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Ultra Performance Liquid Chromatography

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ABSTRACT

Ultra performance liquid chromatography (UPLC) system involves significant technological advances in particle size performance, system optimization, data processing, detector design and control. When all brought together, the specific achievements in each area have created a step-function progress in chromatographic performance. This new technique of analytical separation science uses the principles and practicality of HPLC with increasing the attributes of speed, sensitivity and resolution. Now a day's pharmaceutical industries are in search of new ways to reduce cost and time for analysis of drugs. Analytical laboratories are not exception in this trend. Ultra high performance liquid chromatography (UPLC) with better resolution, assay sensitivity and high sample throughput allows a greater number of analysis to be performed in a shorter period of time and it also impart cost effective advantage over HPLC analysis. So that conventional assay was transferred and optimized for UPLC system. This review introduces the theory of UPLC, and involves some of the most advanced work in the field.

Keywords: Ultra performance liquid Chromatography, High performance liquid chromatography, High pressure liquid chromatography, Liquid chromatography.

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INTRODUCTION

High performance liquid chromatography (HPLC) has proven to be the predominant technology used in laboratories worldwide during the past 30-plus years for various pharmaceutical product analyses. One of the primary promoters for the growth of this technique has been the evolution of packing particle size, which is one of the variables used to investigate chromatographic performance of pharmaceutical product. The "need for speed" has been driven due to large numbers of samples that comes particularly in drug discovery and analytical laboratory[1]. The pharmaceutical industry is under intense pressure to increase productivity and put new drugs onto the market in a shorter period of time. Analytical chemists are challenged to find faster ways of delivering quality data across a range of project needs. A number of approaches are being employed to increase separation throughput.[2]

A new tool for meeting these challenges became generally available in 2004. This new class of separation science is Ultra Performance Liquid Chromatography (UPLC). Which provides improved resolution, speed, and sensitivity and this is achieved through the use of small columns with very undersized particles packing. By using conventional particle sizes, speed, pressures and peak capacity (number of peaks resolved per unit time) can be extended to new limits and compromises with sacrificing retention time. As the particle size is 1.7µm there is a significant gain in efficiency along with resolution and without diminish efficiency, An elevated temperature having the dual advantages of lowering viscosity and increasing mass transfer by increasing the diffusivity of the analyzes, has also been investigated.[3]

Now a day's there is increasing demand for greater pharmaceutical analysis throughput testing by UPLC. An UPLC operates at higher pressure (upto15000psi) and with detector enable to show signal at high data rates. In case of fast eluting peaks in conjunction with new needle design UPLC shows substantially reduce in carryover of sample which is the aids for lowering of limits of quantitation (LOQ). An HPLC method for quality control was optimized to UPLC have attributes to reduce total run time, lower cost per assay and promote instrument uptime.[4] The challenges in developing a new chromatographic particle for analyze separations are described here along with that are more mechanically and chemically stable. Additionally, several new bonded phases provide flexibility during methods development, at variable P^H range that enable the initiation of new products to be brought to market faster. A method development by UPLC simultaneously provides full compatibility between chemistry and instrumentation. It is essential to consider the key parameters that influence peak resolution and ultimately lead to successful

chromatographic methods. The UPLC from Waters Corporation is the first commercially available system that shows the challenge of using elevated pressures and sub-2 µm particles, which makes it a particularly attractive and promising tool for fast liquid chromatography method development[5].

Pharmaceutical separations may depend on three categories, that is high throughput, high productivity, and high resolution, these categories give specific pharmaceutical applications, each of which has discrete separation goals. Thus UPLC has provided a new potential for method development and analysis[6].

In recent scenario this technology has been applied to the study of in vivo drug metabolism, particularly during the analysis of drug metabolites in bile, plasma and urine. The reduction in peak width significantly increases analytical sensitivity by three to five-folds and concomitant increase in peak capacity, significantly reduces spectral overlap resulting in superior spectral quality in both liquid chromatography and liquid chromatography-mass spectroscopy/mass spectroscopy modes. The application of UPLC/MS resulted in the detection of additional drug metabolites, superior separation and improved spectral quality. [7]

Principle

The UPLC is based on the principal of HPLC. It involves stationary phase consisting of particles substance 2 µm, as HPLC columns are typically filled with particles of 3 to 5 µm. According to Van Deemter equation, smaller particles provide not only increased efficiency, but also the ability to do work at increased linear velocity without a loss of efficiency, providing both resolution and speed. Efficiency is the primary separation parameter behind UPLC since it relies on the same selectivity and retentivity as HPLC.

In the fundamental resolution equation [8]

Resolution is proportional to the square root of *N*.

$$Rs = \frac{\sqrt{N}}{4}\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{\kappa}{\kappa + 1}\right)$$

Where *N* is number of theoretical plates, α is Selectivity factor and *k* is mean retention factor. But since *N* is inversely proportional to particle size (*dp*);

$$N \propto \frac{1}{dp}$$

As the particle size is lowered by thrice i.e. from 5 mm to 1.7 mm, *N* is increased by three and the resolution by square root of three i.e. 1.7. *N* is also inversely proportional to the square of the peak width.

$$N \propto \frac{1}{\omega^2}$$

This illustrates that the narrower the peaks they are easy to separate from each other. Also peak height is inversely proportional to the peak width (*w*):

$$H \propto \frac{1}{\omega}$$

So as the particle size decreases to increase *N* and subsequently *Rs*, an increase in sensitivity is obtained, since Narrower peaks also mean more peak capacity per unit time in gradient separations, desirable for many applications such as natural extracts, peptide maps etc. Still another equation comes in to force from the Van Deemter plot when moving toward smaller particles:

$$Fopt \propto \frac{1}{dp}$$

As particle size decreases, the optimum flow rate (F_{opt}) reaches to maximum *N*. But since back pressure is proportional to flow rate, smaller particle sizes require much higher operating pressure and a system properly designed for the same. Higher resolution and efficiency can be level further when analysis speed is the primary objective. Efficiency is proportional to column length and inversely proportional to the particle size:

$$N \propto \frac{L}{dp}$$

Therefore the column can be shortened by the same factor as the particle size without loss of resolution. Using a flow rate three times higher due to smaller particles and shortening the column by one third, the separation is completed in 1/9th the time while maintaining resolution [9]

CHEMISTRY OF COLUMNS

Column researchers and manufacturers have encountered need with the development of more efficient and more reliable packing materials. One of the areas in which improvements have been made is the particle size reduction.

According to chromatographic theory, column efficiency (N) is inversely proportional to particle size (dp). Thus, smaller particles provide higher resolution and lower retention time.

Figure. 1 Van Deemter plot shows the evolution of particle sizes over the last three decades.

In the early 1970s showed that the influence of the particle size of silica gel on column efficiency. This involves the use of smaller particles size resulted in more efficient columns. At that juncture all scientists were used irregularly shaped particles. [10]

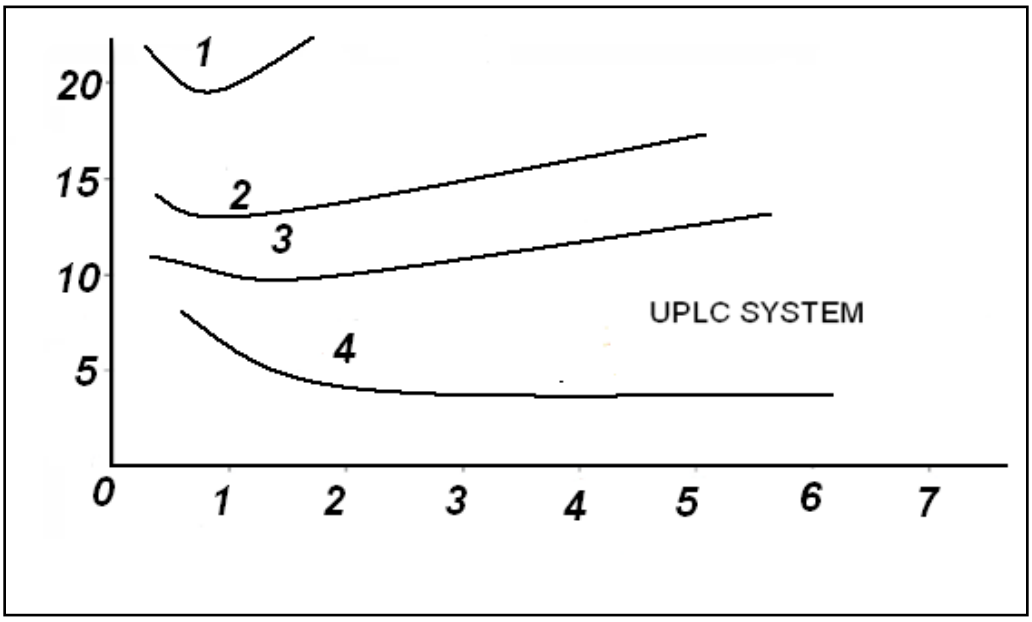

Figure: 1 Van Deemter plot shows the evolution of particle sizes over the last three decades.

(1) 10 µm particle 1970's , (2) 5 µm particle 1980's, (3) 3.5 µm particle 1980's,
(4) 1.7 µm particle 2004's

Types of capillary columns:

1. Packed capillary columns

Packed columns are made by padding the capillary with silica-modified particles of 3–5 µm particles of even smaller sizes 1.5–1.8 µm were successfully employed in UPLC. Such a small particle size provides chromatography systems with higher efficiency, resolution, selectivity, and shorter analysis time; but does not increase the back-pressure. Many research labs pack the columns "in-house". But it is a difficult and skill-demanding process. To ascertain the particles of the same diameter and to avoid undesirable void volumes. The application of packed capillary columns is the most explored option in UPLC.

2. Monolithic Capillary Column

Monoliths are a block of continuous materials made of highly porous rods with two types of pore structures (macropores and mesopores of different sizes), which allow the use of higher flow rates and thus reduces the analysis time. Monolithic polymer columns were first used in the late 1980s, but not available until 2000. Presently four types of monolithic capillary columns can be found: Particle fixed, Silica based, Polymer based, and Molecular imprinted monolith. Now a day there is not much research information on application of monolithic capillary in UPLC.

3. Open tubular capillary columns

In open tubular liquid chromatography column, the capillary wall is coated with highly permeable porous material that serves as the stationary phase. The open tubular capillary has

lower sample loading capacity of the column, because only a small surface area is available for analytes interaction that can result in column overloading causing peak asymmetry and poor efficiency[11].

4. Future scope in small particle technology.

The separation speeds of relatively simple samples by using substance-2 µm porous particle size and shorter columns could result in separations at only a few seconds. The rapid feedback required in on-line process analytical technologies, screening of million-compound libraries in combinatorial chemistry and drug discovery area that might create such a need. In case of higher plate counts longer columns with these smaller particles will be required. Already researcher have demonstrated separations at pressures as high as 7000 bar using nonporous particles with diameters as small as 1 µm packed into nano bore columns (50 µm i.d.) to keep heat generation minimized. Such columns are capable of generating several hundred theoretical plates in minutes. With safety in the routine use of high pressures in the chromatography laboratory is always in a consideration. Nevertheless, the need for further reductions in particle size below the currently available 1.5–1.8 µm particles is required, as manufacturers will respond to provide such columns. Some of the issues surrounding the optimum use of these micrometer-sized particles are the implementation of new column and instrument hardware designs; the development of efficient packing techniques; considerations of particle and packed-column stability; the ability to make stable wide pore packing for the separation of biomolecules. Silica gel-based packing becomes more friable as the pore size increases. Perhaps some of the techniques used to construct silica-organic hybrids, for the synthesis of highly cross linked polymers, or the use of carbon or other inorganic-based packing could alleviate concerns in packing stability[12].

APPLICATION OF UPLC

UPLC with MS

UPLC coupled to quadrupole tandem mass spectrometry(QTMS) which operates with rapid, generic gradients and shows increase in analytical throughput and also shows sensitivity in high throughput pharmacokinetics or bioanalysis studies, the rapid measurement of potential p450 inhibition, induction, and drug-drug interactions had been studied by UPLC-MS/MS.As UPLC based approach can helps to the labs pre-emotively and determine candidate toxicity and drug-drug interactions, it enables organizations to be more confident in the viability of candidate medicines that do progress to late-stage clinical trials. The UPLC/Tandem mass spectrometry

technology is based on the use of columns packed with 1.7µm porous particles enabled to improve in peak resolution, sensitivity and speed of analysis. UPLC/Tandem mass spectrometry chromatograms shows a very sharp peaks with less than 2 s wide at the base, This enhanced efficiency resulted in an increased separation speed of the whole UPLC/TM-MS/MS procedure that required less than 5 min[13].

UPLC in Pharmaceutical Development

Now a day's UPLC is a very attractive tool for the pharmaceutical development laboratory because of high resolution obtained in extremely short period of analysis times, as UPLC provides high throughput, high productivity and high resolution. These categories have specific pharmaceutical applications and distinct separation goals. Now these goals have been achieved by utilizing conventional UPLC with typical column dimensions and particle sizes. The recent introduction of UPLC has provided a new potential for method development and analysis. Pharmaceutical chemists determine the impact of this emerging technology, using sub2 µm particle size column along with increased linear velocities. As mobile phase viscosity must be minimized or the chromatography system must be redesigned to withstand an increased backpressure. Today, there are many commercially available UPLC systems capable of exceeding conventional pressure limits of 400 bars. The advantage of UPLC over conventional HPLC is the capability to increase the speed without sacrificing efficiency. In comparison to traditional HPLC, The research showed that UPLC can decrease run times up to 7 times than HPLC. In addition, for high resolution applications, UPLC achieved significant efficiency advantages over traditional HPLC.

UPLC used in Identification of Metabolite

Biotransformation of New Chemical Entities (NCE) is necessary for drug discovery. When a compound reaches the trial stage, metabolite identification is required and it is necessary for lab to successfully detect and identify all circulating metabolites of a contender drug. Discovery studies are generally carried out *in vitro* to identify major metabolites so that metabolic weak spots on the drug contender molecule can be recognized and protected by changing the compound structure. The key for analysts in metabolite identification is maintaining high sample throughput and providing results to medicinal chemists as soon as they are available. UPLC-MS/MS addresses the complex analytical requirements of biomarker discovery by offering unmatched sensitivity, resolution, dynamic range, and mass accuracy. Tandem quadrupole MS combines with UPLC in ADME screening for sensitivity and selectivity with fast analyses of

samples in matrix to be achieved with minimal cleanup, using MRM (multiple reaction monitoring) for detection and automated compound optimization[14].

UPLC used in Bioanalysis / Bioequivalence Studies

For Pharmacokinetic, Bioequivalence and toxicity studies, the quantitative analysis of a drug in biological samples is an important part of drug development process and this is carried out by UPLC. The drugs having low molecular weight are tested during both preclinical and clinical studies, as several biological matrices are used for quantitative bioanalysis, the most common being used are the blood, plasma, and urine. The primary technique for quantitative bioanalysis of new entity is LC/MS and the sensitivity and selectivity of UPLC/MS/MS at low detection levels generates accurate and reliable data that can be used for a variety of different purposes, including statistical pharmacokinetics (PK) analysis. UPLC/MS/MS develop a robust and compliant assay which has been traditionally the domain of very experienced analysts. UPLC/MS/MS helps in the method development for bioanalytical products. It helps to determine whether new formulations of existing drugs allow the compound to reach the bloodstream at a similar rate and exposure level as the original formulation. UPLC/MS/MS has proven to increase efficiency, productivity, and profitability at bioequivalence laboratories. An application of UPLC/MS/MS in bioequivalence and bioanalysis gives efficient separations with fast acquisition rates using tandem quadrupole MS systems, which easily acquire report and quantify full system data in a compliant environment. And ensure the highest quality results and reliable system operation in regulated environment. UPLC is sophisticated and integrated system for bioanalysis and bioequivalence studies, providing excellent chromatographic resolution and sensitivity along with multiple reactions monitoring[14].

UPLC used in stressed degradation Studies

One of the most important factors that impacts the quality and safety of pharmaceuticals is chemical stability. The FDA and ICH Guideline provide stability testing data to understand how the quality of an API (active pharmaceutical ingredient) or a drug product changes with time under the influence of forced degradation factors such as heat, light, pressure and moisture or humidity. Knowledge of these stability characteristics helps in storage conditions and shelf life, the selection of proper formulations and protective packaging is required for regulatory documentation. Forced degradation, is carried out under even harsher conditions than those used for accelerated stability testing. The test generally performed early in the drug development process, as laboratories cause the potential drug to degrade under a variety of conditions like peroxide oxidation, acid and base hydrolysis, photo stability, and temperature to understand

resulting by products and pathways that are necessary to develop stability indicating methods. The most common analytical technique for monitoring forced degradation experiments is HPLC with UV and/or MS detection for peak purity, mass balance, and identification of degradation products but these HPLC-based methodologies are time-consuming and provide only medium resolution to ensure that all of the degradation products are accurately detected. PDA/MS (photodiode array and MS) used along with UPLC, which allows for faster and higher peak capacity separations of complex degradation product profiles[15].

UPLC separations with the high-speed scan rates of UPLC-specific photodiode array and MS detection will give confidence for identifying degradation products and thus shortening the time required to develop stability-indicating methods[15].

UPLC used in Impurity Profiling

Impurity profiling is an important task for the drug development and formulation process, which helps in detecting, profiling and quantifying drug substances and their impurities in raw materials and final product. As impurity profiling requires high-resolution chromatography which is capable of reliably and reproducibly separating and detecting all of the known and unknown impurities of the active compound. It is critical assignment to accurately measure low-level impurities at the same time as the higher concentration active pharmaceutical component. This activity, however, can be complicated by the presence of excipients in the sample, often resulting in long HPLC analysis times to achieve sufficient resolution. UPLC system and columns specifically gives high-throughput analysis along with high peak resolution. UPLC PDA detector involves two analytical flow cells with maximum flexibility and according to application requirements, as one for maximum chromatographic resolution and a second for high sensitivity. UPLC also ensure the latest peak detection algorithms and custom calculations to optimize data processing and reporting. It also assertively detects impurities in compounds even at trace levels. To characterize impurities, it is often necessary to perform several analytical runs to obtain the necessary MS and MS/MS data. UPLC combines with exact MS, which operate with alternating low and high collision energies, known as UPLC/MS/MS, which has been successfully employed for the identification of drug and endogenous metabolites. The rapid switching of the collision cell energy produces both precursor and product ions of all of the analytes in the sample while maintaining a sufficient number of data points across the peak for reliable quantification.

The sensitivity and flexibility of exact time-of-flight mass spectrometry with alternating collision cell energies, combined with the high resolving power of the UPLC system, allows for the rapid profiling and identification of impurities[16].

UPLC used in Dissolution Testing

Dissolution testing is essential in the formulation and development along with production process in association with quality control. In sustained-release and metered release dosage formulations, testing of higher potent drugs is particularly important where dissolution can be the rate-limiting step in medicine delivery. The dissolution profile is used to demonstrate reliability and batch-to-batch uniformity of the active ingredient and dosage form. Additionally, newer and more potent formulations require increased analytical sensitivity. UPLC provides precise and reliable automated online sample acquisition. It automates dissolution testing, from production to test start, a through data acquisition and analysis of sample aliquots is required for the management of test result in publication and distribution.[16]

Advantages of UPLC

UPLC allows faster laboratory method development with maintaining resolution, selectivity, sensitivity, and dynamic range of LC analysis. As it reduces process cycle times, so that more product can be analyzed with existing resources. It assures quality results with decrease in run time, cost and increases sensitivity of sample analysis along with less solvent consumption. UPLC Increases sample throughput through the use of a novel separation material of very fine particle size and enables manufacturers to produce more material that consistently meet or exceeds the product specifications, potentially eliminating variability, failed batches, or the need to re-work material. It's fast resolving power quickly quantifies related and unrelated compounds.[16, 17]

Disadvantages of UPLC

The performance of UPLC may be similar or even higher that have been demonstrated by using stationary phases of size around 2 µm without the adverse effects of high pressure. The phases of less than 2 µm are generally non-re-generable and thus have limited use as increased pressure it requires more maintenance and reduces the life of the columns[17].

UPLC over HPLC

HPLC and UPLC are both Liquid Chromatography used in separating the components of a compound. To know the difference between HPLC and UPLC, we first need to know what HPLC is. This is because UPLC is a special version of HPLC and can be easily understood if we know what HPLC is. For starters, HPLC stands for High Performance Liquid Chromatography is a technique used to separate different constituents of a compound. It is the most widely used technique to identify, quantify and separate components of a mixture. It uses high pressure to

push solvents through the column. HPLC is widely used in biochemistry for the analysis of constituents of a compound. It is an ideal way for separation and identification of amino acids, nucleic acids, proteins, hydrocarbons, pesticides, carbohydrates, antibiotics, steroids and countless other inorganic substances. HPLC has many advantages over previously used liquid chromatography methods. It allows for higher resolution and speed of analysis, HPLC columns can be reused without repacking or regeneration, better control of parameters affecting efficiency of separation, easy automation of instrument operation and data analysis, and adaptability to large scale procedures. This is also because it supports very small particle sizes and large surface areas and allows for application of high pressure for solvent to flow. In UPLC, which is nothing but ultra high performance liquid chromatography, particle sizes less than 2um can be used. This allows for better separation that particle size of 5um that are used in HPLC. It also allows for a very fast analysis. Though, UPLC is a trademark technology of Waters Corporation, people use it as a general term for a separate technique. Where as the pump pressure in HPLC is 40MPa, in UPLC, this pressure can go up to 100MPa, which is what makes this technique so very exciting and efficient[18].

ADVANCES IN UPLC

(1) PATROL™ UPLC Process Analyzer

Liquid chromatography is a major analytical technique in every QC lab but it is not a major tool for on-line/at-line analysis. As it is too slow process because it requires Long run times along with not rugged enough for continuous operation. It requires Excessive down time and expensive to operation and highly skilled technicians. PATROL™ UPLC process analyzer have “real-time” speed analysis in less than 5 minutes along with user friendly and doesn’t typically require extensive sample handling or sample preparation as during in-process liquid samples which is fully automated, results in multiple chemically-related and unrelated components resolved in a single analysis with broad linear dynamic range with excellent selectivity and sensitivity So as to overcome the problem associated with on time analysis, PATROL™ UPLC process analyzer had been introduced[19].

Applications of PATROL™ UPLC analyzer

PATROL™ UPLC analyzer automatically performs sample dilution as operator introduces sample to this system, during at-line analysis using full barcode level of automation. It analyzes reaction progress and measures concentration of starting material, intermediate, impurities, final product and reaction stopped by analyzer when residual starting material achieved target or

impurities reach a threshold value. Nano ACQUITY UPLC. It gives the most information from very small amounts of highly complex samples and ideal for proteomics and biomarker discovery. It is designed with nano-scale capillary and narrow-bore separation which allows direct nano-flow to attain the highest chromatographic resolution, sensitivity, and reproducibility. It gives 0.25 minute standard deviation run-to-run reproducibility, even over long gradients which increase reliability and ease of use through holistic design, intuitive diagnostics and customized nano-scale fittings with columns. It incorporates the highest level of separation performance with the benefits of simplicity and ease of use. It allows high sequence coverage and dynamic range for analysis of complex proteomic mixtures and protein identification with nano UPLC column and TRIZAIC nano Tile. It significantly increases sensitivity over HPLC and also used for metabolic profiling studies. Nano ACQUITY UPLC provides increased chromatographic performance, resulting in sharper peaks and greater resolution, the increased resolution helps for the shorter analysis time and increased sample sensitivity[20].

(2)UPLC method development and validation for Cefditoren Pivoxil in active pharmaceutical ingredient

The present UPLC method was developed for determination of percentage drug purity of Cefditoren Pivoxil in API using Acetonitrile and Ammonium Acetate buffer (pH 6.7) and Kromacil column C18 (50 × 2.1mm, 3.5µ) as a stationary phase at a flow rate of 0.25 ml/min. Five methods were taken for development and Method 2 was found to be optimized for the determination of percentage purity. The method was validated using system suitability, precision, linearity, robustness, forced degradation and solution stability studies. The proposed method was found to have a good resolution, fast speed and less consumption of solvent as per the standard procedures. The present UPLC method was developed for determination of percentage drug purity of Cefditoren Pivoxil in API using Acetonitrile and Ammonium Acetate buffer (pH 6.7) and Kromacil column C18 (50 × 2.1mm, 3.5µ) as a stationary phase at a flow rate of 0.25 ml/min. Five methods were taken for development and Method 2 was found to be optimized for the determination of percentage purity. The method was validated using system suitability, precision, linearity, robustness, forced degradation and solution stability studies. The proposed method was found to have a good resolution, fast speed and less consumption of solvent as per the standard procedures[21].

(2) Peptide Mapping Using UPLC-UV Method Development Techniques

Peptide mapping or peptide finger printing produced is characteristic for a particular protein and the UPLC technique can be used to separate a mixture of peptides. Normally, recombinant proteins are developed for therapeutic purposes. Peptide mapping is used to confirm the primary structure of a protein, identify post-translational modification, to demonstrate generic stability and analyze potential impurities. Any difference in the structure of a protein should be reflected in a change in retention time for the peptide containing the modification. The relative amounts of the peptide with and without a particular modification are used to measure the fraction of the protein in the particular sample that carries that modification. Changes in area proportions correspond to the fraction of the protein molecules in the sample having a particular modification. Using a UPLC technique, peptide analysis has been shown to give consistent chromatographic separations and reproducible quantitation for peptide mapping in combination with UV, MS or MS/MS detection. In the initial characterization of a protein, it's important to develop a peptide mapping method that resolves modified peptides from native peptides so that all possible modifications may be detected. As development of the biopharmaceutical advances, these peptides must be quantitated. Quantitation is generally expressed as an area or height percent of the native peptides. In this way, the peptide map can provide information on the mixture of protein forms in each sample so that safety and efficacy of the preparation may be assured. The method must, therefore, exhibit excellent sensitivity and linearity for quantitative work. The strategy of peptide quantification includes adding a known amount of a specific peptide (peptide standards) to an actual protein digest to test estimates of quantification. This peptide serves as a surrogate, illustrating the behavior of modified peptides in the digest[22].

(4) Development of a New RP-UPLC Method for the determination of Rabeprazole Sodium in Pharmaceutical Formulation and Application in dissolution Studies

The development of reverse phase ultra-performance liquid chromatographic (RP-UPLC) method for the estimation of Rabeprazole sodium in tablet formulations. Chromatographic separation was achieved on a Waters Acquity BEH C18 (50 x 2.1 mm, particle size 1.7 µm) column using an isocratic method with mobile phase composed of Acetonitrile and phosphate buffer (pH 7.4) in the ratio of 35:65 (v/v). The flow rate was 0.4 ml/min, temperature of the column was maintained at ambient, injection volume was 5 µL and detection was made at 280 nm. The run time was as short as 2 min. Comparison of system performance with conventional HPLC was made with respect to analysis time, efficiency and sensitivity. The developed method was linear for rabeprazole sodium from 0.03 - 30 µg/ml and the linear regression obtained was > 0.999. Precision, evaluated by intra- and inter-day assay, had relative standard deviation (R.S.D)

values within 1.5 %. Recovery data were in the range 98.0 - 101.5 % with R.S.D. values < 1.5 %. The method is precise, accurate, linear, robust and fast. The short retention time of 1.49 min allows the analysis of a large number of samples in a short period of time and, therefore, should be cost-effective for routine analysis in the pharmaceutical industry[23].

(5) Faster Analysis of Radio Pharmaceuticals using UPLC™ in Combination with Low Volume Radio Flow Cell

This application note investigates whether the combination of an UPLC™ with a radioactivity detector would result in faster analyses of PET radiopharmaceuticals with a higher degree of sensitivity and efficiency. 6-fold faster analyses times with much lower backgrounds were achieved using the combination of UPLC™ and Berthold Flow Star radioactivity detector[23].

(6) Improving LC–MS sensitivity through increases in chromatographic performance: Comparisons of UPLC–ES/MS/MS to HPLC–ES/MS/MS

Recent technological advances have made available reverse phase chromatographic media with a 1.7 µm particle size along with a liquid handling system that can operate such columns at much higher pressures. This technology, termed ultra performance liquid chromatography (UPLC), offers significant theoretical advantages in resolution, speed, and sensitivity for analytical determinations, particularly when coupled with mass spectrometers capable of high-speed acquisitions. This paper explores the differences in LC–MS performance by conducting a side-by-side comparison of UPLC for several methods previously optimized for HPLC-based separation and quantification of multiple analytes with maximum throughput. In general, UPLC produced significant improvements in method sensitivity, speed, and resolution. Sensitivity increases with UPLC, which were found to be analyte-dependent, were as large as 10-fold and improvements in method speed were as large as 5-fold under conditions of comparable peak separations. Improvements in chromatographic resolution with UPLC were apparent from generally narrower peak widths and from a separation of diastereomers not possible using HPLC. Overall, the improvements in LC–MS method sensitivity, speed, and resolution provided by UPLC show that further advances can be made in analytical methodology to add significant value to hypothesis-driven research[23].

(7) UPLC-MS Method for the Long-Term Metabolomic Study of Human Serum

A method for performing untargeted metabolomic analysis of human serum has been developed based on protein precipitation followed by Ultra Performance Liquid Chromatography and Time-of-Flight mass spectrometry (UPLC-TOF-MS). This method was specifically designed to fulfill the requirements of a long-term metabolomic study, spanning more than 3 years, and it was

subsequently thoroughly evaluated for robustness and repeatability. We describe here the observed drift in instrumental performance over time and its improvement with adjustment of the length of analytical block. The optimal setup for our purpose was further validated against a set of serum samples from 30 healthy individuals. We also assessed the reproducibility of chromatographic columns with the same chemistry of stationary phase from the same manufacturer but from different production batches. The results have allowed the authors to prepare SOPs for "fit for purpose" long-term UPLC-MS metabolomic studies, such as are being employed in the HUSERMET project. This method allows the acquisition of data and subsequent comparison of data collected across many months or years. The data presented here have demonstrated the complexity of analytical operations performed in metabolomic investigations, specifically sample analysis of serum by UPLC-MS. A range of sources of variation were observed and it is imperative to minimize these variations consistently. We have shown here that, with appropriate control methods in place, UPLC-MS has the potential to serve the required role of a hypothesis-generating holistic acquisition of data related to human serum for thousands of metabolite-related ions. These control measures employ biologically identical quality control (QC) samples which are analyzed periodically throughout the analytical block to assess repeatability[24].

(8) UPLC-MS-based Metabolite Analysis in Tomato

Recent advancements in the performance of hyphenated technologies based on ultra-pressure chromatography and high-sensitivity mass spectrometry have set the stage for a myriad of metabolomics studies in plants and other organisms. In this chapter, we describe the use of a UPLC (Ultra Performance Liquid Chromatography)-qTOF (quadruple time-of-flight) system for profiling semi-polar metabolites in the model fruit plant tomato. Optimized extraction method, instrument parameters and data treatment procedures are provided. The value of UPLC instruments that use small particle size chromatographic columns in terms of resolution, separation and short injection times are presented. When coupled to a TOF mass spectrometer, with high resolution and mass accuracy, good dynamic range and a fast spectral acquisition capacity the system is most suitable, for extensive profiling of hundreds of plant metabolites[24].

(9) Development of a New UPLC®/Ms Method for Systematic Toxicological Analysis

A method has previously been described for the systematic toxicological analysis (STA) of biological specimens. This method comprised a 26 minute HPLC separation, in combination with the collection of full scan mass spectral data and has been successfully applied for the analysis of routine samples in laboratories worldwide over the last 5 years. Since this method

was first described, there have been some significant advances in the available technology. In 2004, the revolutionary separation technique, UPLC® was introduced. We now present our latest STA method. This technique exploits the rapid separation afforded by UPLC combined with the ultra-fast scanning capabilities of the Waters TQ Detector, providing a comprehensive analysis in only 15 minutes – a time-saving of 40 %[24].

(10) A Validated Stability-indicating Reverse Phase HPLC assay method for the determination of Memantine Hydrochloride drug Substance with UV-Detection Using Pre-column derivatization Technique.

This present paper deals with the development and validation of a stability indicating high performance liquid chromatographic method for the quantitative determination of Memantine hydrochloride. Memantine hydrochloride was derivatized with 0.015 M 9-fluorenylmethyl chloroformate (FMOC) and 0.5 M borate buffer solution by keeping it at room temperature for about 20 minutes and the chromatographic separation achieved by injecting 10 µL of the derivatized mixture into a Waters HPLC system with photodiode array detector using a kromasil C18 column (150 × 4.6 mm), 5 µ. The mobile phase consisting of 80% acetonitrile and 20% phosphate buffer solution and a flow rate of 2 milliliter/minute. The Memantine was eluted at approximately 7.5 minutes. The volume of FMOC used in derivatization, concentration of FMOC and derivatization time was optimized and used. Forced degradation studies were performed on bulk sample of Memantine hydrochloride using acid (5.0 Normal (N) hydrochloric acid), base (1.0 N sodium hydroxide), oxidation (30% hydrogen peroxide), thermal (105°C), photolytic and humidity conditions. The developed LC method was validated with respect to specificity, precision (% RSD about 0.70%), linearity (linearity of range about 70–130 µg/mL), ruggedness (Overall % RSD about 0.35%), stability in analytical solution (Cumulative % RSD about 0.11% after 1450 min.) and robustness[25].

(11) Development of a RP-UPLC method for the simultaneous analysis of Secnidazole, Fluconazole, and Azithromycin: Application in pharmaceuticals and human serum.

A novel approach was carried out to develop and validate a rapid and selective analytical method by using reverse Phase Ultra Performance Liquid Chromatographic (RP-UPLC) technique for the simultaneous separation and analysis of secnidazole, fluconazole, and azithromycin in raw materials, their pharmaceutical dosage forms and human serum. The developed analytical UPLC method is superior in technology to conventional HPLC with respect to speed, resolution, solvent consumption and cost of analysis. Elution time for the separation was 10 min in reverse phase mode and ultra violet detection was carried out at 210 nm. Efficient separation was achieved on

BEH C18 sub-2-µm UPLC column using 0.002M Na2HPO4 and Acetonitrile as organic solvent in a gradient program. Benzophenone was used as internal standard. Resolutions between secnidazole, fluconazole, and azithromycin were found to be more than 4.5. The active pharmaceutical ingredient was extracted from tablets using methanol and Acetonitrile (50:50 v/v) as diluent. The calibration graphs were linear for secnidazole, fluconazole, benzophenone and azithromycin. The method showed excellent recoveries for all drugs in bulk and formulated products. The test solution was found to be stable in diluent for 72 h when stored in the refrigerator between 2 to 8 oC. The developed UPLC method was validated that meets the requirements laid down by the International Conference on Harmonization (ICH) guidelines with respect to linearity, accuracy, precision, specificity and robustness, and has been successfully applied to pharmaceutical formulations because no chromatographic interferences from the tablet excipients are found. To the best of our knowledge, a validated reverse phase analytical method for the simultaneous separation and quantification of secnidazole, fluconazole, and azithromycin by using UPLC technique disclosed in this investigation was not published elsewhere[26].

(12) Ultra-performance liquid chromatography coupled to mass spectrometry as a sensitive and powerful technology for metabolomic studies

Metabolomics is the comprehensive assessment of endogenous metabolites of a biological system. These large-scale analyses of metabolites are intimately bound to advancements in ultra-performance liquid chromatography–electro spray (UPLC) technologies and have emerged in parallel with the development of novel mass analyzers and hyphenated techniques. Recently, the combination of UPLC with MS covers a number of polar metabolites, thus enlarging the number of detected analytes in the widely used separation sciences. This technology has rapidly been accepted by the analytical community and is being gradually applied to various fields such as metabolomics and traditional Chinese medicine (TCM). Given the power of the technology, metabolomics has become increasingly popular in drug development, molecular medicine, traditional medicine and other biotechnology fields, since it profiles directly the phenotype and changes thereof in contrast to other “-omics” technologies. Hyphenated UPLC/MS technique is becoming a useful tool in the study of body fluids, represents a promising hyphenated micro separation platform in metabolomics and has a strong potential to contribute to disease diagnosis. This review describes the applications of UPLC/MS in metabolomic research, and comparison role of HPLC/MS, NMR and GC/MS, highlights its advantages and limitations with certain characteristic examples in the life and TCM sciences[27].

(13) Ultra Performance Liquid Chromatography−Atmospheric Pressure Photo-ionization-Tandem Mass Spectrometry for High-Sensitivity and High-Throughput Analysis of U.S. Environmental Protection Agency 16 Priority Pollutants Poly nuclear Aromatic Hydrocarbons

In this work, we demonstrate the utility of ultra performance liquid chromatography−atmospheric pressure photo-ionization-tandem mass spectrometry (UPLC−APPI-MS/MS) for high-sensitivity and high-throughput analysis of United States Environmental Protection Agency (U.S. EPA) 16 priority pollutants polycyclic aromatic hydrocarbons (PAHs). Analyses were performed on a Waters Acquity-TQD equipped with Syagen's PhotoMate APPI source. All 16 PAHs were analyzed on column in approximately 3.5 min with excellent chromatographic separation for all PAH isomers and with low picogram detection limits on column for all analytes using chlorobenzene as a dopant. Dynamic linear ranges were evaluated and found to cover at least 3−4 orders of magnitude. In comparison with the existing U.S. EPA methods, this approach improves instrument sample throughput by at least 10-fold[28].

(14) Studies of UPLC fingerprint for the identification of Magnoliae officinalis cortex processed

This study was carried out with the objective of establishing Ultra Performance Liquid Chromatograph (UPLC) fingerprint for the identification of *Magnoliae officinalis* cortex processed. It was extracted by methanol using an ultrasonic extractor. Twelve samples of *M. officinalis* cortex produced in Zhejiang of China from different places and species were processed with ginger juice; sample solutions were determined by Waters UPLC equipped with BEH C_{18} column and a DAD detector, gradient eluted with formic acid/methanol-formic acid/water as mobile phase. The flow rate was set at 0.3 ml$^{\bullet}$ min^{-1}, while the column temperature was set at 30°C, and the wavelength for detection was set at 240 nm. The characteristic of the common peaks of the UPLC fingerprint for *M. officinalis* cortex processed are obvious. Forty-one common peaks were detected and two of them were identified. The method of UPLC fingerprint established in this experiment was rapid and efficient. It is an effective means for the quality control of *M. officinalis* cortex processed[28].

(15) Development and validation of a UPLC method for the determination of duloxetine hydrochloride residues on pharmaceutical manufacturing equipment surfaces

In pharmaceutical industries, it is very important to remove drug residues from the equipment and areas used. The cleaning procedure must be validated, so special attention must be devoted

to the methods used for analysis of trace amounts of drugs. A rapid, sensitive, and specific reverse phase ultra-performance liquid chromatographic (UPLC) method was developed for the quantitative determination of duloxetine in cleaning validation swab samples. The method was validated using an Acquity UPLC™ HSS T3 (100 × 2.1 mm ^{2}) 1.8 µm column with a isocratic mobile phase containing a mixture of 0.01 M potassium dihydrogen orthophosphate, pH adjusted to 3.0 with orthophosphoric acid and Acetonitrile (60:40 v/v). The flow rate of the mobile phase was 0.4 ml/min with a column temperature of 40°C and detection wavelength at 230 nm. Cotton swabs, moisten with extraction solution (90% methanol and 10% water), were used to remove any residue of drug from stainless steel, glass and silica surfaces, and give recoveries >80% at four concentration levels. The precision of the results, reported as the relative standard deviation, were below 1.5%. The calibration curve was linear over a concentration range from 0.02 to 5.0 µg/ml with a correlation coefficient of 0.999. The detection limit and quantitation limit were 0.006 and 0.02 µg/ml, respectively. The method was validated over a concentration range of 0.05-5.0 µg/ml. The developed method was validated with respect to specificity, linearity, limit of detection and quantification, accuracy, precision, and robustness[29].

(16) Development and validation of an ultra performance liquid chromatography method for venlafaxine hydrochloride in bulk and capsule dosage form

A simple, specific, accurate, and precise ultra performance liquid chromatographic method was developed and validated for the estimation of venlafaxine hydrochloride in tablet dosage forms. A acquity TM BEH column having C18, 100X2.1 mm i.d. in isocratic mode, with mobile phase containing di-potassium hydrogen phosphate: Acetonitrile (30:70 v/v; pH 7.00 with dilute *o*-phosphoric acid) was used. The flow rate was 0.75 ml/min and effluents were monitored at 227 nm. The retention time of venlafaxine hydrochloride was 0.548 min. The method was validated for specificity, linearity, accuracy, precision, limit of quantification, limit of detection, robustness and solution stability. Limit of detection and limit of quantification for estimation of venlafaxine hydrochloride were found to be 6.11 µg/ml and 20.33 µg/ml, respectively. Recoveries of venlafaxine hydrochloride in tablet formulations were found to be in the range of 99.3-99.5%. Proposed method was successfully applied for the quantitative determination of venlafaxine hydrochloride in tablet dosage forms[30].

(17) Development and validation of a RP-ultra performance liquid chromatographic method for quantification of topotecan hydrochloride in bulk and injection dosage form

A simple, very fast, precise and accurate reverse phase ultra performance liquid chromatographic method was developed for the determination and validation of topotecan hydrochloride in bulk

and injection dosage form. A Waters BEH C18, 50X2.1 mm, 1.7 µm particle size column in gradient mode was used with mobile phase comprising of 0.1% v/v orthophosphoric acid in water and Acetonitrile. The analytical column was thermostated at 50° and flow rate was set at 0.4 ml per min, with photo diode array detection at 260 nm. The retention time of topotecan was found 1.38 min. The method was validated in terms of linearity, accuracy, precision and specificity. The calibration curve was found linear between 20 to 60 µg/ml. The limit of detection and limit of quantification were found 0.2353 and 0.7131 µg/ml, respectively. Percentage recoveries were obtained in the range of 98.91% and 99.17%. The proposed method is precise, accurate, selective and reproducible. The ultra performance liquid chromatographic assay procedure, which proved superior because of its greater sensitivity and relatively shorter (4 min) run time, should be an important tool for speedy future analysis of topotecan hydrochloride in bulk and its injection dosage form. It has been revealed that UPLC has great potential to be widely employed for impurity/degradation profiling applications, as well as other development applications. As UPLC increases productivity in both chemistry and instrumentation by providing more information per unit of work as it gives increased resolution, speed, and sensitivity for liquid chromatography. When researcher experience separation barriers with conventional HPLC, UPLC extends the utility of chromatography. The main advantage of UPLC is a reduction of analysis time, along with reduced solvent consumption, high throughput analysis and reduction in cost of analysis. Overall, it appears that UPLC can put forward significant improvements in speed, sensitivity and resolution compared with conventional HPLC[31].

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