Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

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Analytical approaches for determination of COVID-19 candidate drugs in human biological matrices

Rabee Mahdavi [a], Zahra Talebpour [a, b, *]

[a] Department of Analytical Chemistry, Faculty of Chemistry, Alzahra University, Vanak, Tehran, Iran
[b] Analytical and Bioanalytical Research Centre, Alzahra University, Vanak, Tehran, Iran

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ABSTRACT

Since the outbreak of the COVID-19 pandemic, the use of antiviral and other available drugs has been considered to combat or reduce the clinical symptoms of patients. In this regard, it would be necessary to choose sensitive and selective analytical techniques for pharmacokinetic and pharmacodynamic studies, monitoring of drug concentration in biological fluids, and determination of the most appropriate dose to achieve the desired effect on the disease. In the present study, the analytical techniques based on spectroscopy and chromatography with different detectors for diagnosis and determination of candidate drugs in the treatment of COVID-19 in human biological fluids are reviewed during the period 2015−2022. Moreover, various sample preparation and extraction techniques, are being used for this purpose, such as protein precipitation (PP), solid-phase extraction (SPE), liquid-liquid extraction (LLE), and QuEChERS (quick, easy, cheap, effective, rugged, and safe) are investigated.

1. Introduction

A new coronavirus infectious disease (COVID-19) caused by severe acute respiratory syndrome coronavirus (SARS-COV-2) in humans was reported in December 2019 in Wuhan, China [1]. On March 11, 2019, COVID-19 was declared an epidemic by the World Health Organization (WHO) [2]. Since the outbreak of COVID-19, studies on the discovery of new antiviral agents and the repurposing of the existing antiviral drugs for the treatment of COVID-19 have been accelerated [3]. Several classes of drugs including antibiotics, antivirals, nonsteroidal anti-inflammatories, anti-cancers, antimalarials, and immunosuppressants are being studied to evaluate their efficacy and safety in treating the symptoms of patients with SARS-COV-2 [4].

Candidate drugs against COVID-19 inhibit the infection and proliferation of SARS-COV-2 by a variety of mechanisms. These drugs can be divided into the following groups based on their mechanism of action (Fig. 1).

- Drugs that inhibit virus entry into the cell and prevent the virus from entering the host cell by disrupting the interaction of the virus spike protein with the host receptor. These drugs include two classes: protease inhibitors, and angiotensin-converting enzyme 2 (ACE2) inhibitors (Fig. 1A).
- Drugs that prevent the viral genomes from replicating and spreading after the process of membrane fusion (Fig. 1B).

The expert reviews that have been conducted on the proposed drugs for the treatment of COVID-19 since the outbreak until now are reported in Fig. 2 [5–21]. In each of these articles, the therapeutic effectiveness of a number of these drugs has been reported in clinical trials. Investigations have been conducted in different fields of pharmacology, toxicology, pharmaceutics, immunology, microbiology, biochemistry, public health, genetics, molecular biology, and cancer research.

Among them, drugs such as favipiravir, remdesivir, lopinavir, ritonavir, ribavirin, chloroquine, hydroxychloroquine, azithromycin, and dexamethasone for various diseases such as influenza, Ebola, AIDS, hepatitis B and C, and malaria are the most commonly used drugs for COVID-19 treatments [22,23]. The names, mechanisms of action, and effectiveness of the mentioned drugs are listed in Table S1, and other drugs are presented in Table S2. These tables have been compiled based on published studies

* Corresponding author. Department of Analytical Chemistry, Faculty of Chemistry, Alzahra University, Vanak, Tehran, Iran.
E-mail addresses: ztalebpour@alzahra.ac.ir, ztalebpour@yahoo.com (Z. Talebpour).

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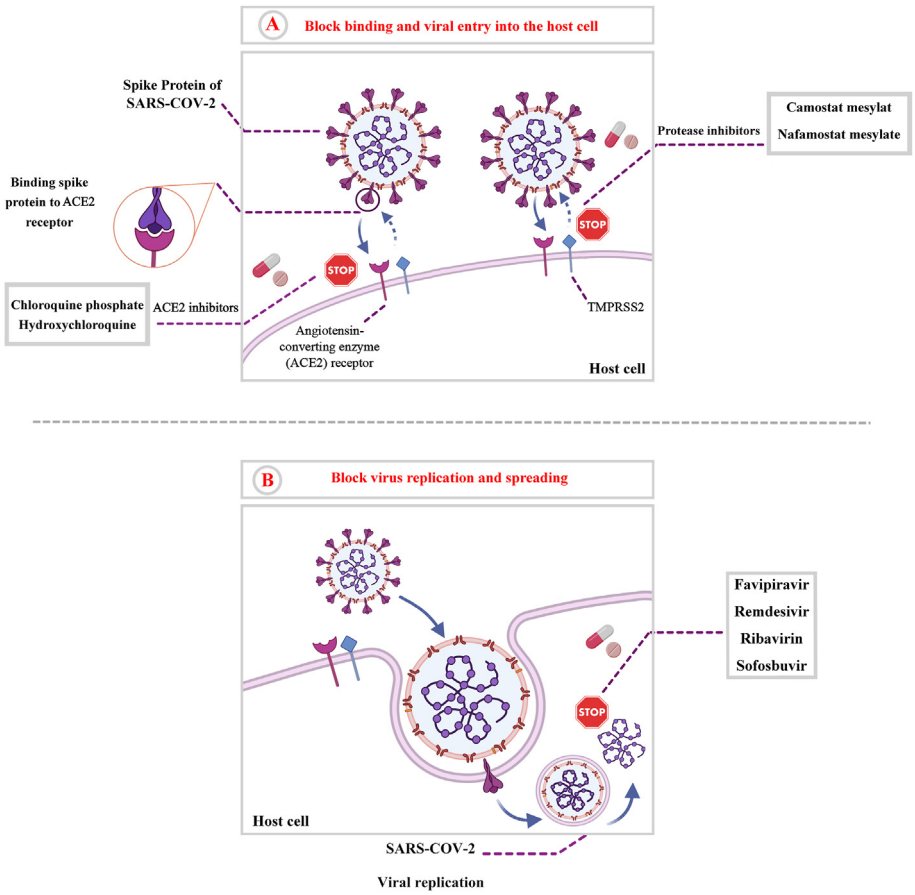

Fig. 1. Inhibition mechanisms of SARS-COV-2 by candidate drugs, A: Block binding and viral entry into the host cell and B: Block virus replication and spreading.

focusing on the use of the first and the most commonly mentioned drugs in the treatment of COVID-19.

Determination of the antiviral drugs in biological matrices during the treatment of COVID-19 is of great importance to optimize the dose of the drug, production, and development of new drugs, toxicity analysis, prevention of therapeutic failures, and therapeutic drug monitoring (TDM) for pharmacokinetic and pharmacodynamic studies. Thus, it would be very necessary and important to provide a suitable, sensitive, and selective analytical method for the quantitative determination of these drugs in biological media with the aim of TDM and identifying the best behaviors for their absorption, distribution, metabolism, and elimination (ADME). Since the mentioned samples are complex, and the concentrations of the drugs under study are very low, specific sample preparation methods must be selected [24,25].

In 2019, a review on the determination of antiviral drugs in wastewater and various environmental samples was presented by Christina Nannou and colleagues [26,27]. In November 2020, Maria A. Acquavia and colleagues provided an overview of the analysis of antiviral drugs currently used to treat SARS-COV-2 and tested in human and animal biological samples [24]. In addition, a review of the reported analytical methods for determining antiviral agents in different matrices was published in 2021 by Ozge Selcuk et al. [28]. Moreover, in February 2022, Jessica Da Ruos et al. reviewed the analytical methods for the determination of the major drugs used to treat COVID-19 [29].

The present study focused on the wide range of drug groups that have been announced for the treatment of COVID-19 during the period 2019–2022 (Table S1). Since many of the drugs used for the treatment of COVID-19 have previously been used for the treatment of other diseases as well, in this study, sample preparation methods and analytical techniques for the determination of these drugs in different matrices were investigated from 2015 to 2022.

2. Characteristics of analytical techniques for analysis of drugs treating COVID-19 in biological matrices

Non-bias and precise analysis methods, as well as high performance and low cost, are prerequisites for the selection of a suitable method for the analysis of trace concentrations of the target compounds. In addition, to perform qualitative and quantitative measurements of the analytes in complex matrices, a high degree of selectivity is required to increase the reliability of the obtained data. Selectivity is assessed at different steps including sample preparation, chromatographic separation, and spectroscopic detection. Spectroscopic techniques despite advantages such as ease, low cost, and low analysis time, do not guarantee high selectivity and sensitivity alone for the determination of drugs in biological matrices [30]. In this regard, chromatography would be considered the main technique used for the determination of drugs in biological matrices due to the versatility, robustness, and flexibility of method development. In both methods, interference from the sample matrix jeopardizes the results of the analysis, and hence sample preparation before analysis would be also required.

Before entering the specialized studies of different analytical methods used for candidate drugs in the treatment of COVID-19, the information on physical and chemical properties of drugs would help to select suitable analysis methods and the associated

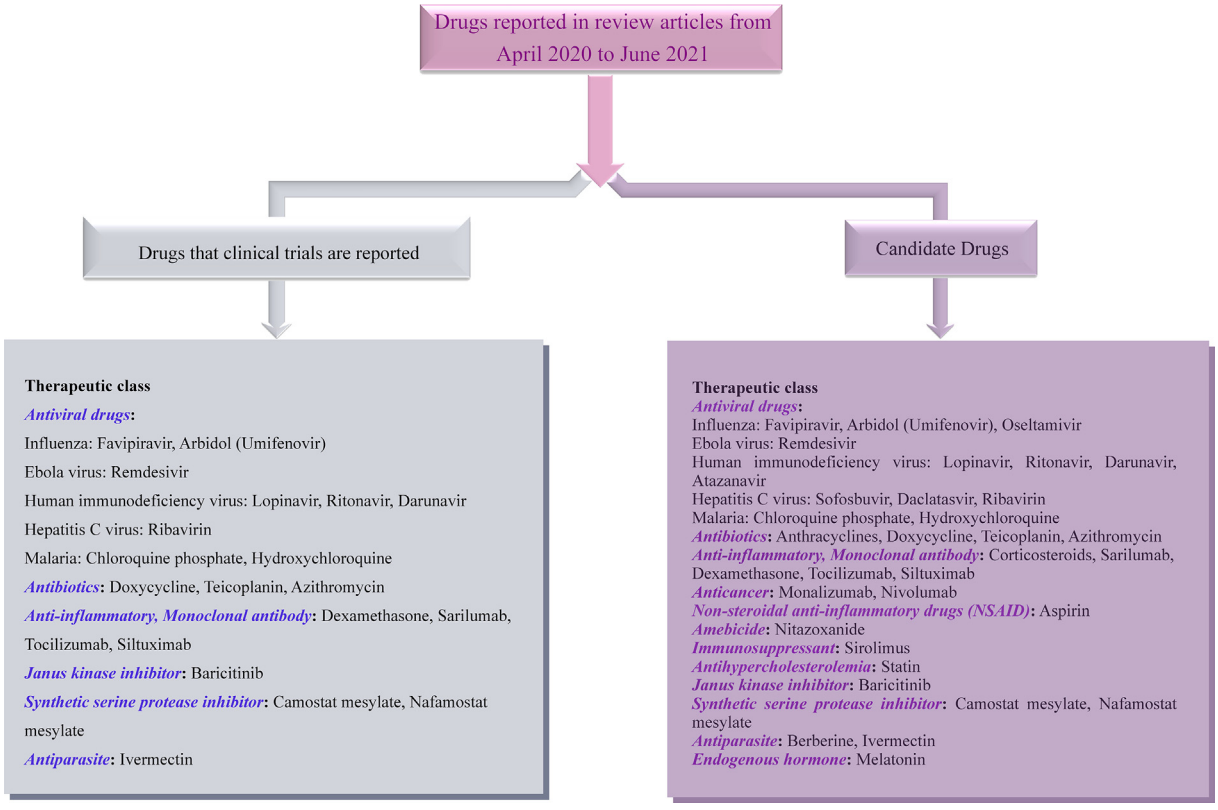

Fig. 2. Overview of candidate drugs and reports of their clinical trials in review articles [5–21].

experimental conditions for the analyte(s) of interest, especially in chromatographic techniques and sample preparation. Thus, some of the physicochemical properties of candidate drugs for the treatment of COVID-19 such as molecular weight, log P, pKa, protein binding percentage, etc. are presented in Table S3, which would be used in different sections of the review [31].

3. Spectroscopic techniques

Among the spectroscopic techniques, only the spectrofluorimetric technique has been reported for the evaluation of drugs in human biological environments. Studies in this field have been conducted on a limited number of candidate drugs for the treatment of COVID-19, such as favipiravir [32], amantadine [33], oseltamivir, ledipasvir, simeprevir, velpatasvir, and daclatasvir [34–40], which have been evaluated by spectrofluorimetric technique in human plasma.

Although oseltamivir and oseltamivir phosphate do not show fluorescence properties, according to the reported studies, oseltamivir phosphate is able to react with derivatizing reagents such as o-phthalaldehyde, ninhydrin, and phenylacetaldehyde, and form a fluorescent product [34,35]. Some drugs such as ledipasvir, simeprevir, and velpatasvir would not require derivatization; however, to increase their native fluorescence, some surfactants or organic solvents have been used as an increaser of the relative fluorescence intensity (RFI). In a research study, to investigate the fluorimetric properties of velpatasvir, different compounds including sofosbuvir, hexadecyl trimethyl ammonium bromide, β-cyclodextrin, Tween-80, methanol, acetonitrile, and dimethyl formamide were used as RFI-increasing agents. Among these compounds, the observed increase in fluorescence produced by methanol was more than that of the other solvents and surfactants [36]. Moreover, Tween-20, as the RFI enhancer, significantly improved the fluorescence intensity of ledipasvir in the presence of sofosbuvir [37].

According to the results obtained by Hamad and his colleagues, simultaneous analysis of simeprevir and ledipasvir in the presence of sofosbuvir would not be possible due to the interference of drug spectra. Under this circumstance, the second derivative of the synchronous fluorescence spectrum was used. For minimum spectral interference, two distinct peaks for each drug with good shape (peak intensity and width) were determined at $\Delta\lambda = 120$ nm [38]. In these studies, the LOD and LOQ values were reported within the range of 0.047–320 and 0.142–980 ng mL^{-1}, respectively [32–40].

4. Chromatographic techniques

Among chromatographic techniques, gas chromatography (GC) is limited to small molecules with low molecular weight due to the volatility criterion. Analyte polarity and thermal stability are two other limiting factors in GC. Therefore, the compounds to be analyzed must be thermostable, non- or semi-polar, and volatile, or achieve these properties by forming an appropriate derivative [41]. Based on the physicochemical properties of the candidate drugs for the treatment of COVID-19 listed in Table S3, most of the compounds have molecular weights of more than 300 Da and log P values below 5. For these reasons, there are very limited reports on the analysis of these drugs by GC or GC-MS [42].

Unlike GC, techniques based on liquid chromatography are considered the most popular methods for the analysis of these drugs in complex biological environments. These techniques are the most ideal and practical analytical methods due to the wide

range and variety of stationary and mobile phases, no limitations in terms of polarity and volatility, the possibility of using different modes from normal to hydrophilic interaction chromatography (HILIC), and the use of a suitable temperature program. In recent years, liquid chromatography has progressed in the field of faster, more efficient, and environmentally friendly separations. Ultra-high performance liquid chromatography (UPLC), miniaturized liquid chromatography (capillary and nano-LC), and multidimensional liquid chromatography (MD-LC) have been significantly highlighted due to their wide range of successful applications in various fields [43].

The UPLC technique has been employed in the analysis of drugs and their metabolic products in biological fluids due to the provision of precise and reliable data in a short period of time. The use of stationary phase particles with smaller sizes, reduction of the physical dimensions of the column (inner diameter and length), and miniaturization of detectors and connections are some of the distinguishing features of these systems compared to traditional ones. The small diameter of the column makes it possible to reduce the flow rate of the mobile phase, which is suitable for increasing the sampling efficiency for electrospray ionization (ESI) sources dramatically as well as reduction of solvent consumption (better detection, less dilution in chromatography, and less waste production). The use of temperature programming to improve efficiency and analysis time is the other advantage of these systems over conventional chromatographic systems [44–46].

In the following sections, the reported articles using various liquid chromatographic techniques would be investigated according to the type of detection technique.

4.1. High/ultra-performance liquid chromatography with spectroscopic detection

The analytical objectives that have been attended by different techniques in previous studies are depicted in Fig. 3. According to our investigations, the determination of the candidate drugs for the treatment of COVID-19 in biological matrices have been performed with the objectives of validation, pharmacokinetic/pharmacodynamic (PK/PD) considerations, therapeutic drug monitoring (TDM), and clinical analysis. Validation by a rate of 41% is the highest goal that has been pursued in studies. The method used in these studies has been validated according to one of the guidelines of the Food and Drug Administration (FDA), the European Medicines Agency (EMA), the International Conference on Harmonization (ICH), and the European Pharmacopoeia (Ph. Eur.), which has been then used for TDM and PK/PD studies [47–51]. In most of the presented reports, the main figures of merit including linearity, sensitivity parameters (limit of detection (LOD) and limit of quantification (LOQ)), accuracy, precision, specificity, carry-over, recovery, robustness, stability studies, and matrix effect have been evaluated to quantify drugs in biological media. Besides, 20% of evaluations have been done focusing on PK, and limited research has also focused on pre-clinical/clinical and bioequivalence studies.

The analysis of candidate drugs for the treatment of COVID-19 independently in their binary or triple mixtures, all performed by HPLC/UPLC techniques, are presented in Table 1, with characteristics of analytical techniques in terms of the type of detector, chromatographic conditions (stationary phase, mobile phase), matrices, and analytical figures of merit.

As shown in Table 1, liquid chromatography with ultraviolet (UV), diode array (DAD), and spectrofluorometric (FLD) detectors has been used for these purposes. The main focus was on the analysis of blood, plasma, and urine samples. Other biological fluids such as serum, breast milk, and peripheral blood mononuclear cells have also been analyzed in some cases. Investigations indicated that reverse-phase liquid chromatography was the first choice for quantification of the candidate drugs in the treatment of COVID-19 (Table 1). The separation of drugs was often performed on the stationary phases of C_{18} and C_8. Based on the physicochemical properties of the drug, the method of sample preparation, and interference of the matrix, different compositions of the aqueous mobile phase have been used.

Sofosbuvir, daclatasvir, ledipasvir, and ribavirin are some of the common antiviral drugs for the treatment of COVID-19, whose separation and simultaneous analysis as binary or ternary compounds have been achieved by various high/ultra-performance liquid chromatography (HPLC/UPLC) and high-performance thin-layer chromatography (HPTLC) with the mentioned goals.

For example, sofosbuvir and daclatasvir were analyzed in human plasma and urine samples by HPLC-UV for PK studies and precise clinical decisions. The use of micelles to minimize the risks and to reduce the cost of analysis was the highlight of this method. By taking one tablet of Gratosovir and Daklanork (each tablet contains 400 mg of sofosbuvir and 60 mg of daclatasvir), the maximum plasma concentration (C_{max}) for sofosbuvir and daclatasvir was reported to be approximately 920, and 820 ng mL^{-1}, respectively [56].

HPLC methods with fluorescence detection are more sensitive, selective, and robust in comparison with the HPLC-UV method. They also provide excellent analytical results with lower application costs compared to MS and MS/MS techniques. Determination of darunavir in peripheral blood mononuclear cells (PBMCs) has been reported by HPLC-FLD [58]. In this method, due to the fluorescence activity of darunavir, there was no need for derivatization. The LOD for darunavir in PBMC cells was declared to be 1.0 ng/10^6 cells., whereas in the determination of darunavir in plasma by HPLC-UV method, sensitivity and selectivity were relatively limited for drug detection at low concentrations, and the reported LOD for this drug in plasma was 0.01 μg mL^{-1} [54]. Despite the ability of FLD, most drug molecules do not show fluorescence, so derivatization reagents are required for non-fluorescent drugs.

In other studies, simultaneous analysis of sofosbuvir and ledipasvir has been performed using RP-HPLC-DAD [61] and UPLC-DAD [67] techniques in human plasma and serum, respectively. In the HPLC-DAD technique, the total analysis time was reported to be 9 min. Gradient elution mode was applied with a flow rate of 1–2 mL min^{-1} and an injection volume of 25 μL. Both drugs represented linearity within the range of 1000–45000 ng mL^{-1}. On the other hand, in the UPLC-DAD technique, the flow rate was adjusted to 0.5 mL min^{-1} and the samples were determined using an injection volume of 5 μL. The whole analysis time was reported to be 1.2 min. The linear range for sofosbuvir and ledipasvir were obtained within the range of 20–1280 and 5–1280 ng mL^{-1}, respectively. Applying a very small injection volume in the UPLC technique is important for the analysis and identification of small sample volumes in biological fluids.

The type of elution mode (gradient or isocratic) is effective to achieve efficient separation and identification of drug compounds. In a study, the isocratic elution was compared to the gradient mode for the simultaneous determination of ribavirin, sofosbuvir, and daclatasvir [64]. The mobile phase was confirmed by gradient elution because of the achievement of sharp symmetrical peaks with good resolution without overlapping with plasma peaks within 20 min. In another study, lamivudine [52] and hydroxychloroquine sulfate [55] were simultaneously determined using isocratic elution. The optimum mobile phase containing small amounts of triethylamine and sodium 1-pentanesulfonate as modifiers reduced the retention time and improved the separation of drug peaks and peak shapes.

High-performance thin-layer chromatography (HPTLC), thanks

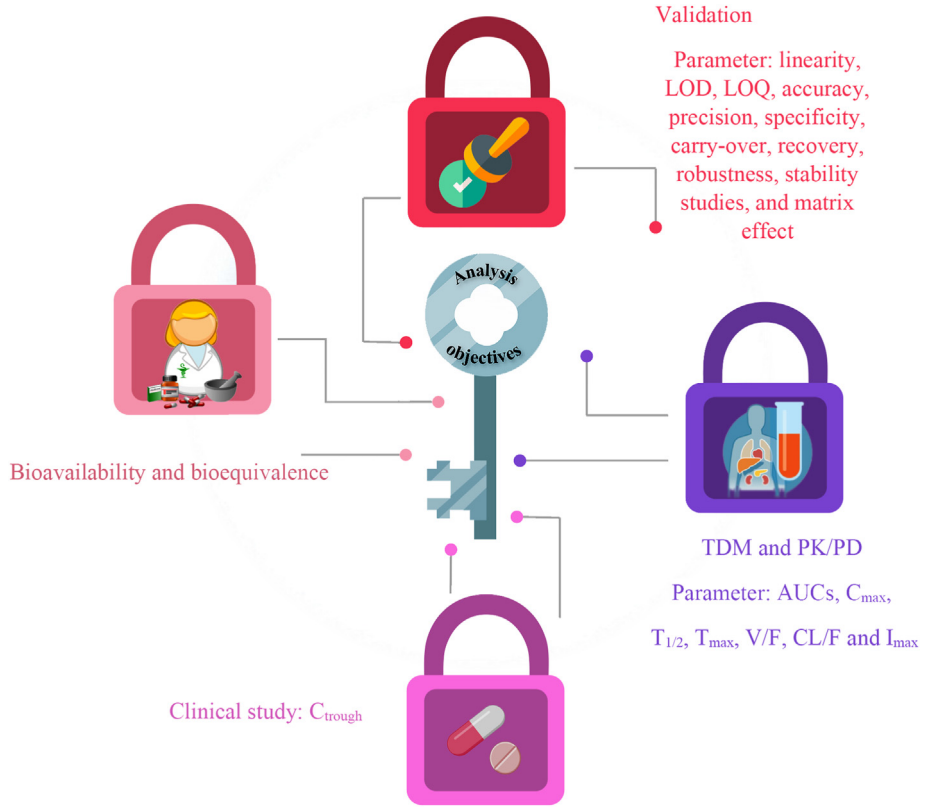

Fig. 3. Objectives of analysis methods in identifying and determining candidate drugs in biological matrices, TDM: therapeutic drug monitoring, PK: pharmacokinetic, PD: pharmacodynamic, AUCs: Area under the plasma drug concentration-time curves, C_{max}: Maximum plasma drug concentration, $t_{1/2}$: Apparent terminal elimination half-life, T_{max}: Time to maximum plasma drug concentration, V/F: Apparent volume of distribution, CL/F: Apparent Oral Clearance, I_{max}: Maximum inhibitory and C_{trough}: Pre-dose trough Concentration.

to its outstanding advantages, is now being considered for the analysis of target compounds in biological samples. The use of the minimum mobile phase volume, less generated waste, the use of green and environmentally friendly solvents, the possibility of analyzing several samples simultaneously, partial clean-up of the sample, less energy consumption, and low-cost functionality are among the advantages of this method [71–73]. As an instance, in a research study reported in 2018, HPTLC with dual-wavelength spectrodensitometry was used for clinical and PK studies of sofosbuvir and daclatasvir simultaneously. In this study, separation using HPTLC was carried out on silica gel 60 F254 aluminum plates (20 × 8 cm) as the stationary phase, and ethyl acetate-isopropanol (85:15, v/v) was used as the mobile phase. The values of the limit of detection (LOD) were 11.3 and 6.5 ng/band, and the values of the limit of quantification (LOQ) were 34.2 and 19.7 ng/band for sofosbuvir and daclatasvir, respectively. These values indicated the high sensitivity and specificity of the procedure for the determination of these drugs in plasma samples without any endogenous interference [71].

The combination of sofosbuvir/velpatasvir is the new antiviral formulation being approved by the Food and Drug Administration (FDA). In 2019, the HPTLC-densitometric method was used for the simultaneous quantitation of sofosbuvir and velpatasvir in their pure form, pharmaceutical formulation, and human plasma. In this study, silica gel 60 F254 pre-coated aluminum sheets (20 × 20 cm) were used as the stationary phase, and the mixture of ethyl acetate-isopropanol (90:10 v/v) as a green mobile phase was applied. Thanks to its high sensitivity and selectivity, this method has the potential for therapeutic drug monitoring and bioavailability studies of sofosbuvir and velpatasvir in human clinical specimens. Dual-wavelength scanning for sofosbuvir and velpatasvir resulted in increased sensitivity and specificity. After ~1 h oral administration, the maximum plasma concentration (C_{max}) for sofosbuvir was 1570 ng mL^{-1}, and the maximum plasma concentration for velpatasvir was reported to be 550 ng mL^{-1} following ~3 h. Prevention of interference from plasma constituent peaks and endogenous sample components indicated high selectivity of the method [72].

Another study was also performed in 2020 by HPTLC technique with densitometric detection on two drugs including sofosbuvir and ledipasvir in plasma and urine samples. The aluminum HPTLC plates of silica gel 60 F254 (20 × 20 cm) with 250 μm thickness and a mixture of ethyl acetate, methanol, water, and glacial acetic acid as the mobile phase were used for separation. The greenness of the method was evaluated by various criteria such as stability, bioaccumulation, toxicity, corrosiveness, hazardousness, and waste production. The low values of LOD in plasma and urine samples (0.21 and 0.27 μg mL^{-1} for sofosbuvir, and 0.05 and 0.03 μg mL^{-1} for ledipasvir, respectively) and LOQ (0.70 and 0.89 μg mL^{-1} for sofosbuvir, and 0.19 and 0.10 μg mL^{-1} for ledipasvir, respectively) indicated the high sensitivity of the method. Degradation of sofosbuvir and ledipasvir was also performed under hydrolytic (alkaline and acidic), photolytic, and oxidative conditions. According to the results, no significant degradation was observed [73].

In another study, simultaneous analysis of emtricitabine, rilpivirine, and tenofovir in urine samples was performed by LC-UV and CE-UV methods. The reported values indicated that the mean recovery and %RSD of the HPLC method (99.7–105.0%, 0.3–0.9%) were lower than those of CE (102.2–105.4%, 2.5–4.2%) [75].

Hydroxychloroquine (HCQ) is one of the candidate drugs relatively effective in treatment of COVID-19. Monitoring the levels of

Table 1
Liquid chromatographic methods for the determination of candidate drugs in the treatment of COVID-19.

Drugs name	Method	Chromatographic conditions		LOD (ng/mL)	Linear range (ng/mL)	Matrices	Ref.
		Stationary phase	Mobile phase				
Lamivudine	HPLC-UV	RP-18e (100 × 4.6 mm)	50 mM Sodium dihydrogen phosphate-triethylamine (96:4, v/v), pH 3.2	10	40 −2560	Plasma	[52]
Simeprevir	HPLC-UV	RP18 (150 × 4.6 mm, 3.5 μm)	Phosphate buffer (pH 6, 52.5 mM) and acetonitrile (30:70, v/v)	20	50 −20000	Plasma	[53]
Darunavir	HPLC-UV	C18	Phosphate buffer (pH 5.9), methanol and acetonitrile (39:22:39, v/v/v)	10	50 −20000	Plasma	[54]
Hydroxy chloroquine sulfate	HPLC-UV	C18 (250 × 6 mm, 5 μm)	Water and (acetonitrile: methanol: 50:50, v/v) mobile phase in 75:25 v/v ratio, phosphoric acid, pH 3.0	240	100 −20000	Plasma	[55]
Sofosbuvir and Daclatasvir	HPLC-UV	C8 monolithic (100 × 4.6 mm)	0.1 M Sodium dodecyl sulfate solution containing 20% v/v n-propanolol and 0.3% v/v triethylamine and pH 6.5 using 0.02 M phosphoric acid	6.3 for plasma 5.06 for urine 3.5 for plasma 5.7 for urine	60-300 for plasma 50-400 for urine 50-300 for plasma 40-400 for urine	Plasma and urine	[56]
Hydroxy chloroquine and metabolites	HPLC-FLD	C18 (150 × 4.6 mm, 5 μm, pore size 100 Å)	Water: methanol: acetonitrile (47:10:43 v/v/v), sodium dodecyl sulfate,3.2 M pH 9.4	1 for all drugs	10 −2500	Whole blood	[57]
Darunavir	HPLC-FLD	C18 (250 × 4.6 mm, 5 μm)	20 mM Potassium phosphate buffer (pH 4.3): Acetonitrile (57:43 v/v)	1.0	5−100[a]	Peripheral blood mononuclear cells	[58]
Hydroxychloroquine	HPLC-FLD	CLC-ODS (15 cm × 6 mm ID)	Acetonitrile-phosphate buffer (13:87 v/v)	NM	200 −2000	Breast milk	[59]
Hydroxychloroquine and Desethyl Hydroxychloroquine	HPLC-FLD	phenyl® (250 × 4.6 mm, 5 μm)	Glycine buffer/sodium chloride (pH 9.7, 100 mM) and methanol (46:54 v/v)	25 12.5	50 −4000 25 −2000	Whole blood	[60]
Sofosbuvir and Ledipasvir	HPLC- DAD	C18(250 × 4.6 mm, 5 μm)	A: water B: Acetonitrile	NM	1000 −45000	Plasma	[61]
Chloroquine and Desethylchloroquine	HPLC- DAD	SB-CN (150 × 4.6 mm, 5 μm)	Phosphate buffer 25 mM, pH 2.60-acetonitrile (88:12 v/v) with 2 mM sodium perchlorate	4	10 −5000	Plasma and whole blood	[62]
Chloroquine, Desethylchloroquine and Primaquine	HPLC- DAD	C18 (250 × 4.6 mm, 5 μm)	Methanol and a buffer of orthophosphoric acid (0.57%), sodium hydroxide (0.087 M), and triethylamine (0.13 mM)	3.28 0.89 21.4	20 −2000[b] 20 −2000[b] 100 −3000[b]	Plasma	[63]
Sofosbuvir, Ribavirin and Daclatasvir	HPLC-DAD	C18 (250 × 4.6 mm, 5 μm)	A: Water B: Acetonitrile	NM	100 −40000 500 −80000 500 −80000	Plasma	[64]
Efavirenz	UPLC-UV	C18 (100 × 2.1 mm, 1.7 μm)	0.1 M Formic acid (containing 0.01 mol/L triethylamine, pH 4), acetonitrile and methanol (30:50:20 v/v)	0.039	78 −10000	Plasma	[65]
Remdesivir	UPLC-DAD	C18 (150 × 4.6 mm, 3 μm)	A: 0.05% Formic acid B: Acetonitrile (52:48)	1.5	5−5000	Plasma	[66]
Sofosbuvir and Ledipasvir	UPLC-DAD	C18 (50 × 2 mm, 1.8 μm)	0.1% Formic acid in water (pH 2.6) and acetonitrile (60:40 v/v)	NM	20 −1280	Serum	[67]
Hydroxychloroquine, Minocycline and Doxycycline	UPLC-UV	BEH Phenyl (50 × 2.1 mm, 1.7 μm)	1% Triethylamine and 1 mM oxalic acid in water adjusted to pH 2.4 with orthophosphoric acid 85%	NM	250 −5000 1250 −10000 1250 −10000	Serum	[68]
Hydroxychloroquine, Desethylhydroxy chloroquine and Desethylchloroquine	UPLC- FLD	C18 (100 × 2.1 mm, 1.7 μm)	Piperazine buffer (46.4 mM, pH 9.8) and acetonitrile (68:32 v/v).	5 9 4	125 −4000 62.5 −2000 50−800	Whole blood	[69]

Table 1 (*continued*)

Drugs name	Method	Chromatographic conditions		LOD (ng/mL)	Linear range (ng/mL)	Matrices	Ref.
		Stationary phase	Mobile phase				
Sofosbuvir and Daclatasvir	UPLC-DAD	BEH C18 (50 × 2.1 mm, 1.7 μm)	Ammonium formate (pH 3.5, 5 mM) and acetonitrile (60:40 v/v)	NM	25 −6400 50 −12800	Plasma	[70]
Sofosbuvir and Daclatasvir	HPTLC with dual wavelength Spectro Densitometer (311, 265 nm)	Silica gel 60 F_{254} aluminum plates	Ethyl acetate-isopropanol (85:15 v/v)	11.3 6.5	40 −640[c] 20 −320[c]	Plasma	[71]
Sofosbuvir and Velpatasvir	HPTLC-UV	Silica gel 60 F_{254} aluminum plates	Ethyl acetate-isopropanol (90:10 v/v)	NM	40 −4000[c] 20 −2500[c]	Plasma	[72]
Sofosbuvir and Ledipasvir	HPTLC-DAD	Silica gel 60 F_{254} aluminum plates	Ethyl acetate: methanol: water: glacial acetic acid (30: 1.5: 1: 0.2% v/v)	210 for plasma 270 for urine 50 for plasma 30 for urine	1000 −20000 200 −6000	Plasma and urine	[73]
Danuravir, Ritonavir, Emtricitabine and Tenofovir	MLC- DAD	C18 (150 × 4.6 mm, 5 μm, pore size 100 Å)	0.06 M Sodium dodecyl sulfate/2.5% 1-pentanol (pH 7)	90 80 110 100	500 −5000 500 −5000 500 −5000 250 −5000	Plasma	[74]

*To facilitate the comparison of the results of the reported studies, units have been converted to (ng/mL).
HPLC: High-performance liquid chromatography, UPLC: ultra-high-performance liquid chromatography, MLC: Micellar liquid chromatography, UV: Ultraviolet-Visible, DAD: diode array detector, FLD: fluorescence detector, RP; reverse phase, NM (not mentioned), HPTLC: high-performance thin-layer chromatography, LOD: Limit of detection, LOQ: Limit of quantification.
[a] (ng/10^6Cells).
[b] (nM).
[c] (ng/band).

this drug and its metabolites in blood samples of the patients treated with this drug is of great importance for the selection of the best dose and duration, due to the narrow therapeutic window of this drug, and has an effective role in its therapeutic response. Therefore, the determination of the exact dose of hydroxychloroquine and its metabolites would be the most critical point for an effective treatment. Recently, hydroxychloroquine concentration levels and its metabolites including desethylhydroxychloroquine (DHCQ) and desethylchloroquine (DCQ) were determined in whole blood using the HPLC-FLD technique. To this end, the separation of HCQ and its metabolites was performed on a phenyl column, the temperature of the auto-sampler was maintained at 4 °C, and a mixture of glycine buffer/sodium chloride (pH 9.7, 100 mM) and methanol (46:54 v/v) was employed as the mobile phase at a flow rate of 1.2 mL min^{-1}. The obtained LOD values were reported to be 25 ng mL^{-1} for HCQ, and 12.5 ng mL^{-1} for DHCQ and DCQ. During the analysis of hydroxychloroquine and its metabolites, no interference with other commonly used drugs as well as endogenous or exogenous compounds was observed in the blood chromatograms of the patients [60].

Other studies conducted for simultaneous analysis of candidate drugs in the treatment of COVID-19 by HPLC/UPLC and the related characteristics of the analytical methods are presented in Table 1.

4.2. High/ultra-performance liquid chromatography-mass spectrometry

Liquid chromatography systems coupled to mass spectrometry (LC-MS and LC-MS/MS) provide unique opportunities to determine drugs in biological samples. The ability to analyze several analytes simultaneously, excellent sensitivity, extraordinary selectivity, reliable identification of new metabolites in biological matrices, confirmation of the identity of known compounds, as well as accurate quantification at very low concentration levels has made this technique a very powerful tool in bioanalysis. Improvements have been made in LC-MS/MS, such as the type and design of the ionization source, ion collectors, collision cells, and hybrid mass analyzers (with higher sensitivity and resolution, and wider dynamic range). These advances in LC-MS/MS have led to further enhancements in identification and analytical capabilities, as well as investigation of the fragmentation behavior of compounds [76,77].

According to our evaluations, the variety of articles regarding the application of LC-MS/MS techniques for the analysis of candidate drugs in the treatment of COVID-19 was great. Thus, their classification was made based on drug groups including anti-Ebola, anti-malaria, anti-influenza, anti-HIV, and anti-hepatitis agents, which are given in Tables 2–4.

Undoubtedly, most of the analyses of candidate drugs for the treatment of COVID-19 have been performed on plasma, serum, dried blood spots, and blood samples. Some studies have also examined samples from hair, tissue, saliva, liver, cerebrospinal fluid, breast milk, and peripheral blood mononuclear cells. According to the analytical goals presented in Fig. 3, in the determination of these drugs, the focus is mostly on full validation of the method, but PK/PD and bioequivalence studies and the investigation of possible drug-drug interactions are also among the targets that have been studied as well. The most common chromatographic column used for the separation of candidate drugs in the treatment of COVID-19 was the C_{18} column. Electrospray ionization (ESI) in positive mode was the preferred ionization method in these

Table 2
Liquid chromatographic methods coupled to MS (LC-MS) and MS/MS (LC-MS/MS) for the determination of candidate drugs in the treatment of COVID-19 from the category of anti-Ebola, Malaria, and Influenza.

Drugs name		Method	Chromatographic conditions			LOD (ng/mL)	Linear range (ng/mL)	Matrices	Ref.
			Mode	Stationary phase	Mobile phase				
Ebola	Remdesivir	UPLC-MS/MS	MRM ESI^+	C18 (150 × 4.6 mm, 3 μm)	A: 0.05% (v/v) Formic acid in ultrapure water B: 100% Acetonitrile with isocratic elution (A: B) 52:48%	0.3	1–5000	Plasma	[78]
	Remdesivir and GS-441524	UPLC-MS/MS	MRM ESI^+	HSS T3 (50 × 2.1 mm, 1.8 μm)	A: Water plus formic acid 0.05% B: Acetonitrile plus formic acid 0.05%	0.24 0.98	3.91–1000	Plasma	[79]
	Remdesivir Remdesivir and GS-441524	LC-MS/MS	MRM ESI^+	RP (100 × 2 mm, 4 μm) C18 (100 × 2.1 mm, 2.6 μm)	1% Formic acid in water (v/v, aqeous) and 1% formic acid in acetonitrile (v/v, organic)	NM 300 2000	0.5–5000 1000–5000000 5000-2500000	Plasma	[80]
		LC-MS/MS	MRM ESI^+		A: 10 mM Sodium formate buffer in 0.1% formic acid B: Acetonitrile starting from 0% of (B) to 100%			Plasma	[81]
	Remdesivir, GS-704277 and GS-441524	LC-MS/MS	MRM ESI^+	HSS T3 (50 × 2.1 mm, 1.8 μm)	A: 10 mM Ammonium formate in 5% methanol, pH 2.5. B: 100% Methanol	NM	4–4000 2–2000 2–2000	Plasma	[82]
Malaria	Hydroxy chloroquine	UPLC-MS/MS	MRM ESI^+	C18 (100 × 2.1 mm, 1.6 μm)	A: 0.01 mol/L Ammonium formate and 0.1% formic acid in water/acetonitrile 95/5 (v/v) B: Acetonitrile	NM	15-1500 for plasma 50-5000 for blood	Plasma and blood	[83]
	Chloroquine and Desethylchloroquine	LC-MS/MS	SRM	SB-CN (50 × 4.6 mm, 3.5 μm)	A: Acetonitrile-ammonium formate 20 mM with 1% formic acid pH 2.6 15/85 (v/v) B: Methanol-acetonitrile 75/25 (v/v)	NM	6–1334	Blood (whole blood, plasma, dried blood spots (DBS)	[84]
	Hydroxychloroquine, Monodesethlhydroxy chloroquine, Desethylchloroquine and Bisdesethylchloroquine	LC-MS/MS	MRM ESI^+	Gold aQ (50 × 3 mm, 3 μm)	Water and methanol acidified with 0.1% formic acid	NM	25–2000	Whole blood	[85]
Influenza	Favipiravir	LC-MS/MS	MRM ESI^+, MRM ESI^-	C18 (50 × 4.6 mm, 5 μm)	A: 0.1% Formic acid in water B: 0.1% Formic acid in methanol	59, 45 (in the positive and negative mode)	48-50000 (in the negative ionization mode) 62–50000 (in the positive ionization mode)	Serum	[86]
	Zanamivir	LC-MS/MS	MRM ESI^+	C18 (50 × 4.6 mm, 5 μm)	0.1% Formic acid and acetonitrile (35:65, v/v)	NM	2.15–64.5	Plasma	[87]
	Amantadine	LC-MS/MS	MRM ESI^+	C18 (150 × 4.6 mm, 4 μm)	Acetonitrile and 10 mM ammonium formate, pH 3.0 adjusted with 0.1% formic acid (80:20, v/v)	0.18	0.50–500	Plasma	[88]

*To facilitate the comparison of the results of the reported studies, units have been converted to (ng/mL).
HPLC: High-performance liquid chromatography, UPLC: ultra-high-performance liquid chromatography, MLC: Micellar liquid chromatography, UV: Ultraviolet-Visible, DAD: diode array detector, FLD: fluorescence detector, RP; reverse phase, NM (not mentioned), HPTLC: high-performance thin-layer chromatography, LOD: Limit of detection, LOQ: Limit of quantification.
[a] (ng/10^6Cells).
[b] (nM).
[c] (ng/band).

studies. Triple quadrupole and quadrupole linear ion traps were the mass analyzers that have been used mainly for the analysis of candidate drugs in the treatment of COVID-19. Determination of drugs has often been performed by multiple reaction monitoring (MRM), which increases the sensitivity and selectivity in quantitative analysis. In limited cases, the mode of selected reaction monitoring (SRM) has also been reported.

Since LC-MS/MS techniques are the most powerful analytical techniques in the field of TDM, sampling approaches emerged as promising tools for sample collection in this field. The use of microsampling methods such as volumetric absorptive microsampling (VAMS) and advanced dried blood spot (DBS) techniques including automated DBS has attracted great attention in the field of TDM. Different microsampling methods along with their advantages are represented in Fig. 4.

VAMS is a polymeric sampling probe that absorbs a constant volume of blood through its tip. The need for low blood volume, overcoming the problems caused by the uneven distribution of blood cells, and homogeneity are among the advantages of this technique [89].

For example, in a research study, the application of VAMS for the determination of hydroxychloroquine and its metabolites in human capillary blood and its comparison with DBS were evaluated with the aim of TDM. In this study, HCQ and the three studied metabolites were measured simultaneously under gradient elution conditions with a flow rate of 0.5 mL min^{-1} and a total run time of

Table 3
Liquid chromatographic methods coupled to MS (LC-MS) and MS/MS (LC-MS/MS) for the determination of candidate drugs in the treatment of COVID-19 from the category of anti-HIV agents.

Drugs name	Method	Chromatographic conditions			LOD (ng/ml)	Linear range (ng/ml)	Matrices	Ref.
		Mode	Stationary phase	Mobile phase				
Tenofovir	LC-MS/MS	MRM ESI^+	C18 (100 × 2.0 mm, 3 μm)	A: 0.1% Formic acid in 5 mM ammonium acetate B: 100% Acetonitrile	NM	10–640	Plasma	[91]
Efavirenz	LC-MS/MS	MRM ESI^-	C18 (100 × 2.1 mm, 3 μm)	A: 1 mmol/L Ammonium acetate in water B: 1 mmol/L Ammonium acetate in acetonitrile	NM	25–5000	Dried Blood Spots	[92]
Maraviroc	LC-MS/MS	MRM ESI^+	C8 (50 × 2.1 mm, 1.7 μm)	A: Water with 0.1% formic acid B: Acetonitrile with 0.1% formic acid	NM	0.5–500	Plasma	[93]
Darunavir and Etravirine	LC-MS/MS	MRM ESI^+	C18 (75 × 2.1 mm, 2.5 μm)	A: Water B: Methanol both containing 10 mM formic acid	0.60 0.62	1.25–125	Peripheral blood mononuclear Cells	[94]
Lopinavir and Ritonavir	LC-MS/MS	ESI^+	C18 (50 × 3 mm, 3 μm)	NM	NM	NM	Plasma	[95]
Tenofovir, Ritonavir and Atazanavir	LC-MS/MS	MRM ESI^+	RP (100 × 2.0 mm, 4 μm)	A: Water B: Acetonitrile both with 0.1% acetic acid added	0.25 0.025 0.001	1–1000	Plasma and peripheral blood mononuclear cells	[96]
Raltegravir, Elvitegravir and Dolutegravir	LC-MS/MS	MRM ESI^+	C18 (50 × 2.1 mm, 3.5 μm)	Acetonitrile/Water (7:3, v/v) containing 0.1% formic acid	NM	5-1500 for plasma 1-200 for CFS	Plasma and cerebrospinal fluid samples	[97]
Nevirapine, Efavirenz and Lopinavir	LC-MS/MS	MRM ESI^+, MRM ESI^-	Kinetex F5 (50 × 2.1 mm, 2.6 μ)	A: Water plus 0.1% formic acid B: Methanol supplemented with 0.1% formic acid	NM	10–10000	Dried blood spots	[98]
Tenofovir, Emtricitabine and Efavirenz	LC-MS/MS	SIM, ESI^+, MRM ESI^-	C18 (50 × 3.0 mm)	Tetrahydrofuran: H2O 60: 40	500	500–300000	Plasma	[99]
Tenofovir, Lamivudine and Nevirapine	LC-MS/MS	MRM ESI^+	C18 (150 × 4.6 mm, 5 μm)	Methanol-Water (80:20, v/v) containing ammonium acetate (2 mM)	160, 30 5, 6 15, 3	416–5000[a] 77–5000[a] 12–5000[a] 15–5000[a] 39–50000[a] 6–50000[a]	Hair	[100]
Etravirine, Maraviroc, Raltegravir and Rilpivirine	LC-MS/MS	SRM	C8, (50 × 2.1 mm, 1.7 μm)	A: Water B: Acetonitrile each containing 0.1% formic acid	NM	1–5000 0.1–1000 1–5000 1–3000	Plasma and tissue	[101]
Abacavir, Tenofovir, Darunavir and Raltegravir	LC-MS/MS	MRM ESI^+	C18 (50 × 1.5 mm, 5 μm)	for Abacavir and Tenofovir; 5 mM Formic acid: Acetonitrile (3:97, v/v) for Darunavir and Raltegravir; 5 mM Formic acid: Acetonitrile (35:65, v/v)	NM	1–10000	Plasma and saliva	[102]
Zidovudine, Efavirenz, Lopinavir and Ritonavir	LC-MS/MS	MRM ESI^+	C18 (150 × 4.6 mm, 5 μm)	MeOH: deionized Water (90:10, v/v) containing ammonium acetate (2 mM, pH 4.5)	18 8 5 6	36–5000[a] 16–5000[a] 10–50000[a] 12–12500[a]	Hair	[103]
Tenofovir, Emtricitabine, Elvitegravir and Rilpivirine	LC-MS/MS	MRM ESI^+	Restek Pinnacle DB Biph (50 × 2.1 mm, 5 μm)	A: 0.1% Formic acid in water B: 0.1% Formic acid in acetonitrile (45:55 v/v)	NM	10–2000	Dried blood spots	[104]
	LC-MS/MS	MRM ESI^+	Phenyl-hexyl (100 × 3 mm, 5 μm)	A: Water B: Methanol both with 0.05% (v/v) formic acid	NM	20-5000 for Dolutegravir Elvitegravir	Plasma	[105]

(*continued on next page*)

Table 3 (*continued*)

Drugs name	Method	Chromatographic conditions			LOD (ng/ml)	Linear range (ng/ml)	Matrices	Ref.
		Mode	Stationary phase	Mobile phase				
Dolutegravir, Elvitegravir, Rilpivirine, Darunavir, Ritonavir, Raltegravir and Raltegravir-β-D-glucuronide						10-2500 for Raltegravir, Raltegravir-β-D-glucuronide, Ritonavir, Rilpivirine 60-15000 for Darunavir		
Entecavir, Lamivudine, Telbivudine and Tenofovir	UPLC-MS/MS	MRM ESI$^+$	HSS T3 (150 × 2.1 mm, 1.8 μm)	A: Water plus formic acid 0.05% B: Acetonitrile plus formic acid 0.05%	0.02 4.9 9.8 7.8	0.039−10 9.8−2500 19.5−5000 15.6−4000	Plasma	[106]
Zidovudine, Lamivudine, Lopinavir And Ritonavir	UPLC-MS/MS	MRM ESI$^+$	C18 (50 × 2.1 mm, 1.7 μm)	A: 0.1% Formic acid in water B: Acetonitrile	NM	12.5−750 50−2500 100−5000 5−250	Breast milk	[107]

*To facilitate the comparison of the results of the reported studies, units have been converted to (ng/mL).
LC-MS: Liquid chromatographic Mass Spectrometry, LC-MS/MS: Liquid chromatographic Tandem Mass Spectrometry, UPLC: ultra-high-performance liquid chromatography, MRM: multiple reaction monitoring, SRM: selected reaction monitoring, SIM: selected ion monitoring, ESI$^+$: electrospray ionization in positive mode, ESI$^-$: electrospray ionization in negative mode, NM (not mentioned), LOD: Limit of detection, LOQ: Limit of quantification.
b (nM).
c (ng/band).
[a] (ng/10^6Cells).

6.5 min. The results indicated that the VAMS sampler exhibited less variability in the volume of absorbed blood compared to DBS. Therefore, it would be possible to obtain more accurate and precise data [90].

Another sampling device based on fully automatic DBS systems with high throughput that can be directly coupled to LC-MS/MS system is DBS-MS 500. This system is different in terms of total capacity and type of sample extraction method. It can accommodate 500 DBS cards in one analysis. This method uses direct elution and horizontal extraction techniques in the integrated step. The analysis of nevirapine, efavirenz, and lopinavir drugs has been reported to be done using a DBS-MS 500 autosampler, which was coupled to an LC system connected to quadrupole ion trap tandem MS. This study aimed to improve the sensitivity of the method and the analysis of a large number of samples in TDM experiments. MRM transitions were measured in the positive mode for nevirapine and lopinavir, and in the negative mode for efavirenz. A comparison of the automatic and manual methods indicated that the obtained results were similar and in agreement with the criteria mentioned in EMA. Thanks to less solvent consumption, the automated method was five times more sensitive than the manual one [98].

Drug Metabolism and Pharmacokinetic card (DMPK) is among the common DBS cards, which are divided into three categories. DMPK-C is one of the categories of this card, which is more suitable for protein-based biomolecules and is less affected by the matrix effect. In a recent study, the LC-MS/MS was used for the quantification of chloroquine and desethylchloroquine in plasma, whole blood, and DBS. Moreover, the effect of using DMPK-C was evaluated and compared with other filter papers. Identification was done using an API 5000 triple quadrupole mass spectrometer in positive mode, and SRM transitions were assessed. The evaluation of filter papers used for DBS revealed that the type and thickness of the filter paper affect the amount of absorbed blood, the homogenous distribution of the blood spot, and the physical size of the blood spot. The results demonstrated that the concentrations of chloroquine and its metabolites were higher in whole blood or DBS compared to plasma due to accumulation in red blood cells. The stability evaluation of chloroquine and desethylchloroquine in all three matrices indicated the possibility of long-term storage. The report of PK studies confirmed the suitability of the method for the analysis of clinical pharmacokinetic samples [84].

In another study, hydroxychloroquine and its major metabolites including DCQ, DHCQ, and BDCQ were analyzed by ion-pairing HPLC-FLD in blood samples of patients with systemic lupus erythematosus. In order to further affirm the suitability of the proposed method, the same blood samples of patients were analyzed by LC-MS/MS technique. The comparison of the two employed analytical techniques was demonstrated by heat map and Bland-Altman plot. A comparison of the obtained concentrations of HCQ and its metabolites displayed a fundamental agreement between these two techniques [57].

In addition, the comparison of the analytical performance characteristics of LC-MS/MS and direct infusion MS/MS, which can be used in TDM of candidate drugs for the treatment of COVID-19, has been evaluated in some studies. As an instance, the analysis of three drugs including efavirenz, emtricitabine, and tenofovir (which make up the single tablet of Atripla) has been carried out using the two mentioned methods. To this end, the mass spectra of the analytes were obtained using a quadrupole ion trap mass analyzer in both positive and negative modes. Three scanning modes including full, SIM, and MS/MS (precursor ion) were used to analyze the individual components of Atripla. By the use of a *t*-test, the average recovery percentages of the two methods were compared. For efavirenz, there was no statistically significant

Table 4
Liquid chromatographic methods coupled to MS (LC-MS) and MS/MS (LC-MS/MS) for the determination of candidate drugs in the treatment of COVID-19 from the category of anti-hepatitis agent.

Drugs name	Method	Chromatographic conditions			LOD (ng/mL)	Linear range (ng/mL)	Matrices	Ref.
		Mode	Stationary phase	Mobile phase				
Telbivudine	LC-MS/MS	MRM ESI^+	C18 (100 × 3.0 mm, 3 μm)	A: 0.1% Formic acid in purified water (v/v) B: Acetonitrile	NM	10–10000	Plasma	[108]
Sofosbuvir	UPLC-MS/MS	MRM ESI^+	C18 (50 × 4.6 mm, 5 μm)	0.5% Formic acid: Methanol (30:70, v/v)	NM	4.063–8000	Plasma	[109]
Sofosbuvir	UPLC-MS/MS	MRM ESI	C18 (50 × 2.1 mm, 1.7 μm)	Acetonitrile-0.1% formic acid (50:50, v/v)	NM	0.25–3500	Plasma	[110]
Sofosbuvir and GS-331007	UPLC-MS/MS	MRM ESI^+	C18 (50 × 2.1 mm, 1.7 μm)	Acetonitrile:5 mM ammonium formate: 0.1% formic acid (85:15:0.1% v/v/v)	0.353	1–1000 10–1500	Plasma	[111]
Sofosbuvir and GS-331007	UPLC-MS/MS	MRM ESI^+	C18 (50 × 2.1 mm, 1.8 μm)	0.1% Formic acid and acetonitrile (50:50, v/v)	NM	10–2500	Plasma	[112]
Sofosbuvir and Velpatasvir	UPLC-MS/MS	MRM ESI^+	C18 (50 × 2.1 mm, 1.7 μm)	Acetonitrile-0.1% formic acid (50: 50, v/v)	NM	0.25–3500 1–1000	Plasma	[113]
Sofosbuvir and Daclatasvir	LC-MS/MS	MRM ESI^+	C18 (50 × 4.6 mm, 5 μm)	5 mM Ammonium formate buffer (pH 3.5): Acetonitrile (50:50, v/v)	NM	0.3–3000 3–3000	Plasma	[114]
Sofosbuvir and Ledipasvir	LC-MS/MS	MRM ESI^+	C18 (100 × 4.6 mm, 5 μm)	10 mM Ammonium acetate, pH 4.0 by acetic acid-acetonitrile-0.1% methanolic formic acid (12: 25: 63, v/v/v)	NM	0.5–2500 5–2100	Plasma	[115]
Sofosbuvir and Velpatasvir	LC-MS/MS	MRM ESI^+	C18 (100 × 4.6 mm, 5 μm)	0.1% Formic acid in water: Acetonitrile: Methanol (30:60:10, v/v/v)	NM	5–5000 10–1500	Plasma	[116]
Elbasvir and Grazoprevir	LC-MS/MS	NM	NM	NM	NM	0.25–500 1.0–1000	Plasma	[117]
Daclatasvir, Asunaprevir and Beclabuvir	LC-MS/MS	MRM ESI^+	YMC Basic S-5, (50 × 2.0 mm, 5 μm)	A: 10 mM Ammonium acetate in water B: 10 mM Ammonium acetate in methanol C: 10 mM Ammonium acetate in acetonitrile	NM	1–1000 1–1000 2–2000	Plasma	[118]
Sofosbuvir, GS-331007 and Ledipasvir	LC-MS/MS	MRM ESI^+	C8 (50 × 4.6 mm,5 μm)	A: Ammonium formate buffer (pH 3.5; 10 mM) B: 40% Acetonitrile, 60% methanol	NM	0.3–3000 3.0–3000 0.1–1000	Whole blood	[119]
Sofosbuvir, GS-331007 and Daclatasvir	UPLC-MS/MS	MRM ESI^+	C18 (50 × 2.1 mm, 1.6 μm)	A: Water containing 0.1% formic acid B: Acetonitrile	NM	11.71–3000 19.53–5000 11.71–3000	Plasma	[120]
Dasabuvir, Ombitasvir and Paritaprevir	UPLC-MS/MS	SRM ESI^+	C18 (2.1 × 50 mm, 1.7 μm)	A: Water: Acetonitrile: Formic acid (95:5:0.1 v/v/v) B: Acetonitrile with 0.1% formic acid	NM	12.5–5000 1.25–2500 5.00–5000	Liver	[121]
Simeprevir, Daclatasvir, Sofosbuvir and GS-331007	LC-MS/MS	MRM ESI^+	C18 (50 × 2 mm)	A: Water plus 0.1% formic acid B: Methanol plus 0.1% formic acid	NM	15.6–2000	Plasma	[122]
Daclatasvir, Elbasvir, Grazoprevir, Ledipasvir, Simeprevir, Sofosbuvir and Velpatasvir	UPLC-MS/MS	MRM ESI^+	BEH C18 (2.1 × 50 mm, 1.7 μm)	A: 10 mM Ammonium formate with 0.005% formic acid in water at pH 4.5 B: 10 mM Ammonium formate with 0.005% formic acid in methanol C: 10 mM Ammonium formate with 0.005% formic acid in a mixture of 10 mM ammonium formate with 0.005% formic acid in a mixture of methanol and acetonitrile (10:90)	NM	10000–5000000 3000–1500000 3000–1500000 7500–1500000 10000–5000000 5000–2500000 7500–1500000	Plasma	[123]
Dolutegravir, Elvitegravir, Rilpivirine, and other thirteen antiretroviral	UPLC-MS/MS	MRM ESI^+ for all drugs except for Efavirenz	HSS T3 (150 mm × 2.1 mm, 1.8 μm)	A: Water plus 0.05% formic acid B: Acetonitrile plus 0.05% formic acid	3.9 351.6	1600–1444000	Plasma	[124]

*To facilitate the comparison of the results of the reported studies, units have been converted to (ng/mL).
LC-MS: Liquid chromatographic Mass Spectrometry, LC-MS/MS: Liquid chromatographic Tandem Mass Spectrometry, UPLC: ultra-high-performance liquid chromatography, MRM: multiple reaction monitoring, SRM: selected reaction monitoring, SIM: selected ion monitoring, ESI^+: electrospray ionization in positive mode, ESI^-: electrospray ionization in negative mode, NM (not mentioned), LOD: Limit of detection, LOQ: Limit of quantification.
a (ng/10^6Cells).
b (nM).
c (ng/band).

difference between the recoveries obtained by the two methods, whereas, for tenofovir and emtricitabine, the evaluations indicated a significant difference in recovery values obtained by the two methods [99].

In situations where a large number of analytes must be monitored for a specific diagnostic purpose in biological environments, LC-MS/MS assays provide the possibility of accurate quantification of several analytes simultaneously. For example, the levels of six drugs including dolutegravir, elvitegravir, rilpivirine, darunavir, ritonavir, raltegravir, and its main metabolite raltegravir-β-D-glucuronide were determined simultaneously in human plasma using isotopically labeled internal standards. The purpose of the evaluation was to monitor drug therapy and drug PK studies. Negative electrospray ionization mode for raltegravir-β-D-glucuronide and positive mode for other drugs were carried out using the MRM strategy. For the separation of the analytes, a gradient elution and a flow rate of 0.5 mL min^{-1} were applied. The response for each drug was confirmed by the selection of two mass transitions, one with the aim of structural verification (secondary ion) and the other transition for quantification (primary ion). Eventually, C_{trough} values in plasma samples of six pregnant women (during the third trimester of pregnancy) who were treated with a combination of the mentioned drugs were reported [105].

The extremely high efficiency of UPLC and the high sensitivity and specificity of MS/MS detection have made UPLC-MS/MS techniques very suitable for the analysis of a wider range of drugs, and provide the requirements for bioequivalence, PK/PD, and TDM studies. In a research study, the determination of 17 drugs (three drugs including dolutegravir, elvitegravir, and rilpivirine, along with 13 other drugs) was performed using UPLC-MS/MS in human plasma. Quinoxaline was selected as the internal standard because of its reasonable price, availability, and good properties in terms of ionization and recovery. Analytes were separated by gradient elution, using the flow rate of 0.5 mL min^{-1} and a total run time of 15 min. All drugs were analyzed in positive ionization mode with triple quadrupole, except for efavirenz, which was detected in negative ionization mode. For each drug, two mass transitions were monitored for quantification and confirmation. C_{trough} and C_{max} for all drugs and the areas under the plasma concentration-time curves (AUCs) for rilpivirine, elvitegravir, and dolutegravir were reported [124].

In addition, seven drugs including daclatasvir, elbasvir, grazoprevir, ledipasvir, simeprevir, sofosbuvir, velpatasvir, and the main metabolite of sofosbuvir (GS-331007) have been quantified in human plasma by UPLC-MS/MS technique. The analyses were performed using a UPLC system coupled to a TQ-S micro tandem mass spectrometer in positive ionization mode for 12 min, and isotope-labeled internal standards were used for quantification. The calibration range was selected to cover the expected concentrations (C_{min} and C_{max}, AUCs) to be used in the clinical evaluation of patients treated with these drugs and to investigate the PK of drugs in clinical studies [123].

Other research studies on simultaneous analysis of candidate drugs for the treatment of COVID-19 in binary, ternary, and multiple

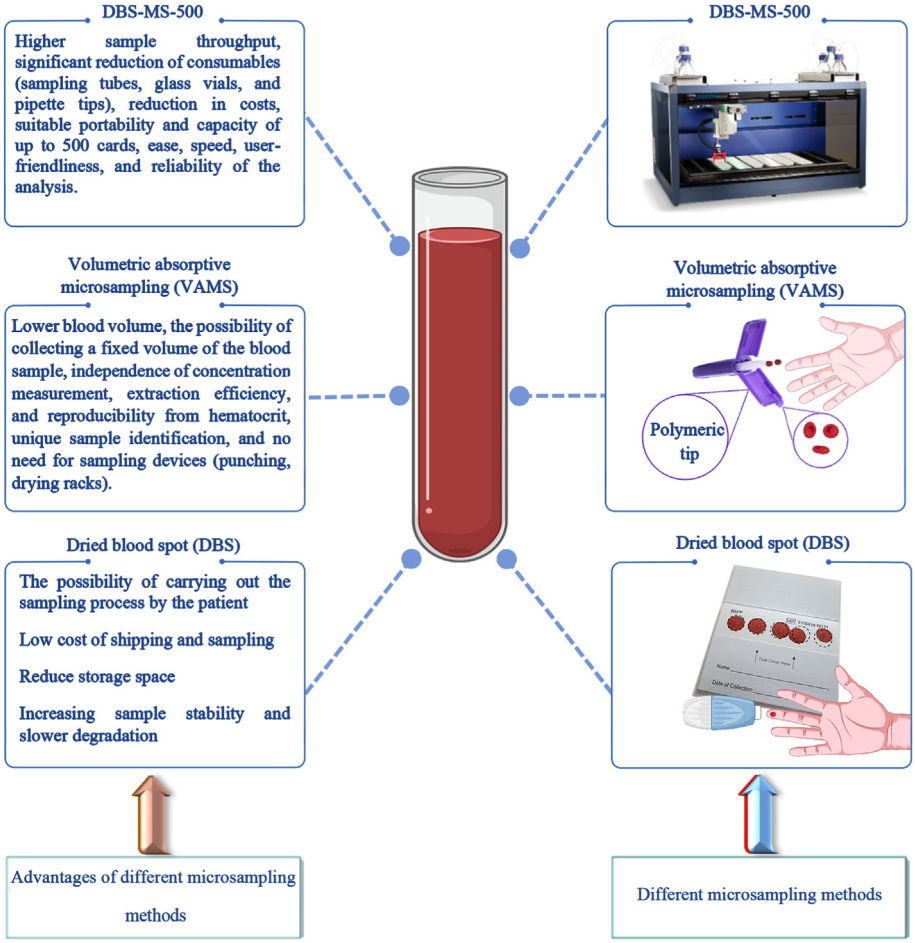

Fig. 4. Different microsampling methods and their advantages.

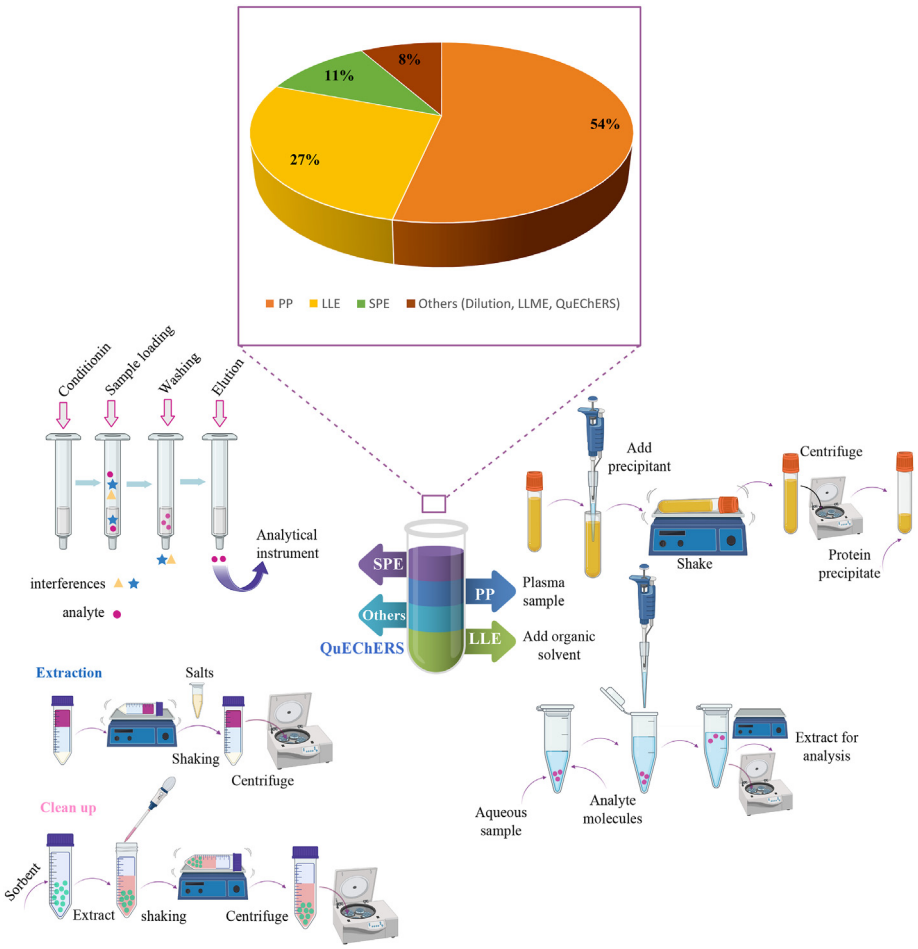

Fig. 5. Percentage and procedure of different sample preparation methods reported for the extraction of candidate drugs, PP: protein precipitation, LLE: liquid-liquid extraction, SPE: solid-phase extraction, LLME: liquid-liquid microextraction, dilution, and QuEChERS: quick, easy, cheap, effective, rugged, and safe.

mixtures by LC-MS and LC-MS/MS techniques, along with the characteristics of the analytical methods and their performance in terms of linear range, LOD, LOQ, and matrices are reported in individual drug groups in Tables 2–4.

4.3. Sample preparation techniques

In biological matrices, the target analytes usually exist in trace amounts below the detection limit of the measuring equipment in the presence of large amounts of other components present within the matrix. Thus, prior to the instrumental analysis, the selection of the appropriate sample preparation method is the most critical step in the entire analytical process. This step aims to clean the sample and transform it into a suitable form for measurement, remove interfering components from such complex matrices, and preconcentrate the sample to improve the detection limit of the method. Sample preparation has a significant impact on the accuracy and repeatability of the obtained data. Therefore, in addition to the achievement of the main objectives, a sample preparation step should be robust, simple, fast, cost-effective, and selective, with high throughput and automation capabilities for the analysis of analytes in biological matrices [125,126].

According to the reports presented in Tables 1–4, liquid chromatography with various detection techniques including UV and fluorescence spectroscopy and MS spectrometry would be the preferred method for the analysis of candidate drugs for the treatment of COVID-19. The percentage of various methods of sample preparation in liquid chromatography with different detectors for the analysis of candidate drugs in the treatment of COVID-19 is depicted in Fig. 5. As can be seen, protein precipitation (PP), liquid-liquid extraction (LLE), and solid-phase extraction (SPE), respectively, are widely used as common and traditional techniques to extract candidate drugs for the treatment of COVID-19 from biological matrices. The removal of proteins and phospholipids, which cause ion suppression, would be one of the most important objectives of sample preparation before analysis with MS detection.

Despite the widespread use of LLE as a traditional method, its disadvantages have created an incentive for the development of innovative LLME. The LLME techniques have been developed towards major reductions in solvent consumption, transfer of target analytes into a few microliters of solvent, and pre-concentration from small volumes of biological fluid. SPE has also been widely used because of its remarkable benefits such as simplicity, low organic solvent consumption, as well as offline and online automation capabilities; however, disadvantages such as long analysis time, high cost due to the consumption of cartridges, and the need for sophisticated equipment have led researchers to the development of miniaturized sample preparation methods [127].

In a research study, vortex-assisted salt-induced liquid-liquid microextraction (VA-SI-LLME) sample preparation method was used to determine remdesivir in human plasma using UPLC-PDA

and UPLC-MS/MS techniques for clinical studies. Factors affecting the sample preparation method including the type and volume of the extraction solvent, extraction time, and the type of salt and its amount were investigated. The type of solvent was selected according to maximum drug extraction capacity, lower density compared to water, the ability to be dissolved in the aqueous phase, and protein precipitation ability in the plasma sample. The highest extraction recovery was obtained at 120 s. Among the investigated salts ($(NH_4)_2SO_4$), $MgSO_4$, Na_2SO_4, and NaCl), the maximum extraction recovery was reported for 2.5 g of ammonium sulfate. This process was carried out with low organic solvent consumption and low cost [66].

Among LLME techniques, dispersive liquid-liquid microextraction (DLLME) has been introduced as a separation method with high extraction speed, remarkable pre-concentration factor, and acceptable recovery. It would not require special equipment and is generally a low-cost and easy operation. Nevertheless, the use of solvents denser than water, which remains at the end of the test tube after centrifugation, is a weakness of many reported DLLME methods. Solidifying and replacing the extraction solvents with low density would be a suitable suggestion to achieve phase separation in DLLME, which is called the solidification of floating organic droplet (DLLME-SFO) [128,129]. It is possible to combine this method with most of the other sample preparation techniques. For an instance, the SPE-DLLME-SFO method (combining the advantages of both methods) has become a powerful tool for the selective determination of trace amounts of analytes in biological matrices.

In a recent study, SPE-DLLME-SFO was used for extraction, preconcentration, and determination of nevirapine, efavirenz, and nelfinavir in plasma samples by HPLC-UV. A C_{18} solid-phase extraction cartridge was used for sample preparation. The optimization of the SPE method was carried out based on the one variable at a time method and the factors of flow rate and breakthrough volume were evaluated. To optimize the DLLME method, first and foremost, the types of extracting and dispersing solvents were optimized separately. In the next step, a two-level fractional factorial design was performed to screen the main effective factors in the extraction process and to investigate their interactions. Finally, the main variables of the DLLME method including the pH of the sample, the volumes of extracting and dispersing solvents, ionic strength, extraction time, and centrifugation time were optimized using the response surface method based on its central composite design [130].

Dispersive micro-solid phase extraction (d-SPE) is one of the miniaturized preparation methods, which would be an alternative to conventional SPE. In this method, by dispersing a small amount of adsorbent (in milligrams) in sample matrices, a close interaction would be created between the adsorbent particles and the analyte, and as a result, the efficiency of the process would increase. The key differences between SPE and d-SPE, such as the difference in availability of the sorbents, the time and method of extraction, and the use of nanomaterials have drawn more attention to the miniaturized format of SPE to overcome its drawbacks. Low consumption of organic solvent and sorbent, high extraction efficiency, and short time requirement are among the advantages of d-SPE [131]. As a matter of the fact, sorbents play a vital role in obtaining high extraction efficiency. Due to the high surface area-to-volume ratio, nanostructured sorbents have a higher absorption capacity compared to other material structures. The use of magnetic nanoparticles as sorbents for the extraction of analytes is very common due to the shorter analysis time and the possibility of easier separation from the matrices. However, disadvantages such as stability and low selectivity, as well as easy agglomeration have caused the modification of their surface with different coatings to be expanded. Metal-organic frameworks (MOFs) have been also used as sorbents and to modify the surface of nanoparticles because of their porous structure, adjustable shape and size, and high specific surface area. Molecularly imprinted polymers (MIPs) are the most prominent polymeric sorbents for the selective determination of target analytes in complex biological samples. These sorbents are prepared by the sol-gel or polymerization method and are molded according to the shape and size of the analyte molecule during the synthesis process using a three-dimensional polymer network containing specific holes for a template molecule. High selectivity and mechanical stability, the possibility of specific absorption of desired compounds, and high resistance under different conditions are the most outstanding advantages of these sorbents [131,132]. In a study, hydroxychloroquine was determined in human serum samples through the d-SPE method using a core-shell Ni magnetic nanoparticles- MIL-100(Fe) with an imprinted layer on its surface as a sorbent. The MIP sorbent was synthesized using the sol-gel method, in which hydroxychloroquine, (3-aminopropyl) triethoxysilane, and tetraethyl orthosilicate acted as template molecule, the monomer, and cross-linker agent, respectively. Satisfactory extraction efficiency, low LOD values, and high enrichment factor have been reported with this method to determine small amounts of drugs in human serum samples using HPLC-UV detection. The combination of the advantages of MOF, MIP, and easy separation by magnetic core has resulted in a high mass transfer rate and short extraction time [133].

Efavirenz was also extracted from human serum and urine samples by MIP nanoparticles as a selective sorbent in the d-SPE technique, which was then determined by HPLC-UV analysis. The sorbent was prepared through miniemulsion polymerization using efavirenz and methacrylic acid as template molecules and monomers, respectively. The possibility of better access to the imprinted cavity and the quick equilibrium of the analyte using imprinted nanoparticles have been established. Removal of interfering peaks from biological matrices, acceptable extraction of analyte from the real sample, and high accuracy and precision for bioequivalence analysis of efavirenz in serum and urine are the reported features of this method [134].

QuEChERS stands for quick, easy, cheap, effective, rugged, and safe, which has gained wide acceptance for the extraction of various analytes including drugs from biological matrices. This method includes three main stages: LLE between the organic and aqueous phases with minimum consumption of organic solvent, salting-out stage to improve the liquid-liquid partition, and clean-up stage by dispersive solid phase extraction (d-SPE) using magnesium sulfate ($MgSO_4$) and a sorbent such as primary secondary amine (PSA). Preventing the loss of the target analyte, increasing the recovery, and concerning the principles of environmentally friendly analytical chemistry are the important advantages of this technique [135].

In a study, QuEChERS, LLE, and PP sample preparation techniques, as well as their binary mixed modes (QuEChERS-PP, QuEChERS-LLE, and LLE-PP) were compared for analysis of efavirenz, emtricitabine, tenofovir, lopinavir, and ritonavir in human plasma using LC-MS/MS based on extraction efficiency, precision, accuracy, limits of detection (LODs), lower limits of quantification (LLOQs), and upper limits of quantification (ULOQs). Among the extraction techniques, QuEChERS was selected as the best sample preparation method for the determination of antiretroviral drugs in human plasma because of its high efficiency and simplicity. In addition, reports have indicated that mixed modes exhibit lower recovery and accuracy [136].

5. An overview of the analysis of drugs evaluated with the specific purpose of treatment of patients with COVID-19 during the outbreak

To deal with COVID-19 during the outbreak and emergency conditions, clinical research studies on drugs including hydroxychloroquine, remdesivir, favipiravir, lopinavir, ritonavir, and azithromycin were carried out to generate reliable PK/PD data, determining the optimal therapeutic dose of the drug, the best duration of treatment, and the evaluation of drug-drug interactions in polytherapy.

The first antiviral drugs approved by the FDA during the COVID-19 pandemic were remdesivir and hydroxychloroquine. In a study, LC-MS/MS was used to determine remdesivir and its metabolites using electrospray ionization in positive mode with a triple quadrupole mass analyzer. Remdesivir-$^{13}C^{6}$ was used as the internal standard to reduce the matrix effect. This method was applied to PK studies in a critically ill patient with COVID-19 who was admitted to the ICU. C_{max} and half-life were evaluated in the treated patient. Since the evaluations in this method have been reported using a minimum volume of plasma (50 μL), this method might be suitable for children as well [81].

In another study, hydroxychloroquine was also determined and validated as an FDA-approved drug by UPLC-MS/MS in blood and plasma samples during the COVID-19 pandemic. HCQ-d5 was used as the internal standard. The analyses were carried out using a C_{18} column under the positive mode of electrospray ionization in MRM mode with a triple quadrupole mass spectrometer for 4 min. In this study, 90 plasma and six whole blood samples from patients with COVID-19 were investigated to evaluate TDM. The LOQ values for plasma and blood samples were reported to be 15 and 50 ng mL^{-1}, respectively [83].

The drug levels of favipiravir were also measured using LC-MS/MS in patients with COVID-19, by a mass spectrometer detector equipped with an electrospray ionization source in MRM and both positive and negative modes for a period of 3.5 min. Atorvastatin was considered as a surrogate internal standard. This method was used to measure the drug levels in random serum samples of 55 patients with COVID-19 who received favipiravir. Out of 55 serum samples, 30 samples were used to compare the serum and plasma. The linear ranges in positive and negative ionization modes were reported to be 50-0.062 and 50−0.048 μg mL^{-1}. The values of LOD and LOQ were determined to be 0.059 and 0.062 μg mL^{-1} for the positive mode, and 0.045 and 0.048 μg mL^{-1} for the negative mode, respectively [86].

In a study, the concentrations of lopinavir and ritonavir were measured by LC-MS/MS method to investigate the population PK of lopinavir in 13 patients admitted to the intensive care unit with an acute respiratory infection. The effect of variables such as age, height, body mass index, and gender on the model was investigated. The presented results indicated that by taking 400 and 100 mg of lopinavir and ritonavir twice a day, respectively, the median concentration of lopinavir in patients was measured between 20 and 30 mg L^{-1}. Moreover, the simulated model revealed that by taking 400 mg of lopinavir twice a day, approximately 40% of patients received less drug than the minimum effective concentration to fight the virus. In this study, due to the limited number of patients, it was not possible to evaluate all PK parameters [95].

In dealing with complex matrices, in some cases, conventional liquid chromatography would not be sufficient. In these cases, the use of multidimensional liquid chromatography (MD-LC) techniques might be a suitable solution. Currently, the two-dimensional liquid chromatography (2D-LC) technique is commonly used to analyze the desired compounds in complex matrices by targeted or non-targeted methods. Using this technique, the need to clean up the sample would be eliminated, thus reducing the overall analysis time [45]. Simultaneous determination of seven drugs including remdesivir and its metabolites GS-441524, chloroquine, hydroxychloroquine, lopinavir, ritonavir, favipiravir, and azithromycin has been performed using two-dimensional isotope dilution LC-MS/MS in human serum. In this study, isotopically labeled drugs were used as internal standards for all analytes. Identification of all analytes and internal standards was carried out by electrospray ionization in the positive mode in MRM using a triple quadrupole mass spectrometer. Patients received the studied drugs according to the National Institutes of Health's COVID-19 treatment guidelines. Six serum samples from patients receiving the mentioned drugs were analyzed using this method. Analytes were determined with different concentration ranges, using a uniform injection volume of 5 μL. Thirty samples of patients under special care who were treated with drugs other than the aforementioned drugs of this study confirmed the selectivity of the assay for the studied analytes [137].

6. Conclusion

Highly sensitive and selective bioanalytical quantification techniques are essential for the simultaneous determination of several drugs, especially in cases where the sample volume is very low and the biological matrices are complex. According to the number of reports presented for the determination of candidate drugs in the treatment of COVID-19, the analytical methods using LC-MS/MS provide reliable results for validation of the method and evaluation of clinical samples. Therefore, this method in the future can be a useful tool to evaluate the optimal therapeutic range and to correct treatment management of COVID-19 with candidate drugs.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Zahra Talebpour reports financial support was provided by Alzahra University. Zahra Talebpour reports a relationship with Alzahra University that includes: board membership. No Conflict of interest.

Data availability

Data will be made available on request.

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To draw the figures, some elements were used from the free section of https://biorender.com/library. The authers appreciate this site.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.trac.2023.116964.

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