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Review

A REVIEW ON TROUBLE SHOOTING IN HPLC AND ITS PREVENTIVE MEASURES

P. Varshitha[1], N. Harichandana[2], Y. Prapurnachandra[3]

[1]Ratnam Institute of Pharmacy, Pidathapolur (V), Muthukur (M), SPSR Nellore Dt.524346 A.P., India.

[2]Department of Pharmaceutical Analysis, Ratnam Institute of Pharmacy, Pidathapolur (V), Muthukur (M), SPSR Nellore Dt.524346 A.P., India.

[2]Department of Pharmacology, Ratnam Institute of Pharmacy, Pidathapolur (V), Muthukur (M), SPSR Nellore Dt.524346 A.P., India.

Corresponding Author:
P. Varshitha

Email: *NA*

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ABSTRACT:

High Performance Liquid Chromatography provide much higher resolution, more accurate quantitative results, as well as shorter analysis times in comparison to the earlier techniques. HPLC has evolved into an indispensable tool in many analytical laboratories and is applied to diverse analytical problems. Trouble shooting HPLC instrumentation and separations require a fundamental understanding of how the instrument functions and how the separation works. Common HPLC problems are caused by component malfunctions like pump, degasser, injector, data system, column, and faulty preparation of the mobile phase or sample preparation. HPLC is a cornerstone technique across diverse scientific domain, yet its effectiveness can be compromised by issues ranging from peak broadening to baseline instability. It navigates through critical aspects such as column selection, mobile phase optimization, sample preparation techniques, and instrument maintenance protocols. Main key to resolve HPLC problem is cleaning of HPLC with appropriate solvent. Best approach for trouble shooting HPLC problems is going with systematic way. At the start of quantitative chromatographic analysis, the first parameter of paramount importance is baseline, especially for the area of any peak. Baseline usually suffers from these errors, namely, high baseline drift, periodic baseline fluctuation, and spikes. Problems can take place in each component, can change the overall performance, and will consume more cost to recover the problems.

HPLC analysis has gone tremendous development from manual instrumentation to the automation instrumentation. Although HPLC method development has been improved by advances in column technology and instrumentation, problems still Arises.

KEY WORDS: HPLC, HPLC troubleshooting, Instrumentation.

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INTRODUCTION:

High Performance Liquid Chromatography (HPLC) is a cornerstone analytical technique in various scientific disciplines, facilitating the separation, identification, and quantification of complex mixtures with unparalleled precision and sensitivity.[1]

Although HPLC method has been improved by advances in column technology and instrumentation, problems still arise. The important segments of HPLC system at the same, whether we use a modular system or a more sophisticated unit.[2]

Validation of analytical methods is important for generating authentic and reliable data in case regulatory submissions which is generated using that method. These methods are essential for a variety of purposes, including

testing for QC release, testing of stability samples, and testing of reference materials and to produce data to support specifications.[3]

Every HPLC system consists of the same important components, no matter if it's a modular system or a specialised all in one unit. Problems can arise in each component and can affect the overall system performance.

PRINCIPLE:

In a separation column between a stationary and a mobile phase, the purification happens. A separation column contains a granular substance with incredibly small porous particles as the stationary phase. On the other hand, the mobile phase is a solvent or solvent mixture that is pushed through the separation column under high pressure. The sample is injected into the mobile phase flow from the pump to the separation column via a valve with a connected sample loop, which is a tiny tube or a stainless steel capillary. As a result of interactions with the stationary phase, the various components of the sample are retained to variable degrees, which cause them to migrate across the column at various rates. After leaving the column the individual substances are detected by a suitable detector and passed on as a signal to the HPLC software on the computer. At the end of this operation a chromatogram in the HPLC software on the computer is obtained, which allows the identification and quantification of the different substances.[4]

Depending on the substrate used i.e. stationary phase used, the HPLC is divided into following types

· Normal Phase HPLC: In this method the separation is based on polarity. The stationary phase is polar, mostly silica is used and the non-polar phase used is hexane, chloroform and diethyl ether. The polar samples are retained on column.

· Reverse Phase HPLC: It is reverse to normal phase HPLC. The mobile phase is polar and the stationary phase is non polar or hydrophobic. The more is the non- polar nature the more it will be retained.

· Size-exclusion HPLC: The column will be incorporating with precisely controlled substrate molecules. Based on the difference in molecular sizes the separation of constituents will occur.

· Ion-exchange HPLC: The stationary phase is having ionically charged surface opposite to the sample charge. The mobile phase used is aqueous buffer which will control pH and ionic strength.

Four major problem areas are covered: peak shape, retention time changes, ghost peaks and problems related to column backpressure. In addition, a section on column care is included – procedures that will help you get maximum lifetimes from your columns. Within each section, several examples are given to illustrate various problems. A set of troubleshooting tables corresponding to each section will help you quickly identify problem causes and solutions. If you are in a hurry, you can go directly to the tables to help you solve an existing problem. Otherwise, we suggest that you read the entire guide so as to pick up some ideas that will help you avoid problems in the future.

Trouble shooting is a form of problem solving, often applied to repair failed products or processes. It is a logical, systematic search for the source of a problem so that it can be solved, and so the product or process can be made operational again. Trouble shooting is needed to develop and maintain complex systems where the symptoms of a problem can have many possible causes.

Having troubles on HPLCs can be incredibly frustrating. For example, You're trying to get some analysis done, and your research can't move forward until you figure out why your tools aren't working. There are some common problems of HPLC columns that pop up from time to time. Knowing what these are and how to fix them can save you hours of frustration. Read on to discover some of these problems and how to address them. We limited this article in basic level. We would recommend receiving consultation with the manufacturer of HPLC system. Main key to resolve HPLC problem is Cleaning of HPLC with appropriate solvent.[5]

Each component of HPLC has the potential to develop issues that could alter overall performance and increase recovery cost Issues may emerge in every part and impact the overall functionality of the system. In order to solve an issue and bring back the operational functionality of a process or product, it entails a logical and systematic investigation to determine the underlying cause of the issue. This methodical approach is especially important and annoying to run into problems with High-Performance Liquid Chromatography (HPLC). Understanding these research analysis issues and their fixes can help you save a lot of time and frustration.

One of the most important steps in fixing HPLC problems is to carefully clean the system using the right solvent. Based on the stationary phase that is used HPLC can be of several types such as Normal, Reverse, Size-exclusion, Ion exchange HPLC. High-Performance Liquid Chromatography (HPLC) is a sophisticated version belonging to

chromatography intended to efficiently separate intricate mixtures of molecules found in biological and chemical systems. Before the development of chromatography, the only methods available for analysis were photometric techniques, colorimetric analysis (UV and visible light detection), gravimetric analysis, and titrimetry (acid-base titration methods).[6]

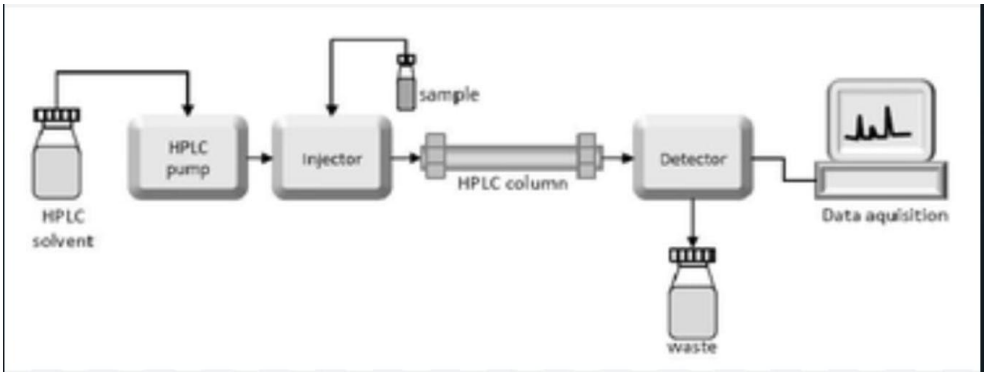

FIGURE NO.1: INSTRUMENTATION FLOW CHART [6]

HPLC is the method of choice for checking peak purity of new chemical entities, monitoring reaction changes in synthetic procedures or scale up, evaluating new formulations and carrying out quality control / assurance of the final drug products

In HPLC, the essential equipment consists of an eluent, reservoir, a high-pressure pump, and an injector for introducing the sample, a column containing the stationary phase, a detector and recorder. 8 The development of highly efficient micro particulate bonded phases has increased the versatility of the technique and has greatly improved the analysis of multi component mixtures. [7]

ADVANTAGES OF HPLC:

✓ Separations are fast and efficient (high-resolution power)

✓ Repetitive and reproducible analysis using the same column.

✓ It provides a means for the determination of multiple components in a single analysis.

✓ Accurate quantitative measurements.

DISADVANTAGES OF HPLC:

✓ Column performance is very sensitive, which depends on the method of Packing.

✓ Very costive, have low sensitivity for certain compounds, and some cannot be detected as they are irreversibly adsorbed.8

METHODOLOGY:

TROUBLE SHOOTING:

Trouble shooting is a form of problem solving, often applied to repair failed products or processes. It is a logical, systematic search for the source of a problem so that it can be solved, and so the product or process can be made operational system.

Trouble shooting is needed to develop and maintain complex systems where the symptoms of a problem can have many possible causes. Trouble shooting is used in many fields such as engineering, system administration, electronics, automotive repair, and diagnostic medicine.

Trouble shooting requires identification of the malfunctions or symptoms with in a system. Then, experience is commonly used to generate possible causes of the symptoms. [9]

TROUBLE SHOOTING STRATEGY AND PROCESS STRATEGY:

Any troubleshooting strategy involves five steps:

1. Identification of the problem
2. Awareness of the cause of the problem
3. Isolation of the extract cause of the problem
4. Rectifying the problem if able
5. Returning the unit to routine use or referring the problem to your maintenance manager.[10]

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TROUBLESHOOTING PROCESS:

1. Gather the facts – not theories.
2. Check the simplest things first – it's easier.
3. To Compare the performance obtained to the expected performance.
4. List possible causes. [3,13]
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. [2]

TROUBLE SHOOTING PROBLEMS:

Mobile phase problems:

The reagents and solvents should be of the highest prescribed quality. Deionized water often contains trace levels of organic compounds and so therefore is not recommended for HPLC use. Ensure that any water used in buffer preparation is of the highest purity. HPLC grade reagents contain no impurities to produce spurious peaks in a chromatogram baseline, whereas AR grade reagents do contain trace levels of impurity, which may produce spurious baseline peaks. Ultra-pure HPLC water (18MΩ resistivity) is generated by passing deionized water through an ion exchange bed. Alternately, HPLC grade water can be purchased from solvent suppliers. [11]

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 DI water. 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. 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. [10]

Pressure problems:

The pressure problem can occur suddenly or be a gradual process. Sudden pressure rises tend to be due to particles from the sample, blocked or damaged tubing or column packed bed collapse. Gradual pressure rises due to particles in the sample, but they can also arise from particles generated in the instrument, for example, debris from vial septa or degrading seals. Pressure problems fall into one of three categories: low pressure, fluctuating pressure, high pressure. The simplest way to troubleshoot pressure problems is using a systematic approach, as highlighted in following tables for high, low or fluctuating pressure. [11]

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 injector. Mechanical problems involving the injector (e.g., leaks, plugged capillary tubing, worn seals) are easy to spot and correct. 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. 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.

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. 9

Column protection: Protect your analytical column from sample and system debris and contaminants to maintain the column performance and efficiency. Guard columns or cartridges are one of the most cost effective and efficient ways of trapping these unwanted system components. A 10 mm length guard for moderate to heavy contamination. Although not an integral part of most equipment, mobile phase inlet filters, pre injector and pre-column filters, and guard columns greatly reduce problems associated with complex separations. All samples are filtered through 0.45µm or 0.2µm syringe filters. The useful life of these disposable products depends on mobile phase composition, sample purity, pH, etc. As these devices become contaminated or plugged with particles. [11]

PROBLEMS AND SOLUTIONS:

INSTRUMENTATION PROBLEMS AND SOLUTIONS:

1.Pump issues:

Table:1 Problem and solution for pump issues [12]

Problem	Solution
Fluctuating flow rates, leaks, or pressure deviations from the pump can affect chromatographic performance.	Check for air bubbles in the pump lines, verify proper seal integrity, and calibrate the pump pressure to ensure consistent flow rates. Replace worn-out seals or damaged components as necessary.

2.Injector problems:

Table:2 Problem and solution for injector problems [12]

Problem	Solution
Injector malfunctions such as sample leakage, incomplete injections, or injection valve sticking can lead to erratic chromatographic behaviour.	Clean and lubricate the injector valve regularly, inspect seals for wear or damage, and ensure proper alignment of injection needles. Use recommended maintenance procedures and replace faulty parts promptly

3.Column related challenges:

Table:3 Problem and solution for column related challenges [12]

Problem	Solution
Column degradation, retention time shifts, or peak broadening can arise from improper column care or aging.	Implement appropriate column care practices, such as flushing with solvent after use and storing columns in recommended conditions. Regularly monitor column performance, and replace worn-out or degraded columns to maintain chromatographic integrity.

4.Detector issues:

Table:4 Problem and solution for detector issues [12]

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Problem	Solution
Detector drift, noise, or sensitivity fluctuations can compromise detection accuracy and reproducibility.	Calibrate the detector regularly using reference standards, optimize detector settings (e.g., wavelength, sensitivity), and clean detector optics to minimize noise. Ensure proper alignment and maintenance of detector components to maximize sensitivity and signal stability.

5.Data Acquisition and Processing Issues:

Table:5 Problem and solution for Data Acquisition and Processing Issues [12]

Problem	Solution
Data acquisition errors, software glitches, or data processing inconsistencies can compromise result accuracy and reliability.	Validate software settings and data acquisition parameters, regularly update software versions, and maintain backups of analytical data. Implement robust data processing protocols, including peak integration algorithms and baseline correction techniques, to ensure accurate and reproducible data analysis.

6.Routine maintenance and Calibration:

Table:6 Problem and solution for Routine maintenance and Calibration [12]

Problem	Solution
Neglecting routine maintenance and calibration can lead to instrument downtime, performance degradation, and inaccurate results.	Establish a comprehensive maintenance schedule, perform regular instrument checks, and calibrate critical components according to manufacturer specifications. Train personnel on proper instrument operation and maintenance procedures to minimize downtime and maximize instrument uptime.

COLUMN-RELATED CHALLENGES AND RESOLUTIONS:

The chromatographic column stands as the heart of High-Performance Liquid Chromatography (HPLC), where analytes undergo separation based on their interactions with the stationary phase.

1.Column degradation:

Over time, columns can degrade due to exposure to harsh mobile phase conditions, sample matrices, or improper storage. To mitigate degradation, employ suitable column care practices, such as flushing with solvent after use, storing columns in recommended conditions, and utilizing guard columns to protect the analytical column from contamination.

2.Peak Broadening and Tailing:

Peak broadening and tailing can result from inadequate column equilibration, overloading, or interactions with sample impurities. Address this issue by optimizing column equilibration time, reducing sample load, or employing sample cleanup techniques such as solid-phase extraction (SPE) to remove impurities before injection. [12]

Table:7 Problem and solution for peak tailing [11]

Problem	Problem cause	Solution
Peak tailing	1. Blocked frit 2. Interfering peak 3. Wrong mobile-phase pH 4. Sample reacting with active sites.	1. Reverse flush column or Replace column. 2. Change mobile-phase and/or column. 3. Adjust pH 4. Add ion pair reagent or volatile basic Modifier or change column.

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Table:8 Problem and solution for peak fronting [11]

Problem	Problem cause	Solution
Peak fronting	1. Low temperature 2. Sample overload	1. Increase column temperature 2. Decrease sample concentration

Table:9 Problem and solution for split peaks [11]

Problem	Problem cause	Solution
Split peaks	1. Contamination on guard or analytical column inlet 2. Sample solvent incompatible with mobile phase	1. a. Remove guard column and attempt analysis b. Replace guard if necessary c. If analytical column is obstructed, reverse and flush 2. Change solvent; whenever possible, inject samples in mobile phase

SAMPLE PREPARATION AND INJECTION ISSUES:

Sample preparation and injection procedures are pivotal stages in High-Performance Liquid Chromatography (HPLC) analysis, exerting significant influence on chromatographic performance and the accuracy of analytical results. The process of sample preparation involves extracting analytes from complex matrices while minimizing interference from matrix components to ensure representative samples. Techniques such as solid-phase extraction (SPE) or liquid-liquid extraction (LLE) are employed to minimize matrix effects, which can compromise chromatographic resolution and sensitivity. [12]

Table:10 Problem and solution for injector leaks [11]

Problem	Problem cause	Solution
Injector leaks	1. Rotor seal failure 2. Loose injection-port seal 3. Improper syringe-needle diameter 4. Waste-line blockage	1. Rebuild or replace injector 2. Adjust 3. Use correct syringe 4. Replace waste line

Baseline problems:

1.Void time noise:

Table:11 Problem and solution for baseline problems [10]

Possible cause	Solution
Air bubble 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

2.Drifting baseline:

Table:12 Problem and solution for baseline problems [10]

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 - contamination buildup and elution	Flush column with strong solvent; clean up sample; use HPLC grade solvent

Pressure problems:

1.Decreacing pressure:

Table:13 Problem and solution for pressure problems [10]

Possible cause	Solution
Insufficient flow from pump	Loosen cap on mobile phase reservoir
Leaking pump check valve or seals	Replace or clean check valves; replace pump seals.

2.Fluctuating pressure:

Table :14 Problem and solution for pressure problems [14]

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

Peak problems:

Table:15 Problem and solution for peak problems [15]

Problem	Possible cause	Solution
Broad peaks	Injection volume too large	Decrease solvent strength of injection solvent to focus solute; inject smaller volume
Ghost peaks	contamination	Flush column to remove contamination; use HPLC-grade solvent

Conclusion:

High Performance Liquid Chromatography has wide variety of applications in many fields such as analysis & separations of pharmaceuticals, biochemistry, analysing the air and water pollutants, monitoring the pesticide levels in the environment. HPLC is made of several critical components. 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. This chapter gives an overview of HPLC system maintenance and summarizes the problems and solutions for HPLC troubleshooting.

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