



High Performance Liquid Chromatography for Clinical Applications

Many forms of chromatography have been used over the years in the clinical laboratory for the separation and quantification of a variety of clinically relevant analytes. (1) High Performance Liquid Chromatography also called High Pressure Liquid Chromatography (HPLC) has emerged as the most important of all chromatography which otherwise includes Thin Layer Chromatography (TLC) and Gas Chromatography (GC). Chromatography methods are based on the separation and isolation of the analyte of interest from all other components of a biological sample prior to detection. The main advantages of chromatography methods over other techniques are their high selectivity, sensitivity, reliability, versatility and generally low cost of operation.

TLC is a very simple method easy to implement that was first developed in the 1940s. It is used today in the clinical laboratory mostly as a screening or semi-quantitative test for amino acids, drugs and lipids in serum or urine. TLC consists of a thin layer of sorbent such as silica gel spread uniformly on a glass plate or plastic sheet. Sample is added as a small spot or band near the edge of the plate which is then placed in a closed chamber containing the mobile phase. The mobile phase migrates up the plate by capillary action and separates the compounds in the sample which can then be visualized by ultraviolet (UV) illumination or spraying with specific reagents. In its basic form, the method has serious limitations in terms of sensitivity and selectivity as compared to modern chromatography such as GC or HPLC and has often time been supplemented or replaced by these techniques.

GC, especially coupled to mass spectrometry (GC/MS) is used in the clinical laboratory primarily for confirmation of drugs and screening of amino acids. For example, GC/MS is commonly used in toxicology to confirm a positive screen for opiates and opioids and can distinguish and simultaneously report the various types, prescribed versus drug of abuse, with high selectivity and sensitivity where an immunoassay method cannot. GC consists of using a gaseous mobile phase or carrier gas to pass a mixture of volatile solutes extracted from the sample through a column containing the stationary phase. Solute separation is based on differences in vapor pressure and interaction with the stationary phase. GC is best suited for the analysis of volatile organic compounds which is also its main limitation for clinical use.

HPLC is the most versatile of all chromatography methods but also the most complex. It was first made available in the laboratory during the 1970s and is currently used for the analysis of amino acids, peptides, proteins, carbohydrates, lipids, nucleic acids and related compounds, vitamins, hormones, metabolites, and drugs. HPLC can be coupled to various detectors such as UV, fluorescence or mass spectrometry (LC/MS and LC/MS/MS) and is routinely used for quantitative analysis in biological samples such as blood, urine and other body fluids. HPLC consists of using a liquid mobile phase to pass under high pressure a mixture of analytes extracted from the sample through a column containing the stationary phase. Analyte separation is based on differences in interaction with both the mobile phase and the stationary phase. Three of the main clinical applications of HPLC are described below.



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Immunosuppressants

Survival rates of transplant patients have increased significantly with the introduction of immunosuppressant drugs such as cyclosporine, sirolimus and tacrolimus. (2-3) However, because of their complex mode of action, variable absorption rate and narrow therapeutic index, patients need to be monitored during treatment in order to prevent both adverse drug reactions and organ transplant rejection events. The diagnostic methods used for immunosuppressant drug monitoring are primarily based on HPLC or immunoassays. One of the major challenges with immunoassays is their metabolite cross-reactivity, even for assays that use monoclonal antibodies. HPLC based methods have been and remain the gold standard for therapeutic drug monitoring of immunosuppressants with unmatched specificity, sensitivity and multiplexing ability with the simultaneous quantitation of several drugs and metabolites from a single sample. HPLC with UV or mass spectrometry detection are routinely used for therapeutic monitoring of immunosuppressants.

Vitamin D

With recent recognition of Vitamin D's broad pathophysiological importance well beyond its effect on bone density, clinical laboratories have seen increase number of requests for testing and many new methods for testing have become available. (4-5) Vitamin D is a group of fat-soluble steroid-derived compounds that play a role in the control of calcium and phosphorous metabolism. It was found to be involved in many conditions such as osteoporosis, cancer, diabetes, cardiovascular diseases, and autoimmune diseases amongst others. Two forms of Vitamin D are biologically active, Vitamin D₂ from dietary sources and Vitamin D₃ which occurs naturally in human. Both forms are metabolized in the liver and kidney to active metabolites. Only the measurement of 25-Hydroxyvitamin D_{2/3} and 1,25-Dihydroxyvitamin D_{2/3} have proven clinical value. LC/MS/MS techniques have emerged as the gold standard for these measurements over immunoassays and HPLC/UV because of their ability to specifically and precisely quantify low levels of the metabolites as well as the different forms of Vitamin D, including D₂ and D₃ simultaneously. Many clinical laboratories today have successfully implemented routine testing using automated or semi-automated LC/MS/MS methods.

Glycolipids

Sphingolipidoses are inherited lipid storage disorders caused by lysosomal hydrolase deficiency leading to the accumulation of unmetabolized glycosphingolipids. (6-7) Amongst the main diseases associated with these lipid metabolism disorders are Niemann-Pick disease, Fabry disease and Gaucher disease. Enzyme replacement therapies and substrate reduction therapies are available for the treatment of Gaucher and Fabry diseases with many trials underway for the other forms. To monitor the effect of these therapies, glycosphingolipids and their metabolic intermediates must be measured. HPLC with UV or fluorescence detection provides a unique and reliable way to simultaneously isolate and quantify glycosphingolipids and their metabolic intermediates. Typically, the glycolipids and intermediates are extracted from patient plasma or urine and derivatized prior to analysis. HPLC has contributed greatly to



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the understanding of the biochemistry of glycosphingolipids and remains the method of choice for diagnosis and follow-up of patients on therapy.

Conclusion

HPLC is a proven method for isolating analytes of interest in complex matrices such as biological fluids. Its use in the clinical laboratory has steadily increased over the past decades as its unmatched analytical performance and versatility allows for testing of many different types of clinically relevant analytes. With the recent advances in detection technology such as mass spectrometry and sample preparation techniques such as bioaffinity chromatography and online automation, HPLC-based methods will likely remain the gold standard of clinical testing for many of the current but also future biomarkers and therapeutic drugs.

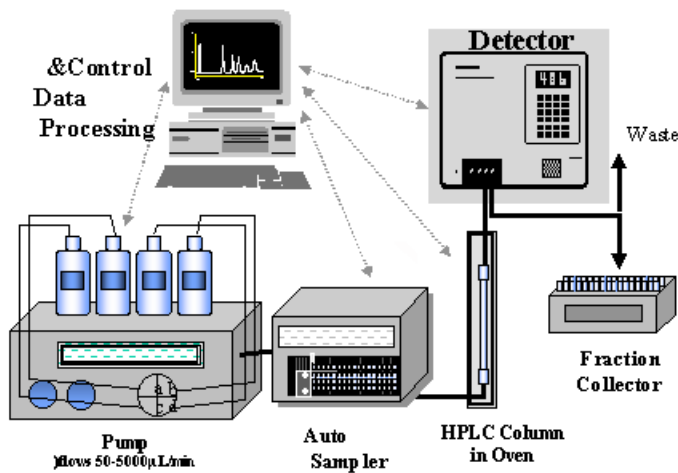


FIGURE 1. The HPLC apparatus includes a solvent reservoir for the mobile phase, a pump, an automatic sampler, an injector, an analytical column, a detector and a computer that controls the system and processes the data

About the author:

Sonia Taktak is a Senior Development Scientist in the Research and Development group at Cambridge Biomedical. Since joining the company in 2011, she has developed and validated several assays including HPLC/UV, HPLC/Fluorescence and ELISAs amongst others. Sonia has experience in performing work



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under both GLP and non-GLP conditions as well as for diagnostics applications. She brings expertise in chromatography in particular HPLC-based methodologies, including LC/MS/MS and in chemistry. Prior to joining Cambridge Biomedical, Sonia worked in the diagnostic field and at the cancer center of MGH as a clinical pharmacology fellow. Sonia holds a PhD in chemistry from Tufts University and has been an active member of the American Association for Clinical Chemistry (AACC) since 2007.

REFERENCES:

1. Burtis CA, Ashwood ER. Tietz Fundamentals of Clinical Chemistry, 5th edition 2001
2. Abushamaa AM. Immunosuppressant TDM Has Successes and Challenges. Clinical & Forensic Toxicology News March 2008
3. Brandhorst G, Oellerich M, Maine G, Taylor P, Veen G, Wallemacq P. Liquid Chromatography-Tandem Mass Spectrometry or Automated Immunoassays: What Are The Future Trends in Therapeutic Drug Monitoring? 2012;58:821
4. Heijboer AC, Blankenstein MA, Kema IP, Buijs MM. Accuracy of 6 Routine 25-Hydroxyvitamin D Assays: Influence of Vitamin D Binding Protein Concentration. Clin Chem 2012;58:543-548
5. Farrell CJL, Martin S, McWhinney B, Straub I, Williams P, Herrmann M. State-of-the-Art Vitamin D Assays: A Comparison of Automated Immunoassays with Liquid Chromatography-Tandem Mass Spectrometry Methods. Clin Chem 2012;58:531-542
6. Ullman MD, Pyeritz RE, Moser HW, Wenger DA, Kolodny EH. Application of "high-performance" liquid chromatography to the study of sphingolipidosis. Clin Chem 1980;26:1499-503
7. Groener JEM, Poorthuis BJHM, Kuiper S, Helmond MTJ, Hollak CEM, Aerts JMFG. HPLC for Simultaneous Quantification of Total Ceramide, Glucosylceramide, and Ceramide Trihexoside Concentrations in Plasma. Clin Chem 2007;53:742-747