B, flow rate and duration on the slide bars. Set Duration to between 0.5 and 1.0 min.

Turn on the pump, position the arrow on the screen in the GO rectangle, click the mouse left hand button. The pump comes on and gradually speeds up to the set flow rate over the period of time set on the duration slide bar. When the pump is up to the set flow rate the number displayed next to the duration slide bar stops changing and stays at 0.25. Click on QUIT. (It is ESSENTIAL) NOT to QUIT before the pumps are up to the set flow rate).

To use a method that is stored on the systems hard disc

From main menu:

Clear (click on all sections). OK

Load

Enter method title

Sample Q (from main menu)

Edit

Fill

Fill sample Q

- enter number of samples (duplicate injections of 1 sample count as 2)
- enter sample name root and first suffix. (Each name plus suffix MUST be unique, that is it must be different from any other in the system. If not, the data from the previous chromatogram with that name will be deleted and replaced by the data from the new one. This applies even if the previous one was obtained by a different method)

- enter file name root and first suffix. (For convenience it is best to use the same root for sample and file names.)
- select ACQUIRE for sample source
- select UNKNOWN for sample type. (NOTE: STANDARD is used when the method is set up to calculate results relative to reference standard chromatograms. This is complex and is best avoided until you are confident in use of the system.)

Replace. The table fills with the sample list.

OK

Edit

Edit Q-wide parameters

Source - set Use Current Sources

Q processing options - set Save Peak Integration

Report options, output - set Printer

format - set Custom Results

Stripchart options - set Printer

Stripchart (at bottom of screen), gives the Q-wide stripchart parameters screen

- set Use Keyed In Values
- set X axis min. 0, X axis max. to the chromatogram run time (eg 15 mins for PenV).
- set Y axis min. 0, Y axis max. to 0.10 (or some other value found by experience to be suitable for the method). (NOTE: Setting Use Keyed In Values and the values for X and Y axis means that all chromatograms are printed on the same scale. If you set Fit To Highest Peak instead of Use Keyed In Values the Y axis will vary for each chromatogram, making visual comparison difficult.)
- set Peak Starts and Ends, Peak Maxima and Baselines
- set Both

OK,OK,OK,OK (returns to main menu)

Run

Execute Methods

Start

OK (to confirm you want to start acquisition)

The system beeps and Waiting To Acq. appears at the bottom of the screen

Inject the first sample

F5 displays the chromatogram on screen as it is acquired.

When the chromatogram is complete Waiting To Acq. appears at the bottom of the screen. Inject the next sample.

Process and Print the chromatogram by:

F3 (to get the Execute Methods screen)

Resume. This prints the chromatogram and the results report (peak areas etc.) that has been defined when the method was developed and saved in the system.

(NOTE: ALL samples in a Q, including the last one, MUST be processed and printed before a new Q is set up. If this is not done the unprocessed chromatogram is saved on disc and it is difficult to get it integrated.)

To view the integrated chromatogram and peak area on screen:

F3

View

Review current chromatogram (OR, File, Load Chromatograms, type in File Name, OK,OK) - the chromatogram is displayed on screen

Show

Peak Data - retention time, peak area etc. is displayed for one peak at a time. Use the mouse to position the green arrow at the bottom of the screen under the peak for which you want the data to be displayed.

NOTE: In normal operation the system will not allow you to change the sample Q, or to CLEAR a method from active memory, while samples which have not been acquired (that is, the chromatograms have not been run) remain in the Q. Consequently, once acquisition has started, ALL samples listed in the Q have to be chromatographed. If you do not want to chromatograph all samples, or you want to add samples to the Q, it should be possible to do this by:

F5 (to detectors screen)

Abort

Abort Acquisition/Control

OK - a box appears asking if you want to clear the Background acquisition and foreground processing lists

OK

You should now be able to go into the sample Q screen through the main menu, delete or add samples and re-start acquisition through the Execute Methods screen in the normal way, but I have not tried this on the Guangzhou system.

To enter a method in the system and save it on disc

From main menu:

Clear (click on all sections), OK

Develop Method - the list of sections is displayed with a x beside each

OK - the Method Title screen appears

type in the title and file name

OK - the Data Acquisition screen appears

- set Trigger to External 1

- leave pre-acq. delay at 0.0

- set Acq. duration to the required chromatogram run time

- set Data Acq. rate to 1.0

- OK (gives channel information screen)

- leave Expansion 1.0, Offset 0.0

- set Range to 1V

- OK (gives Test Option screen)

- select Test

- enter Sample and File name (the same for both)

- OK (gives the Detector screen)

Inject the sample. The chromatogram is displayed as it is acquired. Adjust Expansion and Offset with the arrow up, arrow down, PgUp and PgDn keys to get suitable peak size and baseline position. Each time you press the arrow up key gives a 2 fold expansion, so keying twice gives 4 fold, three times gives 8 fold etc. Note how many times you key for Expansion and for Offset. Look in the Waters manual to find out what value of Offset each PgUp or PgDn keystroke gives. Go back to the Channel Information screen by Alt-F10 (to the Data Acq. screen), OK, and enter the values of Expansion and Offset you have used. (Eg. For Expansion you enter the 2 or 4 or 8 fold figure, not the number of keystrokes)

NOTE: If a large amount of Expansion or Contraction is required the sample concentration is too low or too high. Start again

with a different concentration.

OK,OK,OK - the Gradient screen appears

Clear table (if it is not clear already)

Enter flow rate etc. in gradient table as follows:

- for an isocratic method, enter in line 1;
time 0, flow rate (in ml/min), %B (the system calculates and enters %A)
enter in line 2;
time = end of run, flow rate and %B the same as in line 1, 0 in the Crv. column
- for a gradient method enter the initial conditions in line 1 and the required conditions at various time points in lines 2 onwards. Refer to the manual for definition of curve shapes defined by the number entered in the Crv. column

OK - the Peak Integration screen appears

Auto, OK (gives the chromatogram on screen)

Choose the smallest peak. Draw a rectangle round it with the mouse left hand button pressed. When you let go of the button the peak appears in the lower box. Draw the rectangle very tightly around the peak so that it expands to fill the lower box. (You will probably need several attempts at this until you become familiar with the procedure)

Note the vertical (Y) scale

Save Peak

If the chromatogram contains both small sharp and small broad peaks, repeat with another peak.

Done

Specify Baseline, OK

Choose a section of baseline with no peaks. If the baseline is uneven include an uneven section. Expand it with the mouse to about the same Y scale that the peak was expanded to

Save Baseline

Done - the Integration Parameters screen shows the parameter values the system has calculated from the chosen peak(s) and baseline

Test - to check parameters. The integrated chromatogram is displayed. If satisfactory integration of all peaks is not obtained, EITHER repeat the Peak Save/Baseline Save procedure OR type in new values for some or all of the parameters on the Integration Parameters screen. The new values must be based on previous experience. Test again.

NOTE: This integration procedure must be done with a typical sample chromatogram, eg. a fermentation broth sample, not just with a reference standard chromatogram.

OK - the Component Table screen appears. This shows the chromatogram in the top part and a table below.

Fill

Using peak results

Replace - the component table fills with results from the chromatogram

Type in the peak identity (eg. Pen V, p-OH Pen V etc.) for those peaks whose identity is known, in the Component column of the table.

Peaks that are not of interest can be deleted from the table with Ctrl/Y.

(NOTE: If you want to set up the method to calculate results automatically relative to reference standard chromatograms, you need to complete the Calibration Parameters screen now. Refer to the manual for how to do this.)

OK,OK - the Operators Notebook screen appears

Nearly all of this can be ignored; the only useful item is Stripchart Comment. Use OK to go through the Notebook screens to the last one. Type an appropriate comment into the Stripchart Comment space, eg. Pen V assay. This comment is printed in the header of the chromatogram. The comment needs to be appropriate for all samples you expect to run by this method.

OK,OK - the Report Generator screen appears

This defines the format of the report that is printed with the chromatogram. Select the parameters that you want included in the report and enter the column number where you want that parameter to appear. A suitable format for many methods is: Pk. No., Pk. Start, Pk. End, Retention Time, Type, Peak Area, Area Percent and Component Name as columns 1 to 8 respectively.

OK,OK - the Sample Q screen appears

If you have any samples to run immediately with the new method fill this in as described above on pp 1 and 2.

SAVE the method to disc immediately you have finished the sequence described above. Do not wait until samples have been chromatographed because the information will be lost if a power failure occurs or a fault develops in the computer before it has been saved. To save the method:

Alt/F10 - to main menu

Save

Enter method name

Check that there is an X against all sections of the method

OK,OK

The system gives no visual confirmation that the save operation has been carried out successfully. The only way to find out is to CLEAR the method from active memory and then LOAD it. If subsequently you want to change specific sections of a method (eg. peak integration parameters) you can Edit and Save only that section.

To load chromatograms from disc and re-process them

From main menu:

Sample Q

Edit

Fill

Fill sample Q

- enter sample and file name root and first suffix of chromatograms you want to re-process
- enter Sample Source, load from disc

Replace

F3, to get the Execute Methods screen

Edit

Edit Q wide parameters

- set Review peak Integration
- set Source disc
- set Report and Stripchart options as usual (see p.2)

Reset

Reset for Processing

Start - the first chromatogram is displayed on screen

Show - set Peak Starts and Ends

Peak - this gives options to move peak starts and ends and the baseline using Alt S, Alt E and the mouse. Adjust these as necessary for each peak to obtain satisfactory integration

Quit

Done - the re-processed chromatogram and report should be printed automatically and the next chromatogram in the Q should be displayed. If printing does not occur automatically:

F3 to Execute Methods

Resume

[An alternative procedure to re-process a chromatogram on screen without using the sample Q is as follows:

From main menu:

View

Review Chromatograms

File

Load Chromatograms - type in the File Name

OK,OK,OK - gives the chromatogram on screen

Show - set Peak Starts and Ends

Peak - adjust integration as necessary

File

Save

Print

BUT this only prints the screen, not the full report with integration results that you get using the sample Q method.]

To view the Directory of Chromatograms

From main menu:

View

Review Chromatograms

File

Load Chromatograms

Directory - the list of chromatogram file names is displayed.

This can be several pages long. Use More and

Back to scan it.

To compare chromatograms on screen

From main menu:

View

Compare Chromatograms

File

Load Chromatogram - type in the file name. The chromatogram is displayed in the upper box

Append Chromatogram - type in the file name. The chromatogram is displayed in the lower box

You can compare any 2 chromatograms by typing in the appropriate file names to put one in the upper box and one in the lower box.

System (data) back-up

Operation of the system will slow down when about 1MByte of data is stored. The data that you need to keep must be backed-up (that is, copied) onto a floppy disc and then deleted from the hard disc. With the system in constant use this probably needs to be done about once every month. Refer to Waters technical staff for the procedure to do this - I could find nothing about it in the manual. Always check that data has been transferred correctly to the floppy BEFORE deleting it from the hard disc.

ANNEX 2

HPLC Analysis of Pen V Fermentation and Extraction Samples**Whole Broth**

Stir the broth carefully with a glass rod. Do NOT get air bubbles in it.

Measure 25ml of the broth in a 25ml measuring cylinder. Transfer it to a 250ml volumetric flask or measuring cylinder. Wash the 25ml cylinder with distilled water. Transfer the wash water to the 250ml flask or cylinder. Add distilled water to make the volume up to 250ml. Shake for about 1 minute. Filter some of the mixture through filter paper. Filter some of the filtrate through a fine pore membrane (0.47 μ m or similar) before injecting it into the HPLC.

pH 4.5 Filtrate

Filter the sample through filter paper. Pipette 1ml into a 10ml volumetric flask (or 5ml into a 50ml flask). Add distilled water to make the volume up to 10ml (or 50ml). Filter some of the solution through a fine pore membrane before injecting it into the HPLC.

pH 2.5 Filtrate

Filter the sample through filter paper. Pipette 2ml into a 10ml volumetric flask. Add distilled water to make the volume up to 10ml. Filter some of the solution through a fine pore membrane before injecting it into the HPLC. [NOTE: The pH 2.5 filtrate samples contain low amounts of Pen V].

Acetone Solution of Pen V**1) Solution containing solid**

Filter through paper. Pipette 1ml of filtrate into a 50ml volumetric flask. Add 20ml acetonitrile (HPLC grade). Add distilled water to make the volume up to 50ml. Inject some of this solution into the HPLC. [NOTE: Filtration through a fine pore membrane should not be necessary. If the solution is cloudy prepare another one using 30ml acetonitrile]

2) Clear solution

Proceed as above for a solution containing solid but leave out the filtration through paper.

Filtrate from Pen V (K salt) Filtration

(NOTE: This is an acetone solution)

Pipette 1ml into a 10ml volumetric flask. Add 4ml acetonitrile (HPLC grade). Add distilled water to make the volume up to 10ml. Inject some of this solution into the HPLC. [NOTE: Filtration through a fine pore membrane should not be necessary. If the solution is cloudy prepare more solutions with varying volume of acetonitrile (either more or less than 4ml) to obtain a clear solution].

Acetone Wash of Precipitated Pen V (K salt)

Proceed exactly as above for the Filtrate from Pen V (K salt) filtration.

Solid Pen V (K salt)

Weigh accurately about 60mg sample into a 25ml volumetric flask. Dissolve in, and dilute to volume with, distilled water.

IMPORTANT NOTE

All Pen V solutions for analysis must be injected into the HPLC as quickly as possible after preparation. Whenever possible (e.g. with solid samples) inject into the HPLC immediately after preparation of the solution. NEVER leave Pen V solutions at room temperature for a long time before analysis. If solutions have to be kept before injection, put them in a refrigerator.

HPLC Assay Procedure

Column:	25 or 30cm long, C18, 5 μ m (Waters Microbondapak or other make)
Mobile Phase:	Mix 650ml distilled water, 350ml acetonitrile and 5.75ml glacial acetic acid
Flow Rate:	1.0ml/min
Detection:	254nm
Injection Volume:	10 μ l
Reference Standard Solution:	Weigh accurately about 60mg Pen V reference standard

into a 25ml volumetric flask. If the reference standard is Pen V potassium salt, dissolve it in, and dilute to volume with, distilled water. If the reference standard is Pen V acid, dissolve it in, and dilute to volume with, mobile phase.

Sequence for Standard and Sample Injections:

Make injections in the following order; standard solution 1, standard solution 2, sample solution 1, sample solution 2, sample solution 3, sample solution 4, standard solution 3, sample solutions 5, 6, 7 and 8, standard solution 4 etc.

Whenever possible, especially for analysis of solid samples, make 2 separate solutions of the sample.

ALWAYS inject at least 2 standard solutions, even if you have only one sample to assay. The sequence can be changed if circumstances make this necessary, but it is essential to include several standard solutions in a set of sample solutions.

Calculations

$$\text{Pen V content of liquid samples} = \frac{A_1 \times P \times D}{F} \text{ units/ml.}$$

where A_1 is the area of the pen V peak in the sample chromatogram

P is the purity of the pen V reference standard in units/mg

D is the dilution factor for the sample

[When the procedures given above are followed

$D = 10$ for whole broth and pH 4.5 filtrate

$= 5$ for pH 2.5 filtrate

$= 50$ for acetone solutions

$= 10$ for filtrate from Pen V (K salt) and the acetone wash of precipitated Pen V (K).]

F is the average factor from the reference standard injections, calculated from:

$$F = \frac{A_2 \times V}{W}$$

where A_2 is the area of the pen V peak in the reference standard chromatogram

V is the volume in which the reference standard is dissolved

(=25ml in the procedure given above)

W is the weight of reference standard, in mg

The value of F from the different reference standard chromatograms should be within a range of about 2%. If the range is wider than this more reference standard solutions should be injected to obtain a more reliable average value of F.

$$\text{Pen V content of solid samples} = \frac{A_1 \times V_1 \times P}{F \times W_1} \text{ units/mg}$$

where W_1 is the weight of the sample taken, in mg
 V_1 is the volume in which the sample is dissolved (=25ml in the procedure given above)

Retention Times

Approximate retention times of Pen V and related compounds are:

Penicilloic acid of pen V	4.5 mins
p-OH Pen V	5.1 mins
Phenoxyacetic acid	6.6 mins
Pen V	12.5 mins

ANNEX 3

**HPLC Assay of p-OH Pen V and Phenoxyacetic Acid in
Pen V Fermentation Samples**

Prepare and chromatograph samples as described for analysis of Pen V content.

Phenoxyacetic acid standard solution:

Weigh accurately about 25mg phenoxyacetic acid in a 50ml volumetric flask.

Dissolve it in 20ml acetonitrile and dilute to volume with distilled water.

p-OH Pen V standard solution:

Weigh accurately about 10mg p-OH Pen V in a 50ml volumetric flask.

Dissolve it in 20ml acetonitrile and dilute to volume with water.

[NOTE: p-OH Pen V is very difficult to obtain. Take great care not to use more than is necessary]

Inject the standard solutions just before or just after the fermentation samples.

Calculation

$$\text{Phenoxyacetic acid content} = \frac{A_1 \times 50 \times D}{A_2 \times W_1} \text{ mg/ml of sample}$$

where A_1 is the area of the peak at the phenoxyacetic acid retention time in the fermentation sample chromatogram

A_2 is the area of the peak in the standard solution chromatogram

W_1 is the weight in mg of phenoxyacetic acid dissolved in 50ml

D is the dilution factor for the sample (see the Calculation section in the procedure for assay of Pen V)

$$\text{p-OH Pen V content} = \frac{A_3 \times 50 \times D}{A_4 \times W_2} \text{ mg/ml in sample}$$

where A_3 is the area of the peak at the p-OH Pen V retention time in the sample chromatogram. [NOTE: The p-OH Pen V content is likely to be very low and it is possible that no peak will be visible]

A_4 is the area of the peak in the standard chromatogram

D is the sample dilution factor

W_2 is the weight in mg of p-OH Pen V dissolved in 50ml

NOTE: The sensitivity for p-OH Pen V can be increased by obtaining the chromatograms at 280nm instead of the normal wavelength of 254nm. If you need to use the higher sensitivity you must obtain both the sample and the p-OH Pen V standard chromatograms at 280nm, but you must also obtain the sample chromatogram at 254nm to measure the Pen V and phenoxyacetic acid contents.

You will need to use a lower concentration of p-OH Pen V standard at 280nm. 5mg in 50ml should be suitable.

**High
Performance
Liquid
Chromatography**

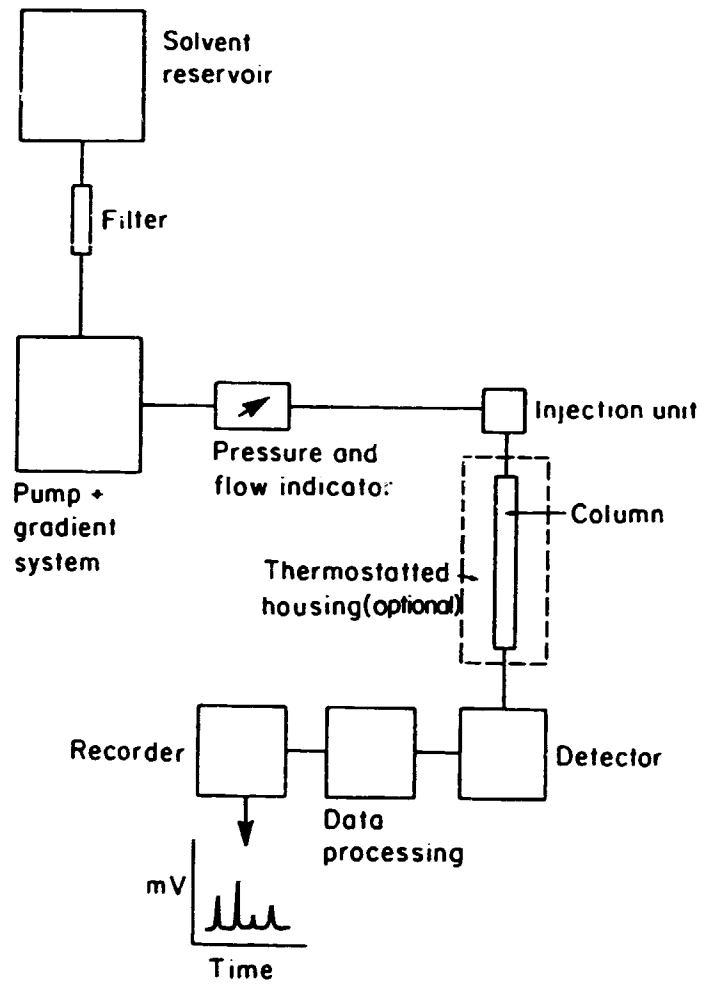
CHROMATOGRAPHY

**A technique for separating mixtures of compounds
by use of their different extent of distribution
between a stationary phase and a mobile phase**

H P L C

Stationary phase: Small particles, usually 3 - 10 μm ,
packed in a column, usually
10 - 30 cm long; 4 - 5 mm i.d.

Mobile phase: A liquid, aqueous or organic or a
mixture, pumped through the column
at high pressure to give flow rates
that are usually 1 - 3 ml/min



Block diagram of a high performance liquid chromatograph

HPLC detectors

UV - fixed wavelength

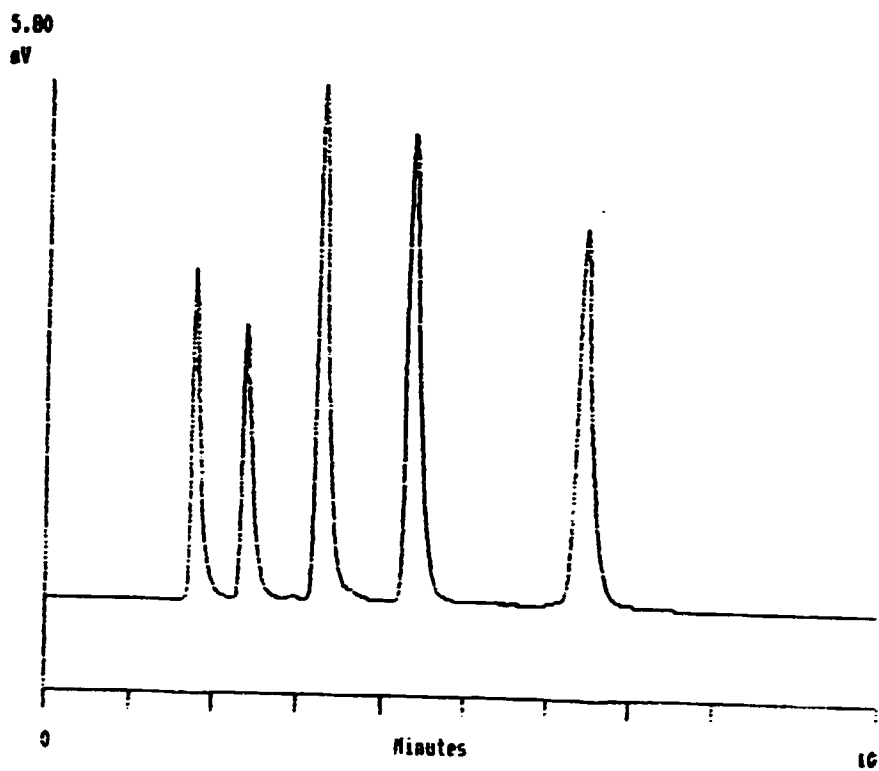
- variable wavelength

- diode array

Electrochemical

Fluorescence

Refractive index



PEAK	IDENTITY	R/TIME
1	URACIL	1.77
2	PHENOL	2.39
3	ACETOPHENONE	3.26
4	METHYL BENZOATE	4.34
5	TOLUENE	6.44

Main uses of HPLC

Qualitative: to identify components in a mixture

- resolve components from each other
- compare R_t with that of known compounds likely to be present
- collect material after elution for identification

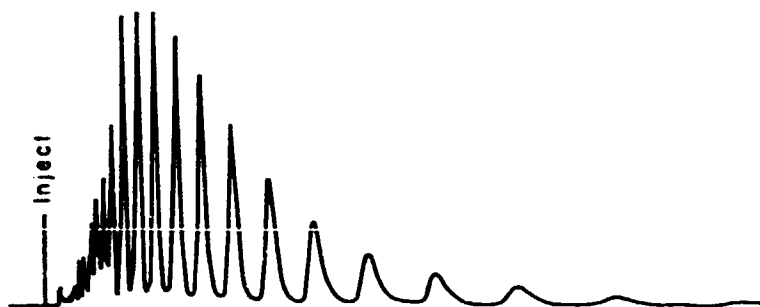
Quantitative: to measure how much of particular compounds is present in a mixture

- resolve the compound(s) of interest from all other compounds in the sample

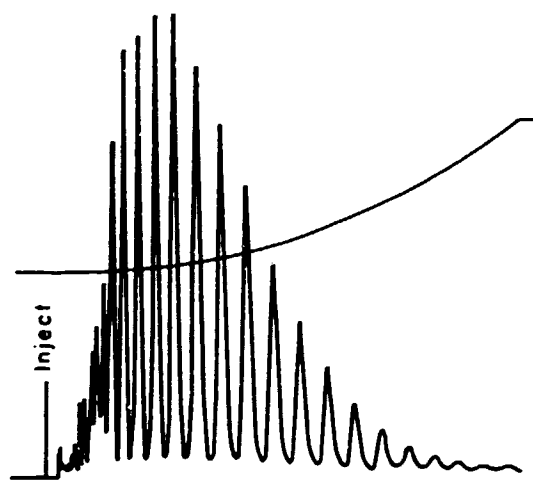
Isocratic and gradient elution

Isocratic - same mobile phase throughout chromatogram

Gradient - mobile phase varies during chromatogram



Chromatogram of Triton X-100. Isocratic elution



Chromatogram of Triton X-100. Gradient elution

Different types of HPLC

Ion exchange

Size exclusion

Normal phase

Reverse phase

Ion exchange HPLC

**Stationary phase: Ionic resin or ionic groups
bonded to silica**

Mobile phase: Aqueous buffer

Sample type: Amino acids

Size exclusion HPLC

Stationary phase: Porous gel or silica

Mobile phase: Aqueous buffer

Sample type: Large molecules, proteins etc.

Normal phase HPLC

- Definition:** Polarity of stationary phase is greater than that of mobile phase
- Stationary phase:** Silica or polar group (NH_2 , CN) bonded to silica
- Mobile phase:** Low polarity organic solvents eg. hexane, chloroform
- Typical samples:** Ketones, alcohols, amines

Reverse phase HPLC

Definition: Polarity of stationary phase is less than that of mobile phase

Stationary phase: Hydrocarbon or polar group (NH_2 , CN) bonded to silica
Synthetic polymer

Mobile phase: Aqueous or aqueous/organic mixture

Sample type: Almost anything

Main stationary phases for reverse phase HPLC

C18 bonded to silica

C8 bonded to silica

CN bonded to silica

NH₂ bonded to silica

[C18 = ODS = octadecylsilane]

**Different makes of C18 stationary phase may
give different results**

Mobile phase components for reverse phase HPLC

Water, pH 2 - 8

Buffer salts (eg. phosphate, acetate)

Organic solvent (eg. CH_3OH , CH_3CN)

Origin of HPLC methods

Develop your own

Pharmacopoeias

Scientific journals

Personal contact

HPLC method development

Consider compound type (ionic etc.)

Choose HPLC type (reverse phase etc.)

Obtain UV spectrum, choose detector wavelength

For most compounds start with C18

Vary mobile phase to get acceptable R_t

Check that other compounds likely to be present
in sample are resolved from compound to be
measured

Validate linearity, precision etc.

Quantitative analysis by HPLC (1)

Peak area or height proportional to amount of compound injected

Area normalisation

Relative to a reference standard

- of the same compound
- of a different compound using a response factor
- with an internal standard

Quantitative analysis by HPLC (2)

Inject a reference standard solution of known concentration

Inject the same volume of a sample solution of known concentration

Measure the area of the peak(s) in both chromatograms

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

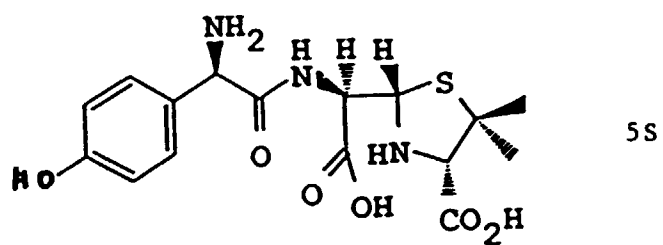
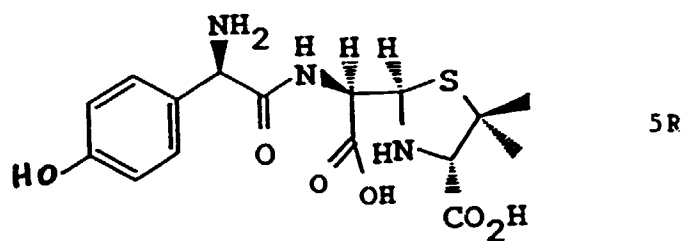
Reference standard and sample must be run on the same day with same equipment and same batch of mobile phase

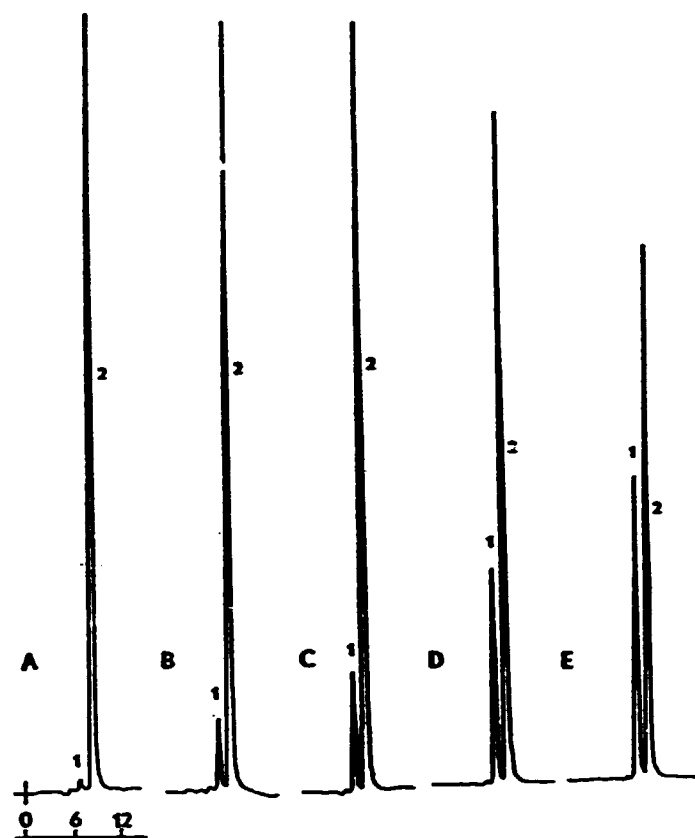
HPLC of stereoisomers

Enantiomers (chiral chromatography)

Diastereoisomers

AMOXYCILLIN PENICILLOIC ACID
DIASTEREOISOMERS





TIME (min)

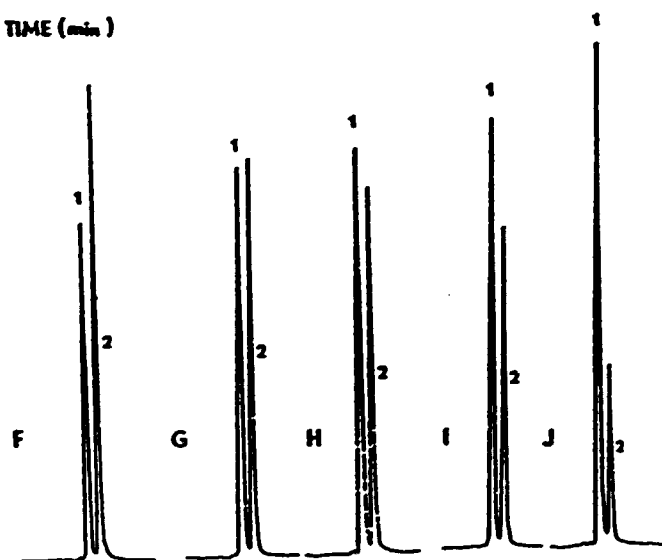


Fig. 1. HPLC scans of amoxicillin penicilloic acids in 0.05 M phosphate buffer (pH 5.4) at room temperature. Conditions: two 150 x 4 mm I.D. Spherisorb® ODS (5 μ m) columns in series; mobile phase: 0.05 M phosphate buffer (pH \approx 5.9); flow-rate: 0.8 ml/min; detection: 228 nm, 0.04 a.u.f.s. Peak identification: 1 = amoxicillin penicilloic acid (α -form), 2 = amoxicillin penicilloic acid (β -form). Time in solution: A = 0.15 h; B = 1.07 h; C = 1.95 h; D = 3.98 h; E = 7.01 h; F = 9.04 h; G = 12.07 h; H = 14.09 h; I = 17.13 h, and J = 31.29 h.

Practical aspects of HPLC

High purity water

High purity solvents

Filter dirty samples

Use a guard column

**Monitor column performance;
replace it when necessary**

Expensive

Backstopping Officer's Technical Comments on the Report of Mr. A.E. Bird

Mr. Bird's report underlines the importance of the validation of quality control assays in a development project such as the Improved Production of Penicillin. Without proper validation, one cannot relate the results obtained by the traditional iodometric and HPLC assays of Penicillin V.

Without going into details of the validation concept, it seems to be important to give the definition of the term as it appeared in the FDA Guidelines on General Principles of Process Validation (20 March 1986): "Process validation is establishing documented evidence which provides a high degree of assurance that a specific process will consistently produce a product, meeting its predetermined specification and quality attributes".

If slightly amended the same definition can stay for testing process validation, in which case the product would be the test result.

The experience gained at the Research Institute of the Guangzhou Pharmaceutical Factory shows that the most important R + D, GLP, GMP, QA and validation documents are missing or at least have not been made available for UNIDO in English. Without the most important basic documentation such as experimental protocols, standard operating procedures, batch production records, testing protocols, testing records, detailed process description, etc., no development work can be properly carried out.

For any further development it is strongly recommended that:

- 1) The flowsheets and the SOPs of the processes should be defined for Penicillin V production and testing;
- 2) The critical operating parameters should be defined;
- 3) The ranges of critical operating parameters should be identified in each direction (end values);
- 4) The critical operating parameters and the actual results of each run, irrespectively whether it had been successful or not, should be recorded.