

Liquid Chromatography

Mohammad Azam Mansoor, *Central Hospital in Rogaland, Stavanger, Norway*

Liquid chromatography, also called high-performance liquid chromatography, is a popular quantitative analytical technique applied in many areas of chemical, biomedical and pharmaceutical sciences. Liquid chromatography currently accounts for about 60% of separation technology applied in life sciences around the world.

Introduction

Chromatographic separations in liquid chromatography (LC) (except size exclusion chromatography) are the consequences of interactions developed between the functional groups of solute molecules, solvent molecules and the stationary phase. The interactions present in LC are hydrogen bonding, van der Waals forces and electrostatic forces, and the modes of LC are classified according to the nature of these interactions. The mode of chromatography best suited for a particular separation depends upon molecular mass, polarity and ionic characters of a solute.

Modes of Liquid Chromatography

Different modes of liquid chromatography have evolved for analyses of a variety of compounds in diverse types of matrices. The modes of liquid chromatography include: normal-phase liquid chromatography (NPLC), reversed-phase liquid chromatography (RPLC), ion-exchange liquid chromatography (IELC) and size-exclusion chromatography (SELC). Selection of a liquid chromatographic mode for a particular analysis also requires selection of a column (stationary phase) and solvents for the mobile phase. Whichever mode is selected for development of a particular method, it is important that certain criteria for method validation are fulfilled.

Normal-phase liquid chromatography (NPLC)

In NPLC or adsorption chromatography, the stationary phase is a polar solid adsorbent based on particles of silica gel, alumina or carbon. Silica gel and carbon particles are also modified covalently with polar groups, for instance aminopropyl ($-\text{NH}_2$), cyanopropyl ($-\text{CN}$) and diol (2OH) functional groups. The adsorbent particles may be fully porous or spherical glass beads covered with an irregular layer of silica gel.

The mobile phase in NPLC is composed of nonpolar, organic solvents (dehydrated), for example methanol, ethanol, 2-propanol, acetonitrile, ethyl acetate, tetrahydrofuran, carbon tetrachloride or hexane, or a binary or a

tertiary solvent system based on two or three nonpolar organic solvents.

In NPLC, polar interactions are exhibited by the polar functional groups ($-\text{OH}$, $-\text{NH}_2$, etc.) on solute molecules and polar groups present on the adsorbent molecules. Solvent molecules also compete with solute molecules to form interactions with adsorbent molecules (for adsorption sites on the stationary phase). Solutes are eluted in order of increasing polarity; thus retention of solute molecules decreases with increasing polarity of a solvent in a mobile phase.

The composition of a mobile phase can be optimized for a particular separation by selecting suitable solvents with 'the right solvent strength'. The solvent strength parameter (ϵ°) for *n*-pentane is 0; those for other organic solvents increasing in the order methanol > ethanol > 2-propanol > acetonitrile > ethyl acetate > tetrahydrofuran > carbon tetrachloride > hexane.

The surface of silica gel used in NPLC is covered with free low energy level hydroxyl groups (OH), (Si-OH) and reactive hydroxyl groups with high energy level (HOH), (Si-OH). The HOH are strong bonding agents and adsorb both polar components of a solute and water to the gel. This double activity of the hydroxyl group is responsible for broad split peaks. To avoid this problem, water or an alcohol is added in the mobile phase to deactivate hydroxyl groups on the gel.

Reversed-phase liquid chromatography (RPLC)

Reversed-phase LC differs from NPLC in that it is based on a nonpolar stationary phase. The most popular column packing material is octadecylsilyl silica (ODS-C₁₈), in which silica is covalently modified by C₁₈ functional group. Octadecyl (C₁₈), octyl (C₈), hexyl (C₆), propyl (C₃), ethyl (C₂), methyl (C₁), phenyl and cyclohexyl functional groups bonded to silica surface render silica (stationary surface) nonpolar and hydrophobic. More than 250 reversed-phase column packing materials have been introduced since 1970.

In RPLC the mobile phase is more polar than stationary phase; water and water-miscible organic solvents such as

Introductory article

Article Contents

- Introduction
- Modes of Liquid Chromatography
- Derivatization
- Applications
- Limits and Possibilities

methanol, acetonitrile and tetrahydrofuran are commonly used.

Since most organic molecules have some nonpolar regions, retention in RPLC arises because water in the mobile phase repels the nonpolar regions of solute molecules and facilitates their interaction with the non-polar functional groups of the silica (stationary phase).

Solute molecules are eluted in order of increasing hydrophobicity or decreasing polarity.

Separation of ionic species by RPLC

Several approaches have been developed for the separation of ionic species by RPLC. In the first approach, ion-pairing reagents (counter-ions) are added in a mobile phase to neutralize ionic species. Neutralized species so formed are separated by common reversed-phase columns. For cations (bases) heptanesulfonic acid and related compounds are used, and for anions (acids) tetrabutylammonium phosphate and related compounds are used as ion-pair reagents.

Partial ionization of solutes due to weak acidic or weak basic character may create problems during chromatographic separation of peaks. Addition of strong acids (for example phosphoric or sulfuric acid) or strong bases (for example ammonium carbonate) may help to sharpen peaks.

RPLC is a popular mode of liquid chromatography because it utilizes an aqueous mobile phase that is compatible with most biological samples. This mode constitutes more than 70% of all liquid chromatographic applications.

Figure 1 shows a typical high-performance liquid chromatography system, and Figure 2 shows an application of LC in biomedicine.

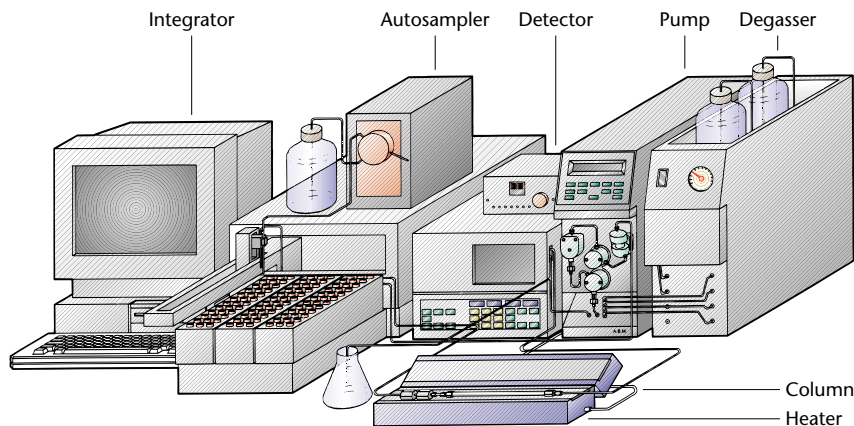


Figure 1 A typical high-performance liquid chromatography system consisting of a degasser, pump, mobile phase delivery system (isocratic or gradient) detector (UV, fluorescence or electrochemical), autosampler and integrator (computer aided). The column may be placed in an oven for constant temperature.

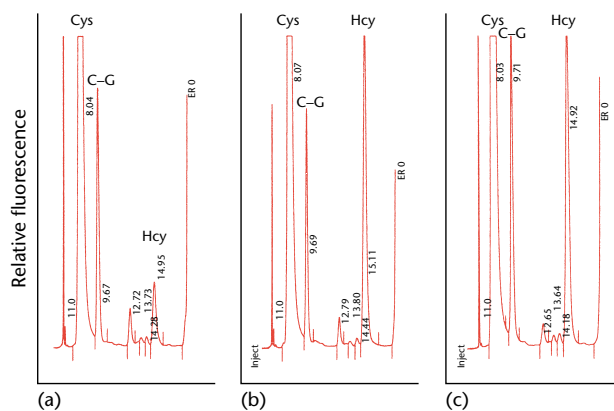


Figure 2 A typical chromatogram of a monobromobimane (mBrB)-derivatized plasma sample of (a) a healthy control subject; (b) an epilepsy patient on anti-epileptic drug, (c) a patient with kidney disease. Samples were analysed according to the method of Mansoor *et al.* (1992). Cys, cysteine, CG, cysteinylglycine, HCY, homocysteine. Column, 150 mm \times 4 mm i.d.; stationary phase, particles ODS 3; mobile phase contains ion-pairing agent tetrabutylammonium phosphate; column temperature 25°C.

Ion Exchange Chromatography (IELC)

In this mode, the stationary phase typically consists of silica on which anionic or cationic groups are immobilized. The anions and cations are classified according to their ability to retain oppositely charged molecules; quaternary salts are strong and amines are weak anion exchangers, whereas sulfonic acids are strong and carboxylic acids are weak cation exchangers.

Chromatographic separation takes place on the basis of the ionic charge of a solute, and is influenced to the extent to which opposite charges are retained on the column. Retention of ionic solute on the stationary phase is affected

by the ionic strength, pH and concentration of the organic modifier in the mobile phase.

The ionic strength is related to the concentration and charge of dissolved ions in the mobile phase. Buffer salts, for example ammonium phosphate, sodium acetate or sodium borate, are commonly used for this purpose. Organic modifiers, for example methanol, acetonitrile or tetrahydrofuran, are added to the mobile phase for elution of ionizable compounds that are insoluble in water and are bound very strongly to the stationary phase.

Size exclusion liquid chromatography (SELC)

In this mode, chromatographic elution of compounds depend upon the molecular size of molecules and their ability to penetrate the pores of the stationary phase. The stationary phase consists of different forms of porous silica gel or polystyrene that behave like a sieve. This allows the smallest molecules to penetrate the pores whereas larger molecules are excluded; consequently, larger molecules elute first and smaller molecules elute later.

The mobile phase is a solvent used merely to dissolve the solute molecules; it serves solely as a carrier for the solute and there are no interactions between the stationary phase, solute molecules and mobile phase.

SELC is usually applied for separation and determination of the molecular mass of a compound or the molecular mass distribution of compounds in a sample. This mode is rarely used for analytical purposes.

Derivatization

Derivatization takes two forms: precolumn and postcolumn derivatization. For precolumn derivatization, undesired components in a sample are removed and sensitive detectable groups are added to increase the detectability of a particular class of compound before the samples are injected onto a column. For postcolumn derivatization, these steps are taken after separation of the components in a sample has taken place.

Applications

- **Biomedical matrices:** RPLC and IELC may be recommended for analyses of amino acids, peptides, proteins, lipids and other compounds in serum, plasma, blood, urine and spinal fluid.

- **Dairy products:** RPLC, IELC and NPLC may be recommended for milk, cheese and other related samples.
- **Oils and fats:** NPLC may be recommended for vegetable oils, animal fats and fat-soluble vitamins and other lipid compounds.

Derivatization procedures will be different for the different matrices.

Limits and Possibilities

Liquid chromatography is a robust analytical technology, but modifications are required to develop methods that reduce consumption of organic solvents, decrease costs and save time.

Higher speed and improved resolution can be achieved by reducing the length and inner diameter of a column, and the average diameter of particles of the stationary phase, and increasing the flow rate of the mobile phase. An unwanted increase in the pressure developed in the system owing to the presence of smaller particles and a higher flow rate can be reduced by raising the temperature of the column. An increase in the temperature by 1°C decreases the retention time by 1–3% for a particular solute.

Methodology development in liquid chromatography specifically aimed at biomedical and pharmaceutical applications should have a focus on automation of the instrumentation.

Further Reading

- Dolan JW (2000) Starting out right, part I. Selecting the tools. *LC-GC* **13**: 12–15. [www.lcgmag.com]
- Dolan JW (2000) Starting out right, part II. Measuring satisfaction. *LC-GC* **13**: 72–76. [www.lcgmag.com]
- Dorsey JG, Foley JP, Cooper WT, Barford RA and Barth HG (1990) Liquid chromatography: theory and methodology. *Analytical Chemistry* **62**: 324R–356R.
- Lim CK (1986) *HPLC of Small Molecules, a Practical Approach*. Oxford: IRL.
- Lough WJ and Wainer IW (eds) (1996) *High Performance Liquid Chromatography, Fundamental Principles and Practice*. London: Blackie Academic and Professional.
- Mansoor MA, Svardal AM and Ueland PM (1992) Determination of the in vivo redox status of cysteine, cysteinylglycine, homocysteine and glutathione in human plasma. *Analytical Biochemistry* **200**: 218–229.
- Snyder LR (2000) HPLC: past and present. *Analytical Biochemistry* **72**: 412A–420A.