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REVIEW ARTICLE

A ANALYSIS ON VARIOUS STRATEGIES OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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A Analysis on Various Strategies of High Performance Liquid Chromatography

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INTRODUCTION

Chromatography is an analytical technique based on the separation of molecules due to differences in their structure and/or composition. In general, chromatography involves moving a sample through the system over a stationary phase. The molecules in the sample will have different affinities and interactions with the stationary support, leading to separation of molecules. Sample components that display stronger interactions with the stationary phase will move more slowly through the column than components with weaker interactions.

Different compounds can be separated from each other as they move through the column. Chromatographic separations can be carried out using a variety of stationary phases, including immobilized silica on glass plates (thin-layer chromatography), volatile gases (gas chromatography), paper (paper chromatography) and liquids (liquid chromatography).

High-performance liquid chromatography (HPLC) is a type of liquid chromatography used to separate and quantify compounds that have been dissolved in solution. HPLC is used to determine the amount of a specific compound in a solution. For example, HPLC can be used to determine the amount of morphine in a compounded solution. In HPLC and liquid chromatography, where the sample solution is in contact with a second solid or liquid phase, the different solutes in the sample solution will interact with the stationary phase as described.

The differences in interaction with the column can help separate different sample components from each other. Basic components of an HPLC system are a solvent delivery pump, sample injection port, column, and detector. HPLC performance requires instruments with characteristics not found in typical LC systems. Solvent delivery must be pulseless and accurately calibrated at flow rates in the 1 mL/minute to 100 µL/minute range.

Syringe pumps are generally acknowledged to be superior to piston pumps for HPLC applications where low, stable flow rates are required. Teledyne Isco Syringe Pumps are excellent HPLC pumps for these high performance applications.

TYPES OF LIQUID CHROMATOGRAPHY

As its name implies, liquid chromatography (LC) has a liquid mobile phase. The great power of liquid chromatography resides in the combination of a wide range of possible mobile-phase properties together with the choice of numerous, significantly different kinds of stationary phases and a wide variety of detectors.

As a result, liquid chromatography really refers to a myriad of combinations, and many of them are classified with more than one name. The names focus on many different aspects of LC methods. For instance, one of the primary classification schemes of LC is by the overall physical shape of the stationary phase, such as column chromatography, thin-layer chromatography (a layer on a plate), and capillary liquid chromatography (a layer on the inner surface of the capillary). Other names arise based on the direction of flow of the mobile phase: ascending chromatography, descending hromatography, and flat-bed chromatography. Classification is also based on the efficiency of the separations, such as high-performance liquid chromatography or high-performance thin-layer chromatography. Sometimes names of LC methods identify the solutes that are separated and detected, such as ion chromatography and amino acid analysis (both usually done on columns).

The types of liquid chromatography also ore named after the general type of interaction that occurs between the stationary phase and the solutes in the eluent. The classifications are denoted normal-phase, reversed-phase, ion- exchange, and gel-filtration (also called exclusion or gel-permeation) chro-matographies. Some stationary phases are designed to interact with specific chemical groups. Chromatography with such site-specific groups on the stationary phase is called affinity chromatography. The stationary phase also may be made with chiral groups linked to it.

Ft is imperative to understand that no LC separation occurs by means of only one type of interaction between the analytes and the stationary phase. There are always interactions besides the named, predominant, type. So if you read "the separation was done by normal-phase chromatography," it means that polar adsorption was the predominant mechanism of interaction with the solid. However, there also was some contribution to the separation from at least one of the other mechanisms discussed here, such as ion exchange or gel filtration.

It is useful to be aware of all these special names. That is, doing effective liquid chromatography is an exercise in manipulating all the equilibria that affect the reaction

$$\text{analyte (mobile phase)} \rightleftharpoons \text{analyte (stationary phase)}$$

Any change in the solvent results in **a** shift of the equilibrium between the mobile and stationary phases. Chromatographic separations can be improved by switching from an isocratic elution (same eluent throughout) to a gradient elution. This is done by mixing two or more different eluents such that the mobile-phase composition changes over time. The gradient always changes the mobile phase toward better desorbing eluents.

In alphabetical order		In numerical order low to high	
Solvent	ε°	Solvent	ε°
Acetic acid	1.0	Pentane	0.00
Acetone	0.56	Petroleum ether	0.01
Acetonitrile	0.65	Hexane	0.01
Benzene	0.32	Cyclohexane	0.04
Carbon tetrachloride	0.18	Carbon tetrachloride	0.18
Chlorobenzene	0.30	Xylene	0.26
Chloroform	0.40	Toluene	0.29
Cyclohexane	0.04	Chlorobenzene	0.30
Dimethylsulfoxide	0.62	Benzene	0.32
Dioxane	0.56	Ethyl ether	0.38
Ethyl acetate	0.58	Chloroform	0.40
Ethylene dichloride	0.49	Methylene chloride	0.42
Ethyl ether	0.38	Tetrahydrofuran	0.45
Hexane	0.01	Ethylene dichloride	0.49
iso-Propanol	0.82	Methylethylketone	0.51
Methanol	0.95	Dioxane	0.56
Methylene chloride	0.42	Acetone	0.56
Methylethylketone	0.51	Ethyl acetate	0.58
Pentane	0.00	Dimethylsulfoxide	0.62
Petroleum ether	0.01	Acetonitrile	0.65
n-Propanol	0.82	Pyridine	0.71
Pyridine	0.71	*iso*-Propanol	0.82
Tetrahydrofuran	0.45	*n*-Propanol	0.82
Toluene	0.29	Methanol	0.95
Water	Large	Acetic acid	1.0
Xylene	0.26	Water	Large

Table 1 Solvent Strength Parameter If for Alumina Supports: ■ The Eluotropic Series

the surface can anchor carbon compounds onto the surface. Some of the groups that have usefully boon bonded to silica surfaces for normal-phase separations are listed in Table 2. These packings with covalently linked surface groups are called bonded phases.

Type	Application
Iminodiacetic acid–Ni^{2+}	Proteins, polypeptides
Nitrilotriacetate–Cu^{2+}	Proteins, polypeptides
Cyclodextrins (cyclic oligo D-glucopyranose)	Racemate separations
Bovine serum albumin (a protein)	Chiral separations
Amino acids (Cu^{2+} in mobile phase)	Chiral separations
Ferrocenylpropyl amine	Chiral separations

Table 2; Representative Bonded-Phase Groups Used for Separations

HPLC SEPARATION AND QUANTIFICATION

High performance liquid chromatography (HPLC) is an important analytical tool for separating and quantifying components in complex liquid mixtures. By choosing the appropriate equipment (i.e. column and detector), this method is applicable to samples with components ranging from small organic and inorganic molecules and ions to polymers and proteins with high molecular weights. The various types of HPLC and their characteristics are summarized in the table 3. In this experiment, we will use reversed-phase partition chromatography.

TYPE	SAMPLE POLARITY	MOLECULAR WEIGHT RANGE	STATIONARY PHASE	MOBILE PHASE
Adsorption	non-polar to somewhat polar	10^0 - 10^4	silica or alumina	non-polar to polar
Partition (reversed-phase)	non-polar to somewhat polar	10^0 - 10^4	non-polar liquid adsorbed or chemically bonded to the packing material	relatively polar
Partition (normal-phase)	somewhat polar to highly polar	10^0 - 10^4	highly polar liquid adsorbed or chemically bonded to the packing material	relatively non-polar
Ion Exchange	highly polar to ionic	10^0 - 10^4	ion-exchange resins made of insoluble, high-molecular weight solids functionalized typically with sulfonic acid (cationic exchange) or amine (anionic exchange) groups	aqueous buffers with added organic solvents to moderate solvent strength
Size-Exclusion	non-polar to ionic	10^3 – 10^6	small, porous, silica or polymeric particles	polar to non-polar

Table 3. Various Types and Applications of HPLC

The system consists of: reservoirs to hold the solvents used to make up the mobile phase a solvent degasser to prevent bubbles in the mobile phase a programmable quaternary pump that mixes the solvents in the prescribed ratios and pumps them through the column and past the detector a column compartment that houses and thermostats the HPLC column (in our case a ZORBAX, reversed-phase C18 column; dimensions 4.6mm x 15 cm) an autosampler that draws prescribed volumes from sample vials and injects them onto the column a diode array detector that monitors the entire UV-vis spectrum of the column effluent at regular intervals.

The goal of any HPLC experiment is to achieve the desired separation in the shortest possible time. Time is critical because time is money and because as we ll see, the more time the sample spends on the column, the more the bands containing the components spread, resulting in reduced resolution. Optimization of the experiment thus usually involves manipulation of column and mobile phase parameters to alter the relative migration rates of the components in the mixture and to reduce zone broadening. These can generally be optimized fairly independently.

RP - HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

Reversed-phase high-performance liquid chromatography (RP-HPLC) involves the separation of molecules on the basis of hydrophobicity. The separation depends on the hydrophobic binding of the solute molecule from the mobile phase to the immobilized hydrophobic ligands attached to the stationary phase, i.e., the sorbent. A schematic diagram showing the binding of a peptide or a protein to a reversed-phase surface is shown in Fig. 1. The solute mixture is initially applied to the sorbent in the presence of aqueous buffers, and the solutes are eluted by the addition of organic solvent to the mobile phase.

Elution can proceed either by isocratic conditions where the concentration of organic solvent is constant, or by gradient elution whereby the amount of organic solvent is increased over a period of time. The solutes are, therefore, eluted in order of increasing molecular hydrophobicity. RP-HPLC is a very powerful technique for the analysis of peptides and proteins because of a number of factors that include: (1) the excellent resolution that can be achieved under a wide range of chromatographic conditions for very closely related molecules as well as structurally quite distinct molecules; (2) the experimental ease with which chromatographic selectivity can be manipulated through changes in mobile phase characteristics; (3) the generally high recoveries and, hence, high productivity; and (4) the excellent reproducibility of repetitive separations carried out over a long period of time, which is caused partly by the stability of the sorbent materials under a wide range of mobile phase conditions. However, RP-HPLC can cause the irreversible denaturation of protein samples thereby reducing the potential recovery of material in a biologically active form.

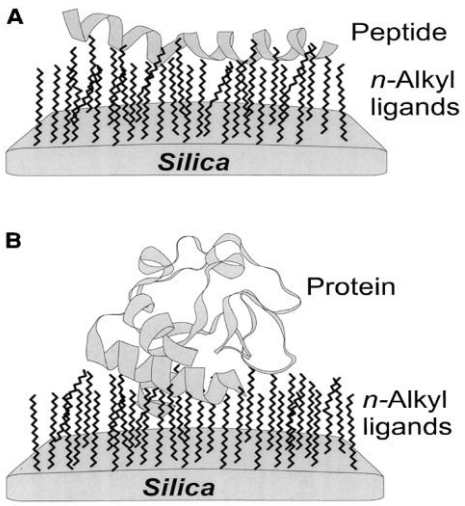

Fig. 1. Schematic representation of the binding of (A) a peptide and (B) a protein, to an RP-HPLC silica-based sorbent. The peptide or protein interacts with the immobilized hydrophobic ligands through the hydrophobic chromatographic contact region.

The RP-HPLC experimental system for the analysis of peptides and proteins usually consists of an n-alkylsilica-based sorbent from which the solutes are eluted with gradients of increasing concentrations of organic solvent such as acetonitrile containing an ionic modifier such as trifluoroacetic acid (TFA). Complex mixtures of peptides and proteins can be routinely separated and low picomolar—femtomolar amounts of material can be collected for further characterization. Separations can be easily manipulated by changing the gradient slope, the operating temperature, the ionic modifier, or the organic solvent composition.

HPLC ANALYSIS OF DRUGS

Analyses of clmgs of abuse are important for the prediction of and protection from the risk to human health, especially for young people. The use of drugs of abuse is increasing world wide and causing serious social problems. In Japan, abuse of methamphetamine (MP) is the most common and arrests for stimulant drug-related offences totaled 14624 in 2003.[15] Recently the use of 4.5-methylenedioxy-metliamplietamine (MDMA), which is called ecstacy. and 4,5-etliylenedioxyamphetamine (MDA) is increasing among the young. Although the arrests for MDMA-related offences numbered 256 in 2003.
which is veiy small compared with those for stimulants. the increase in its use among teenagers is serious. MDMA and MDA are used in tablet form, and thus can be easily taken orally, while MP that is
mainly taken by injection. For the prediction of and protection from abuse of drugs, simple and sensitive methods for qualitative and quantitative analyses are required. Many chromatographic methods
such as thin-layer chromatography, gaschromatograpliy (GC). and high-performance liquid chromatography (HPLC) as well as immunoassays have been developed. Among these. HPLC has been a
secondary choice. However. HPLC essentially can be applied to water-soluble compounds, and thus its use in the forensic and toxicological fields is increasing rapidly.

In this review. HPLC methods developed in the past 5 years and their use in the analyses of stimulant- and MDMA-related compounds in biologic samples are described.

Many types of drugs have been abused and caused serious human health problems. Arrests for the

possession and use of representative drugs of abuse in Japan in 2003 are shown in Fig. 1. MP is the most popular and the arrests of those less than 20 years old for stimulant-related offences totaled 524 in 2003.[2)] On die other hand, although the illegal use of MDMA is less than that of MP. the number of confiscated tablets containing MDMA is rapidly increasing. The total number of MDMA tablets confiscated in Japan was 393062 in 2003.

HPLC is a very versatile method. The most common detection methods are ultraviolet (UV). electrochemical (EC), fluorescence (FL). and MS. For HPLC-UV and -FL methods, a derivatization procedure is generally required to increase sensitivity.

LC-MS is a more versatile method for sensitive determination of many types of drugs including amphetamines. As a conventional method. GC-MS is well known and has generally been used in die forensic and toxicological fields. However. GC also requires derivatization to increase the volatility of target compounds, and thus its use for water-soluble compounds has been limited. On the other hand. LC-MS generally requires 110 derivatization of target compounds. This is an important advantage of analytical procedure in terms of time reduction. As a result. LC-MS is becoming more commonly used
than GC-MS.

Recently, the rapid emergency drug identification high-sensitivity (REMEDi-HS) system utilizing HPLC has been used in emergency hospitals for the detection of drugs of abuse as well as poison
compounds.

QUANTITATIVE ANALYSIS

The quantification methods incorporated in HPLC are borrowed mostly from gas chromatography methods. The basic theory for quantitation involves the measurement of peak height or peak area. To determine the concentration (conc.) of a compound, the peak area or height is plotted versus the concentration of the substance. For peaks that are well resolved, both peak height and area are proportional to the concentration.

Three different calibration methods, each with its own benefits and limitations, can be utilized in quantitative analysis: external standard (std.), internal standard and the standard addition method.

External Standard : The external standard method is the simplest of the three methods. The accuracy of this method is dependent on the reproducibility of the injection volume. To perform this method, standard solutions of known concentrations of the compound of interest are prepared with one standard that is similar in concentration to the unknown. A fixed amount of sample is injected. Peak height or area is then plotted versus the concentration for each compound. The plot should be linear and go through the origin.

Internal Standard : Although each method is effective, the internal standard method tends to yield the most accurate and precise results. In this method, an equal amount of an internal standard, a component that is not present in the sample, is added to both the sample and standard solutions. The internal standard selected should be chemically similar to, have similar retention time and derivatize similarly to the analyte. Additionally, it is important to ensure that the internal standard is stable and does not interfere with any of the sample components. The internal standard should be added before any preparation of the sample so that extraction efficiency can be evaluated.

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