Menthol-assisted homogenous liquid-liquid microextraction for HPLC/UV determination of favipiravir as an antiviral for COVID-19 in human plasma

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ABSTRACT

Favipiravir is a promising antiviral agent that has been recently approved for treatment of COVID-19 infection. In this study, a menthol-assisted homogenous liquid–liquid microextraction method has been developed for favipiravir determination in human plasma using HPLC/UV. The different factors that could affect the extraction efficiency were studied, including extractant type, extractant volume, menthol amount and vortex time. The optimum extraction efficiency was achieved using 300 μL of tetrahydrofuran, 30 mg of menthol and vortexing for 1 min before centrifuging the sample for 5 min at 3467g. Addition of menthol does not only induce phase separation, but also helps to form reverse micelles to facilitate extraction. The highly polar favipiravir molecules would be incorporated into the hydrophilic core of the formed reverse micelle to be extracted by the non-polar organic extractant. The method was validated according to the FDA bioanalytical method guidelines. The developed method was found linear in the concentration range of 0.1 to 100 μg/mL with a coefficient of determination of 0.9992. The method accuracy and precision were studied by calculating the recovery (%) and the relative standard deviation (%), respectively. The recovery (%) was in the range of 97.1–103.9%, while the RSD (%) values ranged between 2.03 and 8.15 %. The developed method was successfully applied in a bioequivalence study of Flupirava® 200 mg versus Avigan® 200 mg, after a single oral dose of favipiravir administered to healthy adult volunteers. The proposed method was simple, cheap, more eco-friendly and sufficiently sensitive for biomedical application.

1. Introduction

The coronavirus outbreak is one of the most intimidating infectious diseases that has been threatening the lives of millions of people. For this reason, the World Health Organization has declared the outbreak a pandemic in March 2020 [1]. Yet, the numbers of infections are still rising to unpreceded limits. The urgent need to discover new drugs to face the COVID-19 pandemic ended up with a number of potential drugs, including hydroxychloroquine, ivermectin, remdesivir, and favipiravir [2]. Favipiravir is an antiviral agent that prevents RNA viral replication [3–5]. The initial results of clinical trials indicated that favipiravir was a good candidate for COVID-19 treatment as it enhanced viral clearance and improved signs and symptoms [6,7]. Favipiravir was first approved in Japan for influenza virus treatment. Recently, several countries gave rapid approval for favipiravir to be included in COVID-19 treatment protocols, including Egypt, Italy, Saudi Arabia, United Arab of Emirates, Japan, Russia, India and Turkey [8].

A few analytical methods have been reported for sensitive favipiravir determination in pharmaceutical formulation or biological fluids, including spectrofluorimetry [9,10] and LC–MS/MS [11–13]. Spectrofluorimetric determination of drugs in biological fluids is not sufficiently selective compared to LC-MS/MS, due to the possibility of interference from endogenous substances and other co-administered drugs. Although LC-MS/MS is highly sensitive and selective, the instrument is sophisticated and the analytical methods are not cost-effective.

HPLC/UV is the most common technique in biomedical analysis and it requires efficient sample treatment before injection. Liquid-liquid extraction is a widely used method for the preparation of biological samples for HPLC/UV determination. However, LLE consumes large amounts of solvents, utilizes non-ecofriendly health hazardous substances and has limited efficiency in the extraction of polar drugs such as favipiravir (log p = 0.25) [10].
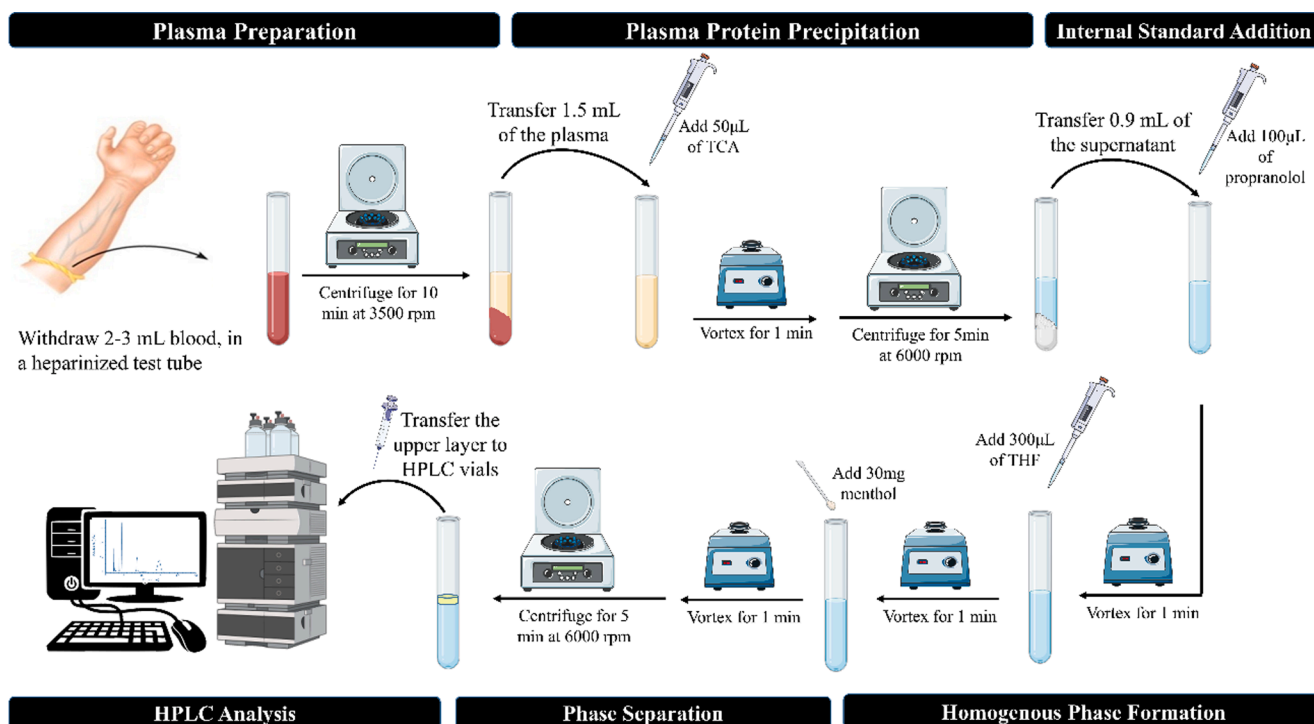

Fig. 1. Procedures of favipiravir sample preparation by the proposed menthol-assisted homogenous liquid–liquid microextraction method.

Homogeneous liquid–liquid extraction (HLLE) is a modified form of LLE in which water miscible organic solvents are used as extractants [14]. In HLLE, the organic solvent is mixed with the aqueous sample to form a homogenous phase, before being separated by addition of salt or sugar. The hydrophilic nature of the extractants in HLLE allows extraction of highly polar analytes [15,16]. Hydrophobic solvent-assisted HLLE (HSLLE) is as a special mode of HLLE in which a hydrophobic solvent (such as chloroform [17], dichloromethane [18] or toluene [19]) is used to induce phase separation instead of salts and sugars. Compared with salting out and sugaring-out, HSLLE gives better phase separation and requires smaller amounts of the phase separating agents. In addition, the polarity of the extractant can be adjusted by proper selection of the phase separating agent, while salts and sugars only expel the extractant from the aqueous sample without affecting its polarity or physico-chemical properties.

In this work, menthol was introduced for the first time as a phase separating agent in HSLLE instead of chloroform. Compared with the reported phase separating agents in HSLLE, menthol was safer to the analyst and the environment. In addition, menthol is more polar than chloroform, dichloromethane and toluene, which makes menthol more suitable for HSLLE of polar drugs. The novel extraction system was applied in a bioequivalence study of Flupirava® 200 mg (European Egyptian Pharmaceutical Industries, Egypt) versus Avigan® 200 mg (Man. by Toyama Chemical Co. Ltd, Japan), after a single oral dose of favipiravir administered to healthy Egyptian adult volunteers. Menthol is more eco-friendly, safer to human, easily recyclable and is available from natural sources.

2. Experimental

2.1. Chemical and reagents

Favipiravir (99.7%) was kindly supplied from Liptis Egypt Pharmaceuticals (new Cairo, Egypt), propranolol (99.8%) was kindly supplied from AstraZeneca (new Cairo, Egypt), acetonitrile, potassium dihydrogen phosphate, phosphoric acid and methanol were purchased from Merck (Darmstadt, Germany), tetrahydrofuran was purchased from

Universal Fine Chemicals (Sanborn, NY, USA), menthol (99%) was purchased from Sigma Aldrich (St. Louis, MO, USA), ethanol, acetone, isopropanol, glycerol and propylene glycol were purchased from Piochem (Giza, Egypt), chloroform was purchased from Fisher (New Hampshire, United States) and trichloroacetic acid was purchased from Fine Chem Limited (Mumbai, Maharashtra 400030, India). Human plasma samples were kindly provided by Vacsera National Blood Bank (Giza, Egypt).

2.2. Instrumentation

The determination of favipiravir was done on a DionexUltiMate 3000 HPLC (Thermo Scientific™, Dionex™, Sunnyvale, CA, USA). The instrument composed of a WPS-3000TSL autosampler, an LPG-3400SD quaternary pump, a VWD-3000 variable wavelength detector and a TCC-3000SD column thermostat. Data processing and acquisition were carried out by Chromeleon 7 software. Tabletop Cyan-CL008 centrifuge (Hulshout, Belgium) was used for protein precipitation and phase separation. The pH values were adjusted by Jenway® 3510 pH-meter (Staffordshire, UK).

2.3. Chromatographic conditions

The chromatographic conditions for favipiravir and propranolol (internal standard) separation were achieved by using a mobile phase consisting of 50 mM phosphate buffer (pH = 2.5) and acetonitrile in a ratio of 60: 40, v/v. The injection volume was 5 μL, the flow rate was 1 mL/min and the detection wavelength was set at 323 nm. Chromatographic separation was achieved on a Thermo® Hypersil ODS C_{18} column (150 mm × 4.6 mm,5 μm), at a column temperature of 30 °C.

2.4. Stock and working solutions

Stock solutions of favipiravir and propranolol (1 mg/mL) were separately prepared in methanol, and stored at 4 °C until use. Working solutions were prepared by appropriate dilutions of stock solutions with double distilled water to obtain favipiravir concentrations in the range

0.1–100 μg/mL, mixed with a constant concentration of 10 μg/mL of propranolol in each solution as an internal standard. All stored solutions were brought to room temperature before use.

2.5. Procedure of plasma extraction

During method development, an aliquot of human plasma (1500 μL) was spiked with different concentrations of favipiravir to attain the desired therapeutic levels of the drug, then a 50 μL of trichloroacetic acid was added. Trichloroacetic acid disrupted the electrostatic interactions, which destabilized the plasma proteins, causing unfolding, intermolecular coalescence and protein precipitation [20]. The tube was vortexed for 1 min and centrifuged for 5 min at 3467g. Then a 900 μL of the clear supernatant was transferred to another 5 mL screw cap glass test tube containing 100 μL of propranolol (10 μg/mL) as an internal standard. The tube was then vortexed for 1 min and a 300 μL of THF was added, vortexed again for 1 min for efficient mixing. After forming the homogeneous phase, 30 mg of menthol was added to the previous homogeneous solution and the tube was vortexed for 1 min, then centrifuged for 5 min at 3467 g to induce phase separation. The upper layer was pipetted (≈20 μL) and was transferred into an HPLC vial for analysis. The same procedures were applied during a bioequivalence study for real sample analysis after plasma preparation, by collecting 2–3 mL of blood samples in a heparinized test tube, followed by centrifugation for 10 min at 1180 g. The procedures of sample treatment and analysis are summarized in Fig. 1.

2.6. Method validation

The HPLC method validation was performed according to the US Food and Drug Administration for bioanalytical method validation guidelines [21] with respect to linearity, range, limit of quantitation (LOQ) accuracy, precision and stability.

2.6.1. Linearity

The calibration curve was constructed by plotting the peak area ratio of favipiravir to propranolol (IS) against the nominal standard concentration. The favipiravir concentrations used were 0.1, 0.25, 0.5, 1 ,5 ,10 ,20 ,40 ,80 and 100 μg/mL. The calibration curve was used to calculate the concentrations of quality control (QC) and study samples. At lower LOQ (LLOQ), the acceptance criteria for the calibrators should be ± 20 % of the nominal standard concentrations, while other QC samples should be ± 15%.

2.6.2. Accuracy and precision

The intra-day accuracy and precision were evaluated by analyzing six replicates containing favipiravir at the four QC levels: LLOQ, low QC (LQC), medium QC (MQC) and high QC (HQC), which were 0.1, 0.75,50 and 85 μg/mL respectively. The inter-day accuracy and precision were done by analyzing six replicates containing favipiravir at four QC samples on three runs. The proposed method's accuracy was expressed as % recovery. According to the FDA guidelines, the recovery (%) should not exceed 15% for all the QC levels except for the LLOQ, for which it was set as ± 20% of the nominal values. Precision was evaluated by calculating the relative standard deviation RSD (%). The acceptance criteria for RSD (%) are ≤ 15% at all levels and ≤ 20% at the LLOQ.

2.6.3. Stability

The stability study was done at different storage conditions for benchtop and freeze–thaw of favipiravir in human plasma. The benchtop stability study was conducted after storing the sample at room temperature for 4 h. The freeze–thaw stability study was conducted as 3 cycles. At each cycle, samples were frozen for 12 h, then LQC and HQC samples were analyzed to assess the stability of favipiravir in different conditions. The results were then compared with freshly prepared samples. Samples were stable if the RSD (%) was within ± 15%
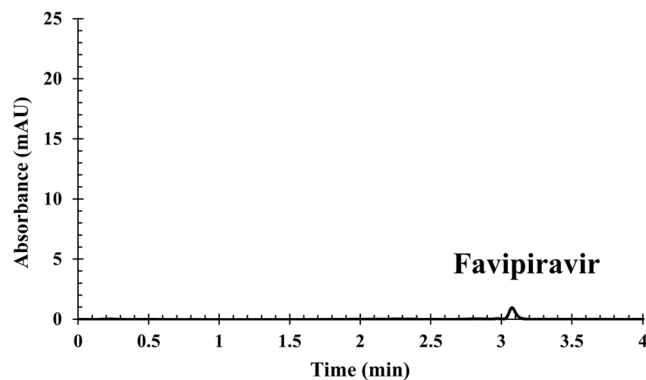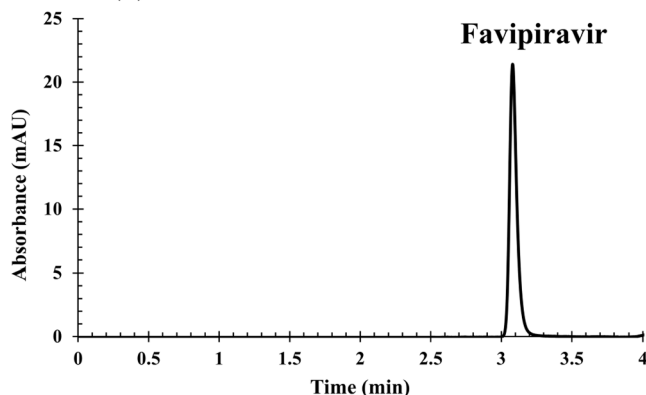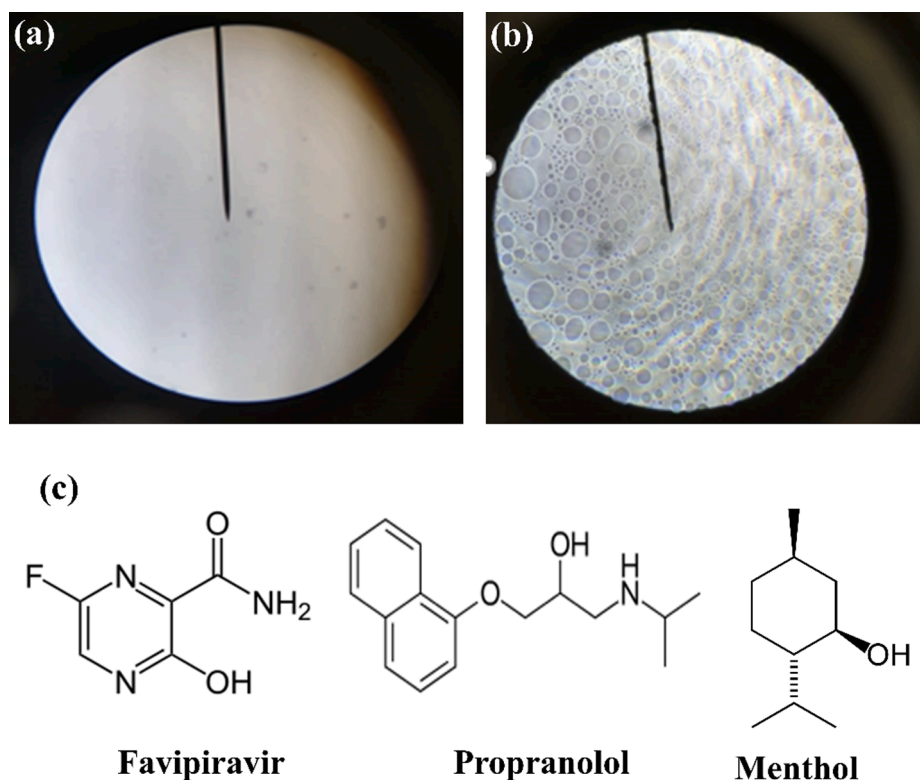

Fig. 2. Chromatographic separation of favipiravir using (a) chloroform-assisted homogenous liquid–liquid extraction and (b) menthol-assisted homogenous liquid–liquid extraction**.** Chromatographic conditions: Column: Thermo Hypersil ODS C18 (250 mm × 4.6 mm, 5 μm) at 30 °C, Mobile phase: acetonitrile: phosphate buffer (50 mM, pH 2.5) (40:60, v/v), Elution: Isocratic, Detection: DAD at 323 nm, Flow rate: 1 mL/min, Injection volume: 5 μL.

compared to the freshly prepared ones.

2.7. Application in a pharmacokinetics study

The HPLC method was developed and validated to support a clinical study which investigate the bioequivalence of one tablet of Flupirava® 200 mg (European Egyptian Pharmaceutical Industries, Egypt) Versus Avigan® 200 mg (Man. by Toyama Chemical Co. Ltd, Japan), after a single oral dose administered to healthy adult volunteers under fasting conditions. The study was an open label, randomized, single dose, two-way crossover bioequivalence study in healthy human Egyptian volunteers under fasting conditions. The inclusion criteria were Egyptian healthy volunteers of both sexes, aged between 18 and 55 years. A pilot study investigating the bioequivalence of Flupirava® and Avigan® 200 mg tablets was performed on four subjects. A single dose of Flupirava® 200 mg (Test product) was administered by each subject followed by a washing-out period of one week before taking a single oral dose of Avigan® 200 mg (Reference product). Blood samples were collected in heparinized tubes at 0, 0.16, 0.33, 0.5, 0.66, 0.83, 1, 1.25, 1.5, 2, 2.5, 3,4, 6, 8, 12, 24 h after oral administration. The collected blood samples were centrifuged at 3500 rpm for 10 min and then transferred directly into 5 mL plastic tubes. The plasma samples were stored at −80 °C until analysis. Model-independent, non-compartmental pharmacokinetic analysis was performed using the PK-Solver 2.0 (China Medicine University, China) [22], depending on concentration versus time data of favipiravir. The estimated parameters were the maximum observed

Fig. 3. The light microscope images of the extracts in chloroform-assisted homogenous liquid–liquid microextraction (a), and menthol-assisted homogenous liquid–liquid microextraction (b). Chemical structures of favipiravir, propranolol as an internal standard and menthol (c).

concentration (C_{max}), the minimum observed concentration (C_{min}), the time at which maximum concentration was observed (T_{max}) and the total area under the curve ($AUC_0 \rightarrow t$).

3. Results and discussion

The reported HSLLE system (water/acetonitrile/chloroform) was used as a starting point for extraction of favipiravir. The amounts were adjusted using the smallest possible volume of chloroform, to induce phase separation. The aqueous samples were mixed with 500 μL acetonitrile to form a homogenous phase before adding 40 μL of chloroform as a phase separating agent. The same procedures were repeated using 40 mg menthol instead of chloroform, and the upper layers in both experiments were collected and injected into the HPLC instrument. It is here worth mentioning that the remaining amount of chloroform were sedimented in the bottom of the test tube, while the residual menthol formed a solid disc at the interface (**Figure S1**). These residues were discarded while the floating upper layers of acetonitrile that contained the analyte were injected.

The extraction efficiencies in both experiments were compared. As shown in Fig. 2, menthol-assisted HLLE was 25 times more efficient in extracting favipiravir compared with chloroform-assisted HLLE. This surprisingly higher extraction efficiency, compared with the reported system, could be explained by investigating the extract under the microscope. As shown in Fig. 3, menthol-assisted HLLE resulted in the formation of vesicles, which could be due to reverse micelle formation. The chemical structure of menthol shows a polar hydroxyl group and a nonpolar chain of carbons, which could urge the menthol molecules to aggregate in the form of reverse micelle to decrease contact surface with the organic extractant. The surfactant-like properties of menthol and the ability to form micelles have been previously reported [23,24]. This interesting property could be exploited to extract hydrophilic analytes, such as favipiravir, which could be entrapped in the polar core of the reverse micelle and be eventually extracted from the aqueous sample.

Accordingly, menthol was used instead of chloroform, not only because of the health hazards of chloroform, but also because of the high enrichment observed with menthol-assisted HLLE.

3.1. Microextraction optimization

Besides using menthol as a phase separating agent, the other variables that may affect the extraction efficiency were studied, including extractant type, extractant volume, menthol amount and vortex time. Optimization of these parameters was performed using one-variable-at-a-time (OVAT). The enrichment factor was the parameter used to evaluate the effect of each variable on extraction efficiency.

3.1.1. Extractant type optimization

The effect of extractant type was studied by trying different water miscible solvents including acetonitrile, acetone, ethanol, methanol, propylene glycol, glycerol, tetrahydrofuran (THF), and isopropyl alcohol. A volume of 500 μL of each solvent was added to 1 mL of aqueous sample, spiked with favipiravir at a concentration of 10 μg/mL. The tubes were vortexed for 1 min, then 40 mg of menthol was added to the previous homogeneous solution, vortexed again for 1 min and centrifuged at 3467 g for 5 min. The results showed that no phase separation was achieved with methanol, propylene glycol and glycerol. The separated floating layers in the other organic solvents were collected and injected into the HPLC instrument, and the enrichment factors were calculated. As shown in Fig. 4a, the maximum enrichment was attained using THF, which could be due to the relatively low polarity of THF, which enhances the probability to form reverse micelles. Consequently, THF was selected as the optimum extractant in the following procedures.

3.1.2. Tetrahydrofuran volume

The extractant volume is one of the most important experimental variables that could affect the enrichment in HLLE. Generally, the analyte pre-concentration is inversely proportional to the volume of
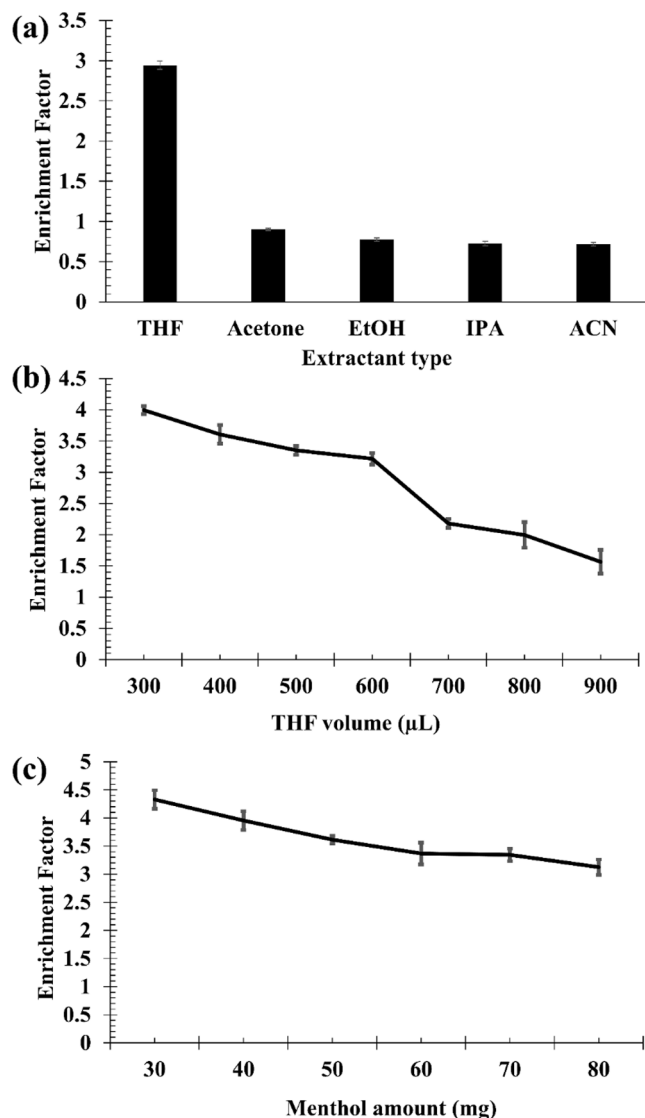
Fig. 4. Effect of extractant type (a), THF volume (b), and menthol amount (c) on the efficiency of menthol-assisted homogeneous liquid–liquid microextraction of favipiravir. Favipiravir concentration: 10 μg/mL, Centrifugation time: 5 min, Chromatographic conditions: Column: Thermo Hypersil ODS C18 (250 mm × 4.6 mm, 5 μm) at 30 °C, Mobile phase: acetonitrile: phosphate buffer (50 mM, pH 2.5) (40:60, v/v), Elution: Isocratic, Detection: DAD at 323 nm, Flow rate: 1 mL/min, Injection volume: 5 μL. The error bar represents the standard deviation of three readings.

extractant [25], and thus small volumes of THF were tested in the range of 100–900 μL. The results (Fig. 4b) showed that the enrichment was improved by decreasing the THF volume down to 300 μL. Using lower volumes of THF was not practically possible because no sufficient phase separation was achieved. The decrease in enrichment factors with the increase in THF volumes could be explained by the dilution effect, which decreases the analyte concentration and reduces the resultant enrichment. Since the volume required of THF is small, and more importantly, the volume retrieved after phase separation is ≈20 μL, the developed method can be referred to as a form of homogenous liquid–liquid microextraction (HLLME). This acronym will be used from now forth to refer to the developed method.

3.1.3. Amount of menthol

Menthol is widely used as a hydrogen bond acceptor (HBA) in preparation of deep eutectic solvents (DES) [26–32]. In addition,

menthol is used as a co-acervating agent in the menthol/water/decyl amine system [33]. Recently, menthol has been used as a liquid extractant after being melted for extraction of benzoic acid from berry juice [34]. The amount of menthol is critical in menthol-assisted HLLME. Using very small amounts of menthol may not be sufficient to induce phase separation, while large amounts could ruin the performance. The effect of menthol amount was investigated in the range of 10–80 mg; no phase separation was observed with 10 and 20 mg, while 30 mg was the lowest amount of menthol able to achieve phase separation. As shown in Fig. 4c, the enrichment was inversely proportional to the amount of menthol with the maximum enrichment attained using 30 mg. Thus, 30 mg of menthol were utilized as the amount of the phase separating agent in the following procedures.

3.1.4. Vortex time

Studying the effect of vortex time during the phase separation step was performed. Different vortex times were investigated in the range of 1 min to 6 min. As shown in **Figure S2**, vortex time had no marked effect on extraction efficiency. So, 1 min was selected as a sufficient vortex time during phase separation. Based on these results, the optimum conditions for extraction of favipiravir using menthol-assisted HLLME included mixing 300 μL of THF with 1 mL of the aqueous sample, and vortexing for 1 min before adding 30 mg of menthol and revortexing for 1 min to ensure efficient mixing before phase separation by centrifugation for 5 min at 3467 g. The developed method was applied for determination of favipiravir in spiked human using propranolol as an internal standard. As shown in Fig. 5a, the separation was acceptable and the retention times were congruent with retention times of real plasma samples from healthy volunteers, enrolled in a favipiravir bioequivalence study (Fig. 5b). The composition ratio of the extract phase was determined using Karl Fischer and GC/MS, and was found to be 66:27:7, w/w/w for THF, menthol and water, respectively. To confirm our hypothesis of the formation of reverse micelle, the extractant was collected and investigated under the microscope under the optimum conditions. As shown in **Figure S2**, the light microscope images showed vesicles of different size and number compared with those formed using ACN (Fig. 3b). **Figure S2** also shows that the reverse micelles formed in THF were smaller in size, larger in number, more homogenous and more organized. The substantially lower dielectric constant of THF compared with acetonitrile and the other tested solvents (**Table S1**) could explain these differences in shape and performance.

3.2. Method validation

The developed method of menthol-assisted HLLME for HPLC/UV determination of favipiravir in human plasma was validated according to the US Food and Drug Administration (FDA) bioanalytical method guidelines [21]. The different validation parameters including linearity, range, accuracy, precision, limit of quantitation and stability were studied.

3.2.1. Linearity, range and limit of quantitation

The calibration curve was constructed by plotting the peak area ratios (favipiravir to propranolol) against concentrations of favipiravir. The linearity range was found to be 0.1 to 100 μg/mL, with a correlation coefficient of 0.9992, which shows an excellent level of linearity. Regression analysis was performed to calculate the slope, the intercept, the standard errors around the slope and the intercept. Limit of quantitation was designated by practically determining the lowest concentration of favipiravir in human plasma that can be measured accurately and precisely. Table 1 summarizes the results of regression analysis of the developed method. These results show that the developed menthol-assisted HLLME method is sufficiently sensitive for determination of favipiravir in human plasma.
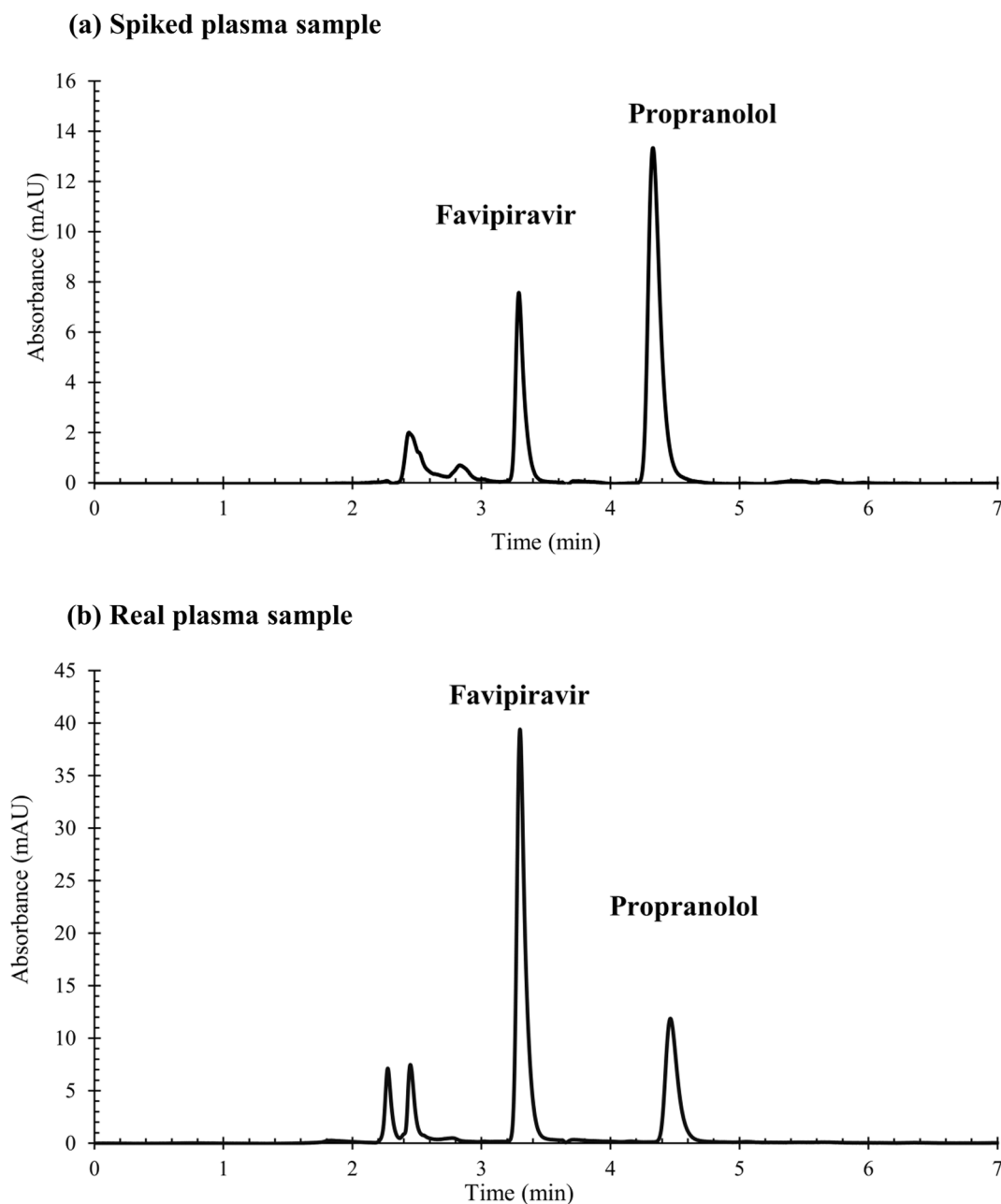
Fig. 5. Representative HPLC chromatograms for favipiravir in spiked plasma samples (a), and in real plasma sample (b) from a healthy volunteer administered one tablet of Flupirava® 200 mg under fasting conditions. The real sample was collected at Tmax (0.5 h). Chromatographic conditions: Column: Thermo Hypersil ODS C18 (250 mm × 4.6 mm, 5 μm) at 30 °C, Mobile phase: acetonitrile: phosphate buffer (50 mM, pH 2.5) (40:60, v/v), Elution: Isocratic, Detection: DAD at 323 nm, Flow rate: 1 mL/min, Injection volume: 5 μL.

Table 1
Regression analysis and quantitative data of favipiravir determination in plasma using menthol- assisted homogeneous liquid–liquid microextraction.

Compound	a	Sa	b	Sb	Sy/x	r	Range (μg/mL)	LLOQ (μg/mL)
Favipiravir	0.126	7.6×10^{-2}	0.433	1.7×10^{-3}	2.2×10^{-1}	0.9992	0.10–100	0.10

a: intercept, *Sa:* standard error of intercept, *b:* slope, *Sb:* standard error of slope, *Sy/x:* residual standard deviation of the regression line, *r:* coefficient of determination, LLOQ: lower limit of quantitation.

3.2.2. Accuracy and precision

Accuracy and precision were evaluated at four levels of QC samples, including LLOQ, LQC, MQC and HQC, each analyzed in six replicates. The recovery (%) was calculated to assess the method accuracy, while repeatability and intermediate precision were evaluated according to the RSDs (%) within-day and between-days, respectively. As shown in

Table 2, the recovery (%) ranged between 97.1 and 103.9%, while the RSD (%) was in the range of 2.03 to 8.15 %. These results show that the method accuracy, intra- and inter-day precision were accepted according to FDA bioanalytical method validation guidelines.

Table 2

Intra-day and inter-day precision and accuracy for favipiravir determination in human plasma using the proposed method (n = 6).

Parameter	Accuracy & Precision					
	Intraday			Interday		
	Added (µg/mL)	Found (µg/mL)	Recovery (%) ± RSD	Added (µg/mL)	Found (µg/mL)	Recovery (%) ± RSD
LLOQ	0.10	0.10	99.89 ± 8.15	0.10	0.099	97.746 ± 7.07
LQC	0.75	0.73	97.33 ± 2.97	0.75	0.75	99.98 ± 3.46
MQC	50.00	51.95	103.90 ± 3.93	50.00	50.48	100.96 ± 4.14
HQC	85.00	85.20	100.24 ± 1.91	85.00	84.38	99.27 ± 2.04
Mean			100.34			99.49
RSD (%)			4.24			4.18

Table 3

Benchtop and freeze/thaw stability results (n = 6).

	Added (µg/mL)	Found (µg/mL)	Recovery (%)	RSD (%)	RE (%)
Bench top	0.75	0.76	101.54	2.12	1.47
	85.00	85.96	101.13	4.76	1.13
Freeze and thaw (1st cycle)	0.75	0.76	102.11	5.27	2.00
	85.00	83.00	97.65	2.97	2.35
Freeze and thaw (2nd cycle)	0.75	0.77	102.32	4.24	2.26
	85.00	87.26	102.66	7.36	2.66
Freeze and thaw (3rd cycle)	0.75	0.76	100.82	4.78	0.80
	85.00	81.77	96.20	5.92	3.79

RE: Relative error.

3.2.3. Stability

The stability of favipiravir was studied in spiked plasma samples, during processing and storage conditions. The stability was investigated at two levels: LQC and HQC, and the results were compared with initial concentration of favipiravir in freshly prepared and immediately processed samples. Table 3 shows the results of stability studies of favipiravir under benchtop and freeze/thaw conditions. The calculated concentrations of 0.75 and 85 µg/mL were within the range of ±15% as recommended by the FDA guidelines, which proves that favipiravir was sufficiently stable for routine analysis and at least three cycles of freeze and thaw.

3.3. Pharmacokinetics application and quantification of favipiravir in human plasma

The validated HPLC method was successfully applied for the determination of favipiravir in plasma samples obtained from a bio-equivalence study after oral administration of a single dose of Flupirava® 200 mg (Test product) and Avigan® 200 mg (Reference product). The C_{max} of Favipiravir was 6.40 µg/mL and 5.83 µg/mL after oral administration of Avigan® 200 mg and Flupirava® 200 mg; respectively. The T_{max} was 0.5 h for both test and reference products. The C_{min} values were 0.44 µg/mL and 0.43 µg/mL for reference and test products. While the $AUC_{0 \to t}$ for favipiravir was 23.04 µg/mL*h and 19.51 µg/mL*h for reference and test products; respectively. Fig. 6 shows the plasma concentration–time profile of Avigan® 200 mg (Reference product) versus Flupirava® 200 mg (Test product).

4. Comparison between the developed method and reported methods for favipiravir determination in biological matrices

A few reported methods were developed for determination of favipiravir in biological matrices. To the best of our knowledge, there is only one reported HPLC/UV method for favipiravir determination in biological samples, but it is not sufficiently sensitive for determining therapeutic levels of favipiravir in plasma due to the limited sensitivity and the high LOQ [35]. The other published methods adopted for favipiravir determination in biological matrices were LC-MS/MS, voltammetry and spectrofluorimetry as indicated in Table 4. Menthol-assisted homogeneous liquid–liquid microextraction achieved comparable and even lower LOQ than some reported LC-MS/MS. This could be due to the sample preparation procedures used in LC-MS/MS determination of favipiravir that involved protein precipitation, which is dramatically affecting the method sensitivity. The low LOQ attained with the spectrofluorimetric techniques are due to the inherent sensitivity of spectrofluorimetric methods. However, spectrofluorimetric methods are not
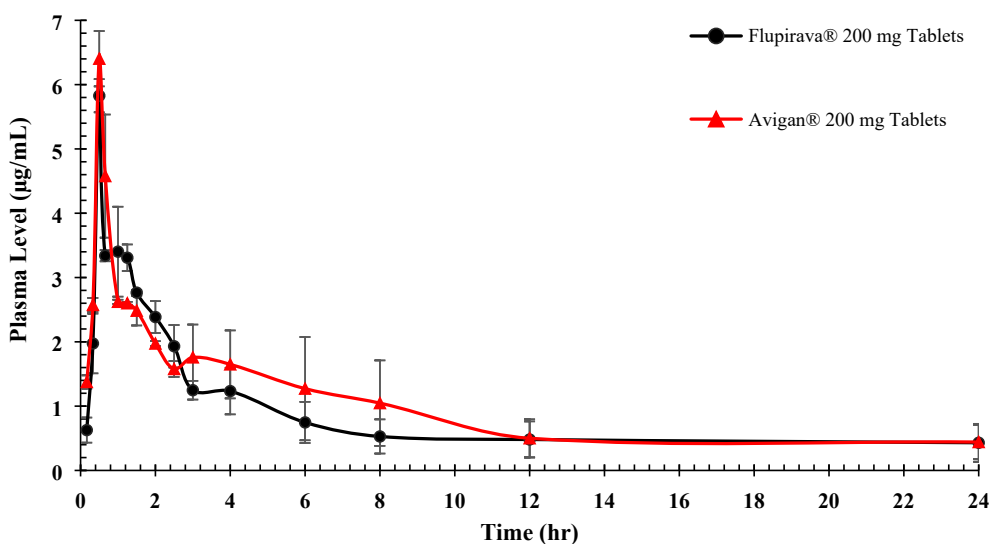
Fig. 6. Favipiravir plasma concentration – time profile of four subjects after a single oral administration of Avigan® 200 mg (Reference product) versus Flupirava® 200 mg (Test product). The error bar represents the standard deviation of the different subjects.

Table 4

Comparison between the developed method and reported methods for favipiravir determination in biological matrices.

Sample	Sample preparation	Detection technique	Linearity(ng/mL)	RSD (%)	LOQ (ng/mL)	Ref
Spiked human plasma	Protein precipitation by methanol using 1000 µL of plasma.	Spectrofluorimetricmethod	40–280	0.85	40	[9]
Spiked human plasma	Protein precipitation by acetonitrile using 1000 µL of plasma.	Spectrofluorimetric Method	20–350	7.60	20	[10]
Human plasma	Protein precipitation by acetonitrile using 200 µL of plasma.	UPLC–MS/MS	250–16000	13.51	250	[12]
Human serum	Protein precipitation and solid phase extraction using 50 µL of serum.	LC–MS/MS	3291–20790	5.57	3291	[11]
Human plasma	Protein precipitation by methanol using 500 µL of plasma.	LC-MS/MS	100–20000	7.11	100	[13]
Human serum	Protein precipitation by acetonitrile using 900 µL of serum.	LC-MS / MS	48–50000	8.00	48	[35]
Human urine	None using 500 µL of urine.	SW-AdSV	10–100 / 100–20000	≤8.80	9.30 / 76	[36]
Human plasma	None using 20 µL of plasma.	SWV	4.5–8640	≤1.53	4.5	[37]
Human plasma	Liquid-liquid extraction by dichloromethane using 1000 µL of plasma.	HPLC/UV	3000–60000	3.04	3000	[34]
Human plasma	Menthol-assisted HLLME using 1500 µL of plasma.	HPLC/UV	100–100000	8.15	100	This work

SWV: Square Wave Voltammetry; SW-AdSV: Square-wave adsorptive stripping voltammetry. Spiked samples were prepared by adding known amounts of favipiravir to blank human plasma.

sufficiently selective for biological samples, especially when other drugs are co-administered. Cyclic voltammetry is highly sensitive, but the instrument is not always available in drug monitoring laboratories and regularity health authorities require separation-based methods for bioequivalence studied. Our proposed method is sensitive enough for real plasma sample analysis, with acceptable accuracy and precision. Higher sensitivities are expected by coupling this sample preparation method with inherently sensitive detectors such as mass spectroscopy and fluorescence. In addition, our menthol-assisted HLLME method is greener, simpler, and cheaper than protein precipitation and solid phase extraction methods. It is also worth mentioning that combining our proposed sample preparation method with sensitive analytical techniques such as LC-MS/MS is expected to substantially improve the sensitivity to unattained levels and to solve extraction problems for other highly polar drugs.

5. Conclusion

A new microextraction technique was developed namely, menthol-assisted homogeneous liquid- liquid microextraction for preparation of favipiravir in human plasma samples for HPLC/UV determination. The method was more economic, simpler and greener compared to the conventional sample preparation methods. In addition, the presented method had comparable or even higher sensitivity compared with the reported LC-MS/MS method. Menthol-assisted homogeneous liquid-liquid microextraction provides a promising microextraction sample preparation technique for polar drug with no need to use expensive and sophisticated instruments like LC-MS/MS. The method was sensitive, accurate, precise and successfully used to extract favipiravir from real human plasma samples that were obtained for a bioequivalence study to investigate a reference and test products of favipiravir as a promising COVID-19 antiviral drug.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author statement

IAA participated in the study design and the results discussion and revised the manuscript. SFH participated in the study design and the results discussion and revised the manuscript. AB conducted the practical work, participated in the study design, the results discussion and the manuscript writing. FRM proposed the study design, participated in the results discussion, manuscript preparation, revision and submission.

Ethical Statement

This study was approved by the Ethical Committee of Scientific Research in the Faculty of Pharmacy, Tanta University, Egypt

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jchromb.2021.123087.

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