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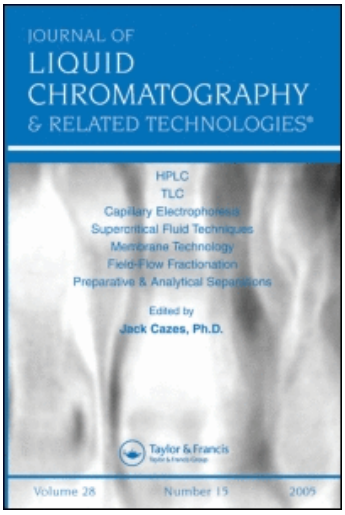

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Review of High-Performance Liquid Chromatography in the Assay of Endogenous Substances in Clinical Chemistry

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REVIEW OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY IN THE ASSAY OF ENDOGENOUS SUBSTANCES IN CLINICAL CHEMISTRY

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ABSTRACT

There is a great potential for HPLC in assaying endogenous substances in the routine clinical chemistry laboratory. This technique coupled with sensitive detectors, can compete in certain instances with RIA and without the need for preparing antisera or labeling the antigen. Furthermore, it can detect small molecular heterogeneity.

In the last few years columns for high speed separation were introduced. Special packing materials with large pores for protein separations became available. The electrochemical detector has been improved by using dual cells.

The technique is very useful in studying the intermediary metabolism of both small and large molecules. Some metabolites have already been studied by HPLC; however, a great number require improved methodology for which HPLC shows great promise,

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e.g., ceruloplasmin in Wilson's disease, ectopic hormones and androgens. HPLC can provide reference methods for many analytes.

Since many related compounds are often detected in the same run, HPLC is useful for profile determination of different disorders, such as the serum profile of ultraviolet absorbing materials in uremia or diabetes.

It is illustrated that HPLC offers the clinical chemist another means to expand his role by bringing into the routine lab some of the more difficult and specialized assays and opens the door for him to contribute basic research concerning the mechanism of many of the human disorders.

INTRODUCTION

Over the past decade, a great wealth of information was gained in different areas of bioscience by the introduction of the technique of high-performance liquid chromatography (HPLC). In clinical chemistry, this technique has been applied mainly in two different areas: therapeutic drug monitoring and to a lesser extent, in endogenous substance (ES) assays. While HPLC offered economy for performing drug assays, immunoassays, because of their simplicity and speed through automation, have dominated the field of therapeutic drug monitoring. Immunoassays have not been applied very extensively to the assay of ES. The potential of HPLC for analysis of ES and expanding the number of tests in the routine clinical chemistry laboratory has not been fully appreciated.

The aim of this review is to summarize what has been done in the past in the area of analyzing ES by HPLC, and more important, to shed light on the areas which need further investigation and

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where the technique will offer new information. The following review will not attempt to list all the tests performed by HPLC but will focus on those areas which are of most interest to the clinical chemist.

Advantages of the Technique

The ES belongs to different classes of chemical compounds; most of these are present in small concentrations. The HPLC offers a simple and effective technique for rapidly separating and quantitating small concentrations of ES in the presence of large contaminants. There are several kinds of packing materials and columns which are suitable for separation of wide varieties of compounds. With appropriate detectors the technique can, in some instances, approach the sensitivity of radioimmunoassays (RIA). However, it does not require preparing antisera or labeling the antigen. Compared to gas chromatography, the HPLC is more suited for separating most of the biological compounds, especially for large and polar molecules. Technically, HPLC is simpler than GC in requiring little or no sample preparation. The basic equipment is relatively inexpensive. The success of HPLC in the routine laboratory depends on dedicated inexpensive equipment (especially pumps) for a single assay. Solvent gradient which allows the technician to perform many assays on a single instrument are desirable in research but not for routine assays.

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The main disadvantage of the HPLC technique, as in the case of other chromatographic techniques, is the high level of skill and knowledge required. It is important to keep in mind that there are many tests which can be performed easier by methods other than HPLC.

Basics

In column chromatography, a chemical mixture is resolved by virtue of the partition of its components between the stationary support of the column and the eluting buffer. Most of the common supports used at the present time are silica, reacted with octadecylsilane, where the separation is based on the hydrophobic attraction between the compound and the stationary phase. This is called reversed-phase separation. Other types of columns, such as ion-exchange and gel permeation, are also in common use. These columns offer special features which make them suitable for specific separations.

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Recent Advances in the HPLC Technique

In the last few years several advances in the HPLC technique occurred:

A. Narrow bore columns. These columns are below 2 mm i.d. Compared to the conventional 4.6 mm i.d. they are about ten times more sensitive, while decreasing solvent consumption by about

tenfolds (1). Unfortunately, these columns require special flow cells and injectors to keep the dead volume to a minimum. These column have not been very popular in clinical laboratories.

B. High speed columns. Speed is very important for routine assays. In general, chromatography is a slow technique compared to the automated immunoassays. In recent years, there has been emphasis on improving the speed of HPLC by using columns with short length (below 10 cm) packed with small particles (5 or 3 μm). Inspite of their short length, these columns have enough high plate numbers with a low pressure drop that it can be operated at a high flow rate, and still produce adequate separation (2,3). Pressure build up is a common problem with the 3 μm columns. This problem can be eliminated by adding an extra 2 mm units or filter in front of the regular 0.5 μm units in the column.

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C. New packing materials. Most of the packing materials have been based on 10 μm silica particles reacted with octodadecylsilane. Recently, 5 and 3 μm silica particles are commonly used.

Packing materials based on synthetic polymers have special advantages over silica. They can be used over a wider range of pH. Basic compounds do not adhere or tail on these columns because of the absence of free silane groups from the packing material. Initially, these columns did not have high plate

numbers but recent improvements gave these columns plate numbers comparable to the silica columns. These packing materials can also be produced more uniformly and with better reproducibility than the silica. Certain compounds such as amino acids and carbohydrates are more suited to separations on the polymeric columns (4). We find the polymeric columns to have better stability compared to silica columns.

D. Microprocessors. Most of the instrument manufacturers have added microprocessors or personal computers to control the solvent gradient, pump speed and manage data handling.

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E. Detectors. Several new kinds of detectors have been adapted to the HPLC including radioisotope counters and mass spectrometers. Ultraviolet detectors are becoming more sensitive. The fluorescent detector is used often in amino acid analysis because of its great sensitivity and the ease of preparing fluorescent derivatives of amino acid (5). The electrochemical detector is becoming common in routine assays for the electroactive compounds such as those containing phenolic groups (6). The detector has been improved by using dual electrode cells (7,8). The new cells with stainless steel auxillary electrode gives better baseline stability and are easier to work with compared to the older cells constructed of plastic. The electrochemical detector is simple enough that it can be constructed in the laboratory with minimum cost (9,10) while providing great selectivity and sensitivity. This

detector, however, requires a great amount of patience and skill for successful operation.

I. Small Molecules

High-performance liquid chromatography is well suited for the determination of small molecules. The widespread use of the technique is mainly due to the durability of the reversed-phase column which can be used with aqueous buffer and has a long lifetime. The technique is useful in studying profiles of a class of metabolites in different disorders such as in uremia (11) and diabetes. Furthermore, it can reveal, from the capacity factors, important structural information about the endogenous matabolites (12). HPLC can be used for purification of small amounts of samples for further chemical studies. The following compounds which are of special interest to the clinical chemist have been studied with HPLC.

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Catecholamines

Catecholamines (CA) and their metabolites are quite difficult to analyze by the traditional fluorescence methods because of their low concentrations and the presence of numerous interfering substances such as drug and natural phenolic compounds. The patient has to be on a special diet for a few days before urine is collected for analysis by the fluorescent methods. On the other hand, HPLC greatly speeds up the analysis of these

compounds, and eliminates the interferences. Several methods have been described for post- and pre-column derivatization with fluorescent detection. Because of its selectivity, sensitivity, and the elimination of the derivatization steps, the electrochemical detection of CA is more popular. Adams, Kissinger and co-workers (6,10) pioneered the use of the electrochemical detector in conjunction with HPLC for the determination of CA. The sensitivity of the technique is greatly enhanced by this detector, especially with dual electrode cells (13) and the 3 um column.

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Urine and serum need to be pretreated and concentrated with alumina (13), boric acid (15), or a disposable ion-exchange column (16) before injection on the HPLC. The latter method, especially in conjunction with Sephadex G-10, gives clean chromatograms (17) but at a higher cost. Serum CA are more difficult to analyze because of their low concentrations. Several methods have been described for the assay of serum CA by the electrochemical detector (15,18). These were found to compare favorably with the radioenzymatic techniques (18).

4-hydroxy-3-methoxymandelic Acid (Vanillymandelic Acid) (VMA) and 4-hydroxy-3-methoxyphenylacetic Acid (Homovanillic Acid) (HVA)

These metabolites are clinically useful for detection of pheochromocytoma and neuroblastoma. Like the CA, they contain an oxidizable group but at a higher voltage (19,20) and differ from

the CA in that they are present in urine in much higher concentrations. The traditional Pisano method for VMA (21), based on the oxidation of VMA to vanillin has many time-consuming steps.

Initially, VMA and HVA assay by HPLC with ultraviolet detection was not entirely successful because of the presence of numerous interfering compounds. Later, it was demonstrated that electrochemical detection for these compounds gives much greater selectivity and sensitivity so that only small amounts of urine need to be injected on the column, leading to increased column-life. Some of these methods are so simple that urine is injected directly on the column without prior sample preparation (18,29,21). Under these conditions the oxidation voltage has to be kept very low to avoid oxidizing the contaminants in the urine in addition to a column with a high plate number. Some of these methods measure both VMA and HVA simultaneously (22,23).

Recently, five methods for VMA assay were compared: Pisano (21), gas chromatography, paper chromatography with diazotization with p-nitroaniline, a commercial disposable column, and the HPLC with electrochemical detection (23). It was concluded that the latter method had the least amount of interferences and was the most suitable for the routine laboratory (24).

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Methoxyhydoxyphenylethyleneglycol (MHPG)

This metabolite has been suggested as an index of the brain metabolism of CA. urinary MHPG has been shown to be useful in

selecting the tricyclic to be administered in depression (25). Usually, it takes a few weeks, based on trial and error, before a patient responds to a certain tricyclic. It was found that patients respond better to amitriptyline when the MHPG level is elevated, while responding better to imipramine when the MHPG level is low (24). Several methods have been described for MHPG analysis by HPLC with electrochemical detection (22,26) which compare well with gas chromatography/mass spectrometry methods (27).

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Indolamines

Because of the importance of serotonin as a neurotransmitter, many methods have been described for the assay of indolamines in different tissues. Carcinoid tumors synthesize excess amounts of these compounds leading to high concentration of 5-hydroxyindolacetic acid in the urine. Because this compound contains an oxidizable hydroxyl group it has been assayed by HPLC with electrochemical detection (22,28,29) and fluorescence detector (30).

Biluribin

In addition to separating the traditional fractions of bilirubin (unconjugated, monoglucuronide and di-glucuronide conjugates), the HPLC revealed a fourth fraction which is tightly bound to protein probably albumin (31,32). Quantitation of the

protein-bound fraction has been shown to be method dependent. It is increased in hepatocellular and cholestatic jaundice and Dubin-Johnson syndrome (33). It becomes a larger fraction when jaundice is subsiding (33).

Steroids

The steroids are present in serum and urine in relatively low concentration. HPLC methods have been described for analysis of cortisol in serum using the ultraviolet detector. However, it was demonstrated that cortisol can be determined with better sensitivity based on its fluorescence characteristics in sulfuric acid (34). A similar HPLC method (35) has been described for urinary free cortisol a compound difficult to measure even by RIA. Urinary free cortisol assays are sensitiv for detecting Cushing's syndrome. This method compared to RIA was found to give about 30% lower values probably due to its greater selectivity (35). Few methods have been described for other steroid analysis by the HPLC (36,37).

Estrogens

Estrogens are characterized by having a phenyl ring which can be detected by fluorescence, ultraviolet spectrophotometry or electrochemically. Electrochemical detection of estrogens after separation by HPLC has been shown to be about 20 times more

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sensitive than ultraviolet detection (9). The advantage of this sensitivity is that small volumes of urine are hydrolyzed, extracted and evaporated. The assay thus is faster and more cost-effective.

Amino Acids

For many years, amino acids have been analyzed quite successfully by the amino acid analyzer which is essentially an expensive, specialized, HPLC instrument. Many workers have tried to adapt the general HPLC instruments while speeding up the analysis by using columns packed with smaller particle size. Amino acid profiles of protein hydrolysate can be completed in less than 16 min on a 3 um column (5). Presently, there are two common methods for amino acids derivatization for HPLC: pre-column and post-column (4,5). The first one is attractive for simplicity and speed but it does not detect the secondary amino acids. The second one requires more complicated instrumentation. O-phthalaldehyde is the derivatizing agent commonly used for amino acids. This derivatizing agent can be detected by fluorescence as well as electrochemically (38). Recently, a simple method for derivatization with phenylisothiocyanate (39) has been developed. This method can also be used with electrochemical as well as ultraviolet detection. Single amino acids such as Taurine (40) and glutamine (41) are easier to determine by HPLC compared to the complete amino acid profile.

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Carbohydrates

The sugars and their corresponding alcohols are important in many disorders. For example, in diabetes mellitus, several sugars other than glucose are elevated and are thought to be involved in the progress of the microangiopathy. In general, the sugars are difficult to separate by HPLC, and also difficult to detect. They require special columns (4) and special detectors. Recently, carbohydrates have been detected by pulsed oxidation at a platinum electrode (42). Sugars are also important for detecting and classifying bacteria. Better methods for detecting sugar and other metabolites based on HPLC probably will speed up bacterial identification (43).

Biopterins

There are several of these related compounds which are widespread in nature, especially in butterflies. They function as coenzymes of many enzymes such as phenylalanine hydroxylase. From a diagnostic point of view they are elevated in tumors and acquired immune deficiency. They have been analyzed by HPLC with fluorescence (44) and electrochemically (7). These compounds need further study in different disorders.

II. Large Molecules

Most of the research for separation of the large molecules by HPLC has been focused upon separation of proteins and peptides.

Traditionally, proteins have been separated by liquid chromatography mainly on ion-exchange and molecular sieve columns. HPLC speeds up the separation of proteins by the use of high plate columns, better equipment and by extending the separation to the popular reversed-phase columns which have been successful in separating small molecules. The use of HPLC for separation and determination of large molecules is more difficult because of the large size, the different charges present on the molecule, and the susceptibility to denaturation. Recently, packing materials with small particle size but with large pores became available for separating proteins (45). Hydrophobic column for protein separation has been introduced recently (46). Protein separation by HPLC has been reviewed recently (47).

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HPLC has been used in studying drugs and small molecules binding to proteins similar to equilibrium dialysis methods (48). HPLC has also been used for molecular weight determination of peptides (49).

Enzymes

In general, enzymes are measured through their catalytic activity by spectrophotometry. Many enzymes have very low turn-over rate or else the reaction product is difficult to distinguish from the substrate such as in the case of dopamine-β-hydroxylase (50), angiotensin converting enzyme (51), or chymotrypsin (52). Assay of these enzymes is greatly facilitated

by separating the reaction products by HPLC, taking advantage of the sensitivity of the special detectors. Post-column reactions for the enzymatic activity have been reviewed (54). Application of HPLC in enzyme assays will be a fruitful area for studying many of the enzymes in different disorders.

Hemoglobins

The design of ion-exchangers with small particles and large pore sizes suitable for protein separations spurred interest in studying hemoglobin variants. The advantages of HPLC over electrophoresis are speed and resolving power with better quantitation. Several methods have been described for determining glycosylated (55) and the other major and minor hemoglobins by HPLC (56,57). Most of the HPLC methods for hemoglobin determination require solvent gradient and complex instrumentation. There is a need for simple methods based on isocratic separation of hemoglobins (58). Quantitation of A2, F and other hemoglobins need further work.

Myoglobin

Myoglobinuria occurs after several kinds of severe muscular injuries and disorders and may provoke acute renal failure which can be prevented by osmotic diuretics. It was found that myoglobin elutes from muscle and serum in two distinct peaks which probably involve a charge difference (59). The method is

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simple enough that serum or urine can be injected directly on the column and eluted isocratically (59).

Concluding Remarks

It is expected that the utilization of HPLC in the routine laboratory to expand in the near future. More metabolites, especially with low concentrations, will be performed with HPLC. New derivatization techniques will be devised to enhance the sensitivity of those compounds persent in low concentration, especially in the areas of fluorescence and electrochemical detection. Proteins and especially hemoglobins will be separated and quantitated by HPLC.

The most important and the least expensive part of the HPLC equipment is the column. Columns with better characteristics such as high plate number, long stability, and low pressure drop will be improved. Short columns packed with smaller particles will be mainly used. The number of columns for specific separation will be increased. Automation of the instrument in general will be enhanced.

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