Review Article

A Review on Trouble Shooting In HPLC and its Solutions

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ABSTRACT
The developments in column packing technology and suitable equipment paved the way for what is now called High Performance or High Pressure Liquid Chromatography (HPLC). The new technique provided much higher resolution, more accurate quantitative results, as well as shorter analysis times in comparison to the earlier techniques .During the sixties, new theoretical insights accompanied by important. Since its introduction, HPLC has evolved into an indispensable tool in many analytical laboratories and is applied to diverse analytical problems. Every HPLC consists of the same basic components .Problems can take place in each component can change the overall performance and also will consume more cost to recover the problems. Troubleshooting HPLC instrumentation and separations require a fundamental understanding of how the instrument functions and how the separation works.

INTRODUCTION

In HPLC numerous problem can arise. In comparison to former days, technology and instrumentation have been improved but typical problems still occur. Every HPLC system consists of the same important components, no matter if it's a modular system or a specialized all-in-one unit. Problems can arise in each component and can affect the overall system performance. With this troubleshooting guide, we provide help for solving typical and frequently found problems in HPLC.[1]

History of HPLC

Prior to the 1970's, few reliable chromatographic methods were commercially available to the laboratory scientist. During 1970's, most chemical separations were carried out using a variety of techniques including open-column chromatography, paper chromatography, and thin-layer chromatography. However, these chromatographic techniques were inadequate for quantification of compounds and resolution between similar compounds. During this time, pressure liquid chromatography began to be used to decrease flow through time, thus reducing purification times of compounds being isolated by column chromatography. However, flow rates were inconsistent, and the question of whether it was better to have constant flow rate or constant pressure was debated. High pressure liquid chromatography was developed in the mid-1970's and quickly improved with the development of column packing materials and the additional convenience of on-line detectors. In the late 1970's, new methods including reverse phase liquid chromatography allowed for improved separation between very similar compounds.

By the 1980's HPLC was commonly used for the separation of chemical compounds. New techniques improved separation, identification, purification and quantification far above the previous techniques. Computers and automation added to the convenience of HPLC. Improvements in type of columns and thus reproducibility were made as such terms as micro-column, affinity columns, and Fast HPLC began to emerge. The past decade has seen a vast undertaking in the development of the micro-columns, and other specialized columns. The dimensions of the typical HPLC column are: 250 mm in length with an internal diameter between 3-5 mm.[2]

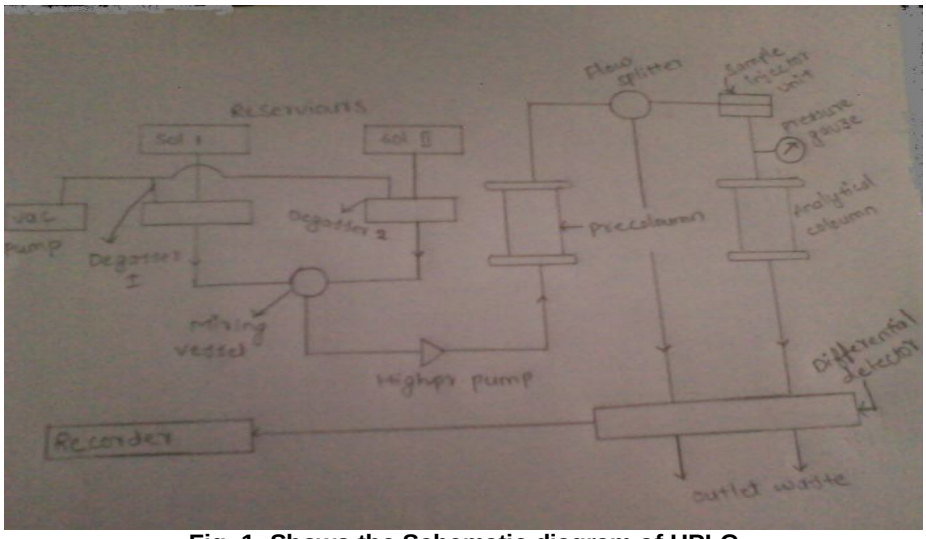

Fig. 1: Shows the Schematic diagram of HPLC

Troubleshooting Strategy and Processes

Strategy

Any troubleshooting strategy involves five steps;

1. Identification of the problem
2. Awareness of the cause(s) of the problem
3. Isolation of the exact cause of the problem
4. Rectifying the problem if able
5. Returing the unit to routine use or referring the problem to your maintenance manager.[3]

Troubleshooting process

To execute the strategy a systematic approach, which will work for any problem, is required. The systematic approach should follow a logical sequence, so that the exact cause of the problem can be found.

1. Gather the facts – not theories.
2. Check the simplest things first – it's easier.
3. Compare the performance obtained to the expected performance.
4. List possible causes.
5. Work through the possible causes in a step-by-step manner checking the outcome from any changes made.
6. As a last resort – get help from elsewhere, for example your instrument supplier help desk or your local technical support department.

Trouble shooting problems

It is important to remember that once the problem is defined and possible corrective action is identified, only one change at a time should be made; after each change, the whole system should be checked again to determine whether the problem still exists or whether the change corrected the problem.

i) Mobile phase problems

Problems that often occur in HPLC are low sensitivity and drift, noise or spikes in the Chromatogram. This phenomenon can often be attributed to problems with the mobile phase. Contaminants in the eluent are especially troublesome in gradient elution. The baseline may rise, and spurious peaks can appear as the level of the contaminated component increases. Water is the most common source of contamination in reversed phase analyses. You should only use high purity deionised (DI) water when formulating mobile phases. However, several common deionizers introduce organic contaminants into the water. To remove these contaminants, pass the deionised water through activated charcoal or a preparative C_{18} column. Use only HPLC grade solvents, salts, ion pair reagents, and base and acid modifiers. Cleaning lower quality solvents is time consuming and trace levels of contaminants often remain and can cause problems when you use a high sensitivity ultraviolet or fluorescence detector. Because many aqueous buffers promote the growth of algae or bacteria, we should discard cloudy

buffers and prepare them freshly. Prevent micro organism growth by adding about 100 ppm of sodium azide to aqueous buffers. Alternatively, these buffers may also be mixed with 10 to 20 % or more of an organic solvent such as methanol, ethanol or acetonitrile. To prevent bubbles in the system, degas the mobile phase before use. We recommend using a constantly working degasser unit. Filtering the mobile phase through a 0.2 or 0.45 µm filter using a vacuum filtration apparatus eliminates dissolved gas. This will also remove particles that could produce noisy baselines or plug the column. Use ion pair reagents carefully. The optimum chain length and concentration of the reagent must be determined for each analysis. In general, increasing the concentration or chain length increases retention time.

It is recommend using concentrations of 0.2 to 10 ml. High concentrations (>50%) of acetonitrile and some other organic solvents can precipitate ion pair reagents. Also, some salts of ion-pair reagents are insoluble in water and will precipitate. This can be avoided by using sodium-containing buffers in the presence of long chain sulfonic acids (e.g. sodiumdodecyl sulfate), instead of potassium-containing buffers. Volatile basic and acidic modifiers, such as tri ethylamine (TEA) and tri fluoroacetic acid (TFA) are useful when we wish to recover a compound for further analysis. These modifiers also avoid problems associated with ion pair reagents. They can be added to the buffer at concentrations of 0.1 to 1.0% TEA and 0.05 to 0.15% TFA. Increasing the concentration may improve peak shape for certain compounds, but can alter retention times[4].

ii) Pump problems

The HPLC pump must deliver a constant flow of solvent to the column over a wide range of conditions. KNAUER HPLC pumps incorporate a dual piston design. Pumping system problems are usually easy to spot and correct. Some of the more common symptoms are erratic retention times, noisy baselines, or spikes in the chromatogram. Leaks at pump fittings or seals will result in poor chromatography. A sure sign of a leak is a buildup of salts at a pump connection. Buffer salts should be flushed from the system daily with fresh DIwater. Run the HPLC system constantly at low flow rates (e.g. 0.1 ml/min) to avoid crystallization effects. To isolate and repair specific problems related to your HPLC system, use the troubleshooting and maintenance sections of the operation manual. Pump seals require periodic replacement. You should perform regular maintenance rather than waiting for a problem to occur. Other locations where problems can occur are the check valves in the pump head. For example when the pump is not able to produce a constant flow/pressure, if this happens, clean the check valves with isopropanol. For example, if this does not work, dismantle the check valves and clean them in an ultrasonic bath using isopropanol. Then refit the check valves in the pump head. Be sure that the valves are inserted in the right direction. If this procedure is not successful, replace check valves. Highly concentrated salts and caustic mobile phases can reduce pump seal efficiency. In some cases, prolonged use of ion pair reagents has a lubricating effect on the pump pistons that may produce small leaks at the seal. Some seals do not perform well with certain solvents. Before using a pump under adverse conditions, read the instrument manufacturer's specifications. To replace seals, refer to the maintenance section of the pump manual.[5]

iii) Injector/injection problems

The injector rapidly introduces the sample into the system with minimal disruption of the solvent flow. HPLC systems currently use variable loop, fixed loop, and syringe-type injectors. Mechanical problems involving the injector (e.g., leaks, plugged capillary tubing, worn seals) are easy to spot and correct. Use a column filter unit to prevent plugging of the column frit due to physical degradation of the injector seal. 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 the mobile phase. Otherwise, be sure the injection solvent is of lower eluting strength than the mobile phase. Be aware that some auto samplers use separate syringe wash solutions. Make sure that the wash solution is compatible with and weaker than the mobile phase. This is especially important when switching between reversed phase and normal phase analyses.[6]

iv) Detector problems

A number of different detectors are available for HPLC systems. The most common are fixed and variable wavelength ultraviolet spectrophotometers, refractive index, and conductivity detectors. Electrochemical and fluorescence detectors are less frequently used since they are more selective. Detector problems fall into two categories – electrical and mechanical/optical. For electrical problems, we should contact the instrument manufacturer. Mechanical or optical problems can usually be traced to the flow cell. Detector-related problems include leaks, air bubbles, and cell contamination. These

usually produce spikes, baseline noise or drift in the chromatograms or low sensitivity. Some flow cells – especially those used in refractive index detectors – are sensitive to pressure. Flow rates or back pressures that exceed the manufacturer's recommendation will break the cell window. Old or defective lamps as well as incorrect detector rise time, gain, or attenuation will reduce sensitivity and peak height. Faulty or reversed cable connections can also be the source of problems.

Problems and solutions

1) No peaks or very small peaks

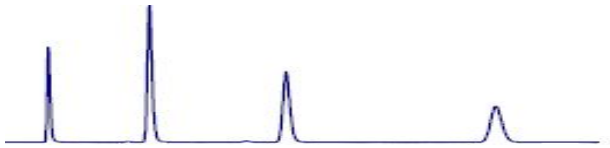

Fig. 2: Shows regular peak

Fig. 3: Shows problematic peak

Table 1: Cause and solutions of no peaks[7]

Possible cause	Solution
Detector off	Check detector
Broken connections to recorder	Check connections
No sample/Wrong sample	Check sample. Be sure it is not deteriorated. Check for bubbles in the vials
Wrong settings on recorder or detector	Check attenuation. Check gain

2) No flow

Table 2: Causes and solutions of no flow

Possible cause	Solution
Pump off	Check reservoirs. Check position of the inlet tubing. Check loop for obstruction or air. Check degassing of mobile phase. Check compatibility of the mobile phase components.
Flow interrupted	Check fittings. Check pump for leaks and precipitates. Check pumps seals.
Leak	Disconnect column and prime pump. Flush system with 100% methanol or isopropanol. Contact servicing if necessary.

3) Column and fittings leaks

Table 3: Causes and solutions of Column and fitting leaks

Problem	Possible cause	Solution
Column end leaks	Loose fitting White powder at loose fitting	Tighten or replace fitting Cut tubing and replace ferrule; disassemble fitting, rinse and reassemble.
Leak at detector	Detector-seal failure	Replace detector seal or gaskets.
Leak at injection valve	Worn or scratched valve rotor	Replace valve rotor
Leak at pump	Pump seal failure	Replace pump seal; check piston for scratches and, if necessary, replace

4) Change in retention time

i) Changing Retention Times

Table 4: Causes and solutions of changing retention times

Possible cause	Solution
Buffer retention times	Use buffer with concentration greater than 20 ml.
Contamination buildup	Flush column occasionally with strong solvent
Equilibration time insufficient for gradient run or changes in isocratic mobile phase	Pass at least 10 column volumes through the column for gradient regeneration or after solvent changes
First few injections - active sites	Condition column by injecting concentrated sample
Inconsistent on-line mobile-phase mixing	Ensure gradient system is delivering a constant composition; compare with manually prepared mobile phase; partially premix mobile phase
Selective evaporation of mobile-phase component	Cover solvent reservoirs; use less-vigorous helium purging; prepare fresh mobile phase
Varying column temperature	Thermostat or insulate column; ensure laboratory temperature is constant.

ii) Decreasing Retention Times[8]

Table 5: Causes and solutions of decreasing retention times

Possible cause	Solution
Active sites on column packing	Use mobile-phase modifier, competing base (basic compounds), or increase buffer strength; use higher coverage column packing.
Column overloaded with sample	Decrease sample amount or use larger-diameter column.
Increasing flow rate	Check and reset pump flow rate.
Loss of bonded stationary phase or base silica	Use mobile-phase pH between pH 2 and pH 8
Varying column temperature	Thermostat or insulate column; ensure laboratory temperature is constant

iii) Increasing Retention Times

Table 6: Causes and solutions of increasing retention times

Possible cause	Solution
Decreasing flow rate	Check and reset pump flow rate; check for pump cavitation; check for leaking pump seals and other leaks in system
Changing mobile-phase composition	Cover solvent reservoirs; ensure that gradient system is delivering correct composition.
Loss of bonded stationary phase	Use mobile-phase pH between pH 2 and pH 8

iv) Slow column equilibration time

Table 7: Causes and solutions of slow column equilibration time

Possible cause	Solution
Reversed phase ion pairing - long chain ion pairing reagents require longer equilibration time	Use ion-pairing reagent with shorter alkyl chain length

5) Baseline

i) Void Time noise

Table 8: Causes and solutions of void Time noise

Possible cause	Solution
Air bubbles in mobile phase	Degas or use back pressure restrictor on detector
Positive-negative - difference in refractive index of injection solvent and mobile phase	Normal with many samples; use mobile phase as sample solvent

ii) Drifting baseline

Table 9: Causes and solutions of drifting baseline

Possible cause	Solution
Negative direction (gradient elution) - absorbance of mobile-phase A	Use non-UV absorbing mobile phase solvents; use HPLC grade mobile phase solvents; add UV absorbing compound to mobile phase B.
Positive direction (gradient elution) - absorbance of mobile phase B	Use higher UV absorbance detector wavelength; use non-UV absorbing mobile phase solvents; use HPLC grade mobile phase solvents; add UV absorbing compound to mobile phase A.
Positive direction - contamination buildup and elution	Flush column with strong solvent; clean up sample; use HPLC grade solvents
Wavy or undulating - temperature changes in room	Monitor and control changes in room temperature; insulate column or use column oven; cover refractive index detector and keep it out of air currents.

iii) Baseline noise

Table 10: Causes and solutions of baseline noise

Possible cause	Solution
Continuous - detector lamp problem or dirty cell	Replace UV lamp (each should last 2000 h; clean and flush flow cell.
Gradient or isocratic proportioning - lack of solvent mixing	Use proper mixing device; check proportioning precision by spiking one solvent with UV absorbing compound and monitor UV absorbance detector output.
Gradient or isocratic proportioning - malfunctioning proportioning valves	Clean or replace proportioning precision valves; partially remix solvents.
Occasional sharp spikes - external electrical interference	Use voltage stabilizer for LC system; use independent electrical circuit.
Periodic - pump pulses	Service or replace pulse damper; purge air from pump; clean or replace check valves.
Random - contamination buildup	Flush column with strong solvent; clean up sample; use HPLC grade solvent
Spikes - bubble in detector	Degas mobile phase; use back pressure restrictor at detector outlet.
Spikes - column temperature higher than boiling point of solvent	Use lower column temperature.

6) Pressure

i) Decreasing Pressure

Table 11: Causes and solutions of decreasing Pressure

Possible cause	Solution
Insufficient flow from pump	Loosen cap on mobile phase reservoir
Leak in hydraulic lines from pump to column	Tighten or replace fittings; tighten rotor in injection valve
Leaking pump check valve or seals	Replace or clean check valves; replace pump seals.
Pump cavitations	Degas solvent; check for obstruction in line from solvent reservoir to pump; replace inlet-line frit

ii) Fluctuating pressure

Table 12: Causes and solutions of fluctuating pressure

Possible cause	Solution
Bubble in pump	Degas solvent; purge solvent with helium
Leaking pump check valve or seals	Replace or clean check valves; replace pump seals

iii) High back pressure

Table 13: Causes and solutions of high back pressure

Possible cause	Solution
Column blocked with irreversibly adsorbed sample	Improve sample cleanup; use guard column; reverse-flush column with strong solvent to dissolve blockage
Column particle size too small (for example 3 micrometers)	Use larger particle size (for example 5 micrometer)
Microbial growth on column	Use at least 10% organic modifier in mobile phase; use fresh buffer daily; add 0.02% sodium azide to aqueous mobile phase; store column in at least 25% organic solvent without buffer
Mobile phase viscosity too high	Use lower viscosity solvents or higher temperature
Plugged frit in in-line filter or guard column	Replace frit or guard column
Plugged inlet frit	Replace end fitting or frit assembly
Polymeric columns - solvent change causes swelling of packing	Use correct solvent with column; change to proper solvent composition consult manufacturer's solvent-compatibility chart use a column with a higher percentage of cross-linking
Salt precipitation (especially in reversed-phase chromatography with high concentration of organic solvent in mobile phase) concentration of organic solvent in mobile phase)	Ensure mobile phase compatibility with buffer concentration; decrease ionic strength and water-organic solvent ratio; premix mobile phase
When injector disconnected from column - blockage in injector	Clean injector or replace rotor

iv) Increasing Pressure

Table 14: Causes and solutions of increasing pressure

Possible cause	Solution
Blocked flow lines	Systematically disconnect components from detector end to column end to find blockage; replace or clean blocked component
Particulate buildup at head of column	Filter sample; use .5 micrometer in-line filter; disconnect and back flush column; replace inlet frit
Water-organic solvent systems - buffer precipitation	Ensure mobile phase compatibility with buffer concentration; decrease ionic strength or water organic solvent ratio

7) Peaks

i) Broad peaks

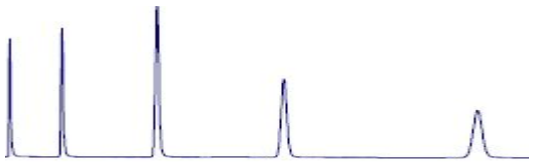

Fig. 4: Regular peak

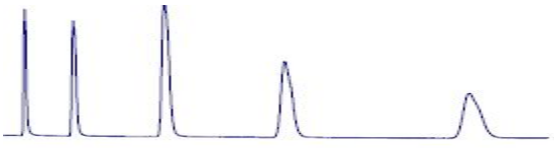

Fig. 5: Problematic peak

Table 15: Causes and solutions of broad peaks

Possible cause	Solution
Analytes eluted early due to sample overload	Dilute sample 1:10 and re inject
Detector-cell volume too large	Use smallest possible cell volume consistent with sensitivity needs; use detector with no heat exchanger in system
Injection volume too large	Decrease solvent strength of injection solvent to focus solute; inject smaller volume
Large extra column volume	Use low- or zero-dead-volume end fittings and connectors; use smallest possible diameter of connecting tubing (<0.10 in. i.d.); connect tubing with matched fittings
Mobile-phase solvent viscosity too high	Increase column temperature; change to lower viscosity solvent
Peak dispersion in injector valve	Decrease injector sample loop size; introduce air bubble in front and back of sample in loop
Poor column efficiency	Use smaller-particle-diameter packing, lower-viscosity mobile phase, higher column temperature, or lower flow rate
Retention time too long	Use gradient elution or stronger isocratic mobile phase
Sampling rate of data system too low	Increase sampling frequency.
Slow detector time constant	Adjust time constant to match peak width
Some peaks broad - late elution of analytes retained from previous injection	Flush column with strong solvent at end of run; end gradient at higher solvent concentration

ii) Ghost peaks

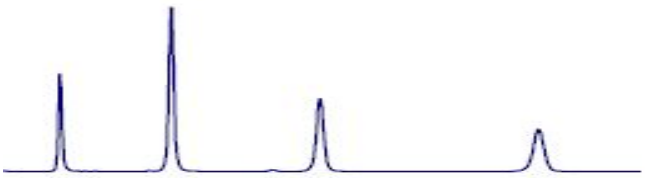

Fig. 6: Shows regular peak

Fig. 7: Shows problematic peak

Table 16: Causes and solutions of ghost peaks

Possible cause	Solution
Contamination	Flush column to remove contamination; use HPLC-grade solvent
Elution of analytes retained from previous injection	Flush column with strong solvent at end of run; end gradient at higher solvent concentration
Ion-pair chromatography - upset equilibrium	Prepare sample in mobile phase; reduce injection volume
Oxidation of trifluoroacetic acid in peptide mapping	Prepare trifluoroacetic acid solutions fresh daily; use antioxidant
Reversed-phase chromatography - contaminated water	Check suitability of water by running different amounts through column and measure peak height of interferences as function of enrichment time; clean water by running it through old reversed-phase column; use HPLC-grade water.
Reversed-phase chromatography - contaminated water	Check suitability of water by running different amounts through column and measure peak height of interferences as function of enrichment time; clean water by running it through old reversed-phase column; use HPLC-grade water.
Unknown interferences in sample	Use sample cleanup or pre fractionation before injection.

iii) Negative peaks

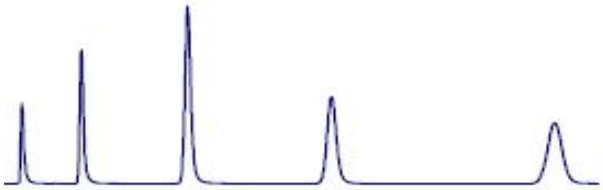

Fig. 8: Shows regular peak

Fig. 9: Shows problematic peak

Table 17: Causes and solutions of negative peaks

Possible cause	Solution
Refractive index detection - refractive index of solute less than that of mobile phase	Reverse polarity to make peak positive
UV-absorbance detection - absorbance of solute less than that of mobile phase	Use mobile phase with lower UV absorbance; if recycling solvent, stop recycling when recycled solvent affects detection

iv) Peak Doubling

Table 18: Causes and solutions of peak doubling

Possible cause	Solution
Blocked Frit	Replace or clean frit; install 0.5-um porosity in-line filter between pump and injector to eliminate mobile-phase contaminants or between injector and column to eliminate sample contaminants
Co elution of interfering compound	Replace or clean frit; install 0.5-um porosity in-line filter between pump and injector to eliminate mobile-phase contaminants or between injector and column to eliminate sample contaminants
Coelution of interfering compound	Use sample cleanup or prefractionation; adjust selectivity by changing mobile or stationary phase
Co elution of interfering compound from previous injection	Flush column with strong solvent at end of ran; end gradient at higher solvent concentration
Column overloaded	Use higher-capacity stationary phase; increase column diameter; decrease sample amount
Column void or channeling	Replace column, or, if possible, open top endfitting and clean and fill void with glass beads or same column packing; repack column
Injection solvent too strong	Use weaker injection solvent or stronger mobile phase

v) Peak Fronting

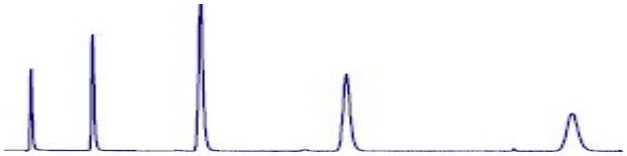

Fig. 10: Shows regular peak

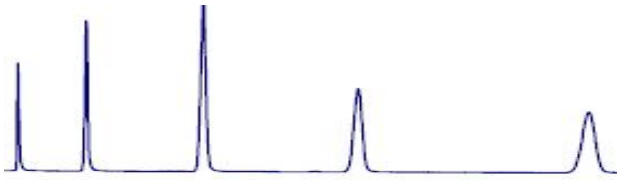

Fig. 11: Shows problematic peak

Table 19: Causes and solutions of peak fronting

Possible cause	Solution
Channeling in column	Replace or repack column
Column overloaded	Use higher-capacity stationary phase; increase column diameter; decrease sample amount

vi) Tailing Peaks

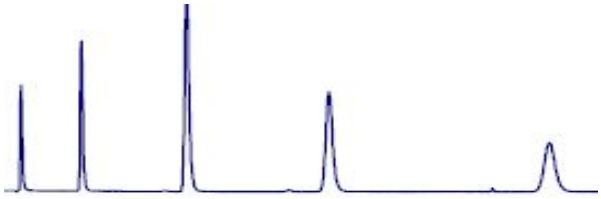

Fig. 12: Shows regular peak

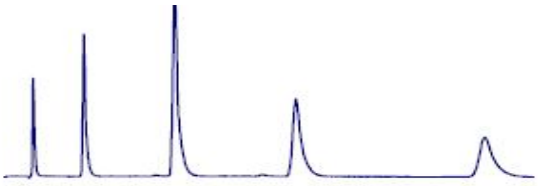

Fig. 13: Shows problematic peak

Table 20: Causes and solutions of tailing peaks

Possible cause	Solution
Basic solutes - silanol interactions	Use competing base such as triethylamine; use a stronger mobile phase; use base-deactivated silica-based reversed-phase column; use polymeric column
Beginning of peak doubling	See peak doubling
Chelating solutes - trace metals in base silica	Use high purity silica-based column with low trace-metal content; add EDTA or chelating compound to mobile phase; use polymeric column
Silica-based column - degradation at high pH	Use polymeric, sterically protected, or high-coverage reversed-phase column; install silica gel saturator column between pump and injector
Silica-based column - degradation at high temperature	Reduce temperature to less than 50 C
Silica-based column - silanol interactions	Decrease mobile-phase pH to suppress silanol ionization; increase buffer concentration; derivatize solute to change polar interactions
Un swept dead volume	Minimize number of connections; ensure injector rotor seal is tight; ensure all compression fittings are correctly seated
Void formation at head of column	Replace column, or, if possible, open top endfitting and clean and fill in void with glass beads or same column packing; rotate injection valve quickly; use injection valve with pressure bypass; avoid pressure shock

8) Spikes

Table 21: Causes and solutions of spikes

Possible cause	Solution
Bubbles in mobile phase	Degas mobile phase; use back-pressure restrictor at detector outlet; ensure that all fittings are tight
Column stored without caps	Store column tightly capped; flush reversed-phase columns with degassed methanol

CONCLUSION

High Performance Liquid Chromatography has wide variety of applications in many fields such analysis & separations of pharmaceuticals, biochemistry, analyzing the air and water pollutants, monitoring the pesticides levels in the environment. Federal and state regulatory agencies use HPLC to survey food and drug products for identifying the narcotics or to check for adherence to label claim, neutraceuticals, forensic department, and clinical diagnostics. HPLC is suited to separating higher molecular weight compounds in order to provide qualitative and quantitative information. HPLC made by several critical components. These guidelines will assist to maintain the HPLC system for routine problems. It leads to reduce the cost and enhance the performance of the system. This article provides common troubleshooting procedures for all types of manufacturers.

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