Chapter

Principles and Applications of Ultra-High-Performance Liquid Chromatography

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Abstract

The science of separation had advanced significantly with the development of ultra-high-performance liquid chromatography (UHPLC), a brand-new type of liquid chromatography. The need for the evolution of HPLC into UHPLC has been driven by the continuously evolving of packing material modifications that affect the separation of mixtures. The separation process of analytes is completed in a substantially decreased amount of time due to the lower particle sizes, which increases surface area of interaction allowing reduction of column length to one-third; thus, shorter columns are employed in UHPLC, which consequently causes the flow rate to be three times higher and subsequently reducing analysis time. Although UHPLC shares the same fundamental idea and instrument layout as HPLC, it differs from HPLC in that it produces narrow peaks and has high spectral quality, allowing for simple compound identification in a variety of analytical applications such as impurity profiling, product formulation, and improved analytical technique and method development. However, high back pressure in UHPLC might lead to decreased column life, and the instrument's higher price compared to HPLC are the disadvantages.

Keywords: HPLC, particle size, UHPLC

1. Introduction

Chromatography is a separation method that distributes a mixture's components between a stationary phase and a mobile phase via several methods, including adsorption, partition, ion exchange, and others [1]. The most popular method to identify, measure, and separate the components in a mixture is liquid chromatography (LC). Later, LC developed into high-performance liquid chromatography (HPLC), which pushes solvents through a column under high pressure [2]. It is an effective LC method for separating mixtures. Additionally, it is utilized to identify and measure pharmaceuticals in biological fluids, final dosage forms, and during the drug research and discovery process. Instrumental developments have been made and are still being

made to improve resolution and other separation-related properties such as speed and sensitivity [3].

The development of UHPLC is a result of the growing need for quick and ultra-quick separation techniques that are more effective and have superior resolution [4]. UHPLC has ushered in a substantial shift by giving analysts new ways to get quick analytical separation techniques without compromising the high-quality outcomes previously attained by HPLC [5].

UHPLC has an astonishingly short analysis time and uses a very small amount of solvent as the mobile phase. Additionally, it significantly increases separation effectiveness and analyte mixture resolution. UHPLC uses column packing particle size of less than 2 microns as its key differentiator from conventional HPLC systems, which use particles between 2.5 and 10 microns in size. Because the smaller particles (2 microns) require a greater pressure to work with, UHPLC systems must be able to perform over 6000 psi, which is frequently the upper limit of conventional HPLCs [5, 6].

2. Principles of ultra-high-performance liquid chromatography

The first UHPLC systems appeared in 2004 [7]. The fundamental idea behind this modification of HPLC is that efficiency is gained as column packing particle size lowers, a particle size reduction of less than 2 μm results in an improvement in efficiency that does not drop at higher linear velocities or flow rates [3, 5].

By boosting chromatographic resolution with the greatest number of resolvable peaks, UHPLC enhances the separation systems of LC. By using small amount of column packing materials and reduction of particle size, analysis becomes faster and more sensitive. Additionally, UHPLC has enhanced instrument designs [8]. It had a shorter analysis time of about 1.5 minutes and it reduces the mobile phase volume usage by at least 80% when compared to HPLC. In general, the development of UHPLC has brought a significant advantage for analysts by providing quick and accurate analytical separation findings [5].

3. Distinct instrumental designs of ultra-high-performance liquid chromatography

The common components of UHPLC system are solvent delivery systems (Pumps), sample injection, columns, column managers, detectors [3].

3.1 Solvent delivery system

UHPLC systems regularly operate at 8000–15000 psi. The delivery system must also counterbalance for various solvents used in isocratic, linear and nonlinear gradient elution modes, and also for solvent compressibility for a wide range of pressures. The two major classifications of solvent delivery systems are constant pressure pump and constant volume pump [2, 9, 10]. Constant pressure is used for column packing, while constant volume pump is mostly used in all common UHPLC applications. HPLC has a pump pressure of 40 Mpa, whereas the UHPLC has a pump pressure of 100 Mpa [7].

3.2 UHPLC columns

UHPLC columns are short in length and have a 150 × 2.1 mm length and diameter dimension respectively with a smaller diameter that ranges from 1 to 2.2 mm [10, 11]. Capillary columns are particularly suitable for UHPLC systems due to lower heat generation and better heat tolerance capability. These columns can operate with pressures higher than 80,000 psi [2]. Charged surface hybrid particle technology, ethylene bridged hybrid particle technology [1, 5, 6], high strength silica particle technology, and peptide separation technology are most commonly used in the construction of columns used in UHPLC [7, 10, 12, 13].

3.3 Column manager

The Column manager adjusts temperature from 10 to 90°C and switches automatically for up to 24 hours to four columns, each with a diameter of 2.1 mm internal diameter and length of 150 mm. It also has the ability to bypass channel for flow injections. Most UHPLC systems contain a binary solvent manager, sample manager with the column heater, detector, and non-compulsory sample organizer. The binary solvent manager employs two individual serial flow pumps to deliver a parallel binary gradient. There is a built-in solvent selection feature valves that allow one to pick among the availed up to four different solvents [14].

3.4 Sample injection

The volume of the sample in UHPLC is usually 2–5 μl. Injection cycle time is 25 s without a wash and 60 s with a dual wash used to further decrease carry-overs. A variety of microtiter plate formats (deep well, mid height, or vials) can also be accommodated in a thermostatically controlled environment when analyte stability and conditions demand. Using the optional sample organizer, the sample manager can perform injection from up to 22 microtiter plates [3]. There is also a direct injection application for biological samples [15].

3.5 The detector

The most common detectors used in UHPLC analysis are UV/visible-based types. Detection of analytes is conventionally based on absorbance [9]. The UHPLC detector ought to have long path length, low volume detection cell, a highest likely sensitive detection, and reliable quantification of the narrow peaks. System volumes should also be lessened to uphold the speed, resolution, and sensitivity of the analysis [10]. Depending on the type of detector, the sensitivity of UHPLC can be increased by 2–3 times more than that of HPLC [2].

4. Applications of UHPLC

UHPLC is playing a substantial role in the advancements of liquid chromatography. This is highly attributed to its ability in providing efficient and fast analysis. It can also be hyphenated with different instruments that make application in immense territories like that of pharmaceutical, toxicological, and food industry. It is helping to determine the nutritional value of certain types of foods. It had wide applications in

different agricultural sectors and in clinical analysis, where it is vital to increase throughput with reduced analysis costs [16].

4.1 Analysis of herbal medicines

Herbal medicines are not pure products with a single active ingredient. Thus, conventional methods for screening and identifying the active ingredients in natural products are inefficient. Traditional natural product discovery, using conventional methods, does not give enough evidence about mode of action until late stage in the discovery course. This causes finding compounds with exceptional biological properties (single compound/API drug discovery) a difficult task [17].

Although herbal medicines are gaining increasing attention, clinical usage studies that evaluate its safety and efficacy based on ***in vivo*** pharmacokinetic data of its main ingredients are limited [18].

Chromatographic fingerprinting of herbal components by UHPLC–MS has become a powerful and widely used technique today. This is because it could systematically profile the composition of herbal medicine samples. It also provides high-quality separations and detection capabilities for active compounds in highly complex samples derived from natural origin herbal remedies [19–21]. The application of UHPLC-HRMS for quality control of traditional Chinese medicine (TCM) includes chemical characterization of TCM, determination of TCM components, chemical fingerprint analysis, identification of the authenticity of TCMs, identification of illegal additives in TCMs, exploring the Quality-Marker (Q-Marker), identification of metabolites, evaluation of the quality of TCMs from different habitats, elucidation of the mechanism of action of TCMs (10).

Table 1 shows typical applications of UHPLC in the analysis of herbal medicines where different methods of UHPLC were employed.

The pulp, seeds, and peel of the seven tropical fruits were tested, and the antioxidant levels and capacities revealed notable variations. Fruits' peels and seeds contain more antioxidant potential and antioxidant chemicals than the pulp, which is deficient in these substances. Most of the samples' antioxidant component levels are reduced by oven and freeze drying process. The results of this study had shown how valuable sources of natural antioxidants in avocado-dried peel and passion fruit seed could be affected by the processing techniques. By using UHPLC-ESI-MS, organic acids (citric, malic, and tartaric) and phenolic compounds were also quantified [17].

By using UHPLC/QTOF MS together with automated identification by the Metabolynx^{TM} instrument, a total of 33 peaks were tentatively identified ***in vitro***, 24 of which were parent components, with nine metabolites being detected: This offered beneficial chemical data for additional pharmacological investigation [22].

The validated UHPLC-ESI-MS/MS method was used in the PK study of 14 ingredients after oral administration of Gumiganghwal-tang tablets, a TCM [18].

More importantly, the application of the UPHLC/MS-network pharmacology method will offer a more trustworthy and convincing tool to identify future prospective targets and biological processing mechanisms of Chinese medicine [19].

In an investigation, acetylcholinesterase inhibition assay ***in vitro*** were utilized to quickly screen and discover acetylcholinesterase inhibitors in the D. auriculatum using UHPLC CQ-TOF-MS and UHPLC-ESI-MS/MS. The technique could be utilized for the quick discovery of novel AChEI from natural compounds and was straightforward, sensitive, and selective. This study had provided scientific experimental basis for the traditional efficacy of the medicinal plant for neurological disease [20].

Experimental Condition	Application	Analytes	Matrix	Type of column	Oven T°	Flow rate	MP	Finding	Ref.
UHPLC–ESI–MS	Antioxidant property	Avocado pineapple, banana, papaya, passion fruit, watermelon and melon	Fruit extracts	*	30°C.	0.20 mL/ min.	*	Natural antioxidants	[17]
UHPLC/QTOF MS	Therapeutic effect of ShenQiWan (SQW) treatment of kidney-yang deficiency syndrome	ShenQiWan (SQW)	Serum	Λ	40°C	0.5 ml/min	Λ	Therapeutic effect on kidney-yang deficiency syndrome	[22]
UHPLC-Q Orbitrap MS	Metabolite profiling and pharmacology	XIAOPI formula extract samples	Rat serum	%		0.5 mL/min	%	Potential critical role of bile acid synthetic pathway	[19]
UHPLC-MS	AchE inhibitory activity characterizing of phytochemical constituents	*D. auriculatum*	Extracts of genus Dichocarpum and other 6 species	#	35°C	0.3 mL/min	#	Significant AChE inhibitory activity	[20]
UHPLC-MS/MS	Herb-Drug interaction	*Polygonum capitatum* extract and Levofloxacin (LVFX).	Urine and fecal matter of rat	$	40°C	0.3 mL/min	$	Drug interaction exists	[21]
UHPLC-Q-Extractive-Orbitrap MS	Interventionl effects	Sanse powder	Rat serum	@	6°C	0.3 mL/ min	@	Protective effects against knee osteoarthritis in rats	[23]

*UHPLC column: *UHPLC BEH C18 1.7 μm particles (2.1 × 50 mm) column, Λ BEH C18 column (100 mm × 2.1 mm i.d., 1.7 μm particle size, %UHPLC HSS T3 column (2.1 mm×100 mm, 1.8 μm, Waters), #C18 column (1.6 μm, 2.1 mm×100 mm, $BEH C18 Column (2.1 mm × 100 mm, internal diameter 1.7 μm), @CSH C18 column (1.7 μm, 2.1 × 100 mm), Mobile Phase: * (Mp-A) formic acid0.1% in Milli-Q water and (MP-B) methanol. Λ (Mp-A) HCOOH to H2O ratio of 1:1000, v/v and (Mp-B) HCOOH to CH3CN of 1:1000, v/v. %(MP-A) 0.1. % formic acid in water and 5 mmol/L ammonium acetate in water (MP-B) was acetonitrile. # (Mp-A) acetonitrile and (Mp-B) water, contains 0.1% formic acid. $ 0.1% aqueous formic acid (Mp-A) and (Mp-B) 0.1% formic acid in acetonitrile @(Mp-A)6:4 acetonitrile: water + 10 mM ammonium formate + 0.1% formic acid and (MP-B) 9:1 isopropanol: acetonitrile + 10 mM ammonium formate + 0.1% formic acid.*

Table 1.
Application of UHPLC in natural and herbal medicines analysis.

UHPLC has been employed to study a herb-drug interaction in animal experiments, that is, rats. It was used to evaluate the tissue distribution and excretion of ***Polygonum capitatum*** extract and Levofloxacin when co-administered on rats [21].

4.2 Analysis of drugs in human plasma

Detection of drugs in biological samples is a requisite to study the pharmacokinetics, toxicity, and bioequivalence of the medicine [5]. The most crucial part of determination of drugs in biological samples (plasma, serum, urine, saliva, etc.) is sample preparation. It is often a baseline for a reliable and fast approach in refining analytical efficiency. Blood sample contains numerous proteins and other possible interfering components that would affect the detection of analytes unless otherwise eliminated. Protein precipitation, solid-phase extraction (SPE), and liquid–liquid extraction (LLE) have been widely employed for sample preparation to recover sufficient analyte from the biological sample matrices [24].

For example, a method was developed and validated for drug adherence measurement of patients with hypertension. The antihypertensive drugs were analyzed from patients' blood using a simple and fast sample preparation protocol with protein precipitation followed by chromatographic separation using a gradient elution on a reversed phase column. Mass spectrometric detection was conducted by applying both positive and negative electrospray ionization (ESI+/ESI−) and selected reaction monitoring mode (MS/MS). Only 50 μl of plasma sample was needed for the simultaneous quantification of all twelve compounds (Amlodipine besylate, hydrochlorothiazide, nifedipine, spironolactone, valsartan, canrenone, enalapril, losartan, losartan carboxylic acid, perindopril and perindoprilate, enalaprilat) within 6-min runtime. Enalapril-d5 was applied as internal standard for all compounds except hydrochlorothiazide in which case the internal standard was hydrochlorothiazide-$^{13}C,d_2$. The method showed a significant advantages of minimal sample volume, clean-up procedure, and a short runtime. The method is now available to monitor drug adherence of patients thus helping to manage resistant hypertension in a hospital setting [25].

Analysis of drugs in human plasma using UHPLC plays another significant role in the therapeutic drug monitoring (TDM) activities. The method is quite simple and rapid (separation of compounds achieved in only 6 min) using just a precipitation method for sample preparation and utilizing low amount of patient plasma (100 μL). Due to these characteristics, the UHPLC-based method allows processing of clinical samples and rendering of results within the same working day. This makes it advantageous and fit for TDM purposes [26].

UHPLC has also been employed in the forensic sector. It analyzes compounds of forensic interest in human plasma. A study was carried out on Diphenidol (DPN), a non-phenothiazinic antiemetic, and antivertigo drug following some cases of suicide and accidental poisoning in China. Even though the exact mechanism of death caused by DPN poisoning has not been fully explained yet it is thought that an excessive amount of DPN may promote successive H1 receptor antagonist and anticholinergic effects, causing depression in the central nervous system and vascular smooth muscle relaxation, resulting in sedation, lethargy, hypotension, convulsions, and respiratory failure. The study had been performed using UHPLC–MS–MS indicated that the postmortem concentration range of heart blood was 0.87 ∼ 99 μg/mL. The method was successfully applied to the detection and quantification of DPN in 15 real forensic cases [27]. **Table 2** lists UHPLC's deployment in drug analysis from human plasma.

Traditional methods are typically time-consuming, non-quantitative, and complex, and the necessary sample size is usually big, making them challenging. To fulfill the requirements of quick clinical detection and forensic identification, it is crucial to design simple, sensitive, and accurate systems. UHPLC analytical technique was effectively utilized for quantitative analysis of diphenidol in tissues where blood samples are unavailable or of poor quality [27].

In comparison with previously published methods, the recently developed UHPLC approach is quick and inexpensive, and has superior selectivity and sensitivity for the simultaneous quantification and pharmacokinetics study of human medications in their pharmaceutical dose forms and in spiked human plasma [28–30].

4.3 Pharmacokinetics and bioavailability study

UHPLC method has been developed and validated for the quantitative measurement and determination of pharmacokinetics and bioavailability of various drugs. This had a significant task in assuring drugs' quality, safety, and efficacy. Besides, since most preclinical studies are done on animals, there has been an indication of potentially massive savings of the number of animals and the amount of compounds used when efficient UHPLC methods are employed [31].

UHPLC is also useful for the study of pharmacokinetics and bioavailability on herbal medicines. UHPLC–MS/MS method was developed for the determination of Uncaria alkaloids (a TCM of dry hooked branch of Rubiaceae) which has shown an antihypertensive, vasodilating, neuroprotective, antidepressant, antiarrhythmic, antiepileptic, and antitumor activities in the blood of mice. The relevant methodological process was verified using UHPLC analytical methods. The results showed that the established UHPLC–MS/MS method is accurate and fast. It takes only 5.5 min to analyze blood sample. It is highly sensitive, and effective for the detection and pharmacokinetic study of Uncaria alkaloids. The pharmacokinetic results showed that the six Uncaria alkaloids metabolized rapidly in mice with a half-life between 0.6 and 4.4 h. The bioavailability study also showed satisfactory oral absorption of each alkaloid [32]. Similar studies were performed on other different herbal medicines [33–35]. **Table 3** depicts typical examples of application of UHPLC in pharmacokinetics and bioavailability studies whereby chromatographic and mass settings are crucial for the method's high sensitivity and short retention period in addition to the extraction and sample preparation stages. With modern technology, such as ultra-performance liquid chromatography based on tandem mass spectrometry, it is now possible to detect drugs with great sensitivity and speed, especially for counterfeit medications with variations in the package or appearance. UHPLC–MS/MS analytical method development was used to investigate the rate of degradation of pharmaceuticals in microbial fuel cells (MFC). Additionally, by observing the voltage produced by the microorganisms and the rate of chemical oxygen demand (COD) elimination, the capability of MFC to treat urine spiked with medications was examined. The performance of MFC and medicinal additives was shown to be related, according to the findings [36].

A study done on Pimavanserin indicates that it passes the blood–brain barrier and approaches a Cmax of 21.9±6.66 ng/g in 2.0 hours, according to pharmacokinetics and brain uptake experiments using the technique. Additionally, it was discovered that the ratio of brain to plasma for pimavanserin (Kbrain/plasma) is 0.16±0.05 and that it is quickly removed. The developed method using UHPLC was linear ($R^2 > 0.99$ over the range of 0.1–300 ng/mL in plasma and 0.25–300 ng/g) in the brain homogenate [39].

Experimental Conditions	Application	Analytes	Sample matrix	Type of column	Oven T°c	Flow rate	MP	Finding	Ref.
UHPLC–MS-MS	Determination of Diphenidol (DPN)	DPN	Human Plasma and pork liver	β	at 4°C	0.3 mL/min	β	Detection and quantification of DPN in 15 real forensic cases	[27]
UHPLC–MS/MS	Assay for the quantification of tofacitinib (TOF)	TOF	Human plasma	∞	40°C	0.25 mL/ min	∞	TOF in human plasma samples was determined	[28]
UHPLC–MS/MS	Simultaneous quantitation of the drug and metabolites	Vorolanib	Human plasma	Ω	40°C	0.40 mL/min	Ω	UHPLC–MS/MS method was developed and validated	[29]
UHPLC–MS/MS	TDM	9 antiepileptic drugs	Human plasma	Λ	40°C	0.40 mL/min	^	3 antiepileptics were not within the effective therapeutic range	[30]

UHPLC Column: β ACQUITY UHPLC BEH C18 column (100 mm × 2.1 mm, 1.7 μm). ∞UHPLC BEH C18 (50 × 2.1 mm, 1.7 μm). Ω Acquity BEH C18 column. Λ Agilent Eclipse XDB-C18 column (50.0 × 2.1 mm, 1.7 μm), Mobile Phase: β (Mp-A)20 mmol/L ammonium acetate, 0.1% formic acid in water and 5% acetonitrile. (Mp-B) was acetonitrile. € acetonitrile: 40% tetrabutylammonium hydroxide., ∞ acetonitrile and 10.0mM ammonium acetate. Ω (MP-A) 0.1 % formic acid in deionized water, (MP-B) was composed of 0.1 % formic acid in HPLC-grade acetonitrile. Λ water and acetonitrile. @ Methanol: water (95:5, v/v).

Table 2.
Application of UHPLC in analysis of drugs in human plasma.

Table 3 describes the utilization of UHPLC for the study of pharmacokinetics of wide array of human medicines where the method was accurate, sensitive, and with a short analysis time.

4.4 Identification of metabolites (metabolomics)

Metabolomics a field of studies dealing with identification of many different metabolites that give virtual devices a far-reaching opportunity of utilizations in the fields of pharmacology, toxicology, enzyme discovery, and systems biology [43]. It intends to identify and quantify the full complement of low-molecular-weight, soluble metabolites in actively metabolizing tissues [44].

It also has an extensive contribution in improving ones understanding of disease mechanisms and drug effects. It improves our ability to predict individual variation in drug response phenotypes. Substantial attention has been developed in the application of metabolomics to describe different pathological states of human diseases such as cancer, diabetes, autoimmune, and coronary diseases, among others [43]. Recent technological advances allow characterizing plenty of metabolites from a small quantity of a biological samples. Numerous experiments conducted on cells and tissue cultures played a significant role in improving our biomedical understanding. This at the same time provides a foundation to interpret the concerns related to metabolic processes of the test subject [45]. UHPLC is considered suitable for metabolite profiling and metabolomics among the various LC platforms, especially for large-scale untargeted metabolic profiling due to its sensitivity, selectivity, and enhanced reproducibility [44]. The sensitivity and selectivity of UHPLC at low detection levels produces a precise and reliable data that can be used for a variety of reasons including pharmacokinetics, toxicity, and bioequivalence studies of different metabolites [46].

A metabolism study is considered to be mandatory in a drug development process. It has a vital role in the development of a new chemical entity (NCE). It aids in identifying the active metabolites in so doing, monitoring the possibility of reactive metabolite formations, augmenting pharmacokinetic and pharmacodynamics properties, comparing preclinical with clinical metabolism profile, understanding clearance, and predicting drug–drug or food-drug interactions at early stages of drug discovery [47]. By the time a NCE reaches the development stage, its metabolites' characterization becomes a critical process. The feeble spots of metabolites of drug candidate molecules are recognized and protected by changing the compound structure early [5]. Various analytical techniques have been coupled with UHPLC for the study of metabolomics. From the possible coupled techniques with UHPLC, nuclear magnetic resonance, the most uniform detection technique, has a lower sensitivity compared to mass spectrometry (MS)—and thus, the detection ability of low-abundance metabolites is restricted. Therefore, high-resolution mass spectrometry (HR-MS) and tandem mass spectrometry (MS/MS) are widely used in metabolomics. HR-MS, such as quadrupole time-of-flight mass spectrometry (QTOFMS), provides accurate mass and specific fragment patterns of MS/MS, which can improve the speed and the efficiency of metabolite identification [48]. Higher sensitivity, greater resolution, and faster separation are all benefits of using UHPLC coupled with quadrupole time of flight tandem mass spectrometry (UHPLC-QTOF/MS) for the evaluation of modified metabolites in intricate components. With the assistance of UHPLC-QTOF-MS/MS, a total of eight metabolites of a newly discovered piperazine-based anticancer molecule (IMID-2) were found and described in various matrices

Experimental Condition	Application	Analytes	Sample matrix	Types of column	Column T°c	Flow rate	MP	Finding	Ref.
UHPLC-MS/MS	Removal efficiency of pharmaceuticals	Trimethoprime	Urine	*	30°c	0.3 mL/min	*	Removal rate achieved was 96±2 %	[36]
UHPLC-MS/MS	Pharmacokinetic study	Celecoxib, dezocine and dexmedetomidine	Beagle plasma	@	45°c	0.4 mL/min	@	UHPLC-MS/MS method was established.	[37]
UHPLC-MS/MS	Pharmacokinetics	Narciclasine, 7-deoxynarciclasine	Rat plasma	Λ	-	0.4 mL/ min	^	Pk of narciclasine and 7-deoxynarciclasine.	[38]
UHPLC-MS/MS	Pharmacokinetics and brain uptake studies in mice	Pimavanserin	Mice brain and plasma	&	-	0.25 ml/min	&	Pimavanserin penetrates the blood-brain barrier	[39]
UHPLC-MS/MS	Pharmacokinetics	Amiodarone, desethylamiodarone, dronedarone, desbutyldronedarone	Rat plasma	€	40°c	0.30 mL/min	€	UHPLC-MS/MS method was developed and PK was described	[40]
UHPLC–MS/MS	Pharmacokinetics Study of Eupatilin	Eupatilin (flavone derived from Artemisia plants)	Rat Plasma	£	40°c	0.4mL/min	£	Pk of eupatilin was determined in rats.	[41]
UHPLC–MS/MS	Determination of ledipasvir, sofosbuvir and its metabolite	Ledipasvir, sofobusvir,	Plasma	¥		0.40 mL/min	¥	Pharmacokinetic investigations of ledipasvir, sofosbuvir in rats	[42]

*UHPLC column: *The Agilent C-18 (2.1 294 mm × 100 mm, 1.8 μm) Zorbex Eclipse plus column for Levofloxacin, @Acquity UHPLC BEH C18 column (2.1 mm × 50 mm, 1.7 μm). Λ A UHPLC HSS T3 (2.1 mm × 100 mm, 1.8 μm), &UHPLC BEH™ C18 column (50 mm × 2.1 mm i.d., 1.7 μm. €Acquity UHPLC BEH C18 (2.1 mm x 50 mm, 1.7 μm). £ BEH C18 column (2.1 mm × 100 mm, 1.7 μm). ¥ Acquity BEH C18 column (2.1 mm × 50 mm, 1.7 μm). Mobile Phase: *0.5% FA in 1mM ammonium acetate and MeOH (1:1).@Acetonitrile (Mp-A) −0.1% formic acid aqueous solution (Mp-B). # Methanol (Mp-A) and 5 mM ammonium formate in water (Mp-B). $ water with 0.1% formic acid (Mp-A) and acetonitrile (Mp-B). Λ acetonitrile and water (with 0.1% formic acid). & (Mp-A) 0.1% formic acid in acetonitrile and (Mp-B) 0.1% formic acid in 20 mM ammonium acetate. € (Mp-A) acetonitrile) and (Mp-B) 0.1% formic acid aqueous solution. £ (Mp-A) acetonitrile and (Mp-B) water with 0.1% formic acid). ¥ Mp-A) acetonitrile), and (Mp- B) 0.1% formic acid.*

Table 3.
Application of UHPLC in pharmacokinetics and bioavailability studies.

including in rat liver microsomes (RLM), human liver microsomes (HLM) and rat S9 fraction (RS9), rat plasma, urine, and feces [47, 49]. Untargeted metabolomics in diseased plants may offer a fresh viewpoint and advance our comprehension of plant defense mechanisms. A relevant method for comparing plant metabolite changes is metabolomics. UHPLC paired with HR-MS is the most extensively used metabolomics technology due to its great sensitivity [48].

Table 4 depicts examples in the study of metabolomics using UHPLC. One study investigated and identified the metabolic characteristics of serum samples from children with urolithiasis and normal controls through UHPLC–MS-based metabolomics study approach. Forty differential metabolites were identified, mainly involved in retinol metabolism, steroid hormone biosynthesis, and porphyrin and chlorophyll metabolism. These results indicated that the metabolic phenotype of serum in patients with kidney stones was significantly different from that found in normal controls. The study provided a new understanding into the potential pathogenesis of urolithiasis, which may help to develop novel therapeutic strategies and preventive interventions [53]. Metabolomics can also be used to identify and quantify metabolites from plants. UHPLC–MS method was developed for chemotaxonomic study of seed accessions belonging to 16 different species of ***Vicia*** (vetch species family Fabaceae-Faboideae). These seeds are produced and consumed worldwide for their nutritional value. Both domesticated and wild taxa were analyzed by chemometrics-based UHPLC–MS method. A total of 89 metabolites were observed in the examined ***Vicia*** accessions. Seventy-eight out of the 89 detected metabolites were annotated. The study shows UHPLC–MS metabolomics method ability to discern the diversity of metabolites at the intrageneric level among ***Vicia*** species [54].

The discoveries of metabolic pathways and metabolites could provide a certain theoretical basis for drug discovery and pharmacological mechanism illucidation research [50].

4.5 Detection of impurities

Identification of impurities in raw materials and the final products is one of the most vital stages of a drug development process [4, 55]. Regulatory authorities give significant attention to impurity profiling. Starting materials, intermediates, precursors, etc., are the most common impurities found in every API unless proper care is taken in every step involved throughout the multi-step synthesis. Sometimes, impurities of intermediates and precursors generate structurally related by-products during synthesis [56].

HPLC with sufficient resolution has been providing an excellent detection and determination of the lowest level of impurities with highly reproducible results. However, due to the presence of excipients, there is prolonged HPLC analysis time so it becomes necessary to perform several analytical runs to get the required data time. Hence, the UHPLC/MS technique is operational at alternate low and high collision energies. The fast change of collision energy produces both precursor and product ions of all analytes present in the sample, which allows rapid identification and profiling of impurities [5]. **Table 5** shows several methods for determining, quantification, and characterization of pharmaceutical, impurities using UHPLC have been devised that are easy to use, quick, appropriate, precise, and accurate. In terms of specificity, system suitability, linearity, limit of detection and quantitation, accuracy, precision, robustness, and solution stability, the suggested technique was fully

Experiment Condition	Application	Analytes	Matrix	Type of column	Oven T°C	Flow rate	MP	Findings	Ref
UHPLC-QTOF-MS/MS	Metabolite profiling	2-[4-(3,4-dichlorophenyl) piperazin1-yl]-1-(imidazo[1,2-a]pyrimidin-3-yl)ethanone} (IMID-2)	Blood, urine and feces of rats	Λ	Ambient	0.4 ml/min	Λ	8 metabolites of IMID-2 were identified	[47]
UHPLC-QTOF-MS	Identification of Metabolites	Rice Leaves	Powdered leaves	#	25°C	0.4 mL/min	#	Secondary metabolites are prostanoids	[48]
UHPLC/Q TOF MS	Metabolic Study of Stable Isotope	Indolinone Derivative compounds	Hepatic cell line	&	45°C	-	&	Seven metabolites were discovered	[50]
UHPLC-QTOF/MS	Metabolomic study from subjects after long-term occupational exposure to low concentration acrylamide	Acrylamide (ACR)	Urine and serum	*	35°C	-	*	New biomarkers of nervous system injury	[49]
UHPLC-Q-TOF/MS	Hepatotoxicity of Emodin and Detoxification of Dihydromyricetin (DMY)	Ampelopsis grossedentata (TCM)	Serum, urine, and liver samples from rats	@	40°C	0.4 mL/min	@	Liver protective effect of DMY	[51]
UHPLC-Q-TOF/MS	To analyze the metabolites in the urine of cardiovascular Heart disease patients	Metabolities associated with CHD	Urine	$	45°C	0.3 ml/min	$	14 biomarkers associated with CHD	[52]

*UHPLC column: Λ SB C_{18} column of (100 x 3mm, 1.8μm). # YMC-Triart C18 column (2.0 × 150 mm, 1.9 μm). & HSS T3 column (100 × 2.1 mm, i.d., 1.8 μm). *Acquity UHPLC™ BEH C18 column (100 mm × 2.1 mm i. d., 1.7 μm). @Acquity UHPLC HSS C18 column (1.7 μm, 2.1 × 100 mm). $ Acquity UHPLC BEH C18 column (2.1 mm × 100 mm, 1.7 μm, Waters). Mobile Phase: Λ Ammonium acetate (Mp-A) and acetonitrile (Mp-B). # 0.1% formic acid in water (Mp-A) and 0.1% formic acid in ACN (Mp-B). & (Mp-A) 0.02% formic acid in ammonium acetate (5 mM) and acetonitrile (Mp-B). * (Mp-A) was 0.1% formic acid in water and (Mp-B) was 100% acetonitrile.@0.1% formic acid—water (Mp-A), (Mp-B) 0.1% formic acid acetonitrile. $ 0.1% formic acid in water (Mp-A) and 0.1% formic acid in acetonitrile in (Mp-B).*

Table 4.
Use of UHPLC in identification of metabolites.

Experimental Conditions	Application	Analytes	Sample matrix	Type of column	Column T^0	Flow rate	Mp	Finding	Ref.
UHPLC	Determination of process related impurities	Azathioprine and related impurities	Solution of drug and impurities	Λ	30°C	0.35 mL/ min	Λ	All the impurities were well resolved within 5 min	[56]
UHPLC-QTrap-MS/MS	Characterization of drug-related impurities	Daptomycin	API solution	*	15°C and 55°C	0.3 mL/min	*	Characterization of drug-related impurities	[57]
UHPLC with QToF-MS/MS	quantification of rosuvastatin (RSV) and its related impurities	RSV and related impurities	RSV dosage form	@	40°C	0.3 mL/min	@	The developed method was validated	[58]
UHPLC-QDa	Detection of potential impurities	Daclatasvir	Drug solution	#	35°C	0.4 mL/ min	#	unknown degradation products were identified	[59]
UHPLC	Determination of impurities in pharmaceutical dosage forms	Glicazide	Forced degradation products	$	45°C	0.7 mL/min	$	UHPLC method was developed and validated	[60]
RP-UHPLC	To determine the drugs and impurities	Mebendazole, quinfamide, its impurities	Drug sample solution	α	40°C	0.38 mL/min and 2μL	α	The method was validated	[61]
UHPLC	Quantitative determination of impurities in drug	(Enzalutamide) ENZ	ENZ soft gel capsule made into solution	π	40°C	0.2 mL min−1	π	Method based developed	[62]

*UHPLC Columns: Λ Acuity UHPLC BEH C18 (100 2.1 mm, 1.7 μm). * BEH C18 Van Guard precolumn (2.1 5 mm, 1.7 μm) and CSH C18 column (150 2.1 mm, 1.7 μm). @BEH C-18 100 x 2.1 mm, 1.7 μm. # BEH phenyl 100 × 2.1 mm, 1.7-μm. $CSH-C18 column (50 mm × 2.1 mm × 1.7 μm), Acquity BEH-C18 column (50 mm × 2.1 mm × 1.7 μm), Phenomenex Kinetex-C18 column (50 mm × 2.1 mm × 1.7 μ) . α Cortecs UHPLC C18, 150 mm x 2.1 mm, 1.6 μm. π ACQUITY CSH C18 (100 × 2.1 mm × 1.7 μm). Mobile Phase: Λ (Mp-A) 0.05% trifluoroacetic acid in water and acetonitrile (Mp-B).*(Mp-A) was 0.2% formic acid and 0.03% ammonia in water, and (Mp-B) was 0.2% formic acid in acetonitrile.@ MeOH and 0.1% TFA in the ratio of 50:50.#(Mp-A) with sodium perchlorate monohydrate and 1-octanesulfonic acid sodium salt with perchloric acid solution and (Mp-B) sodium perchlorate and 1-octanesulfonic acid sodium salt..$ 5mM ammonium acetate buffer of pH 4 and 10% ammonium acetate buffer + 90% acetonitrile in the ratio of 65:35. α (Mp-A) 0.025% Trifluoracetic acid and (Mp-B) BAcetonitrile: Methanol (90:10v/v). π potassium phosphate monobasic buffer and acetonitrile (10 mM, adjusted to pH 4.0 with 1% orthophosphoric acid.*

Table 5.
Application of UHPLC in detection of impurities.

validated. The validation results also indicated positive data for each of the examined parameters [56–63].

4.6 Rapid analysis of dosage formulations

Since disease-causing microorganisms are in more mutation than ever and humans' lifestyle is causing more illness than before, the pharmaceutical industry is under intense pressure to increase productivity and bring new drugs onto the market in a short period. UHPLC system provides accurate and reproducible results in rapid isocratic and gradient methods for drug molecules analysis in dosage forms [34]. This in turn helps in improving pharmaceutical manufacturing efficiency. **Table 6** shows examples of UHPLC application in the analysis of dose formulation.

4.7 Food safety

Food products' safety and quality are a concern for consumers and governments. Analytical information, including surveillance data for both recognized and newly identified contaminants, is also indispensable. However, information about food contamination incidence is still limited [65].

Food additives and mycotoxins are among the major toxic treats in different foods consumed worldwide [64]. Food stuffs are vulnerable to infection and contamination in the field or during storage. Mycotoxins, for instance aflatoxins (Afs), Afs B1 is the most common aflatoxin, and is the most toxic and carcinogenic and ochratoxin A (OTA) along with OTA C is known to cause hepatotoxicity, immunotoxicity, neurotoxicity, teratogenicity, and carcinogenicity. The challenge imposed on food safety due to mycotoxins is given currently a rising attention by the government, regulatory authority, and academia globally [66].

The safety of food components and contaminants can only be guaranteed when a good analysis approach is available. During the past few decades, chromatography has been recognized as one of the methods employed to identify and quantify food contaminants. For both qualitative and quantitative research, this novel procedure enables the isolation, purification, and detection of components from a mixture. UHPLC–MS has lately been utilized to estimate food pollutants and components in order to improve food safety [64].

Sensitivity of UHPLC has reached ppb and ppt levels; thus by these quality analytical results, a food analyst would be more confident in ensuring safe food consumption [65]. It is also ideal for analyzing low-level concentrations of food additives where high sample throughput is required without affecting the method's accuracy and sensitivity [67]. **Table 7** shows applying the rules in the context of complicated matrices, like coffee, which frequently necessitates costly and time-consuming strategies. Without clean-up, the UHPLC–MS/MS approach offered adequate sensitivity and resolution. The performance attributes of the approach include high LOQ, recovery, and precision [66]. Additionally, the UHPLC-ESI-MS/MS technology is utilized to discover other potential microbial metabolites present in samples and to confirm the identity of mycotoxins that have been detected [68]. UHPLC has also been employed to detect adulterated milk powder samples, with an acceptable linear correlation coefficient. It is used to assess food products and their diversity or assure their quality and authenticity [69, 70]. Quantification of essential nutrients from human milk was quantified by UHPLC to direct the diet for lactating women [72].

Experimental Condition	Application	Analytes	Sample matrix	Type of column	Oven To	Flow rate	MP	Finding	Ref.
UHPLC-MS	Rapid analysis of dose formulation	Mefenamic acid (MFA) and chloramphenicol (CA)	Aliquots (1 mL) of the dose formulation	Λ	55 °C	0.1 to 1.2 mL/min	Λ	Analysis of CA and MFA in the dose formulation was done	[64]

*UHPLC Columns: Λ BEH C18 column (2.1 x 50 mm, 1.7 m). Mobile Phase: Λ Water/acetonitrile/formic acid (65/35/0.1; v/v/v). * a buffer with pH 4.5 and acetonitrile (60:40% v/v).*

Table 6.
Applications of UHPLC in analysis of dosage formulations.

Experimental condition	Application	Analytes	Sample matrix	Type of column	Oven T°c	Flow rate	MP	Findings	Ref.
UHPLC-MS/MS	Analysis of aflatoxins and ochratoxin A (OTA)	Raw coffee sample	Aflatoxin B1 aflatoxin B2 (aflatoxin G1 aflatoxin G2 and OTA	β	45°C	0.45 mL/min	β	OTA was found in 3 out of 4 samples commercialised in Morocco.	[66]
UHPLC −MS/MS and UHPLC−QTOF-MS	Determination of multiple mycotoxins	Mycotoxins	Feed stuffs	@	25°C	0.35 mL/min	@	Non-target mycotoxins/ fungal metabolites have been identified	[68]
UHPLC-PDA	Anthocyanins profiling in *Vaccinium* L. berries	Berries	Extracts of bilberries, cranberries and lingon berries	#	30°C	0.5 mL/min	#	Separation and detection of major and minor 406 anthocyanins	[69]
UHPLC	Detection of adulterated camel milk powder	Commercial camel milk powder product	Whey protein solution	α	30°C	0.2 mL/ min	α	8 samples were found to be adulterated with a high level of cow milk	[70]
UHPLC–MS	Analysis of water-soluble azo dyes in soft drinks	Water soluble azo dyes	Soft drinks	Λ	35°C	0.3 ml min1	Λ	Simultaneous determination of 11 water-soluble azo dyes in soft drinks	[71]
UHPLC-MS/MS	Analyzing B-vitamins in Human Milk	Vitamin B's	Breast Milk	Ω	40°C	0.35 ml/min	Ω	Fast quantification of vitamin B's has been developed and validated	[72]
UHPLC-QTOF-MS	Quantitative determination of 20 antimicrobial residues	Tetracyclines, quinolones, sulfonamides and diaminopyrimidines	Edible muscle plus skin tissue of European sea bass	∞	30°C	0.4 mL/min	∞	The proposed method was applied for the analysis of contaminated fish samples	[73]

UHPLC Columns: β ACQUITY UHPLC® BEH C18 analytical column (1.7 μm, 2.1 mm × 100 mm). @ACQUITY UHPLC BEH C18 column (50 × 2.1 mm, 1.7 μm particle size). # ACT, Aberdeen, UK; 100×2.1 mm, 1.7 μm particle size. α BEH 300 C4 column (100 mm x 2.1 mm i.d., 1.7 μm). Λ Acquity UHPLC™ BEH Shield RP18 (2.1 100 mm, 1.7 μm. Ω HSS T3 (2.1 x 100 mm, 1.8 μm). ∞ a C18 BEH column (50 mm × 2.1 mm, 1.7 μm, Waters). Mobile Phase: β (Mp-A)(H2O with 10 mM HCOONH4, pH = 5) and eluent B (MeOH with 10 mM HCOONH4, pH = 5). @(Mp-A)water containing ammonium formate (0.15 mmol/L) and formic acid (0.1%) and MeOH(Mp- B). # (Mp-A) aqueous 10% formic acid solution and (Mp-B) acetonitrile.α(Mp-A)ultra-pure water containing 0.1% (v/v) trifluoroacetic acid (TFA); eluent (Mp-B) was acetonitrile containing 0.09% (v/v) TFA. ΛMethanol and (Mp-B)0.1% (v/v) NH3 and H2O in water (Mp-B). Ω (Mp-A) Acetontrileand (Mp-B) 2.5mmol/L ammonium formate aqueous solution. ∞(Mp-A)0.1% aqueous FA and (Mp-B) methanol with 0.1% formic acid.

Table 7.
Application of UHPLC in food safety determination.

4.8 Application in agricultural sector

UHPLC has also been useful in the agricultural sector for the study of soil components, pesticide residue analysis, and crop analysis. Agricultural products contain not only plant materials but animal products too. Thus, safety and quality of such products need to be studied and quality ensured before consumption [74, 75]. In this direction, a rapid, sensitive, and specific ultra-high-performance liquid chromatography tandem mass spectrometry (UHPLC–MS) method was developed for the analysis of tetracycline antibiotics, including tetracycline (TC), oxytetracycline (OTC), chlortetracycline (CTC), and their 4-epimers (4-epi TCs) in agricultural soil fertilized with swine manure. The limits of detection for the soil extraction method ranged from 0.6 to 2.5 μg kg^{-1} with recoveries of 23.3–159.2%. The method was applied on an agricultural field in an area with an intensive pig-fattening farm. Tetracyclines detected in the soil varied from 2.8 to 42.4 μg kg^{-1}. The results made evident that soil from swine farms can be rigorously contaminated with tetracycline antibiotics and their metabolites [74]. **Table 8** summarizes the effect of fungicide and pesticide treatment on the agricultural lands in which case they were considered and quantified by analyzing the harvested products using UHPLC. Besides seasonal variations on the harvested food, both occurrence and concentration of antioxidant abilities and flavonoids were investigated using UHPLC [75–77].

4.9 Method development and validation

UHPLC has been used broadly for method development and validation purpose. UHPLC plays a crucial role in fundamental laboratory function by increasing efficiency, reducing costs, and improving opportunities for business success. Using UHPLC, analysis times become as short as one minute, methods can be optimized in just one or two hours, thereby appreciably decreasing the time required to develop and validate new analytical method [79]. As an example, a study done for the simultaneous estimation of paracetamol and caffeine capsules dosage form indicated that a method was validated using various validation parameters such as accuracy, precision, linearity, and specificity. Moreover, the results show that the method could find practical application as a quality control tool for analyzing the drug in its capsule dosage forms in pharmaceutical industries offering the above-mentioned advantages [80].

5. Advancements of UHPLC

UHPLC is thought to provide higher rates of efficiency, sensitivity, speed, and resolution of UHPLC devices make the system perfect for use with mass spectrometer. More laboratories are finding UHPLC–MS systems to be practicable due to the accessibility and low cost of a new generation of MS equipment [81]. The software used and created for UHPLC systems, which makes the instrument easy to manage, diagnose, and monitor, is primarily responsible for another breakthrough. This makes a significant contribution to the dynamic data processing and information management that convert the findings of the UHPLC system into useful knowledge. In addition, the recording technology found on the majority of UHPLC columns allows for the recording of column history. Another important development is the availability of sample organizers that multiply system capacity by more than 10 times. Additionally,

Experimental condition	Application	Analytes	Sample matrix	Type of column	Column T°	Flow rate	MP	Finding	Ref.
UHPLC-IMS-QToF	Impacts of Fungicide Treatment	Potato samples	Potato metabolites and extracted pesticides	*	55°C	0.45 mL/min	*	Fungicide usage on the potato tuber metabolome is higher	[75]
UHPLC	Seasonal variations in phenolic compounds	*Cornus stolonifera*	Plant material	@	42°C	0.6 mL /min	@	High phenolic content with profound antioxidant activity	[76]
UHPLC/MS-MS	Analytical detection technology	Pesticide residue	fruits, vegetables and grains	#	350°C	0.24mL/min	#	8 pesticide residues were determined	[77]
UHPLC-QTof-MSE M	Metabolite changes in under drought stress.	The hop (*Humulus lupulus L.*)	leaf samples	Λ	40°C	0.5 mL/min	Λ	Drought treatments produced qualitatively distinct changes	[78]

*UHPLC Coulmns: * UHPLC BEH C18 column (150 × 2.1 mm , 1.7 μm).@ 2.1*100 mm, 1.7 μm ACQUITY UHPLC BEH C18. # UHPLC BEHC18, 1.8 μm, 2.4 mm×55 mm. Λ UHPLC BEH C18, 1.7 μm, 2.1 × 100 mm column attached to an ACQUITY UHPLC BEH C18, 1.7 μm, 2.1 × 5 mm VanGuard precolumn. Mobile Phase: *(Mp-A) Water and (Mp-B) acetonitrile. @(Mp-A) 0.3% phosphoric acid, 5% methanol in 18.2 MΩ/cm water; and (Mp-B) acetonitrile. # (Mp-A) 4.5mmol/L ammonium acetate solution containing 0.2% formic acid (Mp-B) is acetonitrile. Λ (Mp-A) water and (Mp-B),acetonitrile.*

Table 8.
Application of UHPLC in agricultural sector.

some businesses offer a virtual technical support service, which enables them to give customers prompt, proactive help that meets their needs to the fullest extent [14].

Examples of organizers that boost system capacity more than ten times are technologically availed. Thus, UHPLC–MS systems are becoming more practical for more laboratories [82, 83]. Despite its expensive price, these developments will further elevate the UHPLC as a crucial analytical tool for sample studies that will be the analysts' first choice worldwide. Future research on engineering and material science topics might also be able to resolve this issue, making this analytical instrument more accessible to many laboratories.

6. Conclusion

Liquid chromatography has been replaced by UHPLC in a novel approach of improvement. It is a sort of separation technique with nearly identical principles as HPLC. The UHPLC uses small packing particles (less than 2 μm in size), which directly influence the length of the column and, in turn, minimize solvent consumption and shorten analysis times. The quick analysis makes it possible to do several analytical tasks quickly. Additionally, UHPLC has been a popular option for analytical work in recent years due to its better sensitivity and resolution. It is used in many fields because of the excellent quality outcomes that are produced. In addition, UHPLC had a wide range of applications in the fields of bioavailability, pharmacokinetics, natural medicines, metabolomics, food safety, and agricultural sectors. The pharmaceutical industry is benefiting most in the drug discovery process. With the aid of UHPLC, researchers may now complete a range of investigations in a short duration of time while saving money on analysis.

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