

High Performance Liquid Chromatography and its role in identification of *Mycobacteriae*: An Overview

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Introduction

High Performance Liquid Chromatography (HPLC) was developed in the late 1960s and early 1970s. Today, it is widely applied for separations and purifications in a variety of areas including pharmaceuticals, biotechnology, environmental, polymer and food industries¹.

HPLC has over the past decade become the method of choice for the analysis of a wide variety of compounds. Its main advantage over Gas Chromatography (GC) is that the analytes do not have to be volatile, so macromolecules are suitable for HPLC analysis. HPLC is accomplished by the injection of a small amount of liquid sample into a moving stream of liquid – the mobile phase, which passes through a column packed with particles of the stationary phase².

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Separation of a mixture

Separation of a mixture into its components depends on different degrees of retention of each component in the column. The extent to which a component is retained in the column is determined by its partitioning between the liquid mobile phase and the stationary phase. In HPLC, this partitioning is affected by the relative solute/stationary phase and solute/mobile phase interactions¹.

Thus, unlike GC, changes in the mobile phase composition can have an enormous impact on the separation. Since the compounds have different mobility, they exit the column at different times; i.e., they have different retention times, t_R . The retention time is the time between injection and detection.

HPLC samples have to be filtered prior to injection, to remove any particulate matter. This sample should be dissolved in the mobile phase or a solvent weaker than the mobile phase to ensure good chromatographic peak shapes. In HPLC, the sample size (volume) is determined by the internal diameter of the column³.

Detectors

The detector is a device that senses the presence of components different from the liquid mobile phase and converts the information into an electrical signal. There are numerous detectors which can be used in liquid chromatography¹.

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For qualitative identification, matching retention times of known compounds, among the components of an unknown mixture, have to be relied on. It should be remembered that, any changes in operating conditions will affect the retention time which in turn, will affect the accuracy of identification.

Thus, HPLC is most often used when a target compound analysis is performed, where there is a good idea of the compounds present in a mixture, so reference standards can be used for determining retention times. In case of a sample of largely unknown composition, qualitative identification can be determined by liquid chromatography-mass spectrometry. A mass spectrum of any or all peaks in the chromatogram is compared with the spectra contained in the spectral libraries on the HPLC system's computer⁴.

HPLC Systems

HPLCs are simple apparatus, consisting of a mobile phase reservoir which can store enough solvent for many hours of operation. A stainless steel or fritted glass filter removes particulate matter from the mobile phases so that damage will not occur to the pumping system or column. There is a vacuum degassing unit to remove dissolved gasses from the mobile phases. The solvent delivery system consists of a pump for delivering precise, reproducible and a constant amount of the mobile phase, a sample inlet, the column, a detector with associated electronics and an interface such as a computer.

The pumping system is used to provide accurate compositions, flows and the pressure necessary to force the mobile phase through the tightly packed column and often operates at pressures ranging from 500-5000 psi^{1,3,4}.

Solvent Programming

Good separation of a given pair of compounds by HPLC depends on the choice of the column and on the overall efficiency of the system. The relative position of the various components in the sample on the chromatogram is affected by a solute-solvent type of interaction with the column substrate competing with a solute-solvent interaction with the mobile phase.

Column efficiency is concerned with the broadening of an initially compact band of solutes as it passes through the column. For samples with a broad range of retention times, it is often desirable to employ solvent programming, whereby the mobile phase composition is varied continuously or in steps as the separation proceeds.

Basically, the analysis of mixtures of widely varying composition frequently leads to a very wide spread in retention times. The longer the retention time, the broader the peak, so for those components which take a long time to elute, detector sensitivity is diminished and analysis times can be very long.

With solvent programming, successively eluted substances experience stronger solute-mobile phase interactions and so emerge from a column more rapidly than they would under conditions in which the solvent was not varied. So long as there is no peak overlap (*i.e.*, resolution remains tolerable), solvent programming gives superior separation.

HPLC is just one type of liquid chromatography, meaning the mobile phase is a liquid. The reversed phase HPLC is the most common type of HPLC, where the mobile phase is

relatively polar and the stationary phase is relatively non-polar. Thus non-polar compounds are more retained (*i.e.*, have longer retention times) than a polar compound. Other more general types of HPLC include partition, adsorption, ion-exchange, size exclusion and thin layer chromatography.

In general, HPLC is used for the separation of organic, inorganic, biological compounds, polymers and thermally labile compounds by qualitative and quantitative methods. Common specific applications of HPLC include, quantitative and/or qualitative analyses of amino acids, nucleic acids, fatty acids, proteins in physiological samples; measuring the levels of active drugs, synthetic byproducts, degradation products in pharmaceutical industries; measuring the levels of hazardous compounds such as pesticides and insecticides; monitoring environmental samples and purifying compounds from mixtures.

GC, Supercritical fluid chromatography, Capillary electrophoresis, ion chromatography, etc are techniques complementary to HPLC. GC provides analyses of volatile analytes with superior resolution. Supercritical fluid chromatography analyses volatile, non-volatile and thermally labile compounds. Capillary electrophoresis provides superior analyses in many biological and/or pharmaceutical applications. The Ion chromatography provides analyses of ionic compounds, similar to capillary electrophoresis ^{3, 5, 6}.

Identification of *Mycobacteriae* by HPLC:

Principle:

The objective of this method is to identify *Mycobacteriae* by analysis of mycolic acids, which are a part of the Mycobacterial cell wall and contribute to the Acid Fast nature of the cells, using high performance liquid chromatography (HPLC). A suspension of acid-fast bacteria is saponified to cleave the mycolic acids bound to the cell wall. Mycolic acids are then separated by acidification and extraction into chloroform. After conversion to ultraviolet(UV)-absorbing *p*-bromophenacyl esters, the mycolic acids are analyzed on a reverse-phase C18 column using HPLC. A gradient of methanol and dichloromethane (methylene chloride) generated by microprocessor-controlled pumps is used to separate the mycolic acid esters, which are detected with a UV spectrophotometer visualized as reproducible chromatographic patterns containing combinations of different diagnostic peaks.

Pattern recognition is by visual comparison of sample results with mycolic acid patterns from reference species of known *Mycobacteriae*. Correct pattern of interpretation requires training. Computer-assisted pattern recognition technology and high-sensitivity fluorescence detection are being evaluated ^{3, 5, 6, 9}.

Specimens:

Reliable identification is achieved when *Mycobacteriae* are grown under standardized conditions. Accordingly, the cultures to be identified are grown on a Lowenstein-Jensen (L-J) slant, which may be supplemented with additional growth factors for those strains of *Mycobacteriae* that are unable to grow on L-J. A carbol fuchsin/phenol or fluorochrome stain is performed to verify the presence of acid-fast bacilli (AFB).

Visual Interpretation:

Visual Interpretation of the chromatographic patterns has demonstrated an accuracy level of > 91%. Before attempting to identify unknown mycobacteria, it is prudent to maintain chromatographic patterns derived from species whose identity has been verified either by genetic probe or other conventional methodology. Unknown strains must be analyzed with the same method and conditions as the reference samples and controls. Results are interpreted visually by manually comparing the sample chromatogram with the laboratory reference pattern to determine a match^{3, 7, 8}.

Standardized Method for HPLC Identification of Mycobacteria:

Chromatograms are separated into groups of single, double, distinct triple cluster and multi-peak cluster patterns. Patterns producing peaks before 3 min and ending about 6.5 min without any other peaks indicate non-mycobacteria, with the exception of *Tsukamurella*. If the unknown chromatogram matches a reference pattern, the identification is reported and indicated "by HPLC". If a sample chromatogram does not match a reference pattern, the identification is reported and indicated "not identified by HPLC"^{10, 11, 12}.

Single-cluster patterns may be easily confused and should be further evaluated. Several *Mycobacteriae*, including *M. tuberculosis*, *M. bovis* BCG, *M. kansasii*, *M. szulgai*, *M. gordonae*, *M. asiaticum*, *M. marinum*, and *M. gastri* form single clusters of late-eluting mycolic acid peaks that are similar in appearance. Some mycobacteria produce HPLC chromatograms with two clusters of peaks, examples include: *M. avium*, *M. intracellulare*, *M. scrofulaceum*, *M. gordonae* (Chromatotype II), *M. xenopi*, and *M. celatum*. These species are evaluated using the peak height ratio method^{13, 14, 5, 6}.

These groups can contain, but are not limited to the following organisms:

Single	Double	Triple	Multi
<i>M. asiaticum</i>	M. avium	<i>M. simiae</i>	Other mycobacterium species
<i>M. bovis</i>	<i>M. celatum</i>		
<i>M. bovis</i> var BCG	<i>M. chelonae/M. abscessus</i>		
<i>M. gastri</i>	<i>M. fortuitum/ M. peregrinum</i> group		
<i>M. gordonae</i>	<i>M. gordonae</i> (Chromatotype II)		
<i>M. kansasii</i>	<i>M. intracellulare</i>		
<i>M. malmoense</i>	<i>M. mucogenicum</i>		
<i>M. marinum</i>	<i>M. Scrofulaceum</i>		
<i>M. szulgai</i>	<i>M. terrae</i> complex		
<i>M. tuberculosis</i>	<i>M. xenopi</i>		

Reference patterns

The reference patterns presented in the following figures resulted from an inter-laboratory study performed by members of the HPLC steering committee, using the standard method described in the SOP for HPLC Identification of *Mycobacteria*, and were based on a

collection of specimens of known identity. The profiles are median chromatograms inferred from multivariate analysis of a set of specimens from each species. Significant peaks are labelled with adjusted retention times (ART). It should be noted that variations of the median chromatogram occur in different laboratories, therefore the ART are not be used as benchmarks, rather as aids for comparison among species.

In order to identify unknown mycobacteria specimens using HPLC, the laboratory maintains chromatograms of mycobacteria commonly seen in the laboratory. HPLC profiles of unknown mycobacteria are compared to the patterns contained in this spectral library ^{6,9}.

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