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STANDARD OPERATING PROCEDURE (SOP) OF – HIGH PERFORMANCE LIQUID CHROMATOGRAPHY SYSTEM (HPLC)

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ABSTRACT: HPLC is a form of liquid chromatography used for separating compounds that are dissolved in solution. It is a technique for identification, quantification and purification of mixtures for analytical purposes. Applications are found in diverse fields such as in forensic science, pharmaceuticals, food and flavor, clinical tests and in many others. Standard Operating Procedure of HPLC System (Make: DIONEX, Model: ULTIMATE – 3000) has been developed. The different steps involved for operating HPLC System have been successfully explained.

KEYWORDS: Standard Operating Procedure (SOP), HPLC, DIONEX ULTIMATE-3000, blood, urine, pharmaceutical preparations, etc.

INTRODUCTION:

For the separation of different compounds in a given solution chromatographic techniques are used which separates the compounds based on their binding affinities with the stationary or the mobile phase. The mixture is dissolved in the mobile phase which carries it through the stationary phase. In HPLC pump is used to pass the solvent and sample mixture through a column filled with adsorbent leading to the separation of sample components. The adsorbent which is used in column is usually a granular material made up of solid particles(eg : Silica). A detector is used to see the eluted components from the column. The information from the detector is send to computer which generates The purpose of HPLC analysis of any drug is to confirm the identity of a drug and provide quantitative results[1,2,3].The HPLC System (Make DIONEX, Model ULTIMATE -3000) is composed of Chromeleon Software, Vacuum Degasser, Pump, and Column Compartment with 250mm, 5µ, 120Å C-8 Column and Photo Diode Array Detector. The solvents used are usually ACN, Methanol, Ultra Pure Water, IPA in different ratios are used in different ratios.Sample retention time will vary depending upon the

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interaction between the stationary phase, the compounds being analyzed and the solvents used. Compound that have least amount of interaction with the stationary phase and more amount of interaction with the mobile phase will be eluted faster.

Purpose

To develop the standard operating procedure for HPLC make DIONEX, Model ULTIMATE-3000. To ensure its compliance with the provision of Good Laboratory Practice regulations.

Scope

Describes the finest details of the steps to be followed in one of the simplest and precise way required in the analytical technique.

Responsibilities

All the scientific staff members carrying out the HPLC (Make DIONEX, Model ULTIMATE-3000) are responsible for strictly adhering to the procedures given in this text.

DIFFERENT STEPS OF STANDARD OPERATING PROCEDURE OF HPLC

1. **Cold start of HPLC**

1. Generally the instrument is kept "ON'' in the morning and the unit is put "OFF" in the evening.
2. First start the air conditioners
3. Switch on the UPS followed by the Computer.
4. Switch on the ultimate 3000 pump (from the switch which is located at the back of the pump)
5. Switch on the ultimate 3000 column compartment (from the switch which is located at the back of the pump)
6. Switch on the ultimate 3000 Detector (from the switch which is located at the frontof the pump)
7. Go to chromeleon software

2. Purging of Channels

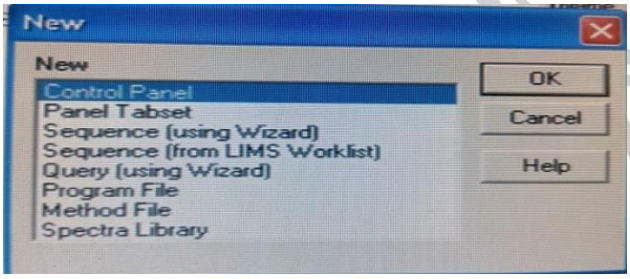
1. Double click on the **Chromeleon software**. Window of last work done will appear.
2. Go to **Default panel tab** set at task bar. Chromeleon server window will appear click on my computer followed by ok.
3. Then click on my computer, click on chromeleon server.
4. **Panel tabset 1** window will appear. Go to Home connect all (Pump, Column compartment and detector). Green signal will appear if connected.
5. Click on the pump, **pump setting window** will appear.
6. Open the **purge valve**, by opening the ultimate 3000 pump compartment (a metal screw is located, loose it) (rotate it in the anticlockwise direction).
7. Click on the motor, green signal will appear if connected.
8. There are four lines present in the system (A, B, C, and D). To purge those lines, set the percentage value of eluent i.e
9. 100% (purge the line whichever is necessary a/c to sample by setting the solvent volume 100%) in each line one by one.
10. For purging line B, C, and D, make it 100% each one by one.
11. Now click on Purge off then following signal will appear

Do you really want to execute this command?

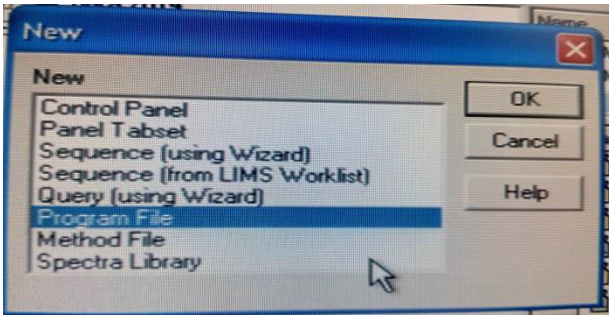
12. Click on OK ,then the icon of Purge: on will turn green
13. Now purging will start which can be seen on ultimate 3000 pump compartment screen
14. After 5 min purging of line will be completed
15. Purging of line A, we have to make **B, C&D** zero.
16. Same procedure is followed for all lines.
17. After completion of purging valve should be close.

3. How to Create New Program

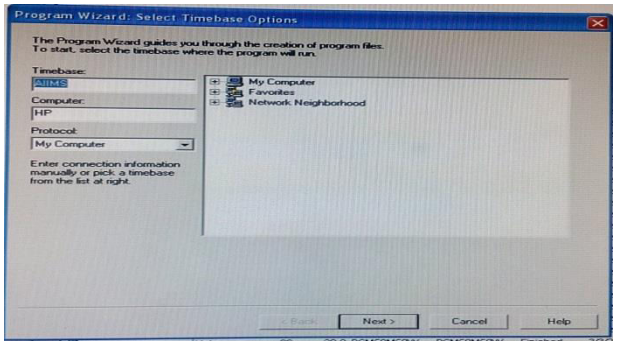
1. Go to file and select New, the following tab set will appear



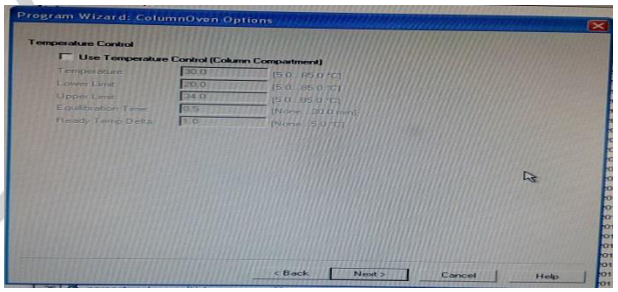
2. Select Program file and click on OK.



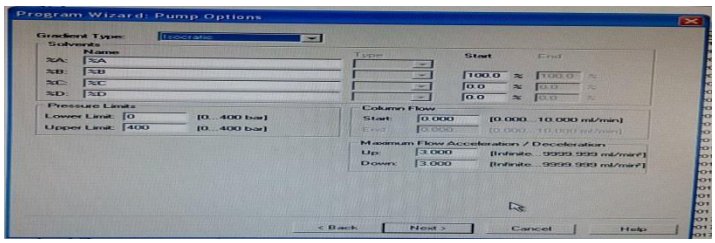
3. After clicking on OK the following window will appears :



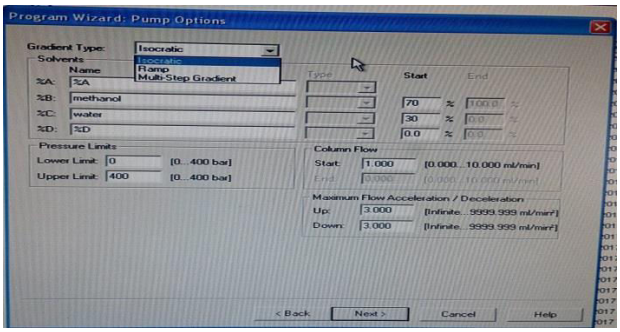
4. Click on Next. Following window will appear: According to the analyte Temperature will be set if required.



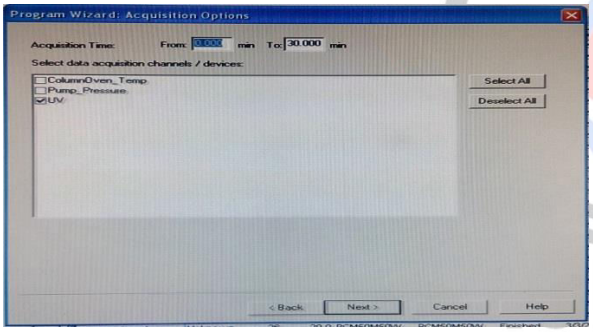
5. Click on Next. Following window will appears : In this section Gradient type, Name of the Solvents, Solvent Percentage, Pressure limit and Column flow will be selected. Maximum flow acceleration/deceleration will be default .



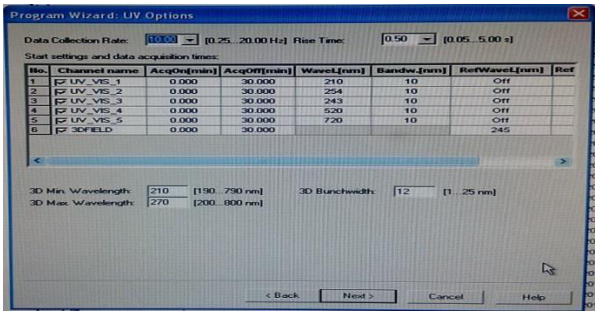
6. Gradient type will be selected according to the analyte. It can be Isocratic, Ramp or Multi gradient.



7. Click on Next. Following window will appear: For data acquisition UV will be selected. Here total run time for single analysis will mentioned as per requirement.

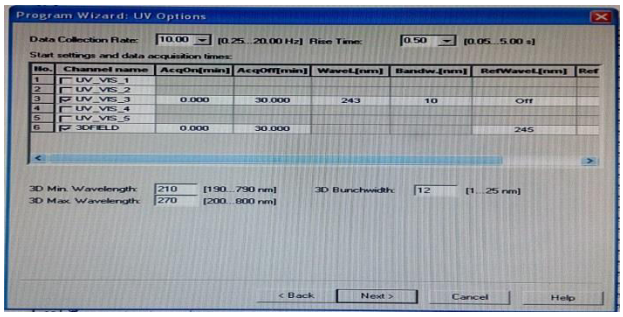


8. Click on Next. Following window will appear:

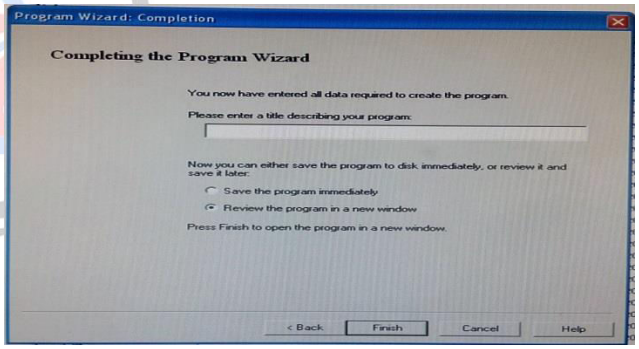


→ Click on desired wavelength and 3D, disconnect rest of the wavelength by just clicking on each one when working on a single wavelength.

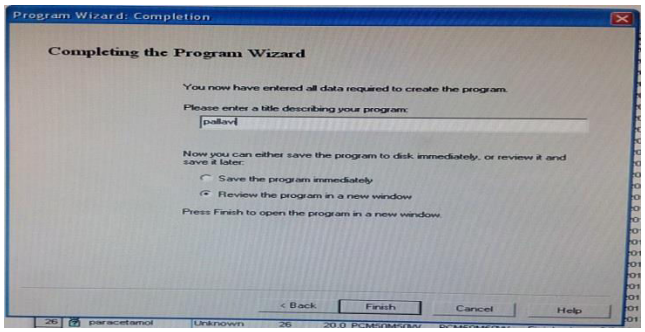
→ Generally UV option is clicked for data acquisition.



9. Click on Next. Following window will appear: Give the name of program and select Review program in new window then open program file and recheck it then click on save in the folder having all the program file.

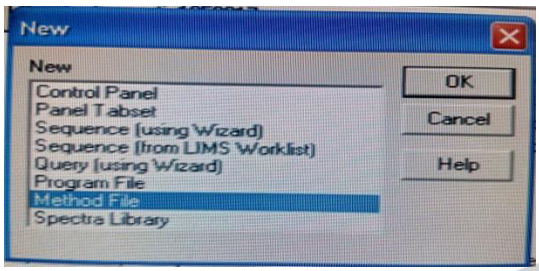


10. Click on Finish the following window will appears. Review this program if changes will required then do it and save this program in program folder located in HP local.

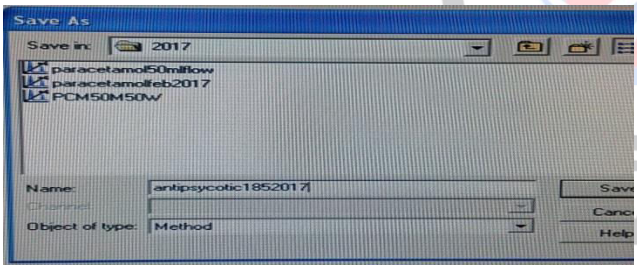


4. How to Create Method file

1. Go to File and click on new, select Method File and click on OK.

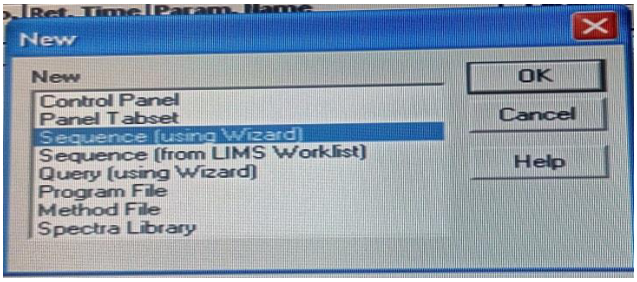


2. A window will open save the window and assign same title as given to the program file then save in the folder having method file.



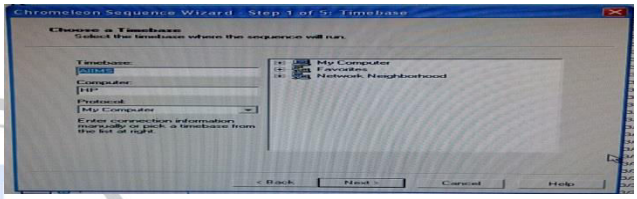
5. How to create new sequence

1. Go to File and click on new, select Sequence (using Wizard).

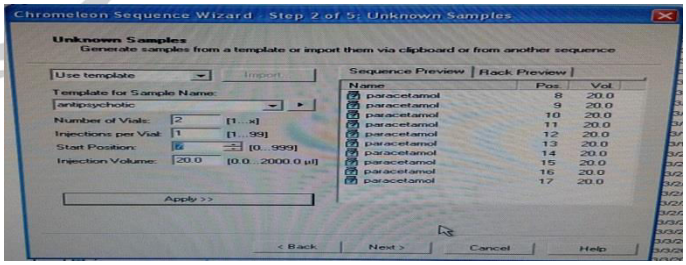


2. Click on OK. Following window will appear.

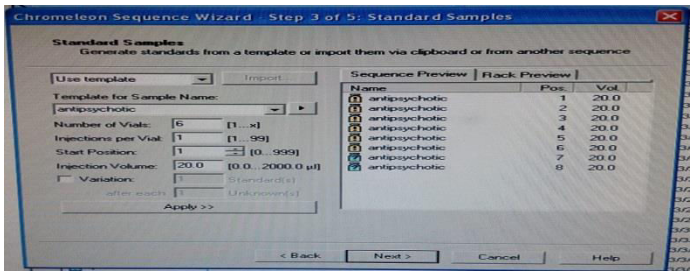
3. Click on Next. Following window will appear:



4. Click on Next. Following window will appear: Select number of vials and inject/vials(for Sample) as per requirement and click on Apply

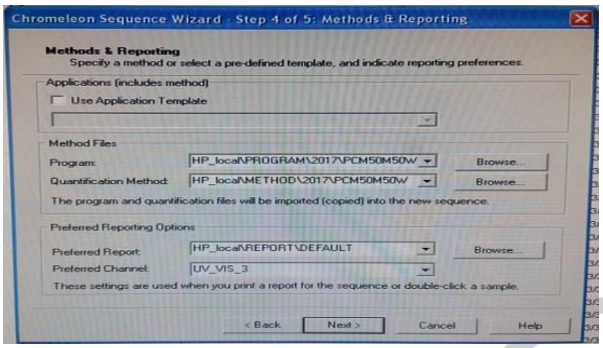


5. Click on Next. Following window will appear: Select number of vials and inject/vials(for Standard) as per requirement and click on Apply.

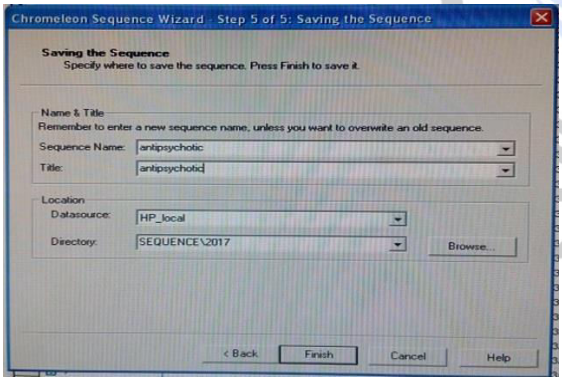


6. Click on Next. Following window will appear : Click on Browse to give select

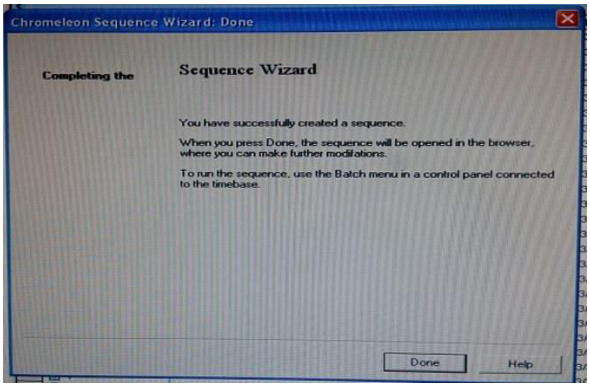
the name of the Program and Quantification Method



7. Click on Next. Following window will appear : Give the name of the Sequence.



8. Click on Finish. Following window will appear :



9. Click on Done. Following window will appear
10. Go to batch and click on start.
11. Start batch window will appear.
12. Add desired batch.
13. Then click on ready check.
14. If ready check is successful then click on ok.
15. Click on start..
16. Green background strip will appear.
17. Now go in panel tab set then double click on PDA and wait for the message on control panel screen. Following message will appear: AZ- Done
18. Now load the sample with the help of 25 micro lit syringe and turn valve from load to inject.

➢ Now wait for the peak to appear on screen

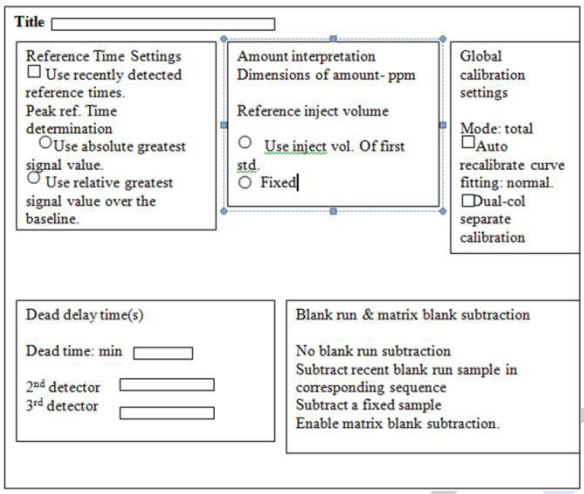
6. Procedure for Peak Decoration: (Quantification)

1. Go to Chromeleon Browser.
2. Go to SEQUENCE and select the concerned sequence.
3. Click on the serial number one.

Chromatogram will appear

Go to the tool bar then select QNT Editor.

4. QNT editor will appear.
5. Click on GENERAL tab at the bottom of the screen. Following screen will appear.
   - Specify Title
   - Specify Dimension of amount as ppm.



6. Click on Detection Sheet. Following window will appear:

No.	Retention Time (min)	Parameter Name	Parameter Value	Channel
1	**0.00**	**Min Area**	**"[signal] min"**	**All Channels**

- Append line
- Specify 'Inhibit Integration' at 0.00 time and set parameter value 'ON' at serial no. 2.
- Specify suitable time limit to remove ghost peaks e.g. 9.00 min(inhibit integration off).
- Specify minimum area to remove smaller peaks at serial no. 1.
- Specify fronting sensitivity factor and tailing sensitivity factor to obtain optimum results.
- Click on SAVE. ☐

7. Go to Peak Table Sheet. Following window will appear:

No.	Peak Name	Retention Time	Window	Standard	Int. Type	Cal. Type	Peak Type	Group	Comments
1.									

- Give Peak Name
- Give average retention time.
- Give window
- Click on SAVE.

8. Go to amount table sheet. Following window will appear:

No.	Peak Name	Retention time	Resp. Factor	Amount of 5ppm	Amount of 10ppm	----	Amount of 1000ppm	Comment
1.								

- Right click on the page →columns → edit amount columns Window will appear.

Edit amount column

- Set assign standards on the basis of 'Name'.
- Click on Auto generate.
- Generate a separate amount column for each standard

Window will appear. Click on apply→ OK.

- All the standards will be seen in amount table sheet→ set amount of ppm used for each standard.
- Click on SAVE.

7. System Shut Down Procedure

1. Go to pump settings in the panel window.
2. Set the flow rate to zero.
   a) Check result window.
   b) Click OK.
3. Wait for flow rate to become zero on pump display screen.
4. Switch off the motor by clicking OFF button.
5. Click on DISCONNECT button.
6. Close pump settings window.
7. Chromeleon PDA detector settings window will appear.
8. Switch off the UV lamp.
9. Click on DISCONNECT button.
10. Close the detector settings window.
11. Chromeleon panel 1 column compartment window will appear.
12. Close the panel window.
13. Switch off the Ultimate 3000 Pump switch located at the backside of the equipment.
14. Switch off the Ultimate 3000 Column Compartment switch located at the backside of the equipment.
15. Switch off the Ultimate 3000 PDA Detector switch located on the front of equipment.
16. Shut down computer.

PRECAUTIONS REGARDING OPERATING HPLC:[4,5,6,7,8]

1. We should purge only those lines which are going to be used.
2. Daily purging is not necessary. If the system is closed for long time purging is must.
3. Always use ultrapure water which should be sonicated before use.
4. All organic solvent should be sonicated well before use.
5. Lines should be dipped properly in the solvent.
6. Bottles for solvents should be cleaned. Always check for any fungal growth or any dirt before use.

TROUBLESHOOTING:[4,5,6,7,9]

7. Only HPLC grade solvents should be used so that the chances of contamination are as low as possible.
8. Buffer salts should be flushed from the system daily with ultrapure pure water so that the lifetime of column can increase.
9. The sample should always be dissolved in the mobile phase to avoid peak broadening, variable peaks and split peaks.
10. Variable peak heights, split peaks, and broad peaks can be caused by incompletely filled sample loops, incompatibility of the injection solvent with the mobile phase, or poor sample solubility. Whenever possible, dissolve and inject samples in mobile phase. Otherwise, be sure the injection solvent is of lower eluting strength than the mobile phase[4].
11. Detector problems fall into two categories — electrical and mechanical/optical. For electrical problems, you should contact the instrument manufacturer. Mechanical or optical problems usually can be traced to the flow cell[4].

DISCUSSION AND CONCLUSION:

In this developed system operation procedure (SOP), all the information regarding how to operate the system, how to resolve problems encountered during analysis and what should be the precautions taken during running the system were gathered in one platform bit by bit. It will be immensely helpful to the laboratory personals to accomplish routine complex analysis and also play an important part to achieve effectiveness, great output and consistency in performance

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