A REVIEW ON HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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Abstract: High performance liquid chromatography (HPLC) is an essential analytical tool in assessing drug product. HPLC methods should be able to separate, detect, and quantify the various drugs and drug related degradants that can form on storage or manufacturing, detect and quantify any drugs and drug-related impurities that may be introduced during synthesis. Today HPLC is widely applied for separations and purify cations in a variety of areas including pharmaceuticals, biotechnology, environmental, polymer and food industries. It is accomplished by injection of a small amount of liquid sample into a moving stream of liquid (called the mobile phase) that passes through a column packed with particles of the stationary phase. The separation of a mixture into its components depends on different degrees of retention of each component in the column. HPLC is just one type of liquid chromatography, meaning the mobile phase is a liquid. This review mainly focuses on the HPLC technique its principle, types, instrumentation and applications. Although several analytical techniques are available for the quantification of steroids, their analysis is challenging due to their low levels and complex matrices of the samples. The efficiency and quick separation of the HPLC combined with the sensitivity, selectivity, simplicity, and cost-efficiency of fluorescence, make HPLC coupled to fluorescence detection (HPLC-FLD) an ideal tool for routine measurement and detection of steroids.

Keywords: HPLC, Types, Application, Analytical method validation, pharmaceutical analysis.

Introduction:

Chromatography is defined as a set of techniques which is used for the separation of constituents in a mixture. This technique involves 2 phases one is stationary phase and another is mobile phase. The separation of constituents is based on the difference between partition coefficients of the two phases. The chromatography term is derived from the Greek words namely chroma which means “color” and graphein means” to write High Performance Liquid Chromatography which is also known as High Pressure Liquid Chromatography. It is a popular analytical technique used for the separation, identification and quantification of each constituent of mixture. HPLC is an advanced technique of column liquid chromatography. The solvent usually flows through column with the help of gravity but in HPLC technique the solvent will be forced under high pressures up to 400 atmospheres so that sample can be separated into different constituents with the help of difference in relative affinities. HPLC depends on pumps to pass a pressurized fluid and an example blend through a section loaded with adsorbent, prompting the partition of the specimen segments. The dynamic segment of the section, the adsorbent, is regularly a granular material made of solid particles (e.g., silica, polymers, etc.) 2 µm to 50 µm in size [3].

The techniques through which the chemical components present in complex mixtures are separated, identified, and determined is termed chromatography. This technique is widely used like spectroscopy and is a very powerful tool not only for analytical methods but also for preparative methods. Compounds of high-grade purity can be obtained by this method.[4] HPLC is recognized from traditional ("low weight") liquid chromatography because operational pressures are fundamentally higher (50 bar to 350 bar), while normal liquid chromatography regularly depends on the power of gravity to pass the portable stage through the segment. Because of the small sample amount isolated in scientific HPLC, column section measurements are 2.1 mm to 4.6 mm distance across, and 30 mm to 250 mm length. Additionally, HPLC segments are made with smaller sorbent particles (2 µm to 50 µm in normal molecule size).[5]

Steroids are biologically active molecules that are available in many natural sources such as, animals, plants and fungi, in addition to being manufactured as therapeutics. Steroids share a common four-fused ring core consisting of 17 carbon atoms and vary according to functional groups attached to the core or side chains, Steroids are key players in many biological, physiological, and pathological events, which are mediated after binding to their cognate tissue receptors. Most prominently, natural steroids play major roles in metabolism, signaling, immunity, reproduction, and salt-retaining activity, because steroids can produce biological action at very low concentrations, analytical methods must be very sensitive and precise particularly when quantifying steroid levels in bio samples. Due to the complex nature matrices of most steroid samples, the methods should also be selective to reliably quantify target steroids and resolve them from other similar endogenous compounds, The efficiency and quick separation of the HPLC combined with the sensitivity, selectivity, simplicity, and cost-efficiency of fluorescence, make HPLC coupled to fluorescence detection (HPLC-FLD) an ideal tool for routine measurement and detection of steroids [6]

Chromatography:

It can be simply defined as follows: “It is the technique in which the components of a mixture are separated based upon the rates at which they are carried or moved through a stationary phase (column) by a gaseous or liquid mobile phase”

Principle of HPLC:

HPLC is a separation technique that involves: The injection of a small volume of liquid sample into a tube packed with tiny particles (3 to 5 micron (µm) in diameter called the stationary phase) where individual components of the sample are moved down the packed tube (column) with a liquid (mobile phase) forced through the column by high pressure delivered by a pump. These components are separated from one another by the column packing that involves various chemical and/or physical interactions between their molecules and the packing particles. These separated components are detected at the exit of this tube (column) by a flow-through device (detector) that measures their amount. Output from this detector is called an "HPLC" In principle, LC and HPLC work the same way except for the speed, and efficiency, sensitivity, and ease of operation of HPLC are vastly superior. Though HPLC retains major of the credits for the analytical side, the earlier one of simple Liquid Chromatography still finds applications for the preparative purposes Figure 1

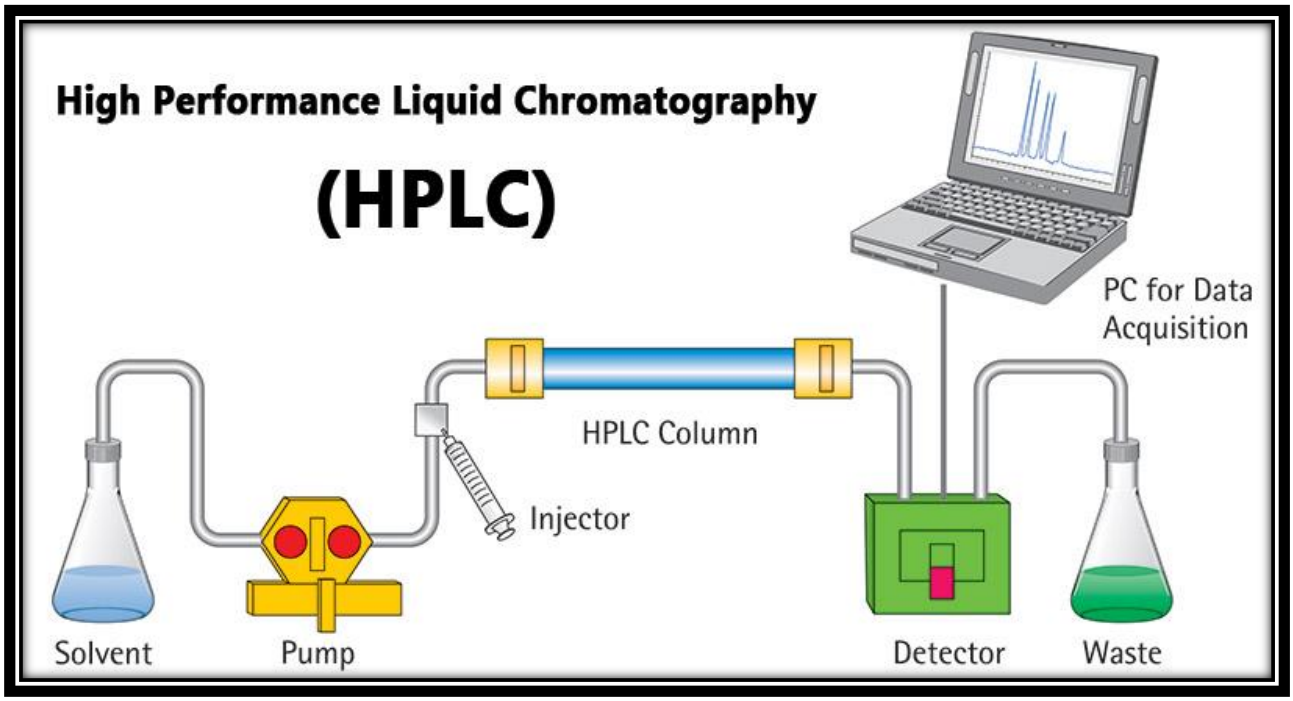

Fig 1 high performance liquid chromatography

As shown in the schematic diagram in the figure above, HPLC instrumentation includes a Solvent reservoir, pump, injector, column, detector, and integrator or acquisition and display system. The heart of the system is the column where separation occurs [4]

TYPES OF HPLC:

Depending on the substrate used i.e., stationary phase used, the HPLC is divided into following types

- **BASED ON MODE OF SEPERATION:**

1. **Normal Phase HPLC**- In this method the separation is based on polarity. The stationary phase is polar, mostly silica is used and the non-polar phase used is hexane, chloroform and diethyl ether. The polar samples are retained on column.
2. **Reverse Phase HPLC**- It is reverse to normal phase HPLC. The mobile phase is polar and the stationary phase is non polar or hydrophobic. The more is the non-polar nature the more it will be retained.
3. **Size-exclusion HPLC**- The column will be incorporating with precisely controlled substrate molecules. Based on the difference in molecular sizes the separation of constituents will occur.
4. **Ion-exchange HPLC**- The stationary phase is having ionically charged surface opposite to the sample charge. The mobile phase used is aqueous buffer which will control pH and ionic strength

- **BASED ON PRINCIPLE OF SEPERATION:**

1. Absorption Chromatography
2. Ion-exchange chromatography
3. Ion-pair chromatography
4. Gel permeation chromatography
5. Affinity Chromatography
6. Chiral chromatography

- **BASED ON ELUTION TECHNIQUE:**

1**. Isocratic elution**: A separation that employs a single solvent or solvent mixture of constant composition.

2. Gradient elution: Here two or more solvent systems that differ significantly in polarity are employed. After elution is begun; the ratio of the solvents is varied in a programmed way, sometimes continuously and sometimes in a series of steps. Separation efficiency is greatly enhanced by gradient elution

- **BASED ON SCALE OF OPERATION:**

1. **Analytical HPLC**
   No recovery of individual components of substance
2. **Preparative HPLC**
   Individual components of substance can be recovere [9]

Instrumentation:
The HPLC instrumentation involves pump, injector, column, detector, and integrator and display system. In the column the separation occurs.

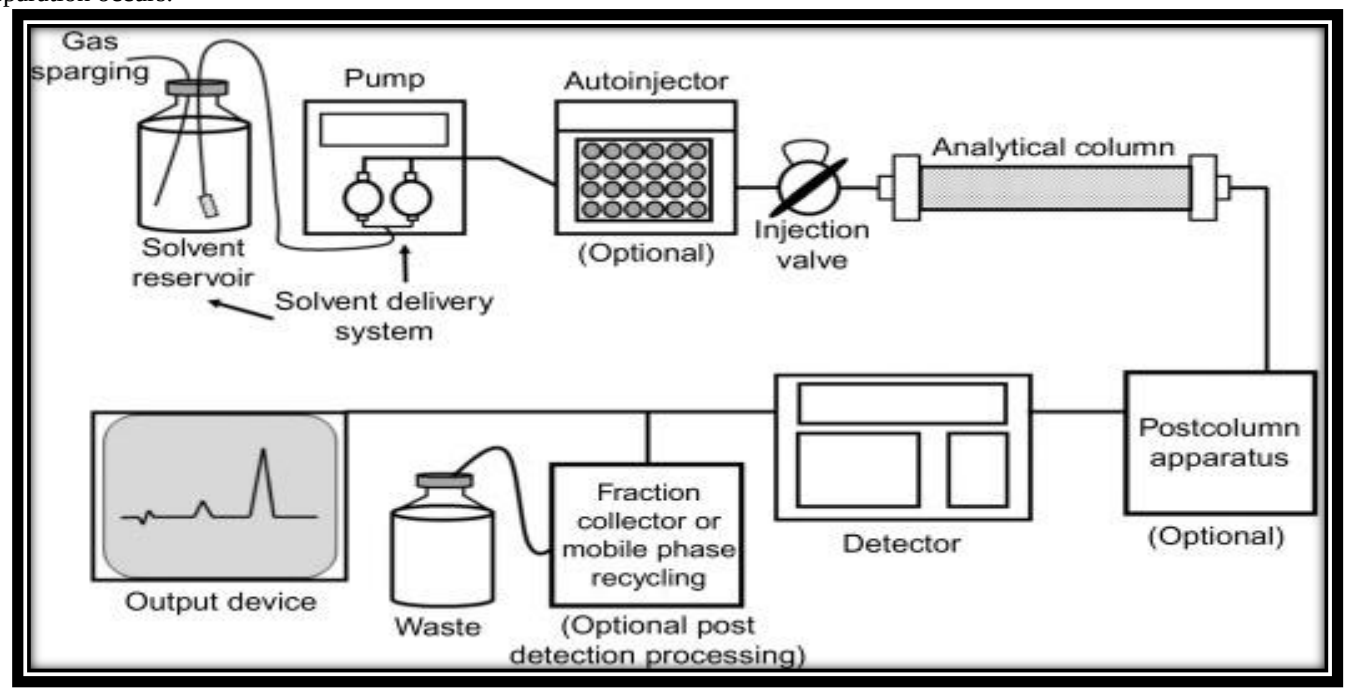

Fig.2 Instrumentation of HPLC:

Parts includes in HPLC:

- **Solvent Reservoir**: The contents of mobile phase are present in glass container. In HPLC the mobile phase or solvent is a mixture of polar and non-polar liquid components. Depending on the composition of sample, the polar and non-polar solvents will be varied.
- **Pump**: The pump suctions the mobile phase from solvent reservoir and forces it to column and then passes to detector. 42000 KPa is the operating pressure of the pump. This operating pressure depends on column dimensions, particle size, flow rate and composition of mobile phase.
- **Sample Injector**: The injector can be a solitary infusion or a computerized infusion framework. An injector for a HPLC framework should give infusion of the fluid specimen inside the scope of 0.1 mL to 100 mL of volume with high reproducibility and under high pressure (up to 4000 psi).
- **Columns**: Columns are typically made of cleaned stainless steel, are somewhere around 50 mm and 300 mm long and have an inward distance across of somewhere around 2 and 5 mm. They are generally loaded with a stationary phase with a molecule size of 3 µm to 10 µm. Columns with inner diameters less than 2 mm are regularly alluded to as microbore segments. Preferably the temperature of the mobile phase and the column should be kept consistent during investigation.
- **Detector**: The HPLC detector, situated toward the end of the column distinguishes the analytes as they elute from the chromatographic column. Regularly utilized detectors are UV-spectroscopy, fluorescence, mass spectrometric and electrochemical identifiers.
- **Data Collection Devices or Integrator**: Signals from the detector might be gathered on graph recorders or electronic integrators that fluctuate in many-sided quality and in their capacity to process, store and reprocess chromatographic information. The PC coordinates the reaction of the indicator to every part and places it into a chromatograph that is anything but difficult to interpret [5]

Sample preparation for HPLC:
Samples in liquid form can be analyzed directly, after a suitable clean-up to remove any particulate materials or after a suitable extraction to remove matrix interferents. In determining polyaromatic hydrocarbons (PAH) in wastewater, for example, an initial extraction with CH_2Cl_2 serves the dual purpose of concentrating the analytes and isolating them from matrix interferents. Solid samples must first be dissolved in a suitable solvent, or the analytes of interest must be brought into solution by extraction. For example, an HPLC analysis for the active ingredients and degradation products in a pharmaceutical tablet often begins by extracting the powdered tablet with a portion of the mobile phase. Gases are collected by bubbling through a trap containing a suitable solvent. Organic isocyanates in industrial atmospheres can be determined in this manner by bubbling the air through a solution of 1-(2-

methoxyphenyl) piperazine in toluene. Reacting the isocyanates with 1-(2-methoxyphenyl) piperazine serves the dual purpose of stabilizing them against degradation before the HPLC analysis while also forming a derivative that can be monitored by UV absorption [4]

The factors which influence the HPLC performance:

Internal diameter:

The internal diameter (ID) of an HPLC column is a critical aspect that determines the quantity of analyte that can be loaded onto the column and also influences sensitivity. Larger columns are usually seen in industrial applications such as the purification of a drug product for later use. Low ID columns have improved sensitivity and lower solvent consumption at the expense of loading capacity.

Particle size:

Most traditional HPLC is performed with the stationary phase attached to the outside of small spherical silica particles (very small beads). Smaller particles generally provide more surface area and better separations, but the pressure required for optimum linear velocity increases by the inverse of the particle diameter squared.

Pore size:

Many stationary phases are porous to provide greater surface area. Small pores provide greater surface area while larger pore size has better kinetics, especially for larger analytes. Pore size defines the ability of the analyte molecules to penetrate inside the particle and interact with its inner surface. This is especially important because the ratio of the outer particle surface to its inner one is about 1:1000. The surface molecular interaction mainly occurs on the inner particle surface.

Pump pressure: Pumps vary in pressure capacity, but their performance is measured by their ability to yield a consistent and reproducible low rate. Modern HPLC systems have been improved to work at much higher pressures, and therefore be able to use much smaller particle sizes in the columns

Temperature:

For proper function of the HPLC the temperature has its own influence. Mostly HPLC columns can work at room temperature or around (25-35 ◦c) are good. But there is also an unexceptional case that requires a higher temperature [4]

Common application areas in the pharmaceutical analysis are:

- To control drug stability.
- Tablet dissolution study of the pharmaceutical dosage form.
- Pharmaceutical quality control.
- Assay
- Related Substances
- Analytical Method Validation
- Stability Studies
- Compound Identification
- Working Standards.[4]

Clinical Applications of HPLC-FLD Techniques:

Quantification of steroids plays an important role in the diagnosis and treatment of endocrine disorders. Diseases, such as cancer, metabolic syndromes, and neurodegenerative diseases, are associated with abnormalities in the endocrine system. Steroids also play an important role in biochemical processes, such as aging, reproduction, and metabolic pathways

1. **Detection of Glucocorticosteroids**

Glucocorticosteroids are widely recognized as markers for adrenal activity. Cortisol can reflect the short-term changes in the activity of the hypothalamic–pituitary– adrenocortical axis (HPA axis), making it a valuable surrogate marker for stress and glucose metabolism

2. **Detection of Steroid Hormones**

Detection of steroid hormones and their metabolites are important in diagnosis of metabolic diseases. Several HPLC-FLD methods were described for the detection of steroid hormones. A study developed a method for monitoring progesterone and 17-hydroxyprogesterone in the serum from pregnant women. The quantification employed fluorescent derivatization using 4,4-difluoro-5,7-dimethyl-4-bora-3a,4adiaza-s-indacene-3-propionohydrazide (BODIPYTM FL hydrazide) [71] (Fi

3. **Detection of Endocrine Disruptive Chemicals (EDCs)**

Detection of Endocrine Disruptive Chemicals (EDCs) The analysis and detection of steroid blood levels is vital for the investigation of food safety and effect on health [42,83]. Many studies have reported the presence of synthetic steroids in our daily food, both from plant and animal sources [61,84–86]. BPA, an industrial chemical, was found in biological fluids because of its ability to leach into food or liquids or through dental sealants into patient's saliva [32,42,61–63]. One of the reasons behind their existence is that farmers increase their profit by using endocrine disruptive chemicals (EDCs) to support the feed conversion and growth rate in animals. Substances with hormonal actions are prohibited in the European community

4. **Pharmaceutical Applications**

Due to its simplicity and versatility, HPLC has become a cornerstone tool in pharmaceutical and biomedical analysis. HPLC is routinely used in drug discovery, development, and manufacturing, and routine assessment for the identification and quantification of drugs, both as active pharmaceutical ingredients and within their formulations [106]. In addition, it is essential for carrying out

product characterizations, including assaying active pharmaceutical ingredients and profiling impurities, as well as degradation products generated by accelerated aging [108]. Moreover, the development of formulations requires studying dissolution properties, stability, and content uniformity of solid dosage forms, as well as conducting assays for the pharmaceutical formulations all of which are carried out using HPLC [106,109]. Although UV is the common detector for these applications, FLD was employed in several assays particularly in steroid analysis due to its sensitivity and selectivity as discussed below

5. **Environmental and Food Applications**:
Environmental and Food Applications Steroids can be found in various environmental samples, including water, plant, and animal samples. Water is the transporter medium and reservoir for various synthetic steroids, whereas plants were found to contain many different natural steroids [17,52,93–95], which can be classified according to their biological relevance. For example, plant physiological steroids can be present as hormones (e.g., brassinolide) and pheromones (e.g., antheridiol), whereas plant allelochemical substances are biologically related to animal hormones such as vertebrate hormones (progesterone) and insect hormones (ecdysone). Other plant steroids can act as protective steroids (e.g., digitoxigenin, solanidine Steroids can be found in various environmental samples, including water, plant, and animal samples. Water is the transporter medium and reservoir for various synthetic steroids, whereas plants were found to contain many different natural steroids [17,52,93–95], which can be classified according to their biological relevance. For example, plant physiological steroids can be present as hormones (e.g., brassinolide) and pheromones (e.g., antheridiol), whereas plant allelochemical substances are biologically related to animal hormones such as vertebrate hormones (progesterone) and insect hormones (ecdysone). Other plant steroids can act as protective steroids (e.g., digitoxigenin, solanidine)

6. **Municipal wastewater treatmen**t:
plants were sampled to investigate the presence of the steroids nonylphenol (NP), octylphenol (OP), nonylphenol polyethoxylates (NPE), 17α-estradiol (E2), and ethinylestradiol (EE2) [5]. SPE disks were used for extraction, followed by HPLC coupled with FLD or competitive radioimmunoassay (RIA). The recorded LODs for HPLC-FLD were 11, 2, and 52 ng/L of water for NP, OP, and NPE, respectively, but were higher than those obtained with HPLC-RIA for E2 and EE2, recorded as 107 and 53 pg/L, respectively

7. **Determination of natural and synthetic estrogenic compounds**
In dairy products was carried out using hollow fiber liquid-phase microextraction coupled to HPLC-FLD/PDA. Estriol, 17β-estradiol, 17α-estradiol, estrone, 17α-ethinylestradiol, diethylstilbestrol, dienestrol, and hexestrol) and 2-hydroxyestradiol were quantified in natural yogurt, a probiotic yogurt-type drink and cheese. The method produced LODs in the low lg/kg or lg/L range with good precision and accuracy.

Applications of recent advances in HPLC:

1. **Phenolic analysis:**
Phenolic analysis 1-D analyses UHPLC and HTLC It has long been known that a reduction in particles size (dp) for packed column HPLC is beneficial. This is due to the fact that small particles provide a more uniform flow and shorter diffusion distances, which increases mass transfer kinetics and implies that high efficiency can be attained in a short time. This knowledge has been the driving force behind the continuous decrease in dp evident from HPLC literature since the 1960s.

Extensive fundamental research has shown the benefits of UHPLC compared with ‘‘conventional’’ HPLC (i.e., 3–5 mm phases and pressures up to 400 bar) to be twofold: (i) improved speed for relatively low-plate counts (due to the characteristics of the small packings referred to above), and (ii) a shift of the optimal performance of any given phase to better performance for high-efficiency analysis. In fact, the combination of small particles and pressure up to 1250 bar (the highest pressures currently available on commercial instrumentation) will provide better results for the analysis up to 100 000 theoretical plates [40, 41]. For conventional HPLC analysis (in the region of 15 000–25 000 theoretical plates), gains in analysis time in the order of three to nine times can be achieved using this approach. UHPLC can also be utilised to improve the efficiency for a given analysis time compared with conventional HPLC although this approach has received less attention to date. The effect of temperature on HPLC separations has been known since the 1980s [42] although it is only in the past decade that the use of temperature as an additional parameter for the improvement of HPLC separations has gained general acceptance. In the past, the benefits of elevated temperature in LC analyses have not been widely explored, partly not only due to the lack of suitable columns and instrumentation [42, 43], but also due to the concerns regarding analyte stability. With the recent availability ofhermally stable stationary phases and suitable instrumentation, HTLC has taken on a new relevance. Analysis temperature affects the selectivity, retention and mobile-phase viscosity in HPLC [12, 42]. The most important effect of an increase in temperature is the reduction of the mobile-phase viscosity and concomitant increase in mass transfer kinetics, resulting in higher optimal flow rates and therefore faster analyses [12, 42]. One approach to benefit from the reduction in mobile-phase viscosity with increasing temperature is to use longer columns to deliver higher efficiency. It should be noted, however, that because of the increase in optimal linear velocity associated with an increase in temperature, this does require higher pressure capabilities. In fact, under pressure-constrained conditions, the primary benefit of elevated temperature is to improve the speed of HPLC separations [12].

A. **Procyanidins:**
Procyanidins (and catechins), being the most abundant class of phenolic compounds in nature, are among the most studied. These compounds also present one of the most severe challenges in terms of chromatographic separation: with an increase in molecular

weight, the number of potential isomers increases exponentially. In fact, especially for highmolecular-weight procyanidins, there is currently no known separation method, as recently summarised [44]: ‘‘polymeric procyanidins y cannot be separated through conventional HPLC.’’ Various researchers have investigated the combination of UHPLC and elevated temperature for improved procyanidin analysis. Most of this work has focussed on reversed-phase (RP) separation on C18 phases using acidified acetonitrile/water phases. For example, Spa´cil et al. [45] achieved a rapid separation of eight tea catechins within 2.5 min using a 100 mm.

B. Flavones and Isoflavones:

Isoflavones are a class of phytoestrogenic flavonoids associated with various protective effects against cardiovascular diseases, cancer, menopausal symptoms and osteoporosis, among others [8, 50]. Flavones, on the other hand, are well known for their potent anti-oxidative activity [51]. It is due to these beneficial properties that significant research effort has been devoted to the analysis of flavones and is flavones in recent years.

C. Anthocyanins:

Anthocyanins (anthocyanidin-glycosides) are coloured phenolic compounds whose analysis by conventional HPLC methods is often problematic. Past research has elucidated the pH-dependant equilibria of the various forms of anthocyanins that occur in solution [59, 60]. The solution chemistry of anthocyanins has important implications for their separation by HPLC. Horva´th and co-workers demonstrated how similar secondary equilibria affect chromatographic separation: for slow inter-conversion reactions (relative to the chromatographic timescale), individual species are separated, whereas fast inter-conversion leads to the detection as a single peak [61]. de Villiers et al. [62] demonstrated that on-column inter-conversion between flavylium cationic and carbinol pseudo-basic species of anthocyanins is responsible for the relatively poor chromatographic efficiency commonly observed for these compounds under conventional RP-LC conditions.[14]

2. Pharmacological studies:

One of the major bioactive coumarins in Chinese licorice (Glycyrrhiza uralensis) is glycycoumarin. After oral administration of this compound to rats (40 mg/kg), 4 and 10 of its metabolites could be determined by HPLC/DAD/ESI–MSn in plasma and urine, respectively [112]. Main routes of metabolism were found to be hydroxylation and glucuronidation, accompanied by hydrogenation and dehydrogenation; however, the majority occurred in unchanged form. The respective structures were tentatively characterized by tandem MS (Finnigan LCQ Advantage ion trap MS), high-resolution MS (IT-TOF from Shimadzu) and after hydrolysis with glucuronidase. Based on these findings a metabolic path- M. Ganzera, S. Sturm / Journal of Pharmaceutical and Biomedical Analysis 147 (2018) 211–233 219 way of glycycoumarin in rats was postulated. In other studies, the metabolism of coumarins from Angelica pubescens [113] and Angelica dahurica [114] was investigated. Most of the target analytes and the assayed matrix (rat plasma) were identical, so that only a few details regarding the more recent publication are mentioned here. Zhao et al. developed a sensitive and specific LC–MS/MS method for investigating the pharmacokinetic properties of sixteen coumarins occurring in A. dahurica [114]. Using a 70% ethanolic extract of the plant they first developed the assay (column: Diamonsil ODS C 18; mobile phase: 0.1% formic acid and acetonitrile; runtime: 20 min; detection: 4000 QTRAP from Applied Biosystems in MRM mode), followed by its validation ($R2 \geq 0.990$; $LOQ \leq 3.1$ ng/mL). Then 6.0 g extract/kg was administered to rats orally, blood samples were collected over a period of 24 h and analyzed by LC/MS. Already 5 min after administration the compounds could be detected in plasma; for most of the coumarins the highest concentration (Tmax) was reachedbetween2and3 h; only marmesin and Columbian tin were found to be resorbed much slower [17]

3. Some historical analysis

The 1970s in HPLC could be characterized as an era of analytical chemical separations. By this I mean small molecule analysis. The application of HPLC to biological molecules really only began at the end of the decade, with work on reversed-phase separation of first peptides (16) and then proteins (17). This focus toward biological separations is part of an interesting history of separations in analytical chemistry and biochemistry, shown in Figure before World War II and especially in the 1950s, many of the separation advances in biochemistry were achieved by scientists in biological fields. Consider people such as Svedberg and his student Tiselius at Uppsala, who were interested in the fundamentals of separations and their application to the purification and analysis of biological substances. With the emergence of GC in the 1960s, analytical chemists became interested in chromatography in a big way, but the focus was on small, volatile species, not biopolymers. There was not a significant interaction between analytical chemists and biochemists at this time. The 1960s and 1970s were a fertile time for biochemists. Gel electrophoretic methods were developed and affinity chromatography was introduced. Both of these areas were largely ignored by the analytical community until the 1980s and 1990s.

The 1990s have seen a merging of analytical chemistry and biochemistry in the separation sciences. HPLC is now a routine tool in protein chemistry. Moreover, capillary electrophoresis, originally begun in the early 1980s by analytical chemists (18), has become a fully developed technique used by analytical chemists and biochemists alike (19). Having lived through the years of development of HPLC and capillary electrophoresis, it is remarkable to me how similar the two histories are. To take only one example, consider the development of separation columns. We have already noted the problems of reproducibility of reversed-phase columns when they first appeared commercially in the mid-1970s. There were many complaints from users, and general acceptance was limited. Only later, when workers learned to improve columns and design additives to the mobile phase to suppress unwanted effects, did reversed-phase columns become widely adopted.

Finally, it is clear to me that the current generation of students in separations or analytical chemistry as a whole need to know a great deal more about the biological sciences than I had to learn in the early 1960s. We are moving into an era where analyses will be performed to an increasing extent on biological molecules. Consider, for example, the field of forensics, where DNA fingerprinting is prominent. Additionally, consider that the information from the Human Genome Project will lead to important diagnostic tests, gene therapy, and population polymorphism screening. To be successful in these and other areas, it is necessary for workers to understand the problems that must be solved. Without proper background, it will be impossible to attack these issues. At the same time, let me also suggest that students in the biological sciences need to learn more about instrumentation and analysis. Today, many of these individuals will end up in industry where careful analysis of product purity and stability, among other analytical issues, will play a significant role in successful product approval by government regulatory agencies. Thus, the circle is closed between the biological sciences and analytical chemistry.

4. **Recent chiral HPLC**:

The majority of enantioselective HPLC systems are composed of a chiral stationary phase and an achiral mobile phase [10]

- **Cyclodextrin-based CSPs:**

Cyclodextrin (CD) CSPs are still widely used in chiral and achiral HPLC as confirmed by the number of papers published since 2010. Commercially available or newly modified (derivatized with various groups) CD CSPs are remaining popular in both basic research and applications.[10]

- **Polysaccharide CSPs:**

Recently, polysaccharide-based CSPs are the most frequently used group of CSPs used in chiral HPLC method development. Many studies reported during the review period discuss the enantiorecognition mechanism [10]

- **Macrocyclic antibiotics-based CSPs:**

Macrocyclic antibiotics-based CSPs represent another powerful group of CSPs with wide application possibilities. They can be used in RP, NP and PO separation modes. The best separation efficiency and selectivity is achieved mainly with PO mobile phases or in RP separation systems [10]

- **Protein-based CSPs:**

Protein-based CSPs are used less frequently for the separation of enantiomers in HPLC. Their main disadvantage is their limited compatibility with mobile phases containing higher amounts of organic modifier. However, they can serve as a model environment for studies of drug interactions in organisms (human body). New simplified, efficient and generic protocols for sample screening on CHIRAL-AGP column (utilizes α1 -acid glycoprotein as CS) for liquid chromatography with MS detection (LC-MS) analyses in RP mode were developed [108]. Chrysanthakopoulos et al. studied the retention behavior of 39 structurally diverse drugs on human serum albumin CSPs (HSA CSP) in RP mode for the calculation/simulation of plasma protein binding data [109]. A Chiral Pak AGP column was successfully used for the separation of a mixture of racemic pharmaceuticals using mobile phase composed of 1% propane-2-ol in 10 mM ammonium acetate, pH 5, compatible with MS detection [10]

- **Pirkle-type CSPs:**

The π-donor or π-acceptor CSPs belong to the oldest applications in chiral HPLC separations. Nevertheless, it is still used in various combinations. Both commercially available and newly π-donor acceptor modified CSPs appear in the literature [10]

- **Crown ether CSPs:**

As these CSPs can be used for a limited group of enantiomers they did not gain an important position among chiral stationary phases with a wide application field. In addition, crown ether-based CSPs were not employed in many papers dealing with liquid chromatography during this review period.[10]

- **CSPs for ligand/ion exchange chromatography:**

LEC represents one of the oldest environments used for the separation of enantiomers. The simplest system can be created using one enantiomer of an amino acid (as chiral ligand) and mostly Cu (II) as a central atom forming the complex with another amino acid as analyte. The click chemistry was applied to immobilize L-prolineamide derivative onto azide-modified silica gel to obtain a novel CSP for ligand exchange chromatography; the CSP was evaluated by enantioseparation of some amino acids [131]. Proton pump inhibitors, i.e., omeprazole, pantoprazole, lansoprazole, rabeprazole were enantioseparated using ligand exchange CSP prepared by bonding (R)-phenyl glycinol derivative, sodium N-[(R)-2-hydroxy-1-phenylethyl]-Nundecylaminoacetate, to silica gel [10]

HPLC Derivatization Methods:

- Enhance detector response
- Improve analyte resolution
- Improve analyte peak shape
- Improve analyte sensitivity
- Establish analyte identity
- Improve analyte stability during analysis
- Change analyte physical properties [9]

Conclusion:

HPLC method is important to separate and quantify the main drug and any reaction impurities. In HPLC, the mobile phase is a liquid. Reversed-phase HPLC is the most common type of HPLC. What reversed-phase means is that the mobile phase is relatively polar, and the stationary phase is relatively non-polar? Therefore, the non-polar compounds. The HPLC is mostly used analytical technique. It is having several advantages. With the use of HPLC one can produce extremely pure compounds. It can be used in both laboratory and clinical science. With the use of HPLC the accuracy, precision and specificity can be increased. The only disadvantage of HPLC is high cost. Since most steroids lack inherited fluorescent properties, various fluorophore labels were introduced to target steroids through different derivatization approaches to enhance steroids quantification. Reaction schemes included in this review illustrated the chemical coupling of the fluorescent label to steroid compounds and highlighted how different steroids can be selectivity targeted depending on the functional group present in the steroid. It can be seen that the system suitability test is a vital tool for the routine quality control of chromatographic assays. Additionally, it can be seen that the sensitivity data generated in a mobile phase optimization study can facilitate the setting of and compliance with these criteria overview of the recent applications of advances in HPLC for polyphenol analysis the recent trend in HPLC of a reduction in particle size, coupled to increases in operating pressure, has also found beneficial application in the analysis of phenolics. The most important advantage of this approach, as applied in the literature reports, is one of the increased throughputs. In addition, separation efficiency and sensitivity may be improved, and in general solvent consumption is largely reduced. Together with the known benefits of RP-LC which remain applicable, such as robust columns and a well-understood separation mechanism.

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