**Practical 2**

**High Performance Liquid Chromatography**

**DATE:30/09/22**

**Aim**

To separate Diclofenac sodium from its combination formulation (Dicloran A) using HPLC.

**Introduction:**

Chromatography is an analytical technique where a sample sample mixture is separated is separated into its individual component. High Performance Liquid Chromatography (HPLC) is one such mode of chromatography. It involves Mass-transfer between stationary and mobile phase. HPLC utilizes a liquid mobile phase and solid / liquid stationary phase to separate the components of a mixture. The sample is first dissolved in a solvent and forced to flow through a chromatographic column under high pressure. In the column, the mixtures separate into its components. The column is packed with the stationary phase composed of irregularly or spherically shaped particles. The amount of resolution is important and is dependent on the extent of interaction between the solute components and both the phases. As a result, HPLC acquires a high degree of versatility not found in other chromatographic system and it has the ability to easily separate a wide variety of chemical mixture.

# Instrumentation

HPLC instrumentation includes a pump, injector, column, detector and data acquisition system. The heart of the system in column, where the separation occurs since the stationary phase is composed of micrometer size porous particles. A high pressure pump is required to move the mobile phase through the column. Eventually each component elutes from the column as a narrow band and is recorded by the recorder. Detection of the eluting compound is important. The response of detector is displayed on a chart recorder or computer screen and is known as chromatogram.

# Stationary Phase

Modern HPLC stationary phases are made up of small rigid porous particles with high surface area. Particle size 3 to 10 micrometers. Particle size distribution as narrow as possible depending on the type of ligand attached to the surface. The adsorbent could be normal phase (-OH, -NH2) or reversed phase and even anion or cation exchanger.

# Mobile Phase

In HPLC, the types of band composition of eluent is one of the variable influencing the separation of the particles should be purity, detector compatibility, solubility of sample, low viscosity, chemical inertness.

The HPLC instrument includes the following components-

* **PUMP**
  1. The role of the pump is to force a liquid (called the mobile phase) through the liquid chromatography at a specific flow rate, expressed in millimeters per min (ml / min).
  2. Normal flow rate in HPLC are in the 1 to 2 ml / min range.
  3. Typical pumps can reach pressure in the range of 6000 – 9000 psi (400 to 600 bar).
  4. During the chromatographic experiment, a pump can deliver a constant mobile phase composition or an interest mobile phase composition (gradient)
* **INJECTOR**
  1. The injector serves to introduce the liquid sample into the flow stream of the mobile phase.
  2. Typical sample volume are 5 to 20 microlitre.
  3. The injector must also be able to withstand the high pressure of the liquid system.
  4. An auto sampler is the automatic version for when the user has many samples to analyse or when manual injection is not practical.
* **COLUMN**
  1. Consider the “heart of the chromatograph” – the column’s stationary phase separates the sample components of interest using physical and chemical parameters.
  2. The small particles inside the column are what cause the high pressure at normal flow rates.
  3. The pump must push hard to move the mobile phase through the column and this resistance cause a high pressure within the chromatograph.
* **DETECTOR**
  1. The detector can detect the individualanalyte that come out (elute) from the column.
  2. A detector serves to measure the amount of these molecules so that the chemist can quantitatively analyse the sample components.
  3. The detector provides an output to a recorder or computer that result in the liquid chromatogram (i.e. the graph of the detector response).
* **COMPUTER**
  1. Frequently called the data acquisition system.
  2. The computer not only control the all the modules of HPLC but it takes the signal from the detector and uses it to the signal to determine the time of elution (retention time) of the sample components and the amount of sample (quantitative analysis).

**PRINCIPLE:**

Reversed phase HPLC is the most popular mode of chromatography. In reverse phase chromatography, the stationary phase is relatively non-polar than the mobile phase. The stationary phase is the liquid coated on inert solid support which is mostly silica. Hence the basic principle of reversed phase HPLC is partitioning between the stationary phase and mobile phase which flows through the column. In reverse phase, retention time is longer for molecules which are more non- polar while polar molecules elutes more readily. The retention time can be increased by adding more water to the mobile phase. This makes mobile phase more hydrophilic relative to the more hydrophobic stationary phase. Similarly, retention time can be decreased by adding more organic solvent to the mobile phase.

# APPLICATION OF HPLC

Preparative HPLC refers to the process of isolation and purification of compounds. Important is the degree of solute purity and the throughput, which is the amount of compound produced per unit time. This differs from HPLC, where the focus is to obtain information about the compound. The information that can be obtained includes identification, quantification and resolution of a compound.

Chemical separation can be accomplished using HPLC by utilizing the fact that certain compounds have different migration rates given a particular column and mobile phase. Thus, the chromatographer can separate compounds from each other using HPLC, the extent or degree of separation is mostly determined by the choice of stationary phase and mobile phase.

Purification refers to the process of separating or extracting the target compound from other (possibly structurally related) compounds or contaminants. Each compound should have a characteristic peak under certain chromatographic conditions. Depending on what needs to be separated and how closely related the samples are, the chromatographer may choose the conditions such as the proper mobile phase, to allow adequate separation in order to collect or extract the desired compound as it elutes from the stationary phase. The migration of the compounds and contaminants through the column need to differ enough so that the pure desired compound can be collected or extracted without increasing any other undesired compound.

Identification of compounds by HPLC is a crucial port of any HPLC assay. In order to identify any compound by HPLC, a detection must be first selected. Once the detector is selected and its set to optimal detection setting, a separation assay must be developed. The parameters of this assay should be such that a clean peak of the known sample is observed from the chromatograph. The identifying peak should have a reasonable retention time and should be well separated from extraneous peaks of the detection levels which the assay will be performed. To alter the retention time of the compound, several parameters can be manipulated. The first is the choice of column, another is the choice column of mobile phase, and last is the choice in flow rate. Identification of a compound by HPLC is accomplished by researching the literature and by trail and error. A sample of a known compound must be utilized in order to assure identification of the unknown compounds. Identification of compounds can be assured by combining two or more detection method.

Quantification of compounds by HPLC is the process of determining the unknown concentration of a compound in a known solution. It involves injecting a series of known concentrations will give a series of peaks that correlate to the concentration of the compound injected.

**Diclofenac sodium** is non-steroidal anti-inflammatory drug (NSAID) which is used to treat minor aches and used as an analgesic to reduced pain. It is available as sodium and potassium salts. It is available as a generic drug in a number of formulations.

# USES OF DICLOFENAC SODIUM

1. Treatment of pain, inflammation disorders.
2. It is used in treatment of various type of arthritis.
3. It is also used in treatment of chronic disorder and acute non-bacterial inflammation of anterior part of eye.
4. It is used in pain management in case of kidney and gall stone and also in case of active migraines.

# SIDE EFFECTS

DFS may cause side effects. Common side effects with DFS are stomach pain, constipation, diarrhea, heart burn or indigestion, headache, nausea, etc.

Contradiction: Hypersensitivity against Diclofenac inflammatory intestinal disorders such as ulcerative colitis, severe renal insufficiency

# ACTION OF DFS

It works by blocking the action of cycloxygenase which is involved in production of prostaglandin. This prostaglandin produced in response to injury or certain diseases and would otherwise go on to cause pain.

# Formulation and Combination Formulation

Dicloran– Single Formulation. It contains only Diclofenac sodium as an API. Dichloran A – Combination Formulation. It contains Diclofenac sodium and paracetamol.

**REQUIREMENTS:**

# A.Apparatus -

* Standard volumetric flask (10ml, 25ml),
* Pipettes (1ml, 5ml),
* 25ml Schott bottles,
* 500ml beakers,
* Hamilton syringe (100µl). **B.Chemicals:**
* Methanol (HPLC grade),
* Acetonitrite (HPLC grade),
* 0.01M KH2PO4

# C.Miscellaneous

* Mortar and Pestle,
* Distilled water,
* Millipore filter,
* Filtering Assembly, **D.Instruments:**
* Sonicator,
* pH meter,
* **Shimadzu Prominence High Performance Liquid Chromatography**

**(HPLC) Gradient System.**

* Sample: Dicloran and Dicloran A tablets
* Standard: Diclofenac sodium (DFS)

**PROCEDURE:**

* Preparation of standard solution of DFS

1. Weigh 25 mg of standard DFS powder and dissolve it in a minimum amount of methanol and make volume up to 25ml in 25ml standard volumetric flask using methanol to make 1000ppm stock solution.
2. Pipette out 1ml of the above solution in a 10 ml of standard volumetric flask and make volume up to 10ml using 10ml methanol to prepare 100ppm solution.
3. Pipette out 1ml of the above solution in a 10 ml of standard volumetric flask and make volume up to 10ml using methanol to prepare 10ppm solution.
4. **Preparation of sample** 1.Weightablet of Dicloron A.
   1. Find out the average weight (so that the average weight corresponds to 50mg of DFS)
   2. Crush the tablet using mortar and pestle.
   3. Weigh the powder which gives **25mg of Diclofenac**and dissolve it in min. amount of methanol.
   4. Make the volume upto 25ml using methanol. Filter the solution through whatmann filter **paper no. 41**, this gives 1000ppm stock solution.
   5. Using this prepare 100ppm, 10ppm solution of Dicloran A.
5. **Preparation of Mobile Phase**

**1.**Prepare mobile phase by adding following solutions **Acetonitrile HPLC grade (ACN)**

# : 0.01M KH2PO4(pH 3.5) in the ratio 70:30(v/v)

1. Sonicate for 10mins.
2. Filter it through filteration assembly and again sonicate for 10mins.

# D.Preparation of Flushing Solution

1. HPLC grade **methanol: HPLC grade water** in the ratio 50:50 (v/v)
2. Sonicate for 10mins.
3. Filter it through filtering assembly and then sonicate for 10mins.

**Standard operating procedure for the operation of Shimadzu prominence hplc system**

# A.Operating procedure

1. Switch on the mains for UPS power supply.
2. Then press the ‘Test’ button on the Smart-UPS RT 2000.
3. Switch on the mains for the Shimadzu Gradient HPLC system.
4. Switch on the mains for the computer. Switch on the CPU.
5. Switch on the grey button with ‘Power’ written on them on the HPLC system. Each one to switch on the LC-20 AD. Pump and SPD-M20 A Detector.
6. Now a red light will start flickering on the instrument and the LED display for pump will get activated.
7. After the instrument stabilizes the red light goes off and a green light indicates that system is ready.

# B.To load the mobile phase reservoirs in the mobile phase reservoir tray

1. After switching on the instrument, keep the required mobile phase components contained in the Schott bottles on the mobile phase reservoir tray. [NOTE: Filter the mobile phase through the 0.2 micron membrane filter and sonicate for half an hour]
2. Dip the desired tubing with solvent inlet filters(A,B,C or D) in the Schott bottles containing mobile phase and dose the mouth of the bottles using aluminum foil to prevent the entry of foreign matter in the mobile phase components.
3. **To purge the hplc system**

Purging is a process in which the mobile phase solvents are circulated at higher flow rates, through the flow lines/tubings of the HPLC system (not through the column) to remove the trapped air bubbles (if any) inside the flow lines; as entry of air in the column is detrimental to the stationary phase in the column which can lead to decrease in column life and poor separation efficiency. So the process of purging is very essential after switching on the instrument as well as the changeover of the mobile phase component.

1. For purging the tubings, rotate the grey knob on the pump of the instrument in the anticlockwise direction in 180 degrees (or half turn)
2. Press ‘Purge’ below the LED display of the pump and purge the tubings for 4-5 mins or till the tubings are free from air bubbles.
3. To stop purging, again press ‘Purge’ button below LED display of the pump and rotate the grey knob on the pump in clockwise direction in 180 degrees (i.e. back to the original position)

# D.To connect the instrument to the software

1. After purging the system, double click on the ‘LC solution’ icon the desktop of the computer.
2. A window opens in which click on the ‘Operation’ mode.
3. Click on HPLC-1 icon which leads to another window where a password will be ask. Don’t enter any password, just click on ‘Ok’.
4. After this 2 beep sounds (one from the pump and the other from the detector), indicates that the instrument is online software. Now the system is online.
5. A new window opens which shows ‘LC Real Time analysis’ and LC: Connected PDA: Connected is highlighted in green in data acquisition mode.

# E.To start an analysis on the system

1. To start with any new analysis first creates a method file.
2. To create a method file go to uppermost toolbar and single click on ‘File’ and select ‘New method file’.
3. Now in the ‘Instrument parameter’s view’ there are two options ‘Normal’ and ‘Advanced’ **Normal**
4. First click on Normal.
5. In this window there are 2 options ‘Simple settings’ and ‘LC time program’.
6. Click on ‘Simple settings’ and feed the parameters.
7. Give LC stop time and click on ‘Apply to all acquisition times’.
8. In the option ‘Pump’ select the HPLC mode i.e. Isocratic or Low Pressure gradient mode.
9. Give ‘Pump a flow’ for Isocratic mode or ‘Total Pump a flow’ for Low Pressure gradient mode.
10. If only 2 ports are in use i.e. A and B then give solvent B conc.
11. In case of use of all the 4 ports; give solvent B, C, and D concentrations accordingly.
12. Now click on ‘Advanced’. **Advanced**
13. In Advanced mode there are 5 different options.
14. First click on ‘Data acquisition’ check the LC stop time.
15. For carrying out gradient analysis click on LC time program and give appropriate gradient program.
16. Click on ‘Pump’ option and enter the pressure limit (Pump A) –max (350kgf/cm2) for C- 18 column.
17. Click on PDA and enter Start and Stop wavelength [in between 190-800 (nm)] as per need of analysis.
18. After feeding all the above information, save the method file.
19. For saving the method file go to uppermost toolbar, single click on ‘File’ and select the option ‘Save Method File as’.
20. Give proper path and name to Method File and save it in the desired folder.
21. After saving method file, click on the ‘Download’ icon on the upper right side of the ‘instrument parameters view’ window, to set those conditions in the instrument.
22. By processing ‘Pump’ button on the LC -20 AD Pump or clicking ‘Instrument ON’ icon in the toolbar, start the mobile phase flushing.
23. By clicking ‘Plot’ icon in the chromatogram window, software starts plotting the chromatogram.
24. Let the mobile phase flush the column for appropriate time so as to stabilize the baseline in the chromatogram window.

# F.To start a single new analysis

1. First step is plotting of chromatogram for the mobile phase by clicking ‘Stop’ icon to the right hand side upper corner of the chromatogram window.
2. Now click on the ‘Single Start’ icon in the vertical acquisition bar at the extreme left hand side of the chromatogram window.
3. This opens a new window for ‘Single Run’ as follows.
4. Fill in all the details such as Sample name, Sample ID etc.
5. Select a method file by clicking a folder icon on the left hand side of ‘Method File’.
6. New window of ‘Select Method File’ opens, now choose accurate path and open the desired method file.
7. Similarly select the appropriate folder to save the data file using the folder icon for ‘Data File’.
8. After feeding all the data in ‘Single Run’ window click ‘Ok’. This opens a new small window which says click ‘Start’ or inject the sample.

# G.To inject the sample in the manual injection

1. Now rinse the sample injection loop using such as methanol i.e. take 50ml of methanol in Hamilton ml syringe, remove the trapped air bubble if any and inject the entire solvent in the loop via manual sample injection port but do not bring the knob from ‘Load’ position to ‘Inject’ position (otherwise the solvent goes to the column).
2. After rinsing the port, now aspirate the sample (little more than 20ml as sample capacity is 20ml) in the Hamilton ml syringe remove the trapped air bubbles, now insert the needle in the manual sample injection part and inject the whole amount of sample in it. Then quickly bring the knob from ‘Load’ position to ‘Inject’ position, so that the sample now goes to the column.
3. After this; software automatically starts the plotting of a chromatogram for the injected sample.
4. To stop the run in between click on ‘Stop’ icon at the upper right hand side of chromatogram window.

# H.Post run analysis

1. Go to ‘LC solution’ window click on ‘Operation Mode’ and single click on ‘Post Run’.
2. ‘LC pasture analysis’ window opens.
3. To open desired data file, single click on ‘File’ in the upper most toolbar.
4. Now click on ‘Open’, select appropriate path and open desired data file.
5. A new window opens which shows a chromatogram view of selected data.
6. To a view a peak table for selected data file, single click on ‘view’ in uppermost toolbar and select a peak table. A peak table view opens.

# I.To create a report format file

1. Single click on ‘File’ in the uppermost toolbar in the post run window, click on ‘new’ and then select ‘Report Format File’
2. The Report Format file opens.
3. Toolbar icons: Addition of a text box; LC/PDA Chromatogram; LC/PDA peak table and Sample information.
4. For adding every component in the report; single click on their respective icons in the toolbar and create spaces for them in the blank report format below by dragging separate boxes for each one of them.
5. After creating a desired report format file go to uppermost toolbar, select ‘File’ click on

‘Save Report Format File as’.

1. Give proper path and save it in the desired folder.

# J.To switch off the system

1. After the analysis is complete always flush the column with HPLC grade Methanol : RO water (50:50) for 30-45mins and finally store the column with 100% HPLC grade Methanol for 30mins before switching off the system.
2. After final Methanol flushing, turn of the mobile phase flow by pressing ‘Pump’ button on LC-20 AD pump and stop plotting the chromatogram.
3. Then exit from the existing windows by cancelling them and finally cancel and close the

‘LC solution’ window, the software gets closed.

1. Refresh the desktop and shutdown the computer and then switch off the mains of the computer.
2. Now to switch off the instrument, press the grey button for power on the LC-20 AD pump and SPD-M20 A detectors and switch them off, the LED display goes off.
3. Then switch off the mains for the instrument.
4. After switching off mains for computer and instrument; switch off mains for UPS and then press the button next to ‘Test’ button on the smart UPS RT 2000.
5. All the green light on the UPS goes off and whole HPLC system with UPS gets shut down.

**Experimental Conditions**

1. Sample:10ppm Std.Diclofinac sodium, 10ppm Dicaloran A
2. Column: **C-18 column**(OctaDecylSilane) type Dimensions of column: a) Diameter- 4.8mm
   1. Length- 250mm
   2. Pore size- 5µ
3. Mobile phase: HPLC grade : 0.01M KH2PO4 (pH 3.5)

Acetonitrile 70 : 30 (v/v)

1. Instrument: Shimadzu prominence HPLC (gradient) system.
2. Software: LC solutions.
3. Detector: Photo Diode Detector.
4. Detecting wavelength: 281nm

Observation table

|  |  |  |  |
| --- | --- | --- | --- |
| Sample | Conc (ppm) | Rt ( min) | Area under Curve |
| Standard | 10 ppm | 4.280 | 352.80 |
| Dichloran A | 10 ppm | 4.295 | 350.834 |

**Calculations:**

Each tablet contains 50mg of DFS.

# Weight of tablet = 150mg

150 mg of 1 tablet contains 50 mg of DFS.

For 25mg of DFS, x = (25 x 150 ) / 50

X= 75 mg = Wt of sample (Dicloran A)

Amount of DFS per tablet:

𝐴𝑈𝐶𝑜𝑓𝑆𝑎𝑚𝑝𝑙𝑒𝐶𝑜𝑛𝑐. 𝑜𝑓𝑆𝑡𝑎𝑛𝑑𝑎𝑟𝑑𝑊𝑒𝑖𝑔ℎ𝑡𝑜𝑓𝑆𝑡𝑎𝑛𝑑𝑎𝑟𝑑

× × 𝑊𝑒𝑖𝑔ℎ𝑡𝑜𝑓𝑇𝑎𝑏𝑙𝑒𝑡

𝐴𝑈𝐶𝑜𝑓𝑆𝑡𝑎𝑛𝑑𝑎𝑟𝑑 𝐶𝑜𝑛𝑐. 𝑜𝑓𝑆𝑎𝑚𝑝𝑙𝑒𝑊𝑒𝑖𝑔ℎ𝑡𝑜𝑓𝑆𝑎𝑚𝑝𝑙𝑒

×

350.834 25

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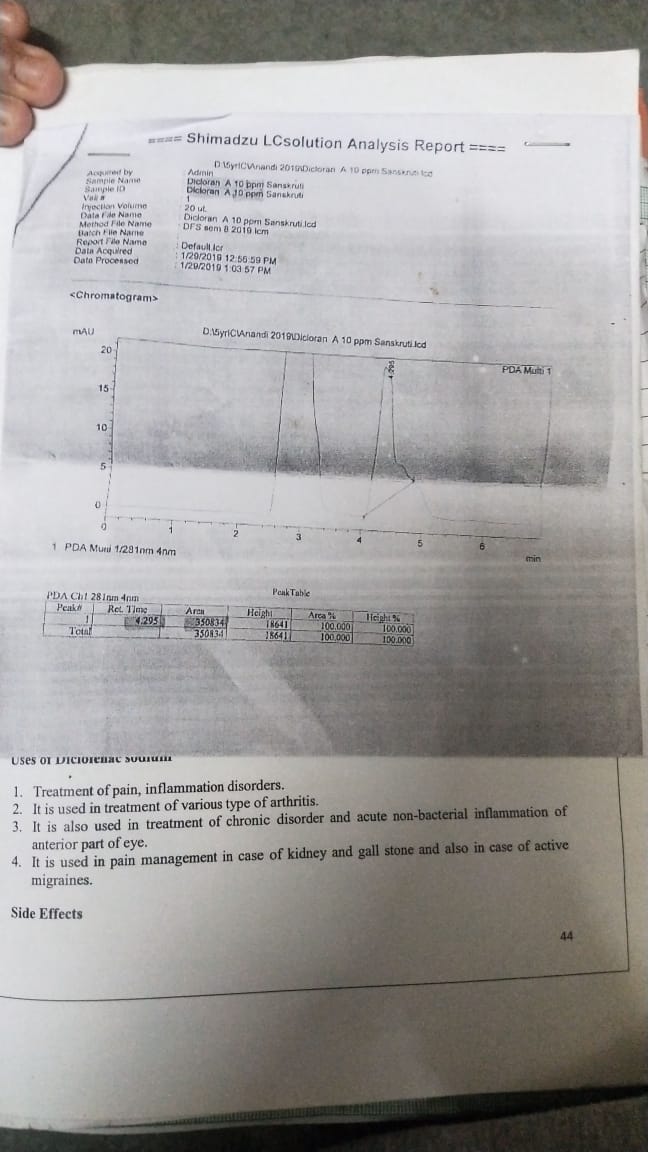
352.80 75

\_\_\_\_mg /tablet

**Result:**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Sample | Conc (ppm) | Rt ( min) | Area under Curve |  | Amount  (in mg) |
| Standard | 10 ppm | 4.280 | 352.80 |  |  |
| Dichloran A | 10 ppm | 4.295 | 350.834 |  |  |

**Conclusion:** HPLC play an important and critical role in the field of pharmaceutical industries and analysis, since it is used to test the products and to detect the raw ingredient used to make them i.e., qualitative and quantitative analysis. Moreover, the importance of HPLC uses in these fields falls under the stringent regulations established by the U.S. Food and Drug Administration (FDA). This obligate all pharmaceutical companies to detect the quality of their products by using the HPLC before allowing them to sell it in the global market

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