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Research Article

Validated green high-performance liquid chromatographic methods for the determination of coformulated pharmaceuticals: A comparison with reported conventional methods

The introduction of sustainable development concepts to analytical laboratories has recently gained interest, however, most conventional high-performance liquid chromatography methods do not consider either the effect of the used chemicals or the amount of produced waste on the environment. The aim of this work was to prove that conventional methods can be replaced by greener ones with the same analytical parameters. The suggested methods were designed so that they neither use nor produce harmful chemicals and produce minimum waste to be used in routine analysis without harming the environment. This was achieved by using green mobile phases and short run times. Four mixtures were chosen as models for this study; clidinium bromide/chlordiazepoxide hydrochloride, phenobarbitone/pipenzolate bromide, mebeverine hydrochloride/sulpiride, and chlorphenoxamine hydrochloride/caffeine/8-chlorotheophylline either in their bulk powder or in their dosage forms. The methods were validated with respect to linearity, precision, accuracy, system suitability, and robustness. The developed methods were compared to the reported conventional high-performance liquid chromatography methods regarding their greenness profile. The suggested methods were found to be greener and more time- and solvent-saving than the reported ones; hence they can be used for routine analysis of the studied mixtures without harming the environment.

Keywords: Caffeine / Chlordiazepoxide / Green analytical methods / High-performance liquid chromatography / Phenobarbitone
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1 Introduction

HPLC is a popular analytical technique that is applied routinely for qualitative and quantitative purposes in both industry and academia. Most of the conventional methods use hazardous organic solvents that are harmful to both humans and the environment and are considered the main source of organic waste. Analysts who are interested in making their methods greener use alternative solvents that are greener and more environmentally friendly.

In modern analytical chemistry, the development of greener methods necessitates considering the green aspects at the stage of method development. This is achieved by designing analytical methods in a way that reduces or eliminates hazardous substances that are used in or generated by the method to be more benign to the environment [1,2], however, most of the used HPLC methods do not consider the impact of application of green methods. Namies´nik [3] presented examples of the application of green chemistry in the analytical laboratory. An environmentally friendly approach compatible with conventional HPLC equipment and columns was described for the development of separation methods for fingerprinting complex matrices [4].

The aim of this work was to highlight the impact of applying green HPLC methods in pharmaceutical analysis and proof that conventional methods can be replaced by greener ones with the same analytical performance characteristics but more eco-friendly. This was achieved by developing validated green HPLC methods for analysis of different pharmaceutical formulations. The suggested methods were designed so that they neither use nor produce harmful chemicals, also not to be corrosive and to produce minimum waste so as to be continuously used in routine analysis and QC laboratories without harming the environment. The developed methods were compared to reported conventional HPLC methods regarding their greenness profile.

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Abbreviations: CAF, caffeine; **CB**, clidinium bromide; **CD**, chlordiazepoxide; **CTH**, 8-chlorotheophylline; **CPH**, chlorphenoxamine hydrochloride; **MB**, mebeverine HCl; **PB**, phenobarbitone; **PZ**, pipenzolate bromide; **SUL**, sulpiride

To achieve our goal, different pharmaceutical formulations treating gastrointestinal tract disorders were selected namely, clidinium bromide (CB) and chlordiazepoxide hydrochloride (CD), phenobarbitone (PB) and pipenzolate bromide (PZ), mebeverine hydrochloride (MB) and Sulpiride (SUL) and chlorphenoxamine hydrochloride (CPH), caffeine (CAF), and 8-chlorotheophylline (CTH). Few HPLC methods were reported for analysis of CB and CD [5–8], for PB and PZ [9, 10], for MB and SUL [11–14] and CPH, CAF, and CTH [15]. It was noticed that these methods did not consider the environmental hazard of the used chemicals or follow any protocol or guidelines for green chemistry application.

2 Materials and methods

2.1 Materials and solvents

Ethanol (HPLC grade; Fisher Scientific, UK), and double distilled water were used.

Pure CB and CD were kindly obtained from E.P.I.CO. 10th of Ramadan City, Egypt. Their percentage purity was reported to be 100.22 and 99.9, respectively. Sugar-coated Librax tablets (each tablet claimed to contain 2.5 mg CB and 5 mg CD) batch number 1104977 manufactured by (E.I.P.I.CO).

Pure PB and PZ were kindly supplied by Kahira Pharm. and Chem. Ind. Cairo, Egypt. Their percentage purity was reported to be 100.88 and 100.34, respectively. Spasmotal drops (Misr for Pharm. Ind.) batch number 316119. Each 1 mL contains 4 mg of Pipenzolate bromide and 6 mg of phenobarbitone.

Pure MB and SUL were kindly supplied by Rameda Pharm. Cairo, Egypt. Their percentage purity was reported to be 99.98 and 100.66. Film-coated Colona tablets (each film-coated tablet contains: Sulpiride 25 mg; Mebeverine HCl 100 mg) batch number 120368 manufactured by Rameda Pharm. Cairo.

Pure CPH, CAF, and CTH were kindly obtained from E.P.I.CO. 10th of Ramadan City, Egypt. Their percentage purity was reported to be 99.82, 99.65, and 99.76, respectively. Emeral tablets (each coated tablet claimed to contain 30 mg CPH, 50 mg CAF, and 20 mg CTH) batch number 1301791 manufactured by (E.I.P.I.CO).

All dosage forms were purchased from the local market.

2.2 Instruments and equipment

2.2.1 HPLC instrument

Chromatography was performed on Agilent 1260 infinity series LC with a 1260 Quaternary Pump, connected with a 1260 VL+ diode array detector. Injection was performed with an 1260 ALS auto-injector fitted with a 100 μL syringe. The instrument was connected to an HP Compaq Home PC, an HP laser-jet p2055d printer, and an HPLC system manager chromatography Chemstation 32 software was used.

2.2.2 Sonicator

A ''BRANSON 5510'' sonicator was used for extraction of drug from pharmaceutical formulation.

2.3 Procedure

2.3.1 Solutions

Standard solutions of each of CB, CD, MB, SUL, CPH, CAF, and CTH (100 μg/mL) were prepared in ethanol.

Standard solutions of each of PB and PZ (200 μg/mL) were prepared in ethanol.

2.3.2 Chromatographic conditions

Chromatographic separations and subsequent quantifications were carried out at room temperature on a Zorbax SB-C_{18} HPLC column (4.6 mm × 75 mm id, 3.5 μm). For both mixtures, CB and CD and PB and PZ an isocratic elution system was employed using mobile phase consisting of ethanol: water (50:50, v/v) with a flow rate of 0.5 mL/min, and injection volume 3.0 μL. Detection was performed at 210.0 and 220.0 nm for CB and CD, and PB and PZ, respectively. A mobile phase consisting of ethanol/water (94.5:5.5, v/v) with a flow rate of 0.8 mL/min, injection volume 3.0 μL was used for MB and SUL and the chromatograms were detected at 220.0 nm. For CPH, CAF, and CTH, separations were carried out at room temperature on a Polaris SI (4.6 mm × 50 mm id, 3 μm) column using ethanol (100%) as a green mobile phase with a flow rate of 0.4 mL/min, injection volume is 1.0 μL. UV detection was done at 220.4, 270.4, and 276.4 nm for CPH, CAF, and CTH, respectively.

2.3.3 Construction of calibration curves

Series of solution mixtures for each previously mentioned pharmaceutical combinations were prepared by mixing volumes from their standard solution and make the appropriate dilution with ethanol to reach final range as follows:

For the mixture containing CB and CD it was 0.5–40 μg/mL for CB and 0.8–30 μg/mL CD, for PB and PZ mixture, it was 1.5–40 μg/mL for PB and 1–35 μg/mL PZ, 1–40 μg/mL for both MB and SUL, and finally for mixture of CPH, CAF, and CTH, it was 1–70 μg/mL CPH, 1.5–35 μg/mL CAF and 1–40 μg/mL CTH. The prepared solutions were analyzed under the previously mentioned chromatographic conditions. The recorded peak areas for each drug were plotted against its corresponding concentrations and regression parameters were calculated.

2.3.4 Application to pharmaceutical preparation

(a) Librax tablets

Ten Librax tablets were accurately weighed after removal of the coat and finely powdered. A weight equivalent to one tablet was transferred into 50 mL volumetric flask and shaken with 35 mL ethanol then followed by sonication for 30 min. The volume was completed to 50 mL with ethanol and filtered. From the previous solution 2.5 mL were transferred into 10 mL volumetric flask and completed to volume with the mobile phase to obtain a solution of 12.5 μg/mL CB and 25.0 μg/mL CD concentrations.

(b) Spasmotal drops

One milliliter from the mixed contents of Spasmotal drops, equivalent to 4.0 mg pipenzolate bromide and 6.0 mg phenobarbitone, was transferred accurately into a 50 mL volumetric flask and completed to the volume with ethanol. Accurate volumes were transferred to 10 mL volumetric flask to obtain a solution of 12.0 μg/mL PZ and 18 μg/mL PB.

(c) Colona tablets

Ten Colona tablets were accurately weighed after removal of the coat and finely powdered. A weight equivalent to one tablet was transferred into 25 mL volumetric flask and shaken with 10 mL ethanol then followed by sonication for 10 min. The volume was completed to 25 mL with ethanol and filtered. From the previous solution 0.1 mL was transferred to 10 mL volumetric flasks and completed to volume with the mobile phase to obtain a solution of 40.0 μg/mL MB and 10.0 μg/mL SUL.

(d) Emeral tablets

Ten Emeral tablets were accurately weighed after removal of the coat and finely powdered. A weight equivalent to one tablet was transferred into 25 mL volumetric flask and shaken with 10 mL ethanol followed by sonication for 15 min. The volume was completed to 25 mL with ethanol and filtered. From the previous solution 0.1 mL was transferred to 10 mL volumetric flasks and completed to volume with the mobile phase to obtain a solution of 12.0 μg/mL CPH, 20.0 μg/mL CAF and 8.0 μg/mL CTH concentrations.

The previously prepared dosage form samples were analyzed using the specified chromatographic conditions and the concentrations were calculated each from its corresponding regression equations.

3 Results and discussion

3.1 Method development

This work aimed to develop greener methods and to prove that they can replace conventional ones in the routine analysis for four selected models. Two aspects were taken into consideration, the safety of the used solvents and the minimization of the produced waste.

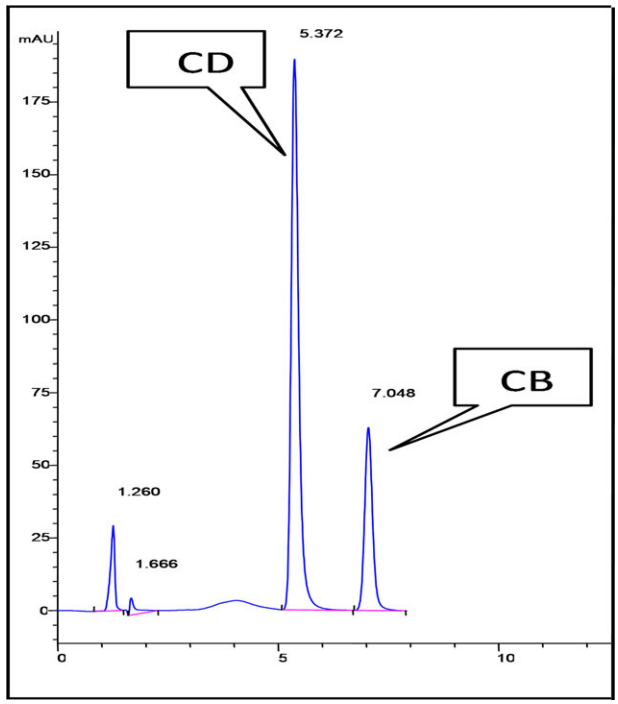

Figure 1. HPLC chromatogram of 15 μg/mL clidinium bromide and 30 μg/mL chlordiazepoxide in synthetic mixture using a green mobile phase consisting of ethanol/water (50:50, v/v) with a flow rate of 0.5 mL/min.

Assessment of green solvents occurred by the Environment, Health, and Safety (EHS) Method, the Life-Cycle Assessment (LCA) Method or Combination of EHS and LCA Method. According to the traditional organic solvents compared by EHS and LCA, EHS-preferred solvents were methanol, ethanol, and methyl acetate. Life-cycle-preferred solvents were hexane, heptane, and diethyl ether [14].

In the selection of a green solvent, four directions can be followed; to substitute the hazardous solvents by others that show better environmental, health, and safety (EHS) properties, to use "bio-solvents," i.e. solvents produced from renewable resources, or the substitution of organic solvents either with supercritical fluids or with ionic liquids [16]. Our methods used the first and second aspects where ethanol and water were the only used solvents.

American Chemical Society Green Chemistry Institute® (ACS GCIPR) considered in criteria solvent guide that ethanol is environmentally the most favorable solvent [15].

Also, solvents are classified in decreasing order of greenness based on several properties for example; water is on the top of the list followed by acetone and ethanol, while benzene and carbon tetrachloride are on the bottom. From an eco-friendly viewpoint, ethanol is highly desirable due to its low toxicity and the fact that it is derived from renewable sources [17]. It also has a low EHS score [16]. So, ethanol and

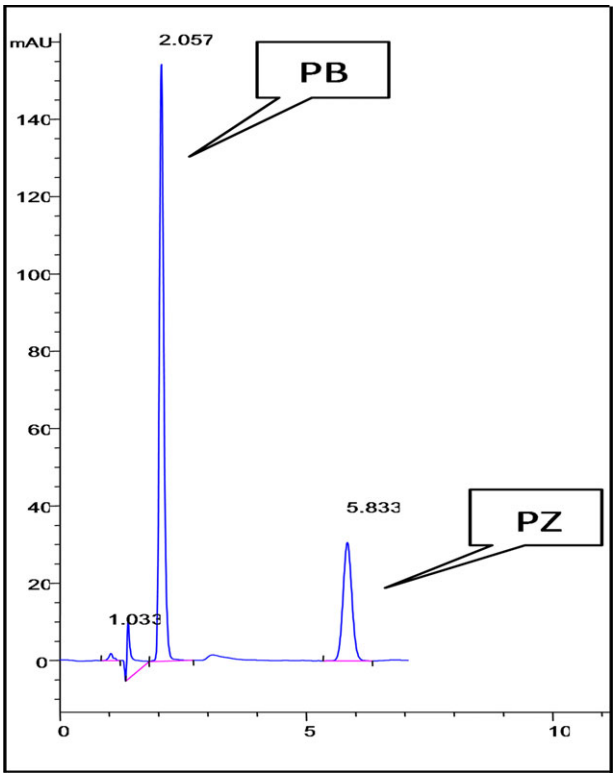

Figure 2. HPLC chromatogram of 24 μg/mL phenobarbitone and 16 μg/mL pipenzolate bromide in synthetic mixture using a green mobile phase consisting of ethanol/water (50:50, v/v) with a flow rate of 0.5 mL/min.

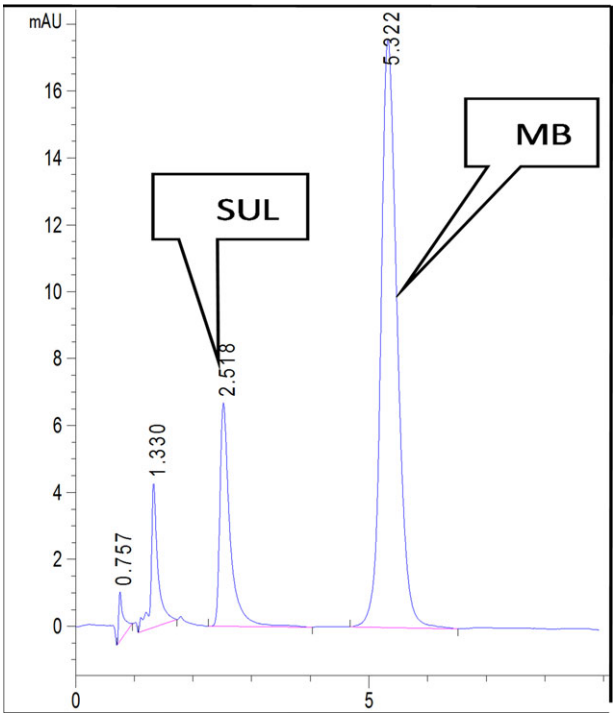

Figure 3. HPLC chromatogram of 20 μg/mL mebeverine HCl and 5 μg/mL sulpiride in synthetic mixture using a green mobile phase consisting of ethanol/water (94.5:5.5,v/v) with a flow rate of 0.8 mL/min.

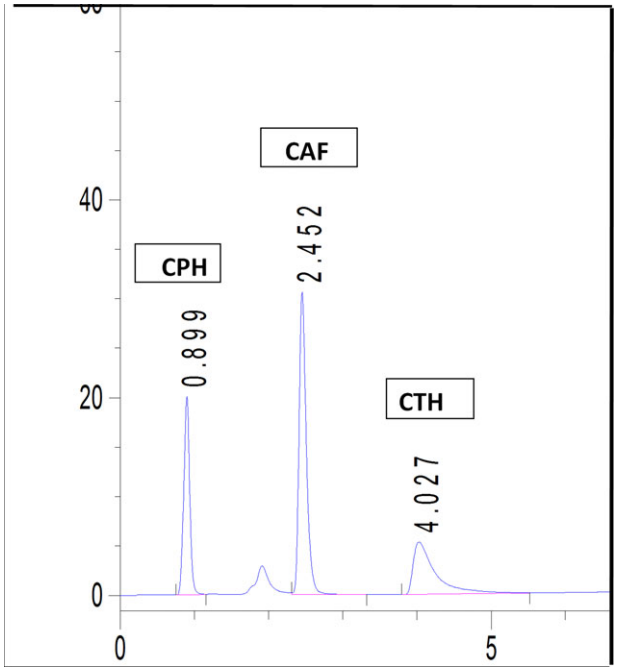

Figure 4. HPLC chromatogram of chlorphenoxamine hydrochloride, caffeine, and 8-chlorotheophylline (5, 10, 10 μg/mL, respectively) in synthetic mixture using a green mobile phase consisting of ethanol (100%) with a flow rate of 0.4 mL/min.

water solvents used in all proposed methods are environmentally favorable according to EHS method and LCA method.

The reported HPLC methods for the selected drug combinations used harmful solvents that are either toxic or not biodegradable. We noticed that acetonitrile which is environmentally not recommendable [16] is common in all the reported methods except two where diethyl ether [9] and ethyl acetate [15] were used, which are less favored than ethanol as they have higher EHS score [16].

Chormatographic separation was achieved on short columns (5 and 7.5cm length) with smaller particle size (3.5 and 3.0) than conventional HPLC and approaches that of UHPLC ones. The separation on such columns was advantageous where the column efficiency is highly increased meanwhile the separation time (6–7 min) and flow rate (0.5 and 0.8 mL/min) decreased. This led to decrease the amount of waste which ranged from 2.4–5.4 mL/run which from the greener point of view is an important measure to be considered. In the reported methods, the run times ranged from 5–25 min with waste production 6–25 mL/run. This shows that the proposed methods save time, solvents and produce smaller amounts of waste.

In the developed HPLC method, a green mobile phase consisting of water and ethanol was used. Good separation and peak symmetry for CB&CD and PB&PZ were obtained upon using ethanol: water in a ratio of (50:50, v/v) at a flow rate of 0.5 mL/min and detection at 210 nm and 220 nm, respectively. The retention times for CD and CB were 5.38 and 7.05 min, respectively (Fig. 1), while for PB and PZ were

Table 1. Assay validation and regression equation parameters of proposed methods for the determination of CB/CD, PB/PZ, MB/SUL, and CPH/CAF/CTH mixtures

Parameters	The proposed method								
	CB	CD	PB	PZ	MB	SUL	CPH	CAF	CTH
Linearity range (μg/mL)	0.5—40	0.8–30	1.5–40	1–35	1–40	1–40	1–70	1.5–35	1–40
Accuracy[a] (Mean ± SD%)	99.6 ± 1.15	100.3 ± 1.12	100.5 ± 0.42	99.8 ± 0.27	99.8 ± 0.85	99.3 ± 0.89	100.1 ± 0.49	99.5 ± 1.52	100.2 ± 0.35
Precision[a]									
(Repeatability)	99.5 ± 1.5	99.9 ± 0.9	99.9 ± 0.9	100.8 ± 0.4	99.3 ± 0.4	98.4 ± 0.5	100.1 ± 0.6	100.3 ± 0.7	100.8 ± 0.8
Intermediate precision	99.7 ± 1.1	99.9 ± 0.2	100.2 ± 0.3	100.1 ± 1.0	99.7 ± 0.3	98.9 ± 0.8	100.2 ± 0.6	99.8 ± 0.7	100.4 ± 0.8
LOD (μg/mL)[b]	0.09	0.22	0.43	0.09	0.10	0.05	0.18	0.44	0.31
LOQ (μg/mL)[b]	0.30	0.72	1.40	0.29	0.33	0.18	0.59	1.47	1.02
Regression									
Slope	47.43	74.25	40.04	23.28	16.95	16.04	6.55	6.59	8.81
Intercept	4.22	35.02	11.78	9.39	−1.42	−0.01	−2.20	2.68	2.32
Correlation coefficient	0.9999	0.9998	0.9998	0.9998	0.9999	0.9999	0.9999	0.9999	0.9998

a) $n = 9$, *Validation was done according to ICH Validation Of Analytical Procedures Text and Methodology.
b) The LOD and LOQ were calculated from $k\ SD/b$ where $k = 3$ for LOD and 10 for LOQ, SD is the SD of y-intercepts, and b is the slope of the calibration curve.

Table 2. System suitability parameters of proposed methods for the determination of CB/CD, PB/PZ, MB/SUL, and CPH/CAF/CTH mixtures

Parameters	The proposed method CB	The proposed method CD	The proposed method PB	The proposed method PZ	The proposed method MB	The proposed method SUL	The proposed method CPH	The proposed method CAF	The proposed method CTH	Reference values[a]
Retention time (min)	7.05	5.38	2.06	5.86	5.32	2.52	3.58	2.47	0.88	
Resolution (R_s)	5.42	16.15	5.11	16.02	7.39	5.54	6.06	6.23	6.41	Rs > 2
Selectivity (α)	1.43	2.06	2.70	7.72	2.77	4.01	3.62	3.10	4.03	α > 1
Symmetry	0.88	0.77	0.77	0.94	0.84	0.52	0.42	0.69	0.62	≤1
Theoretical plates (N)	5149	7829	2809	5440	2118	2071	2169	3293	2134	N > 2000

a) All parameters according to Center for Drug Evaluation and Research, U.S. Food and Drug Administration. Reviewer Guidance, Validation of Chromatographic Methods; FDA, Rockville, MD; (1994).

2.1 and 5.8 min, respectively (Fig. 2). Good separation and peak symmetry for MB and SUL were obtained upon using ethanol: water in a ratio of 94.5:5.5, v/v at a flow rate of 0.8 mL/min and detection at 220 nm. The retention time for MB and SUL were 5.3 and 2.5 min, respectively (Fig. 3). Good separation, and peak symmetry for (CPH, CAF, and CTH) were obtained upon using ethanol (100%) at a flow rate of 0.4 mL/min and detection was carried out for drugs each at its λ max to attain the maximum sensitivity, using diode array detector permits achieving this goal. Retention times for CPH, CAF and CTH were 4.02, 2.45, and 0.89 min, respectively (Fig. 4). This method allowed the simultaneous determination of the three drugs whereas direct spectrophotometry was not possible due to severe overlap of the drugs spectra.

3.2 Validation of the proposed methods

The proposed methods were validated according to the ICH guidelines [18]. The linearity ranges were determined for each compound; the regression equations along with the regression parameters were calculated (Table 1). The LOD and LOQ were calculated based on SD of y intercepts [19].

Precision was performed using three different concentrations of the sample solutions analyzed three times on the same day (repeatability) and on three different days (intermediate precision), and concentrations (15.0, 20.0, and 25.0 μg/mL, 5.0, 15.0, and 25.0 μg/mL, 12.0, 24.0, and 40.0 μg/mL, 5.0, 10.0, and 20.0 μg/mL, 10.0, 25.0, and 35.0 μg/mL, 5.0, 15.0, and 25.0 μg/mL, 5.0, 20.0, and 30.0 μg/mL, 10.0, 20.0, and 40.0 μg/mL and 5.0, 20.0, and

Table 3. Comparison of greenness profile between the suggested and reported HPLC methods

Mixture	Methods	Mobile phase	Run time (min.)	Flow rate (mL/min^{-1})	Waste (g/run) *	Greenness profile**
CB/CD	Proposed	Ethanol/water (50:50, v/v).	6.5	0.5	3.25	
	Reported [3]	0.1 M acetonitrile/methanol/ammonium acetate (30:40:30, v/v).	25	1	25	
	Reported [4]	0.04 Ammonium acetate in 70% acetonitrile solution with 1% dimethyl formamide (pH 6).	20	1	20	
	Reported [5]	Potassium dihydrogen phosphate buffer (0.05M, pH 4.0 adjusted with 0.5% orthophosphoric acid/methanol/acetonitrile (40:40:20,by volume).	120	1	20	
	Reported [6]	Acetonitrile/0.3M ammonium phosphate (32:68 v/v) (pH 4.3).	10	1	10	
PB/PZ	Proposed	Ethanol/water (50:50, v/v).	6.5	0.5	3.25	
	Reported [7]	0.05M ammonium dihydrogen phosphate/acetonitrile: methanol (7:12:1 v/v)	15	1	15	
	Reported [8]	aqueous ammonium formate (pH 3.0, 10mM) and acetonitrile	10	1	10	
MB/SU	Proposed	Ethanol/water (94.5:5.5, v/v).	7	0.8	5.6	
	Reported [9]	Ethanol/diethyl ether/triethylamine (70:30:1,v/v).	5	2	10	
	Reported [10]	Acetonitrile/water (70:30, v/v) at pH 7 .	6	1	6	
	Reported [11]	Elution was accomplished via the application of a dual-mode solvent and flow rate gradient system.	15	Gradient flow rate		
	Reported [12]	Acetonitrile/0.01 M dihydrogen phosphate buffer (45:55 v/v) at pH 4.	10	1.4	14	
CPH/CAF/CTH	Proposed	Ethanol (100%).	6	0.4	2.4	
	Reported [13]	Ethyl acetate/methanol (50:50)/triethylamine pH 9.	30	1	30	

*(runtime × flow rate); **The profile criteria are summarized by four key terms PBT (persistent, bioaccumulative, and toxic), Hazardous, Corrosive, and Waste.

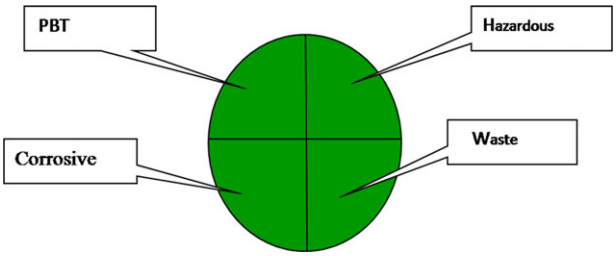

Figure 5. Greenness profile of the suggested HPLC methods.

30.0 μg/mL for CB, CD, PB, PZ, MB, SUL, CAF, CPH, and CTH, respectively). The percentage RSD was calculated (Table 1).

The specificity of the proposed methods was established through the calculation of resolution factor (R_s) of different laboratory mixtures for each combination.

Robustness of the method was studied by deliberately varying certain parameters like flow rate by ± 0.1, changing the ratio of ethanol to water by ± 2 units for each mixture. One factor at a time was changed to estimate the effect. The assay was carried out in triplicate ($n = 3$) at three different concentration levels of each mixture components. Low RSD values were obtained for peak areas and retention time upon applying selected changes, indicating the robustness of the applied methods.

System suitability parameters were evaluated and were found in a good agreement with the USP requirements as presented in Table 2.

The proposed methods were applied for the determination of all components in their dosage forms and the results were compared to reported HPLC methods [7,10,13,15] where comparable results were obtained.

3.3 Greenness profile of the proposed method

To quantify the greenness of an analytical method, the greenness profile symbol (Fig. 5) has been proposed. Greenness profiles have been added to some analytical methods

databases so that one can look at a method's rating and see how green it is. The profile criteria are summarized by four key terms PBT (persistent, bioaccumulative, and toxic), Hazardous, Corrosive, and Waste. Each quadrant is either green or blank depending on the method fit to that particular criterion. Thus, by examining the overall profile, an analyst can quickly compare the greenness of methods [20]. In the proposed methods, water and ethanol are neither defined as PBT nor hazardous by the EPA's Toxic Release Inventory [21, 22]. The pH of the samples and the mobile phases is about 7, i.e. not corrosive and the amount of waste generated is less than 50 g per sample. So according to these criteria, the proposed method passes the four quadrants of the greenness profile (Fig. 5).

A comparison between the suggested and reported HPLC methods is presented in Table 3. It is obvious that the suggested methods are greener than the reported ones with good validation parameters and hence they can be used for routine analysis of the studied mixtures without harming the environment.

4 Conclusion

Green validated HPLC methods can replace conventional HPLC methods for the determination of CB/CD, PB/PZ, MB/SUL, and CPH/CAF/CTH either in their pure powdered form or in their dosage forms. The suggested methods neither use nor produce harmful chemicals. The proposed methods pass the four quadrants of the greenness profile. The methods were validated with respect to linearity, precision, accuracy, system suitability, and robustness. The suggested methods are greener than the reported ones with good validation parameters and hence they can be used for routine analysis of the studied mixtures without harming the environment.

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