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UHPLC in the Analysis of Drug Components

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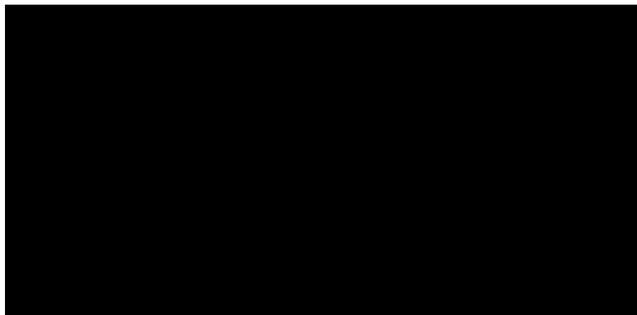
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2013

UHPLC in the Analysis of Drug Components



Submitted By

*RAMA CHANDRA SEKHAR REDDY
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Under the guidance of

Dr. HENNE WALTER

UHPLC in the Analysis of Drug Components

Dissertation submitted to

Governor State University

in partial fulfilment of the requirements for the award of the degree of

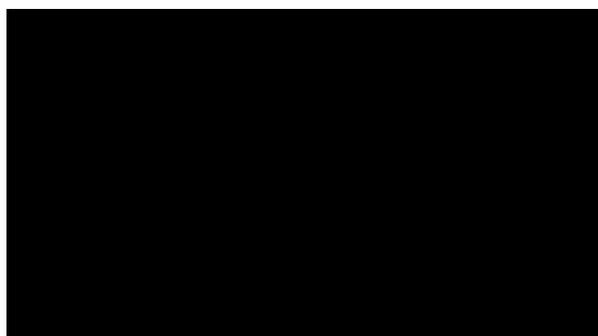
MASTERS in ANALYTICAL CHEMISTRY

Submitted By

RAMA CHANDRA SEKHAR REDDY PULAGAM

Under the guidance of

Dr. HENNE WALTER



MAY 2013

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Abstract

The main objective of this article is to highlight the new trends that have emerged in the enhancement of the HPLC which have given rise to the use of UHPLC. This innovative tool is much more reliable and effective as compared to HPLC, in the investigation of many chemical compounds. In this article, special insight is given on the changes that have been made with regards to both instruments. In this article focus is also placed on the implementation of UPLC in various fields of interest, in order to enhance the processes for which it is utilized. The pros and cons of the utilization of this method are also addressed in this article, along with other relevant characteristics of the same.

Introduction

Ultra Performance Liquid Chromatography, or UPLC for short, have been investigated and demonstrated high relevance and efficiency in the areas of speed and sensitivity of the analysis, as well as in the area of chromatographic resolution. According to **Swartz et al. (2004)**, one of its main characteristics lies in the fact that it utilizes very small particles and decreases the quantity of solvent required for processing. UPLC is a revolutionary tool in the industry that finds its origin in High Performance Liquid Chromatography (HPLC). **Van Deemter et al. (1956)** highlights the fact that the main modification made in the development of UPLC lies in the size of the materials or particles used during the process of separation.

The main idea of HPLC is based on the fact that the efficiency and resolution are enhanced with a reduction in the size of the particulate matter utilized. There is a remarkable increase in efficiency as the size of particles fall below 2.5 micrometres, and unlike the deductions made from the conventional Van Deemter equation, the process is not hampered by the increase in flow rates or in the linear velocity of the mobile phase. (**Van Deemter et al, 1956**) The overall analysis shows that the use of peak intensity, velocity and particles of smaller dimensions can all lead to the achievement of new and improved results. This concept is exactly what defines the principle behind the development of Ultra Performance Liquid Chromatography. **Zhou et al (2005)** points to the fact that the conventional procedure of High Performance Liquid Chromatography (HPLC) is recognized for possessing many beneficial characteristics that include user friendliness, strength, modifiable sensitivity and adequate discrimination.

On the other hand, one of the major backdrops of this procedure is the low efficacy rate in comparison to similar procedures such as capillary electrophoresis or gas chromatography. The main reason behind this backdrop is due to the physical state of the particles; that is, the particles used in HPLC are found in a liquid state and so the coefficients of diffusion are relatively lower in comparison to the other procedures. As proposed by **(Van Deemter et al., 1956)**, the equation illustrates that a reduction of particle size enhances the efficiency. Nevertheless, this process occurs at the expense of a build-up of retrograde pressure which contradicts the pressure limits of the HPLC instruments of 400 bars. As a result, the contradictions arising due to the pressure increase and efficiency have been otherwise resolved with the use of shorter columns and particles of approximately 2 micrometres in size. This solution has been adopted to counterbalance the low efficiency rates at a reasonable load per analysis during the use of the HPLC system.

The efficiency of the HPLC system can be further enhanced by increasing the temperature of the system and by the utilization of larger columns.

Use of the UPLC system

(Gerber et al., 2004) concurs with the ideas of the use of temperature and size of the columns, as ways to increase the efficiency of the HPLC system. An increase in temperature would increase the kinetic energy of molecules and allow them to move further away from each other, thereby decreasing the viscosity of the column particles and increasing the linear velocity of the mobile phase. In addition, the use of larger (Monolithic) columns, with the inclusion of a matrix that supports and allows easy flow of particles, can significantly reduce the build-up of resistance within the column, thereby leading to an overall reduction in the retrograde pressure build-up.

Principle

The main principle involved in the utilization of Ultra Performance Liquid Chromatography is that of the addition of smaller particles of less than 2 micrometres in a stationary phase, as contrasted with the use of larger particles in the High Performance Liquid Chromatography. According to (Swartz et al., 2005), the main idea that regulates this modification is based upon the equation proposed by Van Deemter. In this equation, the association between the parameters of linear velocity and plate height are closely analysed. The graphical representation of this equation is guided by a total of three parameters found in the equation. The main principle of the equation is that the linear velocity improves more significantly with the utilization of smaller-sized particles as compared to larger ones.

The equation can be represented as follows:

$$H=A+B/v+Cv..... (1)$$

In this equation, the constants are represented by the letters A , B and C . v represents the linear velocity. The constant A is not influenced by velocity and it signifies the “eddy” mixing of particles. The value of constant A decreases, with a reduction in particle sized and an increase in uniformity. The second constant B corresponds to the normal trend of molecules to diffuse. This is also referred to as the axial diffusion and it is closely related to the liner velocity in that its level of significance and influence is inversely proportional to the flow rate. The constant C is used to depict the resistance associated with the process of separation. This particular type of resistance is referred to as kinetic resistance, and it is formally defined as the delay that occurs in the alternating movements between the gaseous state and the stationary phase. The relationship is such that an increase in gas flow would lead to an increase the lag time of the molecules in the stationary phase relative to those in the mobile phase. As a result of this analogy, it is evident that the constant C shares a directly proportional relationship with the flow rate v . (Wu et al., 2001)

Taking into consideration the aforementioned, it is clearly visible that the efficacy in processing the materials can be significantly enhanced, together with the agility with which the analysis can be performed, without hampering the overall progress of chromatography.

The development and launching of UPLC has sparked the need for the generation of novel equipment for carrying out liquid chromatography. This equipment would exploit the benefits associated with the achievement of the separation process at significantly higher pressure levels in comparison to that which is used in the HPLC. With this in mind, it is clear that there is a directly proportional relationship between the efficiency and the length of the column used, while the opposite is true about the size of the particles used; that is, the smaller the particles, the greater is the efficiency of the system. (Swartz M. E., 2005).

In the final analysis, it is feasible to decrease both the length of the column and the size of the particles used, while maintaining adequate resolution as required. Wu et al. (2001) achieved significant results in the implementation of UPLC for the discovery of metabolites of drugs that were otherwise difficult to trace. In their study, the authors recorded significant success with regards to the resolution of results and the method of separation.

Sample injection



Fig1. Sample injection system (Swartz M. E., 2005)

Swartz (2005) highlights the importance of the manner in which the sample for analysis with UPLC is injected. One of the backdrops of the commonly used injection valves (**see fig.1**) is the lack of special design and structure to withstand the action of rising pressures within the system. In order to guarantee that the column is carefully guarded against the extreme changes in pressure, the process of introducing the samples must be carried out in a manner so as to prevent the rapid injection of large quantities of samples, as well as the volume that is moved by the system should be kept at a minimum level, so as to avoid the spreading of bands.

According to **Swartz (2005)**, in order to adequately exploit the benefits of a speedy analysis with UPLC, it is essential to consider a rapid injection run time. This overall requirement signifies that a greater sample capacity is also needed. The sensitivity of the system can be further enhanced by administering small quantities of sample with very little leftover particles. In the case of organic sample, the same can be directly introduced into the system.

UPLC Columns



Fig2. UPLC columns (Swartz M. E., 2005).

Swartz (2005) also supports the concept that an increase in the efficiency of the UPLC system, in which a column supporting packed particles of 1.7 micrometres in size, results in the enhancement of the overall resolution. A bonded stage is essential for the disintegration of the sample. The separation of samples via UPLC can be done with the utilization of the phases such as (see **fig.3**), ACQUITY UPLC BEH Shield RP18: in this case a polar group column is installed. ACQUITY UPLCTM BEH C18 and C8: this consist of columns comprising of straight chain alkyl groups ACQUITY UPLC BEH Phenyl: in this phase, the phenyl group is bonded to the silyl group with the aid of a C6 alkyl group.

As can be clearly observed, the different columns provide a distinct mixture of bonding and reaction with the compounds being analyzed. The most commonly known and widely utilized columns are the ACQUITY UPLC BEH C18 and C8 types. These columns are frequently used in UPLC separation analysis because of their broad pH spectrum. They are characterized by possessing multiple ligand attachment properties that give rise to lower chemical stability in more acidic environments. The combination of this characteristic with the higher stability of the BEH particle measuring 1.7 micrometres provides the broad spectrum of pH which facilitates coverage for the analysis and separation of the sample.

On the other hand, ACQUITY UPLC BEH Shield RP18 columns are made with the objective of working hand in hand with the ACQUITY UPLC BEH C18 and C8 phases. With regards to the ACQUITY UPLC BEH Phenyl columns, a trifunctional bond is established between the phenyl ring and the silyl functionality. This ligand ensures that column lifetimes are extensive and that the peak shape remains in optimal conditions. With the formation of a unique structural arrangement on the small particle, the process of selectivity is enhanced.

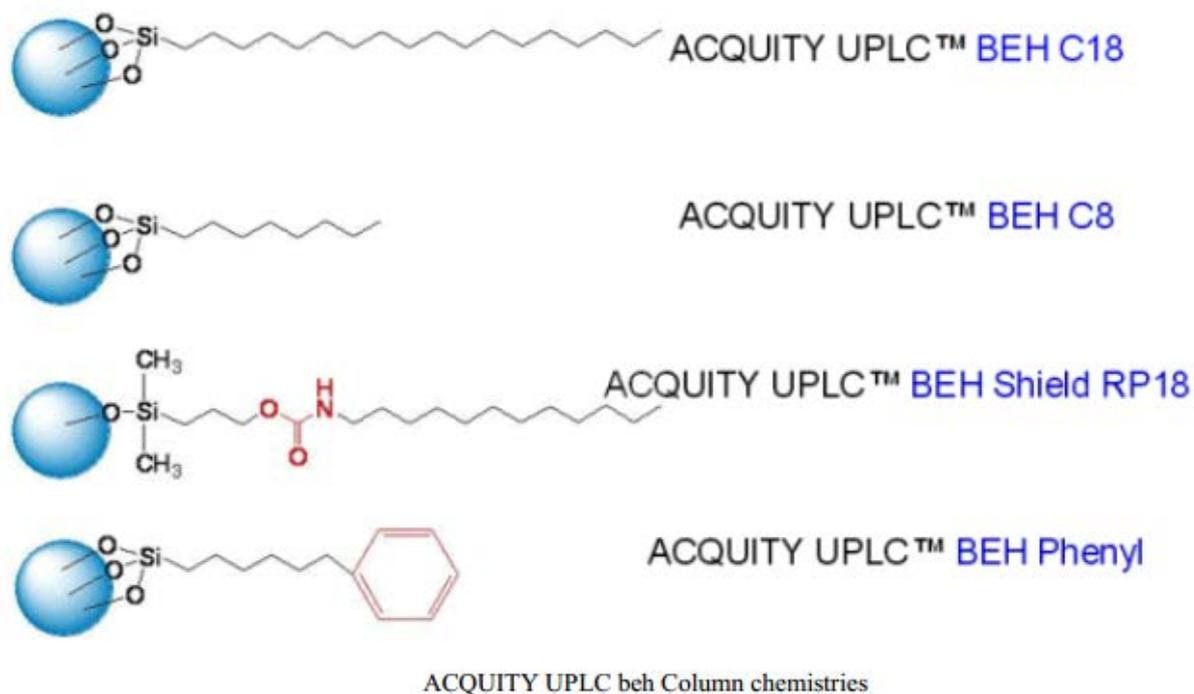


Fig3. ACQUITY UHPLC BEH Column chemistries (Lippert et al., 1997)

The column utilized is designed with an internal diameter of 2.1 mm and to obtain the most optimum level of resolution, a column of 10 cm is required. The same may be shortened to 5 cm in length for a faster separation and better utilization of samples. One of the backdrops of using smaller particles (1.7 micrometres) is the poor capacity of the detector to record smaller peaks with shorter durations (<1 second). This problem can be effectively controlled by modifying the sensitivity levels and sampling rate of the detector. In accordance to the method of detection employed, the sensitivity levels must be much higher when using UPLC as compared to that of separations with HPLC.

The **ACQUITY UHPLC** instrument comprises of two main components with their respective sub-components. These include the following:

Binary solvent manager: driven by two flow serial pumps that aid in the creation of a parallel gradient.

Sample manager: this component includes the column heater, detector, as well as an optional sample organiser.

Within the system, a choice can be made from a selection of four solvents. This is easily facilitated with the use of valves that have been incorporated with the design of the system. The pressure limit reaches approximate values of about 1000 bars and this further enhances the ability to utilize particles below 2 micrometres in diameter. Other novel functions are available in the sample manager. The injection of samples in a pressurized manner permits the maintenance of a low distribution rate and the process is done in an automated fashion with the regulation of special transducers. In addition, a special sampling method (needle-in-needle) is employed with sensors to enhance the quality and precision of the process. The process of carryover is controlled with the use of a washing method. This wash method can vary in accordance with cycle time. Optional plate formats can be fitted into the system. A total of 22 plates can be used to inject samples with the optional sample organizer. The column heater can provide maximum temperatures of 65°C and the same is regulated by the sample manager. The closeness of the source inlet to the column outlet facilitates a reduction in the unwanted spreading of samples. **(Lippert et al., 1997)**. Special detectors, equipped with advanced electronic technology, are utilized for detecting the components of a sample. Given the limitations of the conventional detectors, the introduction of more sophisticated technology provides a solution for proper detection of samples.

In the case of the ACQUITY Tuneable UV/Visible detector, a light guided flow cell similar to an optical fibre, is utilized. This permits the effective transmission of light down the flow

cell. Adequate wiring and routing of the necessary tubing and other connections are established, in order to ensure that the rate of dispersion remains low, as well as to exploit the benefits of leak detectors. (Swartz M. E., 2005)

Difference between HPLC and UPLC assays

	HPLC Assay	UPLC Assay
Column	XTerra C18, 50 × 4.6 mm, 4 μm particles	ACQUITY UPLC BEH C18, , 50 × 2.1mm, 1.7 μm particles
Flow Rate	3.0 mL/min	0.6 mL/min
Needle Wash	Methanol	Strong Needle Wash: 200 μL Methanol; Weak Needle Wash: 600 μL ACN:H ₂ O 10:90
Injection Volume	20 μL	3 μL partial loop fill or 5 μL full loop fill with automatic overfill
Gradient (time in min) (ACN:H ₂ O)	T0 (25:75), T6.5 (25:75), T7.5(95:5), T9 (25:75), T10 (25:75)	T0 (36:64), T1.1 (95:05), T1.3 (36:64)
Flow Rate	3.0 mL/min	0.6 mL/min
Total Run Time	10 min	1.5 min
Total Solvent Consumption (including 0.5 min of delay time in between injections)	Acetonitrile:10.5 mL Water: 21.0 mL	Acetonitrile: 0.53 mL Water: 0.66 mL
Plate Count for Cpd A	2000	7500
USP Resolution	3.2	3.4
LOQ	~0.2 μg/mL	0.054 μg/mL
Carry-over	< 0.05% with needle wash	0.01%
Delay Volume	~720 μL	~110 μL

Fig4. Difference between HPLC and UPLC assays (Chestnut S.,Salisbury J.,2007)

Advantages

Reduces run time while augmenting the sensitivity, Guarantees the selectivity, sensitivity, and dynamic spectrum of the analysis of light chromatography. Manages and keeps the resolution performance in check. Widens the range of Multiresidue Methods, The ability of UPLC to rapidly resolve problems including the quantification of power related and unrelated compounds. Relatively quicker analysis using smaller particles, Reduction in cost of operation. Reduction in the use of solvent. Facilitates a reduction in the cycle time with a corresponding increase in productivity. Augments rate of conversion of samples into processed material. This further enhances productivity and guarantees consistency. Ensures that the analysis of samples occur in a real – time fashion in line with the manufacturing of products. Ensures that the final product is delivered with the expected quality. (Chestnut S.,Salisbury J.,2007)

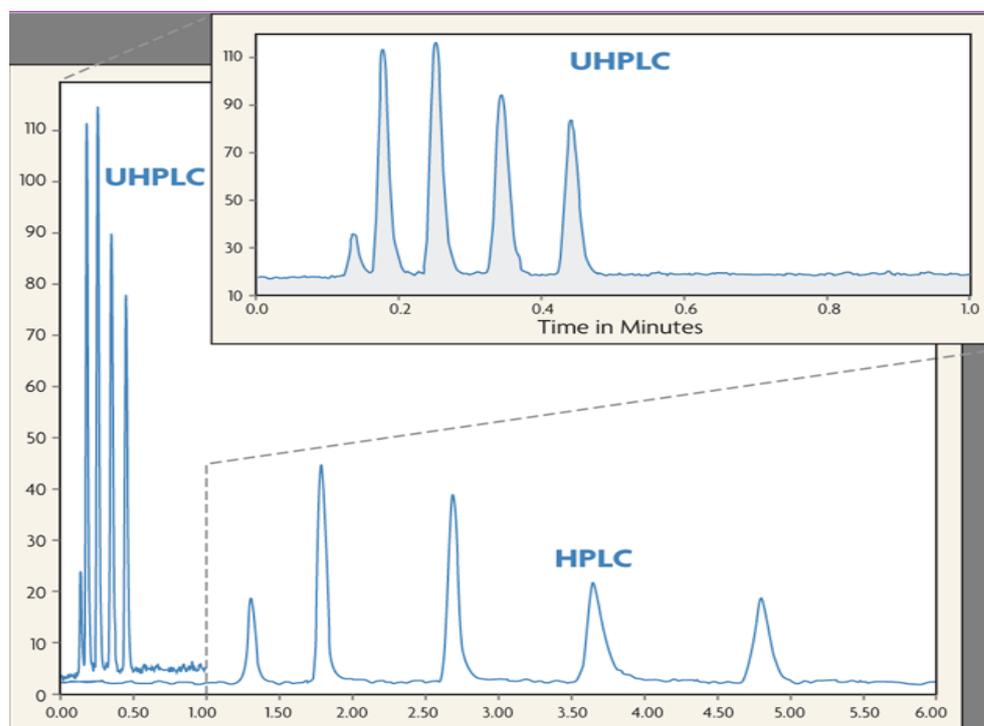


Fig 5. Difference between UHPLC and HPLC run time(Chestnut S.,Salisbury J.,2007)

Disadvantages

The use of higher pressure levels signifies that more attention is needed to ensure that the column remains functional. The columns are also subjected to a shorter life span.

Tubing Slippage



Fig6. Tubing slippage (Chestnut S., Salisbury J., 2007)

Another disadvantage of this system is its ability to succumb to tubing slippage. This is due to the high pressures under which the system operates, associated with pressurized injection, which further stresses the tubing. The fittings are shaped in such a way that the tubing is kept in place in areas of the system that are prone to high pressures. This design strategy can be undesirably opposed with the flow of the fluid throughout the system. The displacement of the tubing can lead to the build-up of dead space and the lowering of the efficiency rate of the system. Due to the gradual movement of tubing, the process may go unnoticed and the detection of the same may be very difficult. This can further lead to the degeneration of the equipment. (Chestnut S.,Salisbury J.,2007)

Tubing ID Compression

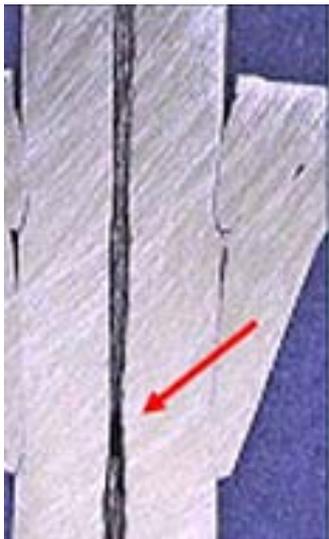


Fig7. ID compression (Chestnut S., Salisbury J., 2007)

In order to reduce the possibility of tubing slippage (see **fig.6**), designers have resorted to the use of metallic parts in substitution of the tubing. Nevertheless, the use of metal also carries certain disadvantages. High pressures tightening torque, needed to ensure that the system is properly sealed, can significantly compress the path and lead to an unwanted reduction in the flow. This can further lead to an increase in retrograde pressures, clogging and turbulence in the flow of liquid. The process is similar to that of tubing slippage in that the diagnostic phase is very difficult.

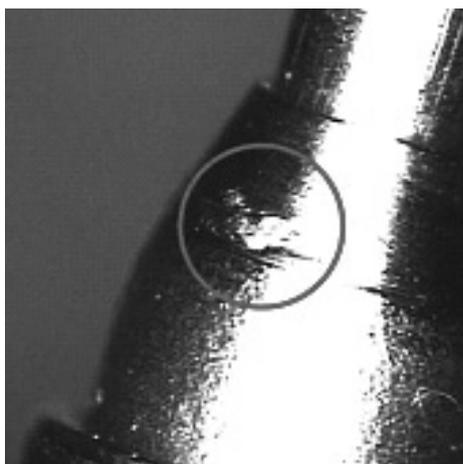


Fig8. Galling (Batts J., 2010)

Apart from the possibility of tubing compression, the torque needed to carefully mount the metal part properly can result in ripping of the material. This process is also referred to as galling and the same may render the receiving port useless. There are instances in which the severely damaged port may seal to other parts of the coupled arrangement and hinder the repair of the same. Damage may also extend to the conical region of the equipment. **(Batts J., 2010)**

Applications of UPLC

Compound Library Maintenance

It is essential to screen libraries containing a wide range of chemical compounds, in order to certify their characteristics and potential for pharmaceutical usage. The certainty of the synthesis of the target compound must be attested. The availability of a complete apparatus is required in the area of testing pharmaceutical ingredients. The same would take into

consideration all of the necessary steps, from the processing of the specimen up to the results, while the more complex analysis would be carried out by other experts. The union of UPLC with special software can facilitate the task of chemists who are seeking to discover or identify certain chemical compounds within a specified timeframe. **(Patil et al., 2011)**

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Laboratories that are dedicated to carrying out analytical studies must seek the optimum efficiency since a high level of results is anticipated and the processes involved are very challenging. As a result of this, special systems, such as the UPLC/MS systems have been developed to ensure that the operations and investigations in the area of medicinal chemistry are properly carried out.

Forced Degradation Studies

The study of the stability of compounds is of vital importance in the pharmaceutical industry, since it determines the effect that such compounds may have on life. The testing of such stability is required before approval of the pharmaceutical compounds can be obtained. This process serves the function of determining beforehand, the way in which drugs would change with time and when they interact with the surroundings. The information obtained from testing stability would further determine particular characteristics such as the conditions under which a product must be stored, as well as the period during which the product can be consumed.

Further testing is also carried out under conditions that simulate more aggressive environments. This process is referred to as stress testing or forced degradation and it is one of the earliest investigations that are carried out during the development of certain pharmaceutical products. The results of such extreme tests aids in the discovery of the

metabolites or bi-products that are derived from reactions and this permits the development of methods directed towards the maintenance of drug stability, should the situation arise. Traditionally, HPLC or other similar techniques have been employed in the monitoring of this process. Nevertheless, these methods carry the disadvantages of narrow resolution spectrum and requiring prolonged periods. The combined utilization of UPLC together with other special techniques, such as photo diode array and MS, facilitates a wider range of results and provides faster and more accurate readings. This would guarantee that all products of degeneration are obtained and that the necessary steps are taken to counteract the same. The end result is the development of a more complete and chemically stable product with greater knowledge of the same. **(Patil et al., 2011)**

Manufacturing / QA / QC

Many factors must be considered when developing new products. In order to ensure that the highest quality of products are attained, special considerations must be taken into account to ensure that the materials used as substrate are pure as possible. This process is known as quality assurance and control and it helps with ensuring that the final products meet the basic stipulated guidelines. The continuation of careful regulation of the stability of products and materials constitutes an essential part of quality assurance and control. UPLC is effectively exploited in the completion of the same. The maintenance of consistency in the production of goods of good standard is necessary for the development of trustworthiness of marketing companies and this provides the opportunity for continued production without constant need for regular auditing. **(Patil et al., 2011)**

Method Development / Validation

Validation refers to the provision of evidence, with a high level of certainty, that a product meets all of the requisite conditions stipulated by the evaluating body. This process is regulated by the Food and Drug Administration. The procedure can be very lengthy given the number of factors to be taken into consideration. The utilization of UPLC reduces the lengthy periods and increases the throughput, among other benefits. The process can also be easily be applied across a greater range of projects involving separation of chemicals. UPLC conditions the speedy creation of methods: this is due to the fact that the time required for sample analysis, using UPLC, is significantly lesser than that which is required for other procedures of separation. As a result, products can be generated and certified at a faster rate.

(Patil et al., 2011)

Certain components of UPLC play a crucial role in provided the requisite data. These components includes: UPLC columns: a broad spectrum of pH and temperatures can be used given the characteristics of the UPLC Calculator: this system is customized to convert from conventional methods to the current ultra-performance method. Column Manager: this component regulates a very vast temperature spectrum and facilitates the utilization of conventional procedures (HPLC) prior to the use of UPLC.

Impurity Profiling

The measuring of chemical compounds and the associated impurities, even before product development is initiated, is very important in the area of drug creation. High resolution chromatography is a key element for the profiling of any impurities found in chemicals. The capacity of the procedure to point out even the lowest levels of impurities translates into great repercussions for pharmaceutical companies. The reason for this lies in the fact that purer versions of products can be produced with better results in their utilization. Two flow cells

are utilized in the UPLC PDA Detector systems. These cells allow for maximum flexibility in terms of resolution and sensitivity. The processing of information is optimum with the peak detection design and routine calculations. The process of identifying drugs and other metabolites of the body is possible with the utilization of the combination of UPLC and exact mass LC/MS. Fast profiling and identification of impurities can be guaranteed by the combination of UPLC and exact mass time-of-flight mass spectrometry. **(Patil et al., 2011)**

Analysis of Natural Products and Traditional Herbal Medicine

Another application of UPLC is for the analysis of natural products and herbal medicines. The understanding of the pharmacological importance of herbs is essential for their screening validation, and use. The active components present in these herbs can be quickly analysed with the combined use of exact mass MS and UPLC, in association with Marker Lynx Software data processing for the statistical analysis of the information obtained. The combined use of these procedures aids in the rapid understanding of the action of these products on and the way in which they can affect the body. The development of drugs from these products can be done using special methods, such as fractionation and purification, in collaboration with bio analytical tools. **(Patil et al., 2011)**

Some specific Applications of UHPLC in Pharmaceutical Sciences

1. Ultra performance liquid chromatography serves as a more advanced version of “HPLC for the analysis of pharmaceutical compounds”. Research carried out by **Toh D. et al, (2010)** with regards to the profiling of raw and steamed Panax notoginseng using ultra-high performance liquid chromatography/time-of-flight mass spectrometry (UHPLC/TOFMS). Results obtained from their analysis showed that the steamed form of the Panax notoginseng, unlike the raw form, can employ to enrich blood and improve anaemic

conditions, through the stimulation of the creation of new blood cells. The conclusion of the study was geared towards the proper utilization of the right form of the herb to achieve the desirable effect. In their study, the use of the UHPLC/TOFMS methods reduced the time needed to complete the study with maintenance of adequate of peak resolution and minimal carry-over effect.

2. Another study carried out by **Deconinck E. et al. (2012)** was centered on the “Development and validation of an Ultra High Pressure Liquid Chromatographic method for the characterization of confiscated illegal slimming products containing anorexics”. In this study, a fully validated UHPLC-DAD method for the identification and quantification of pharmaceutical preparations, containing molecules frequently found in illegal slimming products was developed. The methodology involved the utilization of a HT C18-B column with a gradient. The development and validation of the proposed methodology was done using an Acquity UPLC™ system. The system included a binary solvent manager, a sample manager and a photo diode array detector. The output signal was observed and processed using special software. The method used was capable of fully analysing and characterizing illegal samples, suspected to contain slimming drugs, in runs of only 10 min. The more the use of an ammonium acetate buffer combined with acetonitrile permitted the transfer to a mass spectrometer, allowing even a more thorough investigation. The application of this method significantly reduced time and solvent consumption with increase efficiency as compared to that of the conventional HPLC methods.

3. The development of the column technology makes UHPLC a very practical and reliable technique for the specific analysis of Vitamin D₂ and D₃ (**see fig.9**). With recent advancement in LC column packing and the utilization of smaller particles, the process has been exploited with significant results. The changes made in the ultra-performance system facilitate higher mobile phase flow rates without affecting the separation efficiency that is achieved using

columns packed with larger particles. The UPLC have been used associated with MS in routine clinical analysis. The resulting UHPLC-MS combined technique is suitable for Vitamin D determination because of its ability to differentiate between 25(OH) D₂ and 25(OH) D₃, which is of utmost importance in the monitoring of the vitamin D levels of patients being treated with vitamin D₂.

The appreciation of UHPLC-MS in the clinical testing laboratory is due to the developments in software and instrument control that facilitates clear chromatographic separation associated with the powerful detection and measurement capabilities of mass spectrometry. UHPLC-MS provides quick analysis of biological samples since the volume required is small. The chromera data systems, facilitates control of both the UHPLC and MS Components of the instrument

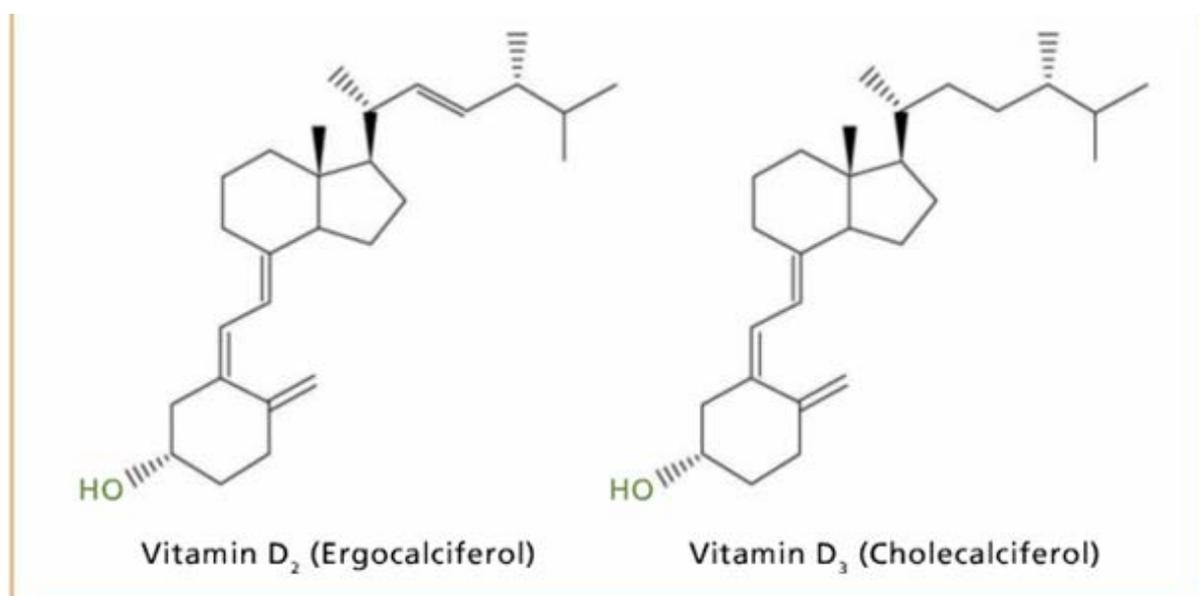


Fig9. Structures of Vitamin D₂, and Vitamin D₃ (Daughterty S., and Green S., 2011)

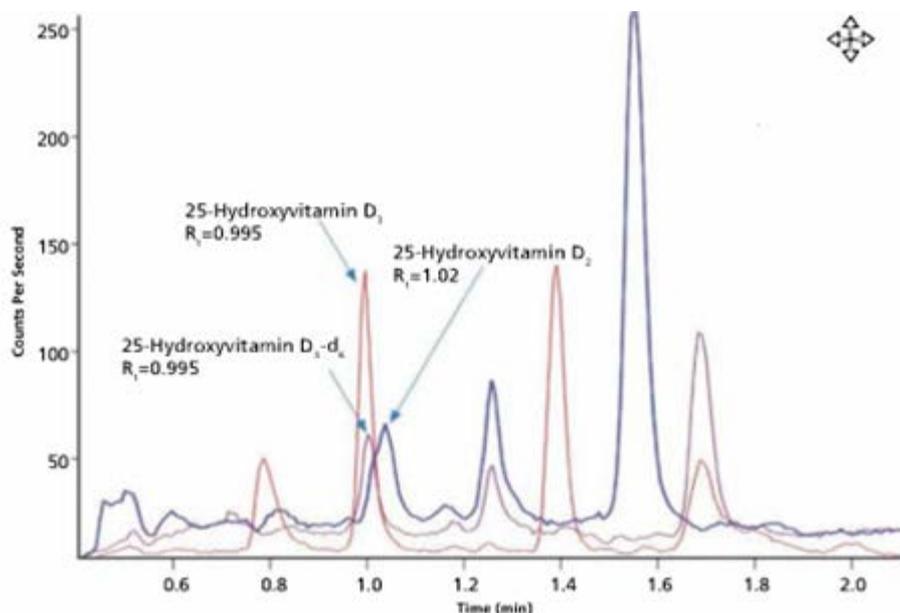


Fig10. Patient sample with separation and detection of 25 hydroxyvitamin D₂ (blue trace), 25-hydroxyvitamin D₃, (red trace) and hydroxyvitamin D₃-d₆ (purple trace) (Daughterty S., and Green S., 2011)

This figure.10 shows the clear peak separation from samples of 25 hydroxyvitamin D₃, 25 hydroxyvitamin D₃-d₆ and 25 hydroxyvitamin D₂. In comparison to other methods, the conditions used for the routine vitamin D testing by UHPLC/SQ MS resulted in highly symmetric peaks with a very low run time of only 6 min, illustrating the agility of the combined UHPLC-MS procedure. Ease and speed of analysis did not interfere with the detection of trace analytes. Results showed that patient samples below 4ng/mL 25 hydroxyvitamin D were detected and quantified without any difficulty. Therefore, the UHPLC-MS is a very precise and advanced technique for detecting and quantifying vitamin D levels within the clinical significant range. The utilization of the combination of improved column design, fast chromatographic separations (UHPLC) with robust single quadrupole mass spectrometry detectors (MS) and sophisticated data systems is currently resulting in the

growing acknowledgement of UHPLC-MS methods as being significantly more useful in bio analytical laboratory. (Daughterty S., and Green S., 2011)

Conclusion

Thus Ultra Pressure Liquid Chromatography has revolutionized the science of Liquid Chromatography and the employment of the same. This technique has significantly modified the manner in which investigation and analysis is done. The discovery and development of UPLC has opened the doors to new insights and uses of chromatography and has reduced the workload and difficulties faced by analysts and scientists. Its multiple advantages outweigh the backdrops of the system and render it very useful in this era. It has been safely utilized in many areas of scientific studies with significant results. It has also demonstrated the capacity to be easily coupled with other advance technologies in order to achieve significantly improved results.

References

1. Swartz, M. E. and Murphy, B. (2004). *Pharmaceutical Formulation Quality* 6(5), 40.
2. Van, J. J., Zuiderweg, E. J., and Klinkenberg, A. (1956). Longitudinal diffusion and resistance to mass transfer as causes of non ideality in chromatography. *Chemical Engineering Science*. 5, 271–289.
3. Zhou, C., Jin, Y., Kenseth, J.R., Stella, M., Wehmeyer, K.R. and Heineman, W.R., (2005). Rapid pKa estimation using vacuum-assisted multiplexed capillary electrophoresis (VAMCE) with ultraviolet detection. *Journal of pharmaceutical sciences*, 94, 576-589.
4. Gerber, F., Krummen, M., Potgeter, H., Roth A., Siffrin, C., and Spöndlin, C., (2004). Stationary phases for ultrahigh pressure capillary liquid chromatography. *Journal of Chromatography*, A1036, 127-133.
5. Swartz, M. E. (2005). *Ultra Performance Liquid Chromatography (UPLC): An Introduction*, *Separation Science Re-Defined*, LCGC Supplement, 8-13.
6. Jerkovich, A.D., Mellors, J.S. and Jorgenson, J.W... (2003). Multidimensional liquid chromatography coupled with mass spectrophotometry. *LCGC* 21, 600–610.
7. Lippert, J.A., Xin, B., Wu, N., and Lee, M.L., J. (1997). Hyper cross-linked polymeric networks. *Journal of Micro column Separation*. 11, 631–643.
8. Batts, J. (2010). Troubleshooting UHPLC connection challenges. Retrieved 6 November, 2012 from http://www.idex-hs.com/about/data_sheets/IDX1156-1-PCO-AI_LR.pdf
9. Toh, D., New, L., Koh, H., and Chan, E. (2010). Ultra-high performance liquid chromatography/time-of-flight mass spectrometry (UHPLC/TOFMS) for time-

- dependent profiling of raw and steamed *Panax notoginseng*. *Journal of Pharmaceutical and Biomedical Analysis*, 52(1), 43-50.
10. Deconinck, E., Verlinde, K., Courselle, P., and Beer, J. (2012). A validated Ultra High Pressure Liquid Chromatographic method for the characterisation of confiscated illegal slimming products containing anorexics. *Journal of Pharmaceutical and Biomedical Analysis*, 59(5), 38-43.
 11. Chesnut, S., & Salisbury, J. (2007). The role of uhplc in pharmaceutical development. *Journal of Separation Science*, 30(8), 1188-30.
 12. Patil, V., Tathe, R., Devdhe, S., Angadi, S., & Kale, S. (2011). Ultra performance chromatography review. *International Research Journal of Pharmacy*, 2(6), 39-44.
 13. Zhao, Y. Y.; Su, Q.; Cheng, X. L.; Tan, X. J.; Bai, X.; Lin, R. C. Pharmacokinetics, bioavailability and metabolism of rhaponticin in rat plasma by UHPLC-Q-TOF/MS and UHPLC-DAD-MSn. *Bio analysis* 2012, 4, (6), 713-23.
 14. Baranowska, I.; Magiera, S.; Baranowski, J. UHPLC method for the simultaneous determination of beta-blockers, is flavones, and flavonoids in human urine. *J Chromatogr Sci* 2011, 49, (10), 764-73.
 15. Dousa, M.; Gibala, P.; Radl, S.; Klecan, O.; Mandelova, Z.; Brichac, J.; Pekarek, T. Identification, preparation and UHPLC determination of process-related impurity in zolmitriptan. *J Pharm Biomed Anal* 2012, 58, 1-6.
 16. Chesnut, S. M.; Salisbury, J. J. The role of UHPLC in pharmaceutical development. *J Sep Sci* 2007, 30, (8), 1183-90.