HPLC: Highly Accessible Instrument in Pharmaceutical Industry for Effective Method Development

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Abstract

The analytical technique of High Performance Liquid Chromatography (HPLC) is used extensively thought the pharmaceutical industry. It allows simultaneously both qualitative and quantitative information of a drug. It is used to provide information on the composition of drug related samples. The information obtained may be qualitative, indicating what compounds are present in the sample. The information obtained may be qualitative, providing the actual amount of compounds in the sample. HPLC is used in all the different stages in the creation of new drug discoveries and also used routinely in the drug manufacturing process. The HPLC techniques is highly accessible to the author’s knowledge this review represents the most exhaust description of instrumentation, method development, recent advancement, area of application. This article reviews the most recent advances in sample preparation, separation and the mass spectrometric aspects of high-throughput for method development.

Keywords: High Performance Liquid Chromatography; Method development; Pharmaceutical analysis

Introduction

High-performance liquid chromatography (sometimes referred to as high-pressure liquid chromatography), HPLC, is a chromatographic technique that can separate a mixture of compounds and is used in biochemistry and analytical chemistry to identify, quantify and purify the individual components of the mixture. HPLC typically utilizes different types of stationary phases, a pump that moves the mobile phase(s) and analyte through the column and a detector to provide a characteristic retention time for the analyte. The detector may also provide additional information related to the analyte, (i.e. UV/Vis spectroscopic data for analyte if so equipped) [1]. Analyte retention time varies depending on the strength of its interactions with the stationary phase, the ratio/composition of solvent(s) used and the flow rate of the mobile phase. It is a form of liquid chromatography that utilizes smaller column size, smaller media inside the column and higher mobile phase pressures [2].

With HPLC, a pump provides the higher pressure required to move the mobile phase and analyte through the densely packed column. The increased density arises from smaller particle sizes. This allows for a better separation on columns of shorter length when compared to ordinary column chromatography. The sample to be analyzed or separated is introduced, in small volumes, into the stream of mobile phase [3,4]. The solution moved through the column is slowed by specific chemical or physical interactions with the stationary phase present within the column. The velocity of the solution depends on the nature of the sample and on the compositions of the stationary (column) phase. The time at which a specific sample elutes (comes out of the end of the column) is called the retention time; the retention time under particular conditions is considered an identifying characteristic of a given sample [5,6]. The use of smaller particle size column packing (which creates higher backpressure) increases the linear velocity giving the components less time to diffuse within the column, improving the chromatogram resolution. Common solvents used include any miscible combination of water or various organic liquids (the most common are methanol and acetonitrile). Water may contain buffers or salts to assist in the separation of the sample components, or compounds such as trifluoroacetic acid which acts as an ion pairing agent [7].

History of HPLC

Liquid chromatography was initially discovered as an analytical technique in the early twentieth century and was first used as a method of separating colored compounds. This is where the name chromatography, chroma means color, graphy means writing, was derived [8]. A Russian botanist used a rudimentary form of chromatographic separation to purify mixtures of plant pigments into the pure constituents. He separated the pigments based on their interaction with a stationary phase, which is essential to any chromatographic separation. The stationary phase he used was powdered chalk and alumina, the mobile phase in his separation was the solvent. After the solid stationary phase was packed into a glass column (essentially a long, hollow, glass tube) he poured the mixture of plant pigments and solvent in the top of the column. He then poured additional solvent into the column until the samples were eluted at the bottom of the column [9,10]. The result of this process most crucial to his investigation was that the plant pigments separated into bands of pure components as they passed through the stationary phase. Modern high performance liquid chromatography or HPLC has its roots in this separation, the first form of liquid chromatography. The chromatographic process has been significantly improved over the last hundred years, yielding greater separation efficiency, versatility and speed.

Instrumentation of HPLC

Instrumentation is required to enable the follow of the mobile phase through the stationary phase and also to convert the separated
Component into meaningful full information (Figure 1). The HPLC system is a highly complicated system consisting of different components given in the (Table 1,2).

Mobile phase reservoir

The mobile phase usually a glass container. The containers need to be of an appropriate size so that it can contain enough mobile phase for the analysis being performed. PTFE tubing connects the content of the reservoir with the HPLC system. This tubing is typically of outer diameter (OD) 1/8 inches and the inner diameter is (ID) 1/16 inch. The size of the tubing in the HPLC system usually measured using the empirical system of inches. At the end of the tubing which is contact with the mobile phase there is usually a filter (10µm) to remove any particulate matter, this is also act as 'sinker' to hold the tubing at the bottom of the container. The lid to the container needs to allow a space for tubing, purpose made lid can be purchased [11]. It is important not to seal the reservoir tightly to avoid the creation of vacuum. The number of lines available on the instrumentation to perform reversed phase gradient elution more than one line is required so that the proportion of the organic component in the mobile phase can be increased throughout the analysis. For isocratic (1-line is available), binary (2-lines are available), ternary (3 lines are available).

Degassing

In HPLC degassing of solvents, mobile phase is very essential for proper operation and retrieval of uniform data. Degassing removes entrapped air bubbles from the solvents. Improper degassing leads to many problems like fluctuation in pressure, improper peaks, improper detection, clogging of column etc. Degassing is done by degasser techniques like ultrasonic, filtration etc. Ultrasonication Here gas is removed by either bursting of gas bubbles immersing ultra sonic generator into the solvent or by placing the solvent container into ultra sonicator tray [12,13]. This is the commonest technique for degassing of HPLC solvents.

Pump

Because of the small particles used in modern HPLC, modern LC pumps need to operate reliably and precisely at pressures of 10,000 p.s.i. or at least 6,000 p.s.i. To operate at these pressures and remain sensibly inert to the wide variety of solvents used HPLC pumps usually have sapphire pistons, stainless steel cylinders and return valves fitted with sapphire balls and stainless steel seats. For analytical proposes HPLC pumps should have flow rates that range from 0 to 10 ml/min., but for preparative HPLC, flow rates in excess of 100 ml/min may be required. It is extremely difficult to provide a very constant flow rate at very low flow rates [14]. If .1% is considered acceptable then for 100ul/min a flow variation of less than .1ul/min is required. This level of constancy is required because most HPLC detectors are flow sensitive and errors in quantization will result from changes in flow rate [15].

Sample valve injector

Since sample valves come between the pump and the column it follows that HPLC sample valves must also tolerate pressures up to 10,000 p.s.i. For analytical HPLC, the sample volume should be selectable from sub-micro liter to a few micro liters, whereas in preparative HPLC the sample volume may be even greater than 10 ml. To maintain system efficiency the sample valve must be designed to have very low dispersion characteristics, this is true not only for flow dispersion but also for the less obvious problems of dispersion caused by sample adsorption/desorption on valve surfaces and diffusion of sample into and out of the mating surfaces between valve moving parts [16,17]. It goes without saying that the valves must deliver a very constant sample size but this is usually attained by the use of a constant size sample loop.

Column component

HPLC columns are packed with very fine particles (usually a few microns in diameter). The very fine particles are required to attain the low dispersion that give the high plate counts expected of modern HPLC. Plate counts in excess of 25,000 plates per column are possible with modern columns, however, these very high efficiencies are very rarely found with real samples because of the dispersion associated with injection valves, detectors, data acquisition systems and the dispersion due to the higher molecular weight of real samples as opposed to the common test samples. Packing these small particles into the column is a difficult technical problem but even with good packing a great amount of care must be given to the column end fittings and the inlet and outlet connection to keep dispersion to a minimum [18]. Some

**Table 1: Component of HPLC system**

| HPLC system component | Description               |
|-----------------------|---------------------------|
| Mobile phase reservoir| Stock of mobile phase for required analysis |
| Degasser              | Degasses the mobile phase |
| Pump                  | Solvent delivery system, enable the follow of mobile phase |
| Sample valve Injector | Sample delivery system, introduces the sample to the system |
| Column Component      | Used to control the temperature of the column |
| Detector              | Detect each component in the mixture for after eluted from column |
| Data Acquisition      | Convert the date from the detector to a meaning full result |
| Waste                 | Collection of liquid waste |
state of the art systems are now ‘chip’ based and may use no particles at all. Some limited use has been made of HPLC for preparative purposes using half inch to one inch diameter columns. LC columns, in general, achieve their separation by exploiting the different intermolecular forces between the solute and the stationary phase and those between the solute and the mobile phase [19]. The column will retain those substances that interact more strongly with the stationary phase than those that interact more strongly with the mobile phase. The basic intermolecular forces that are exploited in the HPLC are the same as those discussed in The Mechanism of Chromatographic Retention and The Thermodynamics of Chromatography of the Chrom-Ed series. The main consideration with HPLC is the much wider variety of solvents and packing materials that can be utilized as because of the much lower quantities of both which are required. In particular very expensive optically pure compounds can be used to make Chiral HPLC stationary phases and may even be used as (disposable) HPLC solvents [20].

**Detector**

LC detectors have been extensively discussed in Liquid Chromatography Detectors and HPLC detectors use the same detection principals with extra care being given to the small solute elution volumes that result from the combination of high column efficiencies with small volumes. In order to give an accurate chromatographic profile the detector sampling (cell) volume must be a small fraction of the solute elution volume. If the detector volume were larger than the elution volume then you would have peaks that appeared with flat tops as the whole peak would be resident in the detector at the same time. This means that as column volumes decrease and system efficiencies increase the volume of the detector cell volume must also decrease [21,22]. This is of course at odds for the requirement for detector to maintain high sensitivity as this is usually dependant on having a larger cell volume. Again, this requires the very careful design of modern detectors (Table 3).

**Data acquisition**

Data acquisition was discussed in Liquid Chromatography Detectors and the only extra consideration required for HPLC is the higher sampling rate needed for the rapidly eluting narrow peaks of the HPLC chromatogram. Although the theoretical number of samples needed for good quantitation are actually quite small, for real systems a hundred samples or more per peak is recommended; thus, for a 4 sec wide peak, a rate of 25 samples per second may be required. The same data analysis and reporting software can be used as in ordinary LC [23].

**Recent Advancement in HPLC**

Recent advances in both ionization methods and mass spectrometers have resulted in powerful new techniques for the study of drug metabolism and disposition. The interest in high-performance liquid chromatography/mass spectrometry (HPLC/MS) is the result of the lack of a sensitive universal detector for HPLC. Although it is not the ideal detector, HPLC/MS has become a reliable technique for xenobiotic analysis. The application of HPLC/MS to studies of the pharmacology and toxicology of molecules of mass < 1,500 daltons is most advantageous in three areas: development of specific methods for trace analysis, detection and characterization of metabolites and studies of interactions between drug molecules and peptides/proteins. We have used HPLC/MS to study the deposition of cyclosporine and its metabolites in needle biopsy samples from kidney and liver in which sample size is severely limited.[24,25]The limit of detection in the single-ion monitoring mode was 500 fg (450 amol), which is about a thousand fold lower than UV limits of detection.

UPLC refers to Ultra Performance Liquid Chromatography. It improves in three areas: chromatographic resolution, speed and sensitivity analysis. It uses fine particles and saves time and reduces solvent consumption. UPLC is comes from HPLC. HPLC has been the evolution of the packing materials used to effect the separation. An underlying principle of HPLC dictates that as column packing particle size decreases, efficiency and thus resolution also increases. As particle size decreases to less than 2.5 μm, there is a significant gain in efficiency and it’s doesn’t diminish at increased linear velocities or flow rates according to the common Van Deemter equation. By using smaller particles, speed and peak capacity (number of peaks resolved per unit time) can be extended to new limits which is known as Ultra Performance [26,27]. The classic separation method is of HPLC (High Performance Liquid Chromatography) with many advantages like robustness, ease of use, good selectivity and adjustable sensitivity. Its main limitation is the lack of efficiency compared to gas chromatography or the capillary electrophoresis due to low diffusion coefficients in liquid phase, involving slow diffusion of analytes in the stationary phase. The Van Deemter equation shows that efficiency increases with the use of smaller size particles but this leads to a rapid increase in back pressure, while most of the HPLC system can operate only up to 400 bar. That is why short columns filled with particles of about 2 μm are used with these systems, to accelerate the analysis without loss of efficiency, while maintaining an acceptable loss of load [28].

Liquid chromatography–mass spectrometry (LC-MS, or alternatively HPLC-MS) is an analytical chemistry technique that combines the physical separation capabilities of liquid chromatography (or HPLC) with the mass analysis capabilities of mass spectrometry. LC-MS is a powerful technique used for many applications which has very high sensitivity and selectivity [29]. Generally its application is oriented towards the general detection and potential identification of chemicals in the presence of other chemicals (in a complex mixture). The limitations of LC-MS in urine analysis drug screening are that it often fails to distinguish between specific metabolites, in particular with

| TYPE     | SAMPLE POLARITY            | MOLECULAR WEIGHT RANGE | STATIONARY PHASE                                | MOBILE PHASE            |
|----------|----------------------------|------------------------|-------------------------------------------------|-------------------------|
| Adsorption | non-polar to somewhat polar | 10⁸ - 10¹⁰             | non-polar liquid adsorbed or chemically bonded to the packing material | non-polar to polar      |
| Partition (reversed-phase) | non-polar to somewhat polar | 10⁸ - 10¹⁰             | highly polar liquid adsorbed or chemically bonded to the packing material | relatively non-polar    |
| Partition (normal-phase)   | somewhat polar to highly polar | 10⁷ - 10⁹              | ion-exchange resins made of insoluble, high-molecular weight solids functionalized typically with sulfonic acid (cationic exchange) or amine (anionic exchange) groups | aqueous buffers with added organic solvents to moderate solvent strength |
| Ion Exchange               | highly polar to ionic        | 10⁵ - 10⁶              | non-polar to ionic                               | polar to non-polar      |
| Size-Exclusion             | non-polar to ionic           | 10⁷ – 10⁹              | small, porous, silica or polymeric particles     |                         |

Table 2: Different type of HPLC which are used for Method development for Pharmaceutical industries
hydrocarbon and its metabolites. LC-MS urine analysis testing is used to detect specific categories of drugs however gas chromatography (GC-MS) should be used when detection of a specific drug and its metabolites is required [30].

Liquid chromatography linked to tandem mass spectrometry (LC-MS/MS) has played an important role in pharmacokinetics and metabolism studies at various drug development stages since its introduction to the pharmaceutical industry. Newly introduced techniques such as ultra-performance liquid chromatography with small particles (sub-2 μm) and monolithic chromatography offer improvements in speed, resolution and sensitivity compared to conventional chromatographic techniques. Hydrophilic interaction chromatography (HILIC) on silica columns with low aqueous/high organic mobile phase is emerging as a valuable supplement to the reversed-phase LC-MS/MS. Sample preparation formatted to 96-well plates has allowed for semi-automation of off-line sample preparation techniques, significantly impacting throughput [31]. On-line solid-phase extraction (SPE) utilizing column-switching techniques is rapidly gaining acceptance in bioanalytical applications to reduce both time and labor required to produce bioanalytical results. Extraction sorbents for on-line SPE extend to an array of media including large particles for turbulent flow chromatography, restricted access materials (RAM), monolithic materials and disposable cartridges utilizing traditional packing’s such as those used in Spark Holland systems. In the end, this paper also discusses recent studies of matrix effect in LC-MS/MS analysis and how to reduce/eliminate matrix effect in method development and validation [32-34].

**HPLC in Different Area of Pharmaceutical**

HPLC can be used in both qualititative and quantitative applications that are for both compound identification and quantification. Normal phase HPLC is only rarely used now, almost all HPLC separation can be performed in reverse phase. Reverse phase HPLC (RPLC) is ineffective in for only a few separation types; it cannot separate inorganic ions (they can be separated by ion exchange chromatography). It cannot separate polysaccharides (they are too hydrophilic for any solid phase adsorption to occur), nor poly-nucleotides (they adsorb irreversibly to the reverse phase packing). Lastly, incredibly hydrophobic compounds cannot be separated effectively by RPLC (there is little selectivity). Aside from these few exceptions, RPLC is used for the separation of almost all other compound varieties. RPLC can be used to effectively separate similar simple and aromatic hydrocarbons, even those that differ only by a single methylene group. RPLC effectively separates simple amines, sugars, lipids and even pharmaceutically active compounds [3]. RPLC is also used in the separation of amino acids, peptides and proteins. Finally RPLC is used to separate molecules of biological origin. The determination of caffeine content in coffee products is routinely done by RPLC in commercial applications in order to guarantee purity and quality of ground coffee. HPLC is a useful addition to an analytical arsenal, especially for the separation of a sample before further analysis.

**Specialty Chemical Analysis**

The development stage, where HPLC is used to characterize products of the chemical synthesis, by analyzing the active pharmaceutical ingredients (API), their impurities and/or degradation products generated by accelerated aging. The development of formulation requires also studies of the dissolution properties of solid dosage forms as well as assays of the pharmaceutical formulations. Method for the verification of system’s cleanliness during the manufacturing process are developed and used at this stage [36,37]. All the HPLC methods that have been finalized at the developmental stage are validated and transferred to the manufacturing laboratories for a quality control analysis.

In-process control method monitors the progress in the manufacturing of an active pharmaceutical ingredient or its formulation. HPLC is frequently employed for the in-process assay of active pharmaceutical ingredient synthesis. The results signal the production chemist/pharmacist whether to proceed with a subsequent unit operation. The decision about whether to use an in-process control test in a manufacturing is established during process development and is based on scientific judgment. In recent years the FDA issued recommendations for process analytical technology (PAT) [38]. The guidance intended to describe a regulatory framework that will encourage the voluntary development and implementation of innovative pharmaceutical development, manufacturing and quality assurance [39]. The new LC ultra performance/rapid technologies are emerging as the technology of choice for the in-process control assay due to their extended efficiency, sensitivity and speed of the analysis.

**Pharmaceutical Analysis**

To understand the purpose of HPLC analytical method it is necessary to consider the applications of HPLC in pharmaceutical analysis. There are wide verity of application throughout the processing of new drugs, from the initial drug discovery to manufacture of formulated products which will administered to the patients [40-42]. In the majority of cases the use of reversed phase (RP) HPLC conditions and UV detection has been reported. Several general studies support the selection of the optimal chromatographic conditions investigated
Effective HPLC Method Development

HPLC analytical method development is a critical process when using HPLC for pharmaceutical analysis. A method needs to separate the desired components satisfactorily, they need to generate the required result and they must be reproducible and robust so that they can be used time after time without problems. The selection of suitable HPLC conditions to achieve a desired separation is often both confusing and time consuming in order to have an efficient method development process, the following three things to keep in mind [48].

The 3 critical components for a HPLC method are: sample preparation, HPLC analysis and standardization (calculations). During the preliminary method development stage, all individual components should be investigated before the final method optimization. This gives the scientist a chance to critically evaluate the method performance in each component and streamline the final method optimization [49].
factors should be established for the significant related substances during the method validation. To limit the workload during method development, usually 3 or less significant related substances should be selected in a method [52].

Other related substances: These are potential degradation products that are not significant in amount. The developed HPLC conditions only need to provide good resolution for these related substances to show that they do not exist in significant levels.

Resolution

A stability indicating method must resolve all significant degradation products from each other. Typically the minimum requirement for baseline resolution is 1.5. This limit is valid only for 2 Gaussian-shape peaks of equal size. In actual method development, Rs = 2.0 should be used as a minimum to account for day to day variability, non-ideal peak shapes and differences in peak sizes [53].

Limit of Quantization

The desired method LOQ is related to the ICH reporting limits. If the corresponding ICH reporting limit is 0.1%, the method LOQ should be 0.05% or less to ensure the results are accurate up to one decimal place. However, it is of little value to develop a method with an LOQ much below this level in standard practice because when the method is too sensitive, method precision and accuracy are compromised.

Precision, Accuracy

Expectations for precision and accuracy should be determined on a case by case basis. For a typical related substance method, the RSD of 6 replicates should be less than 10%. Accuracy should be within 70% to 130% of theory at the LOQ level [54].

Analysis time

A run time of about 5-10 minutes per injection is sufficient in most routine related substance analyses. Unless the method is intended to support a high-volume assay, shortening the run time further is not recommended as it may compromise the method performance in other aspects (e.g., specificity, precision and accuracy) [55].

Adaptability for automation

For methods that are likely to be used in a high sample volume application, it is very important for the method to be "automatable". The manual sample preparation procedure should be easy to perform. This will ensure the sample preparation can be automated in common sample preparation workstations.

Sample solvent selection

This stage focuses on the selection of the sample solvent (for extraction) and the proper sample preparation procedures. Investigate the effect of sample solvents of different % organic, pH, extraction volume and extraction procedure on accuracy, precision, sensitivity (LOQ) and the changes in the chromatography (e.g., peak shape, resolution). Whenever possible use the mobile phase in the sample preparation (Figure 2). This will ensure that there will not be any compatibility issues between the sample solution and the HPLC conditions [56].

Accuracy: To investigate the accuracy in sample preparation (i.e., extraction efficiency), prepare a spiked solution by adding known amounts of related substances into a sample matrix. Compare responses of the spike solutions and the neat standard solutions to assess the recovery from the sample preparation. In this stage, since only one particular step is being investigated (i.e., sample preparation), close to theoretical recovery should be observed at this point (e.g., 90-110%) [57].

Precision: Use the stressed sample to represent the worst case scenario and perform replicate sample preparations from the same sample composite. Investigate the consistency of the related substance profile (i.e., any missing peaks?) and the repeatability results from these preparations [58].

Standardization

Area % method: If the response of the active pharmaceutical ingredient is linear from LOQ to the nominal sample concentration, use the % area approach where the related substance is reported as % area. This is the most straightforward approach and doesn’t require the preparation of standard solutions. It also has the highest precision since preparation to preparation variation will not affect the results. However, in order to ensure the concentration is linear within this range, the sample concentration is usually limited and this will reduce the method sensitivity (i.e., increase LOQ). In general, use this approach as long as the desired LOQ can be achieved [59,60].

External Standard method: Use the external standard method if the response of the active pharmaceutical ingredient is not linear throughout the whole range, or the desired LOQ can’t be achieved by the area % method. The concentration of standard solution should be high enough to ensure the standard solution can be prepared accurately and precisely on a routine basis, it should be low enough to approximate the concentration of related substance in the sample solution. In general, the standard concentration should correspond to about 5% of related substances [61].

Wavelength Selection and Relative Response Factor: Generate the linearity plot of API and related substances at different wavelengths. At this point, Photodiode Array Detector can be used to investigate the linearity of the active pharmaceutical ingredient and related substances in the proposed concentration range. By comparing the linearity slopes of the active pharmaceutical ingredient and the related substances, one can estimate the relative response factors of the related substances at different wavelengths. The optimum wavelength of detection is the wavelength that gives the highest sensitivity (A_max) for the significant related substances and minimizes the difference in response factors between those of the active pharmaceutical ingredient and the related substances. After the optimum wavelength is determined, use a highly stressed sample (e.g., 5% degradation) to verify that the selected wavelength will give the highest % related substance results [62,63].

Optimization method

After the individual components of the method are optimized, perform the final optimization of the method to improve the accuracy, precision and LOQ. Use an experimental design approach to determine the experimental factors that have significant impact on the method. This is very important in determining what factors need to be investigated in the robustness testing during the method validation [64]. To streamline the method optimization process, use Plackett Burman Design (or similar approach) to simultaneously determine the main effects of many experimental factors. Some of the typical experimental factors that need to be investigated are: HPLC conditions: % organic, pH, flow rate, temperature, wavelength, column age. Sample preparation: % organic, pH, shaking/sonication, sample size, sample age. Calculation/standardization: integration, wavelength, standard concentration, response factor correction [65-67].
Validation of HPLC method

Robustness: Method validation should be treated as a “final verification” of the method performance and should not be used as part of the method development. Some of the typical method validation parameters should be studied thoroughly in the previous steps. At this point, the robustness experiments should be limited only to the most significant factors (usually less than 4 factors) [68-70]. In addition, unlike the final method optimization, the experimental factors should be varied within a narrow range to reflect normal day to day variation. During the method validation, the purpose is to demonstrate that the method performance will not be significantly impacted by slight variations of the method conditions.

Linearity, Accuracy, Response Factor: Linearity, accuracy and response factors should be established for the significant related substances during the method validation. In order to limit the workload of method development, usually less than 3 significant related substances should be selected in a method. Therefore, the other related substances should not be included in these experiments [71].

System suitability criteria: It is advisable to run system suitability tests in these robustness experiments. During the robustness testing of the method validation, critical method parameters such as mobile phase composition and column temperature are varied to mimic the day-to-day variability. Therefore, the system suitability results from these robustness experiments should reflect the expected range. Consequently, the limits for system suitability tests can be estimated from these experiments [72].

Conclusion

The optimized HPLC method described here provides fast and reliable analyses; it is a valuable tool during drug formulation development. Additionally, significant savings in time and money can be achieved. By designing chromatographic conditions to fit the needs of a specific experiment, analytical laboratories can save as much as 93% in time, relative to their original assays. Reproducible quality HPLC results can only be obtained if attention has been paid to the method development, validation and the system’s suitability to carry out the analysis.

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