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REVIEW ON -HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

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ABSTRACT
Chromatography is defined as a set of techniques which is used for the separation of constituents in a mixture. This technique involves 2 phases' stationary and mobile phases. The separation of constituents is based on the difference between partition coefficients of the two phases. The chromatography term is derived from the greek words namely chroma (colour) and graphein (to write). The chromatography is very popular technique and it is mostly used analytically. There are different types of chromatographic techniques namely Paper Chromatography, Gas Chromatography, Liquid Chromatography, Thin Layer Chromatography (TLC), Ion exchange Chromatography and lastly High Performance Liquid Chromatography (HPLC). High performance liquid chromatography (HPLC) is a modern application of liquid chromatography. High performance liquid chromatography guarantees a high sensitivity and, at the same time, this technique has its gas analogue. The principle of HPLC is the same as that of liquid chromatography (LC), liquid–solid chromatography (LSC) and liquid–liquid chromatography (LLC). High performance liquid chromatography is the most recent technique. This review mainly focuses on the HPLC technique its principle, types, instrumentation and applications.

KEYWORDS: Ion-Exchange, precipitation, reproducible, HPLC, inositol phosphates.

INTRODUCTION

High-performance liquid chromatography (HPLC) is an instrumental form of liquid chromatography that employs stationary phases consisting of small particles, thereby achieving more efficient separations than those used in conventional liquid chromatography. Since its origin in the late 1960s, it has been known by several different names, including high-pressure liquid chromatography, because of the high pressures required to force the mobile phase or solvent through the stationary phase, and high-resolution liquid chromatography, because of the good resolution achieved using this technique.[1]

Two techniques related to HPLC, fast-protein liquid chromatography (FPLC) and supercritical fluid chromatography (SFC), are also considered. The basic theory of the chromatographic process and the factors that affect separation efficiency are discussed in the section on Chromatography.

HPLC stands for High Performance Liquid Chromatography. Before HPLC was available, LC analysis was carried by gravitational flow of the eluent (the solvent used for LC analysis) thus required several hours for the analysis to be completed. Even the improvements added in later time were able to shorten the analysis time slightly. Those classical/initial LC systems are called "low pressure chromatography" or "column chromatography".[2]

In 1970s in the US, Jim Waters founded Waters Corporation and started to sell HPLC instruments. This promoted the use of HPLC in practical analysis areas. The LC systems that Waters Corporation developed used high-pressure pump that generates rapid-flow of eluent, and thus resulted in dramatic improvement in the analysis time. Compared to the "low pressure chromatography" the newer types were called "high pressure liquid chromatography". Therefore it was used to be thought that HPLC stands for High Pressure Liquid Chromatography, however nowadays it is a common agreement that HPLC stands for High Performance Liquid Chromatography. Another big change from Tswett's date was the data acquisition methods. Instead of observing the changes of layers by eyes, detector system was coupled to the LC and out-put was recorded on paper chart. If we were to demonstrate Tswett's analysis result on a chart (chromatogram), it will be like figure3. Initially, HPLC system was referred to Waters Corporation's system. Still now, Waters Corporation is the HPLC pioneer, but there are several other companies that manufacture and sell HPLC systems. Technically speaking, the word LC represents all the Liquid

Chromatography, including low pressure LC, however most LC systems used these days are HPLC thus often the word LC is used as comparable as HPLC.[3]

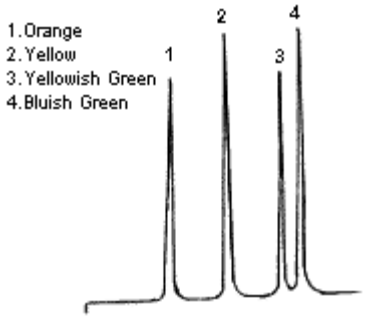

Figure 1: Representation of Tswett's LC analysis.

Components of HPLC

Typical HPLC system consists of followings (Figure 2). Details of each are explained below.[4-5]

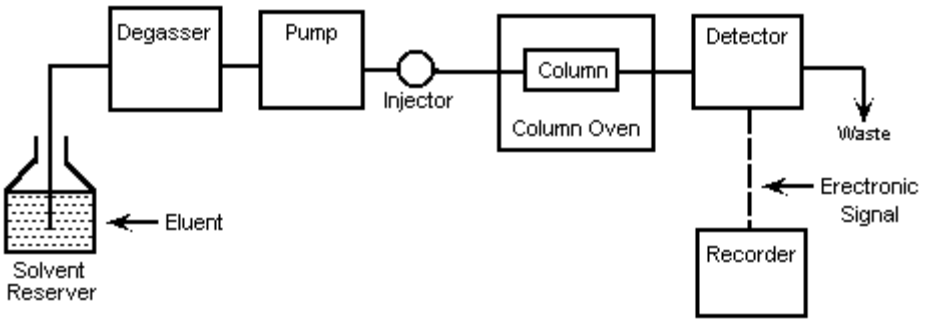

Figure 2: Components of HPLC system.

1. Pump

In the earlier state of HPLC development, the pump was the most important part of the system. The development of HPLC can be said that it was a development of pump system. Pump is positioned in the most upper stream of the LC system and generates a flow of eluent from the solvent reservoir to the system. In the earlier stage of LC development, to be able to generate the high pressure was the one of the most important system requirements. However, nowadays, the high pressure generation is a "standard" requirement and what is more concerned nowadays is to be able to provide a consistent pressure at any condition, to provide a controllable and reproducible flow rate. Since a change in the flow rate can influence the analysis largely.

Most pumps used in current LC systems generate the flow by back-and-forth motion of a motor-driven piston (reciprocating pumps). Because of this piston motion, it produces "pulses". There have been large system improvements to reduce this pulsation and the recent pumps create much less pulse compared to the older ones. However, recent analysis requires very high sensitivity to quantify a small amount of analytes, and thus even a minor change in the flow rate can influence the analysis. Therefore, the pumps required for the high sensitivity analysis needs to be highly precise.

2. Injector

An injector is placed next to the pump. The simplest method is to use a syringe, and the sample is introduced to the flow of eluent. Since the precision of LC measurement is largely affected by the reproducibility of sample injection, the design of injector is an important factor. The most widely used injection method is based on sampling loops. The use of auto sampler (auto-injector) system is also widely used that allows repeated injections in a set scheduled-timing.

3. Column

The separation is performed inside the column; therefore, it can be said that the column is the heart of an LC system. The theory of chromatography column has not changed since Tswett's time; however there has been continuous improvement in column development. The recent columns are often prepared in stainless steel housing, instead of glass columns used in Tswett's experiment. The packing material generally used is silica or polymer gels compared to calcium carbonate used by Tswett.

The eluent used for LC varies from acidic to basic solvents. Most column housing is made of stainless steel, since stainless is tolerant towards a large variety of solvents. However, for the analysis of some analytes such as biomolecules and ionic compounds, contact with metal is not desired, thus a polyether ether ketone (PEEK) column housing is used instead.

4. Detector

Separation of analytes is performed inside the column, whereas a detector is used to observe the obtained

separation. The composition of the eluent is consistent when no analyte is present. While the presence of analyte changes the composition of the eluent. What detector does is to measure these differences. This difference is monitored as a form of electronic signal. There are different types of detectors available.

Detectors for HPLC

UV, VIS, and Photo Diode Array detectors

The UV, VIS, and Photo Diode Array detectors are categorized as absorbance detectors. They provide good sensitivity for light-absorbing compounds at ~pg level. They are easy to operate and provide good stability. UV detector is a very commonly used detector for HPLC analysis. During the analysis, sample goes through a clear color-less glass cell, called flow cell. When UV light is irradiated on the flow cell, sample absorbs a part of UV light. Thus, the intensity of UV light observed for the mobile phase (without sample) and the eluent containing sample will differ. By measuring this difference, the amount of sample can be determined. Since the UV absorbance also differs depend on what wavelength is used, it is important to choose an appropriate wavelength based on the type of analyte. A standard UV detector allows user to choose wavelength between 195 to 370 nm. Most commonly used is 254 nm. Compared to a UV detector, a VIS detector uses longer wavelength (400 to 700 nm). There are detectors that provide wider wavelength selection, covering both UV and VIS ranges (195 to 700 nm) called UV/VIS detector. PDA detects an entire spectrum simultaneously. UV and VIS detectors visualize the obtained result in two dimensions (light intensity and time), but Photo Diode Array adds the third dimension (wavelength). This is convenient to determine the most suitable wavelength without repeating analyses.[6-10]

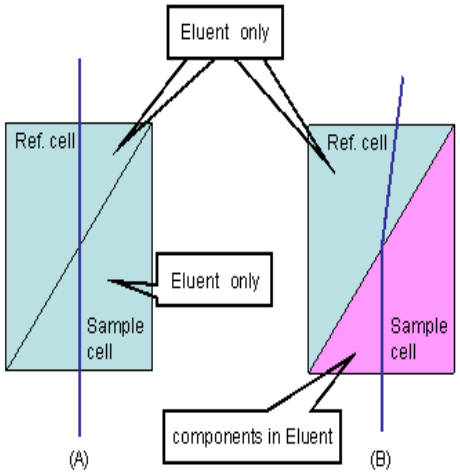

Fig. No. 3: Photo Diode Array detectors.

Refractive-Index Detector

Refractive Index detector measures change in reflex index. A glass cell is divided into two chambers (cells). The effluent from LC column flow through the "sample cell", while other cell called "reference cell" is filled with only mobile phase. When the effluent going through the sample cell does not contain any analyte, the solvent inside both cells are the same. When a beam is irradiate on the cells, the observed beam will be straight in this case. However, in a case the effluent contains any components other than mobile phase; bending of the incident beam occurs due to the reflex index difference between the two solvents. By measuring this change, the presence of components can be observed.

Refractive Index detector has lower sensitivity compared to UV detector, and that's the main reason why Refractive Index is not as commonly used as UV. However there are some advantages over UV detector.

It is suitable for detecting all components. For an example, samples which do not have UV absorption, such as sugar, alcohol, or inorganic ions obviously cannot be measured by a UV detector. In contrast, change in reflective index occurs for all analyte, thus a RI detector can be used to measure all analyte.

It is applicable for the use with solvent that has UV absorbance. A UV detector cannot be used with solvent which has UV absorbance. Sometimes the organic solvent used for GPC analysis absorbs UV, and thus UV detector cannot be used.

It provides a direct relationship between the intensity and analyte concentration. The amount of UV absorbed depends on each analyte, thus the intensity of UV detector peak does not provide information on the analyte concentration. While intensity observed by a RI detector is comparable to the concentration of analyte. Because of those advantages, RI is often used for the detection of sugars and for SEC analysis.

Evaporative Light Scattering Detector

ELSD provides good sensitivity for non-volatile analytes at ng level. The column effluent is nebulized and then evaporated to make it form fine particles. The analyte is then radiated with a laser beam and the scattered radiation is detected. The target sample includes lipids, sugar, and high molecular weight analytes. It is used in the similar way as a RI detector, but can provide more sensitive detection with stable base line. Another advantage is that ELSD can be used for the gradient method whereas RI cannot.

Multi-Angle Light Scattering Detector

For the SEC analysis, MW of analyte is estimated from the calibration curve drown using a set of known standards. However, by using a MALS, MW can be determined directly without the need of calibration curve. Also MALS can provide an absolute MW of the analyte with very low detection limit.

Mass Spectrometer
The analytes are detected based on their MW. The obtained information is especially useful for compound structure identification. However, its use is not limited to structure identification and can be used to quantify very low detection limit of elemental and molecular components.

Conductivity Detector
Solutions containing ionic components will conduct electricity. Conductivity detector measures electronic resistance and measured value is directly proportional to the concentration of ions present in the solution. Thus it is generally used for ion chromatography.

Fluorescence Detector
The advantage of fluorescence method is its high sensitivity for selective groups of compounds at fg level. By using a specific wavelength, analyte atoms are excited and then emit light signal (fluorescence). The intensity of this emitted light is monitored to quantify the analyte concentration. Most pharmaceuticals, natural products, clinical samples, and petroleum products have fluorescent absorbance. For some compounds which do not have fluorescence absorbance or low absorbance, they can be treated with fluorescence derivatives such as dansyl chloride. The system is easy to operate and relatively stable.

Chemiluminescence Detector
Similar to Fluorescence, but instead of using a light source to excite the analyte atoms, the excitation is initiated by chemical reaction. Since it is not relied on the external excitation source, the noise is small, results in high signal to noise ratio, i.e. it provides even higher sensitivity than Fluorescence.

Optical Rotation Detector
Specific for the optical Isomer measurement. The column can separate R- and L- type optical isomers, but the general detectors (e.g., UV) cannot distinguish which is R and L. Optical Rotatory detector provides this information.

Electro Chemical Detector
There are several different types of Electro Chemical. The detection is based on amperometry, polarography, coulometry, and conductrometry. They offer high sensitivity, simplicity, convenience, and wide-spread applicability. It is especially suitable for the use with semi-micro or capillary type system.

5. Recorder
The change in eluent detected by a detector is in the form of electronic signal, and thus it is still not visible to our eyes. In older days, pen (paper)-chart recorder was popularly used. Nowadays, computer based data processor (integrator) is more common. There are various types of data processors; examples include a simple system consisting of in-built printer and word processor, and a personal computer type consisting of display monitor, keyboard, and printer. Also there are software that are specifically designed for LC system. It provides not only data acquisition, but features like peak-fitting, base line correction, automatic concentration calculation, molecular weight determination, etc.

The components introduced so far are the basics of LC system. Below are some optional equipment used with the basic LC system.[11-15]

6. Degasser
The eluent used for LC analysis may contain gases such as oxygen that are non-visible to our eyes. When gas is present in the eluent, this is detected as a noise and causes unstable baseline. Generally used method includes sparging (bubbling of inert gas), use of aspirator, distillation system, and/or heating and stirring. However, the method is not convenient and also when the solvent is left for a certain time period (e.g., during the long analysis), gas will dissolve back gradually. Degasser uses special polymer membrane tubing to remove gases. The numerous very small pores on the surface of the polymer tube allow the air to go through while preventing any liquid to go through the pore. By placing this tubing under low pressure container, it created pressure differences inside and outside the tubing (higher inside the tubing). This difference let the dissolved gas to move through the pores and remove the gas. Compared to classical batch type degassing, the degasser can be used on-line, it is more convenient and efficient. Many of new HPLC unit system contain a degasser.

7. Column Heater
The LC separation is often largely influenced by the column temperature. In order to obtain repeatable results, it is important to keep the consistent temperature conditions. Also for some analysis, such as sugar and organic acid, better resolutions can be obtained at elevated temperature (50 to 80°C). It is also important to keep stable temperature to obtain repeatable results even it is analyzed at around room temperature. There are possibilities that small different of temperature causes different separation results. Thus columns are generally kept inside the column oven (column heater).

How Does HPLC Work?
In column chromatography a solvent drips through a column filled with an adsorbent under gravity. HPLC is a highly improved form of column chromatography. A pump forces a solvent through a column under high pressures of up to 400 atmospheres. The column packing material or adsorbent or stationary phase is typically a granular material made of solid particles such as silica or polymers. The pressure makes the technique much faster compared to column chromatography. This allows using much smaller particles for the column packing material. The smaller particles have a much greater surface area for interactions between the stationary phase and the

molecules flowing past it. This results in a much better separation of the components of the mixture.

The pressurized liquid is typically a mixture of solvents such as water, acetonitrile and/or methanol and is referred to as the mobile phase.

The components of a mixture are separated from each other due to their different degrees of interaction with the absorbent particles. This causes different elution rates for the different components and leads to the separation of the components as they flow out the column. Compared to column chromatography, HPLC is highly automated and extremely sensitive.

Types of HPLC

The two most common variants are normal-phase and reversed-phase HPLC.

A. Normal-Phase HPLC

The column is filled with tiny silica particles, and a non-polar solvent, for example, hexane. A typical column has an internal diameter of 4.6 mm or smaller and a length of 150 to 250 mm. Non-polar compounds in the mixture will pass more quickly through the column, as polar compounds will stick longer to the polar silica than non-polar compounds will.

Reversed-Phase HPLC

The column size is the same. The column is filled with silica particles which are modified to make them non-polar. This is done by attaching long hydrocarbon chains (8–18 C atoms) to its surface. A polar solvent is used, for example, a mixture of water and an alcohol such as methanol. Polar compounds in the mixture will pass more quickly through the column because a strong attraction occurs between the polar solvent and the polar molecules in the mixture.

Non-polar molecules are slowed down on their way through the column. They form varying degrees of attraction with the hydrocarbon groups principally through van der Waals dispersion forces and hydrophobic interactions. They are also less soluble in the aqueous mobile phase components facilitating their interactions with the hydrocarbon groups. Reversed phase HPLC is the most commonly used form of HPLC. The actual separation of each component in the sample is carried inside a column; however this separation needs to be "collected" for us to be able to see it. The detectors are used for this purpose. The separated components are monitored and expressed electronically. There is no universal detector that can monitor all compounds and there are many detectors used for LC analysis.[16-19]

REFERENCES

1. Yost R.W., Ettre L.S., and Conlon R.D., 'Practical Liquid Chromatography, An Introduction', Perkin-Elmer, Norwalk, 1980.
2. Giddings J.C., 'Dynamics of Chromatography 1. Principles and Theory', Marcel Dekker, New York, 1965.
3. Ahuja S., Scypinski S. Handbook of pharmaceutical analysis, Separation science and technology, Vol 3, Academic press, 2001; p. 95-118, 374, 445-448.
4. Gilbert M.T., 'High-performance Liquid Chromatography', Wright, Bristol, 1987.
5. Hara S., Mori S., and Hanai T., 'Chromatography. The Separation System', Maruzen, Tokyo, 1981.
6. Hatano H. and Hanai T., 'New Experimental High Performance Liquid Chromatography, Kagaku Dojin, kyoto, 1988.
7. Heftmann E., 'Chromatography', 5th edn, Journal of Chromatography Library, Vol.51a, part A Fundamentals and techniques' Elsevier, Amsterdam, 1992.
8. A.M. Krstulovic and P.R. Brown, 'Reversed-Phase High-Performance Liquid Chroma tography', Wiley, New York, 1982.
9. Lindsay S. and Kealey D., 'High Performance Liquid Chromatography', Wiley, Chichester, 1987.
10. Meyer V.R., 'Practical High-Performance Liquid Chromatography', Wiley, Chichester, 1994.
11. Sewell P.A., Clarke B., and Kealey D., 'Chromatographic Separations', Wiley, Chiche- ster, 1987.
12. Smith R.M., 'Retention and Selectivity in Liquid Chromatography', Journal of Chro matography Library, Elsevier, Amsterdam, 1995; 57.
13. Snyder L.R. and Kirkland J.J., 'Introduction to Modern Liquid Chromatography', Wiley, New York, 1979.
14. Snyder L.R., Kirkland J.J., and J.L. Glajch, 'Practical HPLC Method Development', 2nd edn, Wiley, New York, 1997.
15. Guideline for Submitting Samples and Analytical Data for Methods Validation. U.S. Department of Health and Human Services, Food and Drug Administration, Rockville, MD, Feb. 1987; 7-28.
16. A Guide to Validation in HPLC, R.A. van Iterson Drenthe College, Holland, pp- 1-15.
17. HPLC for Pharmaceutical Scientists, edited by, Yuri Kazakevich and Rosario Lobrutto, Wiley Interscience, A John Wiley and Sons INC. Publication, 2007; p.352,353.
18. Skoog DA, Holler, Nieman. Molecular Spectroscopy In: Principles of Instrumental Analysis. Thomson Asia Pte. Ltd., Singapore; 5th Ed., 2004; 342-344.
19. Weston A., Brown PR. High Performance Liquid Chromatography, Separations in High Performance Liquid Chromatography, Instrumentation for HPLC. In: HPLC and CE – Principles and practice. Academic Press, USA, 1997; p. 1-4, 8-11, 24-26, 29-32, 71.