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Eco-friendly UPLC-MS/MS analysis of possible add-on therapy for COVID-19 in human plasma: Insights of greenness assessment

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ABSTRACT

Facing the pandemic COVID-19 is of highest priority for all researchers nowadays. Recent statistics indicate that the majority of the cases are home-treated. Two drugs of interest, Guaifenesin and Bromohexine HCl, are among the add-on therapy for treatment of COVID-19 mild cases, which has raised the need for their simultaneous determination. The analysis of the two drugs of interest was described using ultra-performance liquid chromatography–tandem mass spectrometric (UPLC–MS/MS) in plasma of healthy human volunteers using tetryzoline HCl as an internal standard (IS) after liquid–liquid extraction. The applied chromatographic conditions were Kinetex C_{18} (100 Å, 2.6 μm X 50 mm X 4.6 mm) column and a mixture of methanol: water (95: 5, v/v) as a mobile phase at flow rate 1 mL/min. The positive ionization mode was used for detecting the ions, by observing the pairs of transition m/z 199 < 125 for GUF, m/z 377 < 114 for BRM and m/z 201 < 131 for IS. The linearity range was from 50 to 1500 ng/mL for GUF and 0.5–50 μg/mL for BRM. Limit of detection (LOD) was found to be 35.16 and 0.43 ng/ml for GUF and BRM, respectively. The method was validated according to FDA guidance. The proposed method was assessed to be more eco-friendly versus the reported method using the greenness assessment tools: National Environmental Methods Index (NEMI), Assessment of Green Profile (AGP), Green Analytical Procedure Index (GAPI) and Eco-Scale. The proposed method was applied for the application of a pilot pharmacokinetic study.

1. Introduction

By the end of year 2019, the focus of scientists and researchers all over the world were attracted towards confronting the outbreak of pandemic COVID-19. According to WHO, the total number of confirmed cases was around 89 million cases by the beginning of year 2021 [1]. Various symptoms were reported to accompany COVID-19 such as fatigue, fever, diarrhea, cough and short breath [2]. Over the past year, several therapies were described against COVID-19 including Chloroquine/ Hydroxychloroquine [3] and antiviral agent (Remdesivir), in addition to adjuvant therapies such as corticosteroids [4], anticoagulants [5] mucolytic and expectorants [6].

This work describes the determination of two drugs, bromhexine HCl (BRM) and guaifenesin (GUF), which are commonly used for the treatment of cough as a symptom of COVID-19. Moreover, the potential of BRM as an add-on therapy was recently discussed [7]. Add-on therapy is defined as a treatment which bolster or support the effectiveness of a previous one. The transmembrane protease serine 2 (TMPRSS2) enzyme is responsible for breaking or priming of the S protein required for the viral fusion to the host cell at the ACE2 receptor followed by viral entry and replication, as shown in Supplementary Material Fig. 1SM. Several studies showed that at maximum clinical doses of BRM, (TMPRSS2) is strongly and specifically inhibited [8,9], which can block the pulmonary virus infection [10]. Through the second wave of pandemic, the number of COVID-19 cases among children has increased [1], where the role of BRM becomes remarkable due to the reported studies of the use of BRM in pediatric COVID-19 cases along with its rare side effects [11,12]. Meanwhile, the medical centers for Universities of Nebraska–Lincoln [13] and Maryland [14] recommended the use of preparations containing GUF such as Mucinex® for the home treatment and relief of symptoms.

Guaifenesin (GUF) is (RS)-3-(2-methoxyphenoxy) propane-1,2-diol. GUF is commonly used as an expectorant. It helps to relief the chest congestion and cough caused by common cold and bronchitis through reducing the viscosity of mucus in the respiratory tract which makes the cough easier. Bromhexine (BRM) is 2,4-Dibromo-6-([cyclohexyl

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(methyl)amino] methyl) aniline. BRM is used as a mucolytic as it works by loosening the mucus. BRM increases the production of mucus in the airways, thus decreases the viscosity of phlegm and promotes the coughing out. GUF and BRM are over-the-counter medications usually co-administrated due to their synergistic effect in treatment of chest congestion and cough. The chemical structures are shown in Supplementary Material Fig. 2SM.

Chromatography is the benchmark among other analytical techniques for several reasons including: providing reliable analytical data with good accuracy and precision [15,16]; the possibility of separation and identification of countless mixtures with complexity such as enantiomers [17–20] or natural extracts [21–23] and the availability of different detection approaches that serves the diversity of samples' nature such as ultraviolet detection [24,25], fluorescence [26] or mass spectrometry [27]. Ultra-Performance liquid chromatography (UPLC) is an effective technique defined by smaller particle size, extended capacity with decreased time and solvent consumption. The maximum benefit is achieved when merging the possibilities of UPLC with MS/MS detection which is defined by its sensitivity and specificity. UPLC coupled with MS/MS detection is the method of choice for analysis of different matrices such as biological tissues or fluids [28], food samples [29,30], environmental samples [31–33].

After comprehensive literature review, GUF and BRM were determined by several chromatographic methods either alone [34,35] or in combination with other drugs or metabolites [36–47]. Although GUF and BRM were usually administered simultaneously, only one chromatographic method (HPLC/MS/MS method) was reported for their simultaneous determination of both drugs in spiked human plasma [48], but this method was not applied on real human samples after drugs' administration.

The aim of this work is to develop a sensitive, selective, robust, reliable and efficient UPLC/MS/MS method for simultaneous determination of the co-administered GUF and BRM, followed by a pilot pharmacokinetic study. In addition, A comparative study was conducted between the developed UPLC/MS/MS and the reported method in terms of analytical greenness using the assessment tools (National Environmental Methods Index (NEMI), Assessment of Green Profile (AGP) and Green Analytical Procedure Index (GAPI) and Eco-Scale).

2. Experimental

2.1. Chemicals and reagents

Guaifenesin (GUF) (purity 99.91%), Bromhexine HCl (BRM) (purity 99.83%) and Tetryzoline HCl (TEZ) (IS) (purity 99.86%) were kindly supplied by NODCAR, Egypt. The standards were checked for purity using the official BP methods [49]. The solvents: methanol, acetonitrile and tertiary butyl methyl ether (TBME) were obtained of HPLC grade from Sigma-Aldrich (Milwaukee, USA). Blank human plasma was supplied from VACSERA, Egypt.

2.2. Pharmaceutical formulations

Mucinex® tablet is manufactured by Reckitt Benckiser Pharmaceuticals, England, and is claimed to contain 500 mg of GUF; while Ezolvin® tablet is manufactured by Julphar, UAE, and is claimed to contain 8 mg of BRM.

2.3. Instrumentation

Waters UPLC MS/MS system equipped with acuity Ultra performance LC, Quaterny Pump, 1290 infinity sampler and Kinetex C_{18} column (50 mm X 4.6 mm) with particle size 2.6 μm and porosity 100 A°. TSQ triple quadrupole 6420-mass spectrometer. Mass lynx V 4.1 software was used.

2.4. Chromatographic and spectrometric conditions

An isocratic elution was applied using the mobile phase consisting of methanol: water (95:5, v/v) at flow rate 1 mL/min. The mobile phase was degassed using ultrasonic bath before application. Positive-ion electrospray ionization (ESI +) was applied. Auto-tuning of the mass spectrometer was performed using the software Mass lynx V 4.1. The mass spectrometric parameters were listed in Table 1. LC-MS/MS in MRM mode was performed as m/z 199 < 125 for GUF, m/z 377 < 114 for BRM and m/z 201 < 131 for IS.

2.5. Standard and working solutions

Stock standard solution of GUF and BRM were prepared in methanol to obtain concentrations of (100 μg/mL) and (1000 μg/mL), respectively. Dilutions were done using methanol to prepare the working standard solutions of concentrations (10 μg/mL) and (100 μg/mL) for GUF and BRM, respectively. For IS, stock solution was prepared in methanol (100 μg/mL) and the working solution was prepared by dilution with methanol to obtain a concentration (1 μg/mL).

2.6. Calibration curves and quality control samples

A volume of 450 μL of control human plasma was spiked with 25 μL from the working standard solutions of each drug of increasing concentration to from plasma samples with concentrations of (50–1500 ng/mL) for GUF and (0.5–50 μg/mL) for BRM. Three levels of quality control samples were prepared in blank plasma: low (LQC), medium (MQC) and high (HQC) with concentration of 1.5, 15 and 40 ng/mL for GUF and 150, 600 and 1200 μg/mL for BRM, respectively.

2.7. Sample preparation

The plasma samples were spiked with certain aliquots of both drugs and internal standard, then 5 mL of tertiary butyl methyl ether (TBME) was added for liquid–liquid extraction. The samples were vortexed for 4 min followed by 5 min of centrifugation (4000 rpm) at 10 °C. Into a Wassermann tube, the clear supernatant was carefully transferred and heated to 60 °C to be concentrated then reconstituted with methanol. A final aliquot of 2 μL was injected into the UPLC– MS/MS system.

2.8. Method validation

Method validation was performed according to FDA guidance for industry and bioanalytical method validation [49] with respect to the following parameters:

2.8.1. Selectivity

The evaluation of selectivity was done using six batches of blank plasma which were spiked with GUF and BRM at the LLOQ level, then analyzed as previously described. Then, the spiked plasma chromatograms were compared versus the blank plasma chromatograms. The LLOQ is the lowest concentration of an analyte which can be quantitatively estimated with satisfactory precision (RSD ≤ 20%) and accuracy (Recovery 20–80%) without interference from endogenous plasma

Table 1
LC/MS–MS parameters selected for the quantification of Guaifenesin and Bromohexine using Tetrazoline as internal standard.

Analyte	Precursor (Da)	Product (Da)	Dwell (See)	Cone (v)	Coll. Energy (V)	Ion mode
Guaifenesin	199	125	0.058	30	25	ESI+
Bromohexine	377	114	0.058	25	25	ESI+
Tetrazoline	201	131	0.058	30	20	ESI+

constituents.

2.8.2. Linearity and range

The calibration curves in plasma were established by plotting the ratios of peak areas of each drug / IS versus its corresponding concentration. Linearity was assessed using the analysis of linear regression, then concentrations of analytes were calculated via linear regression equation. Linearity is judged as acceptable when the correlation coefficient (r) is higher than 0.99, the variation of the calculated concentrations from the nominal values is within ± 15% and the variation for LLOQ samples is within ± 20%.

2.8.3. Accuracy and precision

In order to evaluate the intra-day and inter-day accuracy and precision, six different samples of each QC level (LLOQ, LQC, MQC and HQC) were analyzed on the same day and on different days. Accuracy was assessed through calculating the recovery % of blind samples. On the other hand, precision was judged by the calculating relative standard deviation (RSD%) of nine determinations. The accepted variation is 15% for all QC concentrations, while for LLQC samples, 20% deviation from the nominal value is allowed.

2.8.4. Extraction recovery

A comparison was carried out between the ratio of peak areas of the spiked GUF and BRM in blank plasma before extraction (pre-extracted samples) versus after extraction (post-extracted samples) using six replicates at each QC levels (LQC, MQC and HQC).

2.8.5. Matrix effect

The matrix effect was used to judge the influence of plasma components on the suppression or enhancement of the ions' signals of GUF, BRM and IS. The ratio of peak areas of post-extracted samples of both drugs were compared to those of neat samples (pure stock solutions) at LQC and HQC levels using six batches of different blank plasma. The calculated RSD% should not exceed 15%.

2.8.6. Stability studies

Four stability studies were conducted in order to evaluate the stability of the analytes in a simulation to the expected conditions during the mass-sample analysis. These experiments were carried out on six replicates (n = 6) of each of the LQC and HQC levels for the studied drugs GUF and BRM.

- *Short term stability* was conducted by defrosting the QC samples at room temperature and leaving them for 6 h before analysis.
- *Freeze-thaw stability* was repeated for three cycles. Each cycle was performed by leaving the frozen samples to thaw at room temperature for 2 h followed by overnight freezing at −86 °C.
- *Post-preparative stability* was assessed where the QC samples were left in the autosampler for one day at room temperature before their analysis.
- *Long term stability* was investigated by storing the QC samples at −86 °C for 30 days followed by their analysis.

The stability of the investigated samples was evaluated by comparing the mean recovery % of samples kept under these mentioned stability conditions versus those of freshly prepared samples having the same drug concentrations. Stability of samples are accepted when the mean recovery % at each QC level is within acceptable limits (±15%) and with RSD not exceeding 15%.

2.9. Pilot pharmacokinetic study

The validated proposed UPLC-MS/MS method was applied for the determination of GUF and BRM in the plasma samples of three healthy Egyptian volunteers, followed by collecting blood samples (500 μL) from their forearm vein into heparinized polyethylene tubes. The samples were collected at the intervals: 0.00 (pre-dose), 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 10 and 24 h after oral administration of Ezolvin® (8 mg of BRM) and Mucinex® (600 mg of GUF). The samples were directly centrifuged for 25 min at 4000 rpm and then plasma samples were stored at −86 °C till its analysis. The samples were treated as under "Extraction procedure"The concentrations of the GUF and BRM in plasma were calculated. The plasma concentration–time curves were plotted followed by computing different pharmacokinetic parameters.

3. Results and discussion

This work described the determination of the mixture of bromhexine HCl (BRM) and guaifenesin (GUF), commonly co-administrated or put together in a single formulation due to their synergistic effect to treat the chest congestion and cough, which is considered one of the main symptoms for COVID-19. The importance of this mixtures arises from the recently reported studies about the possibility of using it as an add-on therapy for COVID-19 patients especially home -treated mild cases and children [7,9,11].

The novelty of the proposed UPLC-MS/MS method was the analysis of the cited drugs in real human plasma samples after administration of both drugs through a pilot pharmacokinetic study. The method was developed, optimized and validated. The greenness of the proposed method was assessed and compared against the reported method where the proposed method preceded the reported one via four tools of greenness.

3.1. Method development and optimization

The separation and quantitation of trace concentrations of different samples in the plasma and their extraction from the plasma matrix with acceptable recovery represent a challenge in bioanalysis. Two reversed phase columns were tested for this elution: Acquity UPLC HSST3 column (100 Å, 1.8 μm, 50 mm X 2.1 mm) and Kinetex C_{18} (100 Å, 2.6 μm X 50 mm X 4.6 mm), where the later one showed better resolution between the cited drugs. Several trials were done using several mobile phase compositions. Acetonitrile: water (90:10, v/v) was tried but GUF showed a tailed peak while BRM showed a forked peak. Methanol: water (60:40, v/v) was tried but the chromatogram of GUF and BRM indicated forked and tailed peaks, respectively. Another trial was done using higher amount of methanol (methanol: water, 80:20, v/v), where the GUF peak was sharper with small noise but the BRM peak was also tailed. Finally, Kinetex C_{18} (100 Å, 2.6 μm X 50 mm X 4.6 mm) column was used with a mobile phase of methanol: water (95:5, v/v). The resulted chromatograms showed sharp and symmetric resolved peaks with improved sensitivity of the analytes. Positive ion mode in ESI source using multiple reaction monitoring (MRM) was applied. The MRM of the protonated precursor parent ions $[M + H]^+$ were measured at *m/z* 199 for GUF, 377 for BRM and 301 for internal standard (IS). The following product ions were selected at 125, 114 and 131 for GUF, BRM and IS, respectively, Fig. 1. The total runtime was found to be around 2 min which is relatively much shorter than the reported method [48] where the runtime reached 6 min.

3.2. Extraction procedure

Liquid-liquid extraction is advantageous when compared to other extraction procedures such as protein precipitation and solid-phase extraction (SPE) because it is easily applied as it requires small sample size and consumes small quantity of organic solvents. Also, it reduces manipulating steps, cost and time. It is suitable for extraction from different matrices such as biological fluids [50] and food samples [51,52].

Different solvents were tried for the liquid–liquid extraction of spiked drugs from plasma matrix. The first trial was done using butanol

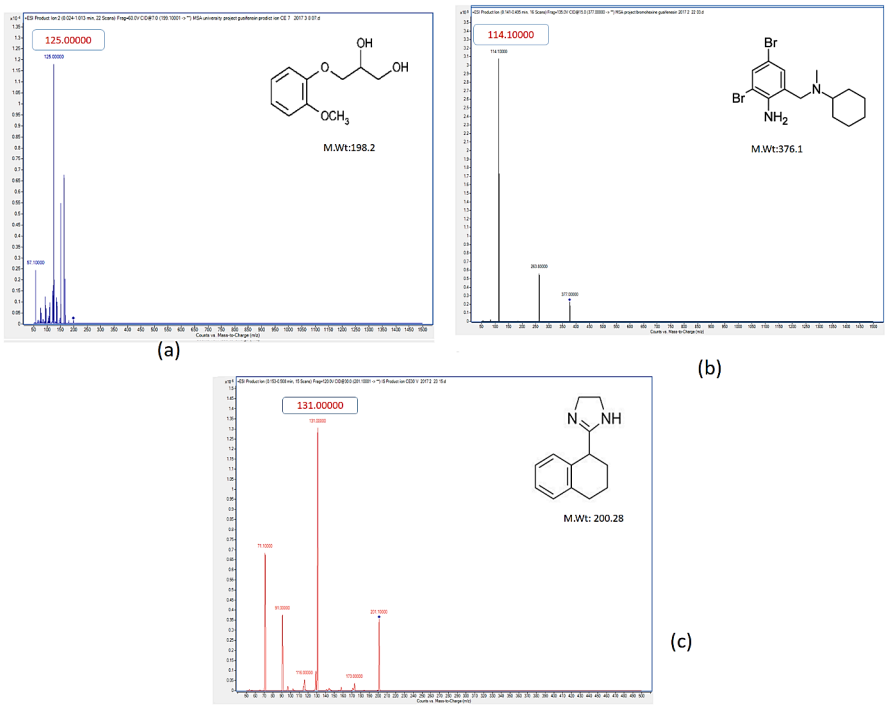


Fig. 1. Product ion spectra of $[M + H]^+$ of: The positive ion ESI-MS/MS spectra of (a) GUF; (b) BRM and (c) IS.

and hexane (50:50, v/v) but low recovery% of both analytes was observed. Additional trials were done using diethyl ether (DEE) and dichloromethane (DCM) (70:30, v/v), and DEE: DCM (70:30, v/v, pH 5.8) but the trials was also rejected due to low recovery% and weak separation of each drug. The highest recovery % of both drugs was achieved by using tetra butyl methyl ether (TBME) with accepted linearity response at correlation coefficient equal to 0.9988 and 0.999 for GUF and BRM respectively, in comparison to other solvents as shown in Supplementary Material Fig. 3SM.

3.3. Method validation

3.3.1. Selectivity

A comparison was done between the chromatograms of six blank plasma samples and spiked plasma with the two drugs and the IS. No interference was detected at their retention time, as the background noises were <20% of LLOQ for GUF and BRM and <5% of the IS response as shown in Fig. 2.

3.3.2. Linearity and range

The GUF and BRM displayed linear calibration curves over the range (50–1500 ng/mL) and (0.5–50 ng/mL) respectively. The blank plasma (without IS) were compared to the zero plasma (with IS) were assayed to confirm the absence of any interference from human plasma, showing no interference with the two studied drugs. The deviation of HQC, MQC and LQC was within ± 15% while for LLOQ was within ± 20% from the nominal concentration, so the results were acceptable.

The limit of detection is defined as the lowest concentration of analyte that can be distinguished from zero. LOD was found to be equal to 35.16 and 0.43 ng/ml for GUF and BRM, respectively.

3.3.3. Accuracy and precision

The accuracy and precision were determined via intra-day and inter-day analysis four concentrations (LLOQ, LQC, MQC and HQC) of GUF and BRM using 6 replicates (n = 6). The intra-day accuracy was ranged between 103.74 and 113.36% with RSD of 3.90–5.69% for GUF, and 99.33–105.77% with RSD of 2.47–8.03% for BRM. While the inter-day

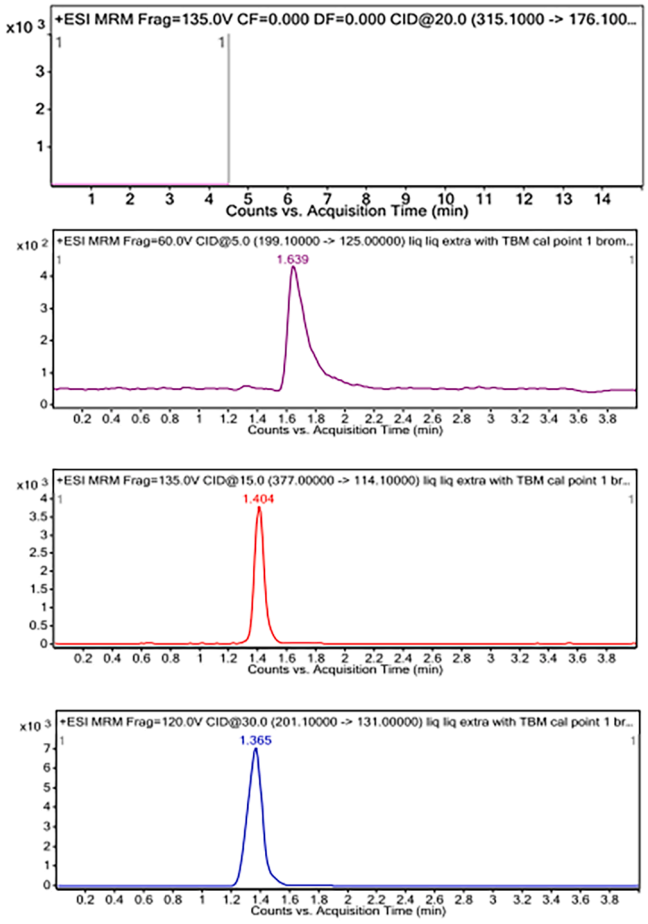


Fig. 2. Mass chromatograms of (a) blank plasma, plasma spiked with (b) GUF (150 ng/mL), (c) BRM (25 μg/mL) and (d) IS (444 ng/mL) using isocratic elution of methanol: water (95:5, v/v) at flow rate 1 mL/min.

accuracy was ranged between 100.24 and 109.40% with RSD of 4.09–7.15% for GUF, and 104.40–106.40% with RSD of 5.71–8.30% for BRM. The results were summarized in Supplementary Material Table 1SM.

3.3.4. Extraction recovery

The peak areas of both drugs for the pre-extracted samples at three levels of LQC, MQC and HQC was compared by the peak areas of the drugs for the post-extracted plasma samples using six replicates to assess the extraction recovery. The obtained recoveries indicated the efficiency of the extraction procedure. The mean extraction recovery was 97.48% with RSD of 9.02% for GUF and 89.99% with RSD of 10.73% for BRM. The results were shown in Table 2.

3.3.5. Matrix effect

A comparison was performed using six different blank plasma batches having the same concentration for LQC and HQC level between the peak areas in the post-extracted plasma with that of the plasma spiked with the standard drugs and the IS to assess the matrix effect. The results indicated that the ionization of the GUF, BRM and IS in the ion source was not affected by the constituents that may present in the co-eluted matrix. In addition, the obtained data in Table 2 confirm the effectiveness of sample processing procedure in removal of any possible interference from the matrix.

3.3.6. Stability studies

The chemical properties of the drug, storage conditions and the drug container system affect the drug stability in the biological fluid. The recovery% ± RSD of three determinations of each of low and high QC levels were used to express the drugs stability in human plasma. The demonstrated results in Supplementary Material Table 2SM proved the good stability of GUF and BRM in plasma samples as all data were within the acceptable limits.

3.4. Application to human plasma samples

The proposed UPLC-MS/MS method was effectively applied for determination of the co-administered GUF and BRM in healthy Egyptian volunteers who were administered simultaneous doses of Mucinex® (600 mg of GUF) and Ezolvin® (8 mg of BRM) oral tablets. The goals of the study and its possible risk were informed clearly to the volunteers. In addition, the protocol was approved by the center of applied research and advanced studies (CARAS), Cairo university (Code: MS09011901). In order to avoid any possible interaction from food or drink, all samples were done under fasting conditions. The volunteers were healthy according to laboratory tests and physical examination. Before the volunteers' inclusion to study, the consent forms were signed by them. The inspected pharmacokinetic parameters involved; C_{max} (ng/mL), t_{max} (h), $t_{1/2}$ (h), AUC_{0-t}, $AUC_{0-\infty}$ (ng.h/mL) and elimination rate constant; K_{el} (h^{-1}). The mean plasma concentration- time curves of GUF and BRM were demonstrated in Fig. 3, while the pharmacokinetic results were calculated in Table 3.

3.5. Greenness assessment

The concept of green analytical chemistry (GAC), as discussed by Gałuszka et al. [53], is controlled by 12 main principles aiming for eco-friendly procedures with highest accuracy and reliability for analysis of pharmaceuticals and/or biological fluids. The greenness of analytical methods plays an important role, in particular when developing chromatographic methods due to the use of varieties of organic solvents and high energy equipment [16,24,54]. The greenness can be evaluated using four tools: National Environmental Methods Index (NEMI), Assessment of Green Profile (AGP) and Green Analytical Procedure Index (GAPI) and Eco-Scale.

The NEMI qualitative tool provides limited data about the tested method [55]. The method is presented by a pictogram divided into four quadrants (a –d) which are green-shaded or left blank. The quadrant is green when: a) None of the reagents used are defined as persistent, bio-accumulative, and toxic (PBT) by EPA-TRI (Environment Protection Agency's Toxic Release Inventory) [56]; b) All the applied chemicals are not listed as C, D, F, or I hazardous wastes in EPA list [57]; c) when the pH range is (2–12) and d) when the maximum limit for produced waste is 50 g. By assessing the proposed method and comparing it versus the reported one, it was found that proposed method has better profile with three green quadrants (a, b, c), while the reported method showed only two green quadrants (a and c), and quadrant b is left blank due to the use of formic acid which is labeled as C and T hazardous in EPA list as shown in Table 4. Quadrants "d" for both methods were left blank as the generated waste generated will exceed the maximum limit.

AGP is a semi-quantitative tool which provides more data than NEMI. It is presented by a pictogram divided into five risk potentials: a) health, b) safety, c) environmental, 4) waste, and 5) energy [58] with a score from (1–3) : 1) green, 2) yellow and 3) red. These scores are calculated according to National Fire Protection Association (NFPA) codes and standards [59]. Environment and waste pentagrams are scored according to its yield (<50 g, 50–250 g and > 250 g). Energy pentagram is scored in terms of solvent evaporation by the apparatus. By comparing the proposed and reported methods as shown in Table 4, the proposed method showed two green potentials (b and e), while reported method showed a yellow "b" safety potential due to the use of formic acid and ammonia solution as part of the mobile phase. Both methods showed yellow (a, c, and d) representing a medium health potential, low energy consumption and low environmental impact.

Wasylka [60] presented GAPI tool to elaborate much data than previous tools. Each method is presented by five pentagrams subdivided into smaller sections in order to assess 15 analytical aspects, as in Supplementary Material Table 3SM. The shade of each section is red - yellow - green according to impact from high – medium - low. Both proposed and reported methods showed much detailed profiles than the previous tools, where red pentagrams were measured for the first time. Resemblance of GAPI pentagrams of both methods were observed regarding the type of method, the sample (collection – preservation – transport - storage – preparation), instrumentation and waste treatment. The proposed method transcended the reported one in terms of safety hazard of solvents use, due to the use of formic acid and ammonia in the reported method, number of grams of used solvents and generated waste (aspects

Table 2
Extraction Recovery and matrix effect data for the determination of Guaifenesin and Bromohexine in human plasma.

	QC Level	Guaifenesin		Bromohexine		IS	
		[a]Recovery%	[a]RSD	[a]Recovery%	[a]RSD	[a]Recovery%	[a]RSD
Extraction recovery	LQC	99.67	9.56	89.33	12.74	102.94	8.59
	MQC	91.25	6.75	90	11.61		
	HQC	101.53	10.76	90.64	7.84		
Matrix effect	LQC	102.47	8.93	95.87	10.31	96.32	10.22
	HQC	101.85	9.44	101.63	7.22		

[a] Average of six separate determinations.

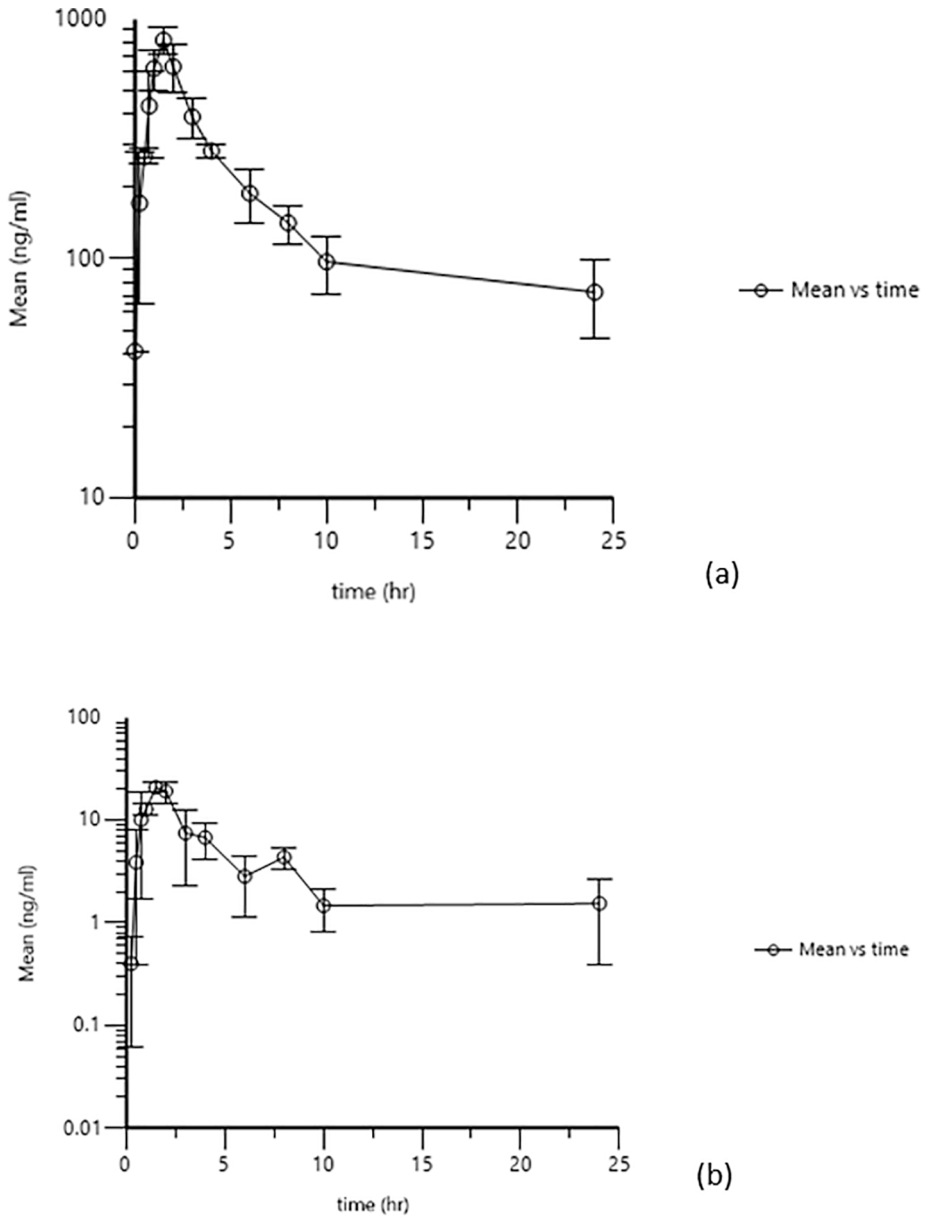


Fig. 3. Mean plasma concentration–time profile of (a) GUF and (b) BRM following administration of oral dose of Mucinex ® and Ezolvin ® to three healthy volunteers (n = 3).

Table 3
Pharmacokinetic parameters for Guaifenesin and Bromohexine after oral administration of Ezolvin® (8 mg of Bromohexin) and Mucinex® (600 mg of Gufenesin) to Egyptian healthy volunteers.

Parameters	Proposed Method	
Analyte	Guaifenesin	Bromohexine
Dose (mg)	600	8
C_{max} (ng/mL)	818.12	22.597
t_{max} (h)	1.5	1.417
$t_{1/2}$ (h)	10.222	8.845
AUC $_{0\text{-}t}$	4103.714	88.105
AUC $_{0\text{-}\infty}$	5172.445	113.207
AUC%	82.04347	80.04127
AUC% $_{Extra}$	17.95653	19.95873
K_{el} (h^{-1})	0.073	0.093

Cmax = Plasma maximum concentration, Tmax = Time of the maximum plasma concentration, t1/2 = Plasma concentration half time, AUC 0-∞ = Area under the plasma concentration curve extrapolated to infinite time, Kel = Elimination rate constant. The study was performed on 3 healthy volunteers (n = 3).

9, 11 and 14), as shown in Table 4.

The Eco-Scale [61] is the only quantitative tool that can compare the greenness of several methods on a common scale from (0 – 100). Penalty points are calculated for every factor in a method, then sum of penalty points is subtracted from 100 to calculate its eco-scale. The penalty points are calculated for several aspects including: the hazard and amounts of the reagents used; the method of extraction; energy, occupational hazard and the waste produced by the instruments. An ideal green method will have eco-scale of 100, excellent is > 75, acceptable is > 50 and inadequate is < 50. The Eco-Scale for the reported and proposed methods was calculated as shown in Table 5, it was found that the eco-scale of the proposed method was equal to be 78 points (excellent) versus 70 points for the reported one. The reported method suffered from a lower Eco-Scale due to the use of several non-green solvents such as formic acid and ammonium solution, in addition to the longer runtime (6 min in comparison to 2 min for the proposed method) and higher flow rate which accordingly increased the amounts of reagents used and the waste produced.

Table 4
Greenness assessment tools of the proposed and reported methods for the simultaneous determination of BRM and GUF in human plasma.

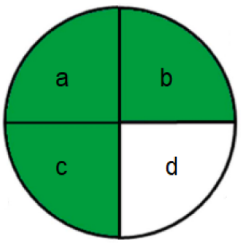
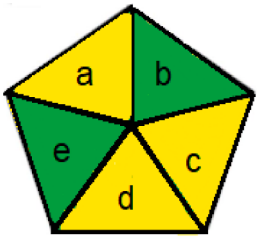
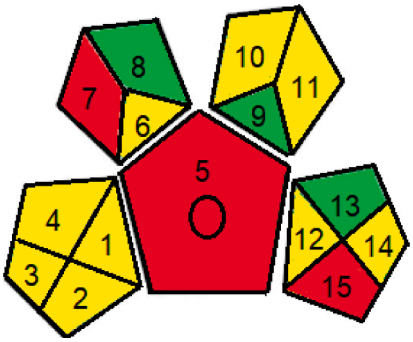
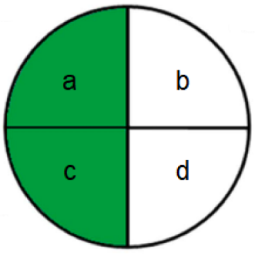
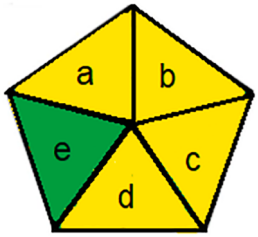
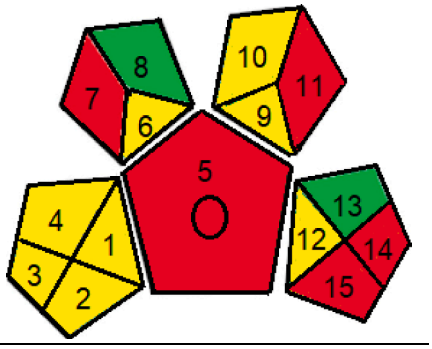
	NEMI	AGP	GAPI
Proposed method			
Reported method [28]			

Table 5
Total penalty points (PPs) for estimating the greenness of the proposed and reported methods.

	Penalty points (PPs)	
	Reported method	Proposed method
Reagents		
Water	0	0
Tetra butyl methyl ether	—	4
Methanol	12	12
Formic acid	6	—
Ammonia solution	4	—
SPE	2	—
liq-liq extraction	—	2
Instrument		
Energy	1	1
Waste	5	3
Total PPs	∑ 30	∑ 22
Eco Scale total score	70	78

4. Conclusion

The proposed UPLC-MS/MS method was successfully established and validated to determine the co-administered GUF and BRM drugs as an add-on therapy for mild home-treated COVID-19 cases. The results obtained by the proposed method were satisfactory and indicated its accuracy, precision and effective extraction with no interference from the matrix. The proposed UPLC-MS/MS method was applied to determine the plasma concentrations GUF and BRM in a pharmacokinetic study involving Egyptian healthy volunteers showing no difference of the studied pharmacokinetic parameters versus the reported method. Finally, the proposed UPLC-MS/MS method was found to be more eco-friendly than the reported one using four assessment tools.

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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.

Appendix A. Supplementary data

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References

[1] https://covid19.who.int/.
[2] M. Khalili, M. Karamouzian, N. Nasiri, S. Javadi, A. Mirzazadeh, H. Sharifi, Epidemiological characteristics of COVID-19: a systemic review and meta-analysis, MedRxiv (2020).
[3] I. Ali, O.M.L. Alharbi, COVID-19: disease, management, treatment, and social impact, Sci. Total Environ. 728 (2020) 138861.
[4] J.M. Sanders, M.L. Monogue, T.Z. Jodlowski, J.B. Cutrell, Pharmacologic treatments for coronavirus disease, (COVID-19): a review, JAMA 323 (2020) (2019) 1824–1836.
[5] R.D. McBane, V.D. Torres Roldan, A.S. Niven, R.K. Pruthi, P.M. Franco, J. A. Linderbaum, A.I. Casanegra, L.J. Oyen, D.E. Houghton, A.L. Marshall, N.N. Ou, J.L. Siegel, W.E. Wysokinski, L.J. Padrnos, C.E. Rivera, G.L. Flo, F.E. Shamoun, S. M. Silvers, T. Nayfeh, M. Urtecho, S. Shah, R. Benkhadra, S.M. Saadi, M. Firwana, T. Jawaid, M. Amin, L.J. Prokop, M.H. Murad, Anticoagulation in COVID-19: a systematic review, meta-analysis, and rapid guidance from mayo clinic, Mayo Clin. Proc. 95 (2020) 2467–2486.
[6] Z. Esam, Protective potential of expectorants against COVID-19, Med. Hypotheses 142 (2020) 109844.
[7] M. Depfenhart, D. de Villiers, G. Lemperle, M. Meyer, S. Di Somma, Potential new treatment strategies for COVID-19: is there a role for bromhexine as add-on therapy? Intern. Emerg. Med. (2020) 1.
[8] T. Li, L. Sun, W. Zhang, C. Zheng, C. Jiang, M. Chen, D. Chen, Z. Dai, S. Bao, X. Shen, Bromhexine hydrochloride tablets for the treatment of moderate COVID-19: an open-label randomized controlled pilot study, Clin. Transl. Sci. 13 (2020) 1096–1102.
[9] D. Stepanov, P. Lierz, Bromhexine hydrochloride: potential approach to prevent or treat early stage COVID-19, (2020).

[10] S. Habtemariam, S.F. Nabavi, S. Ghavami, C.A. Cismaru, I. Berindan-Neagoe, S. M. Nabavi, Possible use of the mucolytic drug, bromhexine hydrochloride, as a prophylactic agent against SARS-CoV-2 infection based on its action on the transmembrane serine protease 2, Pharmacol. Res. 157 (2020) 104853.

[11] Q. Fu, X. Zheng, Y. Zhou, L. Tang, Z. Chen, S. Ni, Re-recognizing bromhexine hydrochloride: pharmaceutical properties and its possible role in treating pediatric COVID-19, Eur. J. Clin. Pharmacol. (2020) 1–3.

[12] Y. Wang, Y. Zhang, X. Chen, K. Xue, T. Zhang, X. Ren, Evaluating the efficacy and safety of bromhexine hydrochloride tablets in treating pediatric COVID-19: A protocol for meta-analysis and systematic review, Medicine 99 (2020).

[13] https://health.unl.edu/covid-19-vs-flu-and-common-cold.

[14] https://www.umms.org/coronavirus/what-to-know/treat-covid-at-home.

[15] S.M. Elgizawy, N.Y. Hassan, H.M. Lotfy, S.S. Saleh, Comparative study of RP–HPLC versus TLC– spectrodensitometric methods applied for binary mixtures of fluoroquinolones and corticosteroids, Acta Chromatogr. 26 (2014) 439–456.

[16] D. Mohamed, M.A. Hegazy, G.M. El-Sayed, S.H. Youssef, Greenness evaluation of different chromatographic approaches for the determination of dextromethorphan, phenylephrine & brompheniramine in their pharmaceutical formulation, Microchem. J. 157 (2020) 104893.

[17] H.Y. Aboul-Enein, I. Ali, Comparative study of the enantiomeric resolution of chiral antifungal drugs econazole, miconazole and sulconazole by HPLC on various cellulose chiral columns in normal phase mode, J. Pharm. Biomed. Anal. 27 (2002) 441–446.

[18] H.Y. Aboul-Enein, I. Ali, Optimization strategies for HPLC enantioseparation of racemic drugs using polysaccharides and macrocyclic glycopeptide antibiotic chiral stationary phases, Il Farmaco 57 (2002) 513–529.

[19] H. Aboul-Enein, I. Ali, HPLC enantiomeric resolution of nebivolol on normal and reversed amylose based chiral phases, Die Pharmazie 56 (2001) 214–216.

[20] I. Ali, L. Naim, A. Ghanem, H.Y. Aboul-Enein, Chiral separations of piperidine-2,6-dione analogues on Chiralpak IA and Chiralpak IB columns by using HPLC, Talanta 69 (2006) 1013–1017.

[21] E.M.H. Abdelraheem, S.M. Hassan, M.M.H. Arief, S.G. Mohammad, Validation of quantitative method for azoxystrobin residues in green beans and peas, Food Chem. 182 (2015) 246–250.

[22] S.M. Wabaidur, A. AlAmmari, A. Aqel, S.A. Al-Tamrah, Z.A. Alothman, A.B. H. Ahmed, Determination of free fatty acids in olive oils by UPHLC–MS, J. Chromatogr., B 1031 (2016) 109–115.

[23] A.a. Al-Rifai, A. Aqel, T. Al-Warhi, S.M. Wabaidur, Z.A. Al-Othman, A.Y. Badjah-Hadj-Ahmed, Antibacterial, antioxidant activity of ethanolic plant extracts of some convolvulus species and their DART-ToF-MS profiling, Evidence-Based Complementary Alternative Med. 2017 (2017) 5694305.

[24] S.S. Saleh, H.M. Lotfy, G. Tiris, N. Erk, Y. Rostom, Analytical tools for greenness assessment of chromatographic approaches: application to pharmaceutical combinations of Indapamide, Perindopril and Amlodipine, Microchem. J. 159 (2020) 105557.

[25] H. Salem, N.Y. Hassan, H.M. Lotfy, S.S. Saleh, Column performance study of different variants of liquid chromatographic technique: an application on pharmaceutical ternary mixtures containing tetryzoline, J. Chromatogr. Sci. (2014), https://doi.org/10.1093/chromsci/bmu109.

[26] S.N. Muchohi, N. Thuo, J. Karisa, A. Muturi, G.O. Kokwaro, K. Maitland, Determination of ciprofloxacin in human plasma using high-performance liquid chromatography coupled with fluorescence detection: application to a population pharmacokinetics study in children with severe malnutrition, J. Chromatogr., B 879 (2011) 146–152.

[27] Q. Jiang, Y. Liu, Y. Wang, Y. Sun, B. Li, Z. Li, T. Lu, S. Wang, Z. He, Simultaneous determination of entecavir and lamivudine in rat plasma by UPLC-MS/MS and its application to a pharmacokinetic study, RSC Adv. 6 (2016) 70990–70998.

[28] H.M. Lotfy, S.S. Saleh, N.Y. Hassan, H. Salem, Development and validation of impurity-profiling UPLC method for the determination of sodium cromoglicate and tetryzoline hydrochloride: application on rabbit aqueous humor, J. Chromatogr., B 1006 (2015) 121–129.

[29] N.A. AlFaris, S.M. Wabaidur, Z.A. Alothman, J.Z. Altamimi, T.S. Aldayel, Fast and efficient immunoaffinity column cleanup and liquid chromatography–tandem mass spectrometry method for the quantitative analysis of aflatoxins in baby food and feeds, J. Sep. Sci. 43 (2020) 2079–2087.

[30] Y.M.G. Al Shamari, A.A. Alwarthan, S.M. Wabaidur, M.A. Khan, A.A. Alqadami, M. R. Siddiqui, New Ultra Performance liquid chromatography-mass spectrometric method for the determination of allura red in soft drinks using corncob as solid phase extraction sorbent: analysis and food waste management approach, J. King Saud Univ.-Sci. 32 (2020) 1135–1141.

[31] M.R. Khan, S.M. Wabaidur, Z.A. Alothman, R. Busquets, M. Naushad, Method for the fast determination of bromate, nitrate and nitrite by ultra performance liquid chromatography–mass spectrometry and their monitoring in Saudi Arabian drinking water with chemometric data treatment, Talanta 152 (2016) 513–520.

[32] M.R. Khan, S.M. Wabaidur, R. Busquets, M.A. Khan, M.R. Siddiqui, M. Azam, Identification of malachite green in industrial wastewater using lignocellulose biomass composite bio-sorbent and UPLC-MS/MS: a green environmental approach, Process Saf. Environ. Prot. 126 (2019) 160–166.

[33] A.A.H. Hakami, S.M. Wabaidur, M. Ali Khan, Z. Abdullah Alothman, M. Rafatullah, M.R. Siddiqui, Development of ultra-performance liquid chromatography-mass spectrometry method for simultaneous determination of three cationic dyes in environmental samples, Molecules 25 (2020) 4564.

[34] H. Patil, S. Sonawane, P. Gide, Determination of guaifenesin from spiked human plasma using RP-HPLC with UV detection, J. Anal. Chem. 69 (2014) 390–394.

[35] M.R. Rao, M. Kumar, S. Aghav, G. Sukre, Development and validation of HPTLC method for determination of bromhexine hydrochloride in human plasma, Res. J. Pharmacy Technol. 5 (2012) 1054–1057.

[36] A. Ozdemir, H. Aksoy, E. Dinç, D. Baleanu, S. Dermis, Determination of guaifenesin and dextromethorphan in a cough syrup by HPLC with fluorometric detection, Revue Roumaine de Chimie 51 (2006) 117.

[37] K. Bucktowar, A review on bromhexine hydrochloride and the different analytical methods used for its determination, Int. J. Pharma Bio Sci. 8 (2017) 43–49.

[38] V.D. Rohit, J. Tandel, P. Chauhan, S. Shah, A novel stability indicating RP-HPLC method development and validation for estimation of Phenylephrine hydrochloride and Bromhexine hydrochloride in their tablet dosage form, J. Curr. Pharma Res. 6 (2016) 1860–1876.

[39] A. Kumar, S. Nanda, A validated high performance liquid chromatographic method for estimation of bromhexine and terbutaline in bulk and tablet dosage forms, Pharm. Methods 2 (2011) 218–222.

[40] V. Jain, M.C. Sharma, Validated RP-HPLC method for determining the levels of bromhexine HCl, chlorpheniramine maleate, dextromethorphan HBr and guaiphenesin in their pharmaceutical dosage forms, J. Taibah Univ. Sci. 10 (2016) 38–45.

[41] H.M. El-Sayed, H. Hashem, Quality by design strategy for simultaneous HPLC determination of bromhexine HCl and its metabolite ambroxol HCl in dosage forms and plasma, Chromatographia 83 (2020) 1075–1085.

[42] G. Bazylak, L.J. Nagels, Simultaneous high-throughput determination of clenbuterol, ambroxol and bromhexine in pharmaceutical formulations by HPLC with potentiometric detection, J. Pharm. Biomed. Anal. 32 (2003) 887–903.

[43] J. Liu, X. Chen, Y. Hu, G. Cheng, D. Zhong, Quantification of the major metabolites of bromhexine in human plasma using RRLC–MS/MS and its application to pharmacokinetics, J. Pharm. Biomed. Anal. 51 (2010) 1134–1141.

[44] X. Chen, J. Huang, Z. Kong, D. Zhong, Sensitive liquid chromatography–tandem mass spectrometry method for the simultaneous determination of paracetamol and guaifenesin in human plasma, J. Chromatogr. B 817 (2005) 263–269.

[45] Q.-H. Ge, Z. Zhou, X. Ziu, J. Yu, Simultaneous determination of dextrorphan and guaifenesin in human plasma by HPLC, Chin. J. Pharmaceut. 35 (2004) 32–34.

[46] T.H. Eichhold, D.L. McCauley-Myers, D.A. Khambe, G.A. Thompson, S.H. Hoke II, Simultaneous determination of dextromethorphan, dextrorphan, and guaifenesin in human plasma using semi-automated liquid/liquid extraction and gradient liquid chromatography tandem mass spectrometry, J. Pharm. Biomed. Anal. 43 (2007) 586–600.

[47] J. Wen, H. Zhang, C. Xia, X. Hu, W. Xu, X. Cheng, J. Gao, Y. Xiong, A sensitive liquid chromatography–electrospray ionization–mass spectrometry method for the simultaneous determination of pentoxyverine citrate and guaifenesin in human plasma—application to pharmacokinetic and bioequivalence studies, Biomed. Chromatogr. 24 (2010) 351–357.

[48] T. Komarov, D. Bogdanova, O. Miskiv, A. Aleshina, I. Shohin, Y.V. Medvedev, N. Bagaeva, I. Korenskaya, Development and validation of salbutamol, bromhexine, ambroxol and Guauaifenesin determination in human plasma by HPLC-MS/MS method, Drug Dev. Registr. 8 (2019) 61–74.

[49] Food and Drug Administration, Center for Drug Evaluation and Research. Guidance for Industry, Bioanalytical Method Validation. USA, (2018).

[50] R. Heydari, Z. Lotfi, M. Ramezani, Simultaneous determination of zidovudine and lamivudine in plasma samples using miniaturized homogenous liquid–liquid extraction and high-performance liquid chromatography, J. Anal. Chem. 73 (2018) 1105–1110.

[51] N.A. AlFaris, J.Z. ALTamimi, Z.A. ALOthman, S.M. Wabaidur, A.A. Ghafar, T.S. Aldayel, Development of a sensitive liquid-liquid extraction and ultra-performance liquid chromatography-tandem mass spectrometry method for the analysis of carbaryl residues in fresh vegetables sold in Riyadh, J. King Saud Univ.-Sci. 32 (2020) 2414–2418.

[52] M.R. Khan, Z.A. Alothman, A.A. Ghfar, S.M. Wabaidur, Analysis of aflatoxins in nonalcoholic beer using liquid–liquid extraction and ultraperformance LC-MS/MS, J. Sep. Sci. 36 (2013) 572–577.

[53] A. Gałuszka, Z. Migaszewski, J. Namieśnik, The 12 principles of green analytical chemistry and the SIGNIFICANCE mnemonic of green analytical practices, TrAC, Trends Anal. Chem. 50 (2013) 78–84.

[54] A. Mohammad, Green Chromatographic Techniques: Separation and Purification of Organic and Inorganic Analytes, Springer Science & Business Media, 2013.

[55] L.H. Keith, L.U. Gron, J.L. Young, Green analytical methodologies, Chem. Rev. 107 (2007) 2695–2708.

[56] U.S.E.P.A. (EPA), Persistent, Bioaccumulative, and Toxic (PBT) Chemicals under TSCA Section 6(h), USA, 2019.

[57] C.o.F.R.-. EPA, Identification and listing of hazardous waste (2016).

[58] D. Raynie, J. Driver, Green Assessment of Chemical Method, 13th Annual Green Chemistry & Engineering Conference, Maryland - USA, ASC, 2009, p. 34.

[59] N.F.P.A. (NFPA), List of NFPA Codes & Standards, 2019.

[60] J. Płotka-Wasylka, A new tool for the evaluation of the analytical procedure: green analytical procedure index, Talanta 181 (2018) 204–209.

[61] K. Van Aken, L. Strekowski, L. Patiny, EcoScale, a semi-quantitative tool to select an organic preparation based on economical and ecological parameters, Beilstein J. Organ. Chem. 2 (2006) 3.