JETIR.ORG ISSN: 2349-5162 | ESTD Year : 2014 | Monthly Issue
JOURNAL OF EMERGING TECHNOLOGIES AND INNOVATIVE RESEARCH (JETIR)
An International Scholarly Open Access, Peer-reviewed, Refereed Journal

HPLC TROUBLESHOOTING: A REVIEW

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ABSTRACT:

High-Performance liquid chromatography is a critical analytical technique widely used for separating, identifying, and quantifying components in a mixture. Despite its robustness, various issues can arise during HPLC operations, impacting the accuracy and reliability of results. Troubleshooting is needed to develop and maintain complex systems where the symptoms of a problem can have many possible causes. In this article we provide a comprehensive overview of typical HPLC problems and their potential causes, including solutions and preventive measure to enhance performance and ensure accurate analysis.

Key Words: Troubleshooting, HPLC, Resolution, Retention Factor, Selectivity, Column Back Pressure, Retention time variation, Incorrect peak shapes.

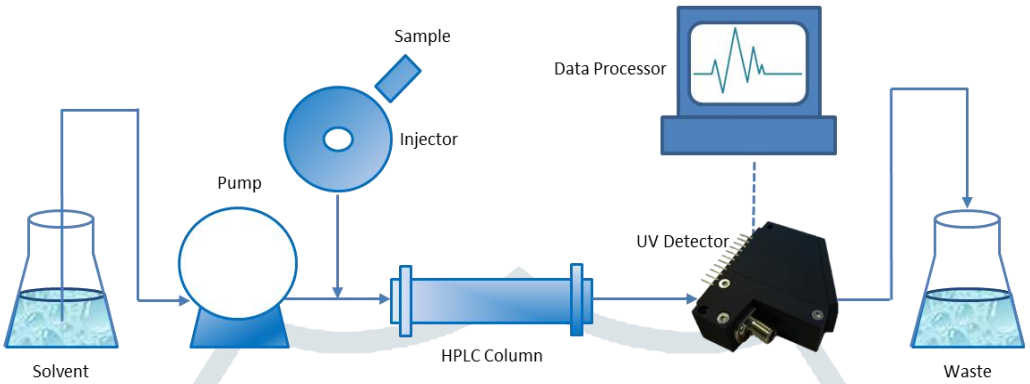
INTRODUCTION:

HPLC is an advanced form of liquid chromatography that makes use of excessive-pressure pumps to push a liquid mobile phase through a column packed with a stationary phase. This setup permits for the separation of numerous additives in a sample based totally on their interactions with the desk bound segment and their solubility in the cell section[16]. When the sample is injected too the HPLC instrument the separation of the complex compound into the simple molecules occurs. This method is highly automated and extremely sensitive. It is used in biochemistry and analytical chemistry to identify, quantify and purify the individual components of a mixture[16].

Stationary phase: The substance on which adsorption of the analyte the substance to be separated during chromatography takes place. It can be solid, a gel, or a solid liquid combination[14].

Mobile phase: Solvent which carries the analyte a liquid or a gas. In liquid chromatography, mobile phase is a liquid solvent containing the sample as a mixture of a solvent[14].

INSTRUMENTATION:



A typical instrument of HPLC consists following parts:

1. Pump-solvent delivery system
2. Mixing unit, gradient controller and solvent degassing
3. Injector – manual or auto injectors
4. Guard column
5. Analysis column
6. Detector
7. Computer

Resolution (Rs): The most important thing in HPLC is to obtain the optimum resolution in the minimum time. A resolution value of 1.5 or greater between two peaks will ensure that the sample components are well (baseline) separated to a degree at which the area or height of each peak may be accurately measured[20].

Retention Factor(k): The retention factor is equal to the ratio of retention time of the analyte on the column to the retention time of a non-retained compound. The non-retained compound has no affinity for the stationary phase and elutes with the solvent front at a time t0, which is also known as the 'hold-up time' or 'dead time'[20].

Selectivity (Separation) Factor (α): The selectivity (or separation) factor (α) is the ability of the chromatographic system to 'chemically' distinguish between sample components. It is usually measured as a ratio of the retention (capacity) factors (k) of the two peaks in question and can be visualized as the distance between the apices of the two peaks[20].

parameters affecting selectivity in reversed phase HPLC:

PARAMETER	USAGE
Organic solvent	Changing to a different solvent e.g. methanol to acetonitrile in reversed phase HPLC will alter the selectivity.
Mobile phase Ph	Can alter the degree of ionization of some analytes affecting their hydrophobicity
Solvent strength and additives	Can be adjusted to affect selectivity as well as retention factor
Stationary phase	One of the most popular ways to alter the selectivity of a separation
Temperature	Can have an effect with certain analytes in reversed phase and chiral HPLC

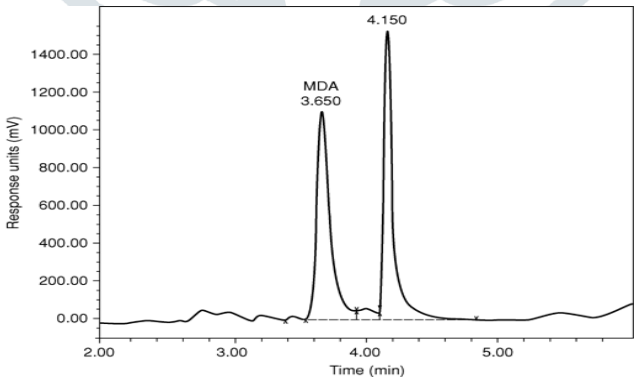
AT THE TIME OF METHOD DEVELOPMENT PROBLEMS ARISES DUE TO:

1. Problems with the peak shapes
2. Problems with the column pressure
3. Problems with retention issues.

HPLC TROUBLE SHOOTING:

1. PROBLEMS WITH PEAK SHAPE:

Peak tailing[1,3]:



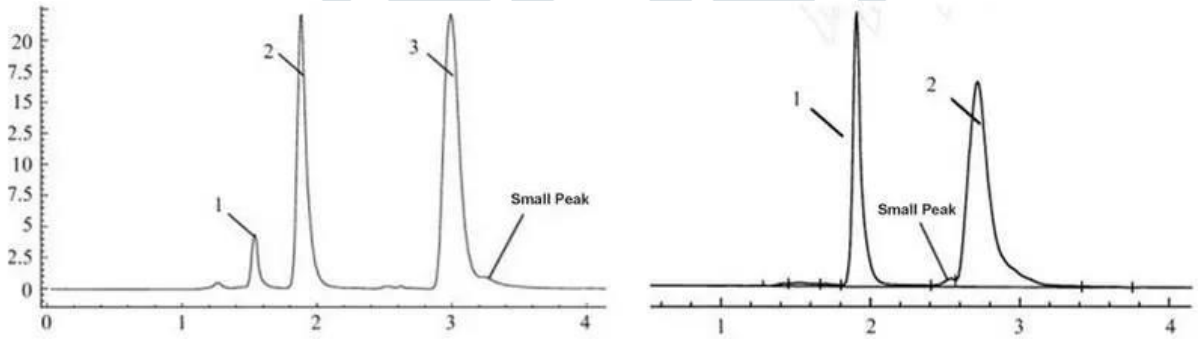
Cause	Trouble shooting
The peak tailing is a common problem with the RP-HPLC it occurs due to the interaction between the acidic silanol groups present on the surface if the silica particles within the column, this is due to the low purity of the column.	To reduce the Peak tailing one, can add the TEA (Triethylamine) of small proportions to the mobile phase, TEA is very effective which don't allow the ionization of the surface of silica present in the column.

1. Insufficient buffer or mobile phase 2. The mobile phase additives can also cause the peak tailing[1]. 3. The buffer or mobile phase additives causes the alteration in the pH of the column and causes the ionization and leads to the peak tailing in the chromatogram. 4. Majorly this error occurs while the analysis of the proteins and peptides. 5. Distortion for all peaks in chromatogram. 6. The main principle of the HPLC is to separate the molecular mixture to simple compounds but in this case the molecules forms frit at the head of the column cause separation of the sample via different path and causes distortion of the peak	1. To control this type of trouble one can, employ the Trifluoracetic acid (TFA) while the separation of the proteins takes place because the TFA supress the ion pairing of the sample proteins with the silanol[1]. 2. TFA of 0.1% concentration is majorly used as this amount would not affect analysis of the sample protein mixture. 3. To overcome this the mobile phase should run the mobile phase at high flow rate like 0.5, 1.0, 2µml/min. 4. for a period of 1hr to allow the removal of frit at the column head, but in now a days usage of the sophisticated equipment. 5. It consist 0.5µm porosity in line filters it shed off the particles prior to enter into the column and prevents the systems backpressure and distortion of peak in the chromatogram.

No peaks[1,8]:

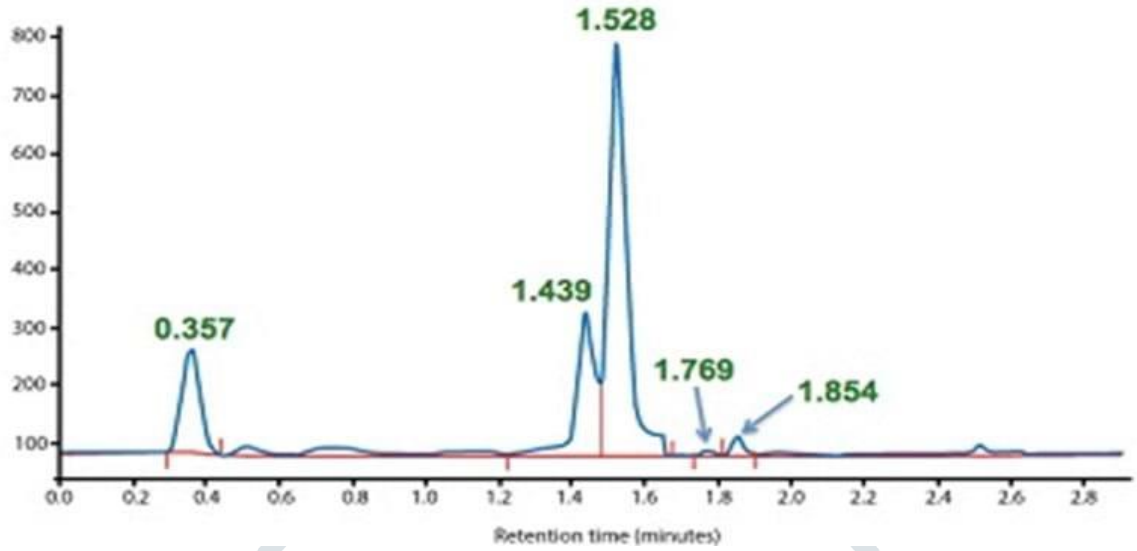
Cause	Trouble shooting
1. The detector lamp is off[1]. 2. The wire between detector and indicator are lose. 3. The mobile phase does not flow through the column[1]. 4. No peak an also occurs due to the leaks and air trapped in the HPLC system[8].	1. In this case one should check the connection[1]. 2. Check the detector electrical connection. 3. flow of the sample within the column and the system[1]. 4. The systems for the fittings to avoid the leaks[8].

Very Small Peaks[8]:



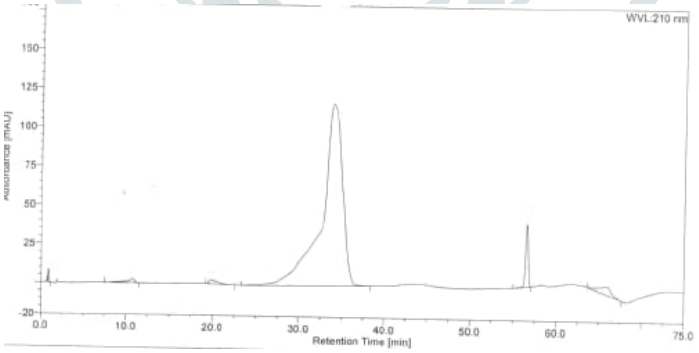
Cause	Trouble shooting
1. This occurs when the air is entrapped in the head of the pump[8].	1. Disconnection of the column's inlet for checking the flow, purge pump at the high flow rate. 2. Losing the valves to let the escape of the air present in the system[8].

Split peaks[1]:



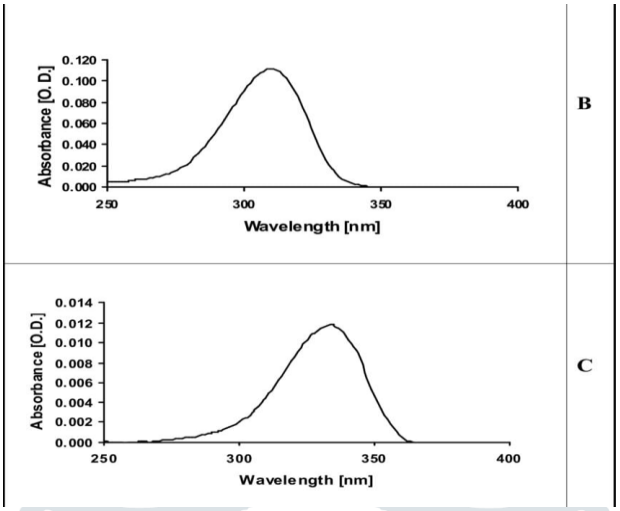
Cause	Trouble shooting
1.The split peak occurs due to contamination partially blockage of the column small void at the column inlet and mainly compatibility of the sample with the column[1].	1.This occurs when the analytical column is obstructed then one can flush the column which may leads to removal of the clogged particle[1]. 2.If the problem still not resolved replace the column.

Fronting peaks[1]:



Cause	Trouble shooting
The fronting of the peaks occurs due to column overloaded sample solvent incompatibility[1].	1. Inject the smaller volume of the sample, dilute the sample with the 1:10 ratio, adjust the solvent[1]. 2. Whenever this error occurs run the HPLC grade the Ethyl acetate at 2-3 times with the standard flow rate[1].

Peak broadening[1,4]:



Cause	Trouble shooting
The broadening of the peaks occurs due to column overload, high viscosity samples and poor sample solubility[1].	1. Check the sample preparations to ensure whether it is well dissolved or not. 2. Use a column with the small particle size, if necessary, change the stationary phase[4].

2. PROBLEMS WITH PRESSURE:

Column back pressure[1,8,6]:

Cause	Trouble shooting
1. Occurs due to column blocked with irreversible adsorbed sample, small particle size and microbial growth on column[8]. 2. This pressure is caused by resistance developed by the frictions between the mobile phase and the stationary phase filled in the column[1].	1. Losing the fittings of the HPLC system which helps to reduce the back pressure developed. 2. Using the in-line filters: These are used in the latest expensive sophisticated equipment; the filters contain the 0.5µm porosity frit to filter particles originating from the mobile phase helps to prevent the blockage oof the column[1]. 3. Guard cartridges: This gives two-fold protection to the column and HPLC system in case if in line filters are absent these are used which trap the

	particles to don't enter the column and helps to improve the column health[8]. 4. Column flushing: High flow rate of the mobile phase for prolonged period cause the removal of the buffer particles present in the column.

High pressure[8,4,12]:

Cause	Trouble shooting
High pressure in an HPLC system can be a sign of several underlying issues such as: 1. **Blockages or clogs:** Clogged columns are the main cause of high pressure[8] 2. **Verify mobile phase composition:** High viscosity or incorrect composition of mobile phase increases the pressure[12]. 3. **Inspect the pump system:** Issues with the pump such as malfunctioning results in high pressure[4]. 4. **Air bubbles:** Air bubbles in the system can lead to the high pressure in the system[12]. 5. **Inspect connections and tubing:** Kinked or blocked tubing can rise the pressure[1]. 6. **Flow rate:** Operating the system at high flow rate can cause the rise in system pressure.	1. To overcome this, flush the column with an appropriate solvent[8]. 2. Prepare the mobile phase correctly and degas. The mobile phase viscosity should be appropriate[12]. 3. Check valves and piston seals. Ensure the pump is functioning correctly[4]. 4. Air bubbles can be prevented by degassing the mobile phase before use[12]. 5. Inspect all the tubing and connections for kins, blockages, or damages[1]. 6. Maintain the flow rate at recommended range for your column and method.

Low pressure[15,1]:

Cause	Trouble shooting
Low pressure in the HPLC system can be caused by pump malfunctioning, column issues and leaks[1].	1. Find out the issues with the pump mechanism or settings. If necessary, repair or replace the pump[1]. 2. Check if the column is properly installed and connected as such that there should be no leaks at the pumps[15]. 3. If any leaks are there in the system address them by tightening or replacing fittings[1].

Fluctuating pressure[3]:

Cause	Troubleshooting
Pressure fluctuations can be caused by inconsistent mobile phase flow, air bubbles in the system and leaks in the system.	Ensure that mobile phase flow rate is consistent and set according to the method specifications. Inspect all the connections, fittings and tubing for leaks. Ensure that mobile phase is degassed.

RETENTION TIME VARIATION:

When the retention time varies it occurs due to the change in the mobile phase composition, column chemistry, column temperature or flow rate it also occurs due to the errors in on-line mixing of the isocratic or gradient mobile phase also causes the retention time problems[18].

Leaks in HPLC system[15]:

Cause	Trouble shooting
It occurs due to lose or damaged fittings, worn out or damaged seals, incorrect tubing installation, pump issues, detector cell leaks, temperature fluctuations.	Checking the system for any leaks and if found tighten the fittings and checking the pump fittings, inlet and outlet. Changing the pump seals if necessary.

Changes in the mobile phase composition[6]:

Cause	Trouble shooting
It occurs due to degassing issues, contamination, mobile phase preparation errors, solvent evaporation, solvent quality, buffer concentration changes, pump or plumbing issues, mobile phases aging.	A small change in the mobile phase composition causes the change in the retention time of analytical compound, if it occurs so the parameters of the system should be change or a new mobile phase as per the procedure should be employed.

Column temperature fluctuation[5]:

Cause	Trouble shooting
It occurs due to incorrect thermostat settings, poor column insulation, airflow issues, column location, column aging, power supply issues.	High temperature causes high efficiency of the column hence using the reliable column oven leads optimum results or better results.

Air trapped in the system:

Cause	Trouble shooting
It occurs due to inadequate degassing, leaks or loose fitting, mobile phase preparation, pump issues, column installation, tubing or connection issues, sample injection.	Purging the system helps to escape the air entrapped in the system. Losing the fittings also escape the air

Column overloading[5]:

Cause	Trouble shooting
It occurs due to excessive injection volume, inadequate sample preparation, incorrect column selection, column aging, sample matrix effects.	Injection of smaller amount of the analyte concentration of 1:10 ratio (Solute: Solvent).

Sample solvent incompatibility[5]:

Cause	Trouble shooting
Incompatible solvent properties, solvent strength miscibility, buffer or additive incompatibility, changes in PH.	If the solvent is not suitable to the solute compound altering the solvent helps to the solubility of the solute and makes sample solution to make in use.

Unexpected retention time[18]:

Cause	Trouble shooting
Column equilibration, change in sample matrix and system leaks can cause the unexpected retention time.	Before running the samples allow the column to equilibrate with the mobile phase, samples should be prepared in a consistent matrix and ensure that they are comparable to standards, inspect the system for leaks.

Peak elution time problems[5]:

Cause	Trouble shooting
Possible causes for this problem are mobile phase flow rate issues, column temperature fluctuations and sample solvent issues.	To overcome this problem, stabilize the flow rate, maintain stable temperature and use the same solvents for all samples to maintain consistent elution time.

Loss of resolution[5]:

Cause	Trouble shooting
Resolution can be loss by column degradation, incorrect mobile phase and flow rate issues.	If the column is degraded or damaged replace it, to enhance resolution optimize the mobile phase composition and PH, check for the flow rate if it is correct.

CONCLUSION:

In High-Performance Liquid Chromatography many problems arise during method development. This article emphasises common troubleshooting areas include problems with peak shape, column pressure and retention issues. The main causes of these problems are from equipment malfunctions, such as pump inconsistencies, column deterioration, or detector faults, as well as from operator errors or sample related factors. To overcome these problems regular maintenance, proper calibration and adherence to optimized operating conditions and common trouble shooting methods are necessary. By understanding and addressing the challenges associated with HPLC, users can maximize the potential of their techniques and achieve high quality analytical outcomes.

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