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Green UHPLC Method for Simultaneous Determination of Febuxostat and Diclofenac in Pharmaceutical Dosage Form and Human Plasma

Shimaa A. Mahmoud[1]*, Amira M. El-Kosasy[2], Fatma A. Fouad[1]

[1]Pharmaceutical Analytical Chemistry Department, Faculty of Pharmacy, Al-Azhar University, Cairo, Egypt
[2]Pharmaceutical Analytical Chemistry Department, Faculty of Pharmacy, Ain Shams University, Cairo, Egypt
Email: *Shimaa.ahmed22@azhar.edu.eg

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Abstract

Eco-friendly Ultra-high-performance liquid chromatography (UHPLC) with green aqueous-organic mobile phase was applied for the simultaneous determination of febuxostat (FEB) and diclofenac (DIC) with the composition of water:ethanol (85:15 v/v) utilizing phenomenex Kinetex C_{18} column (4.6 × 100 mm 2.6 μm), flow rate 1 ml/min and UV detection at 280 nm with linear ranges of 0.4 - 4.0 μg/mL and 0.5 - 5.0 μg/mL for FEB and DIC, respectively. The proposed method was also successfully applied to analyze the two drugs in pharmaceutical dosage form and human plasma. The results obtained were validated and statistically analyzed and found to be in accordance with those given by reported methods. Moreover, the greenness of the developed method is assessed using suitable analytical Eco-Scale and GAPI tools and comparison with the previously published methods have been carried out to indicate the priority of the proposed method. UHPLC is considered eco-friendly method regarding uses of safe solvents, simple, accurate and short time of analysis.

Keywords

Febuxostat, Diclofenac, Ultra-High-Performance Liquid Chromatography, Eco-Scale, Green Analytical Procedure Index Tools

1. Introduction

Febuxostat (FEB) is 2-[3-cyano-4-(2-methyl propoxy) phenyl]-4-methyl-1, 3 thiazole-5-carboxylic acid [1], Figure 1(a). It is a xanthine oxidase inhibitor that

is used in the treatment of hyperuricemia and chronic gout [2]. It is a nonpurine selective inhibitor of xanthine oxidase. FEB was found to be superior to allopurinol in reducing the serum uric acid levels [3]. To decrease inflammation and control pain in gout attacks, some dosage forms containing a NSAID such as diclofenac potassium (DIC) are co-formulated. DIC is a potassium salt of 2-(2, 6-dichloranilino) phenyl acetic acid [1], **Figure 1(b)**. The literature review revealed few analytical methods for simultaneous analysis of FEB and DIC. These methods include: spectrophotometry [4] [5] [6] [7], HPTLC methods [7] [8] and HPLC methods [9] [10]. FEB is not official in any pharmacopoeia while DIC is official in British, United States and European Pharmacopoeias [11] [12] [13]. All these methods have threats to the environment as they are using or applying hazardous solvents and chemicals, and the green analytical chemistry principles were not implemented.

In modern analytical chemistry, establishing a greener method implies bearing in mind the green aspects from the early stages of method development to ensure the reduction or elimination of hazardous substances that are either utilized in or produced by the method, thus, turning it to be a much safer method to the environment [14] [15]. Recently, UHPLC has been frequently proposed as an alternative to HPLC, which means introducing an environment-friendly approach to drug analysis achieved by reducing the consumption of solvents. It also offers greater chromatographic resolution and higher sensitivity as well as requiring less time due to faster analysis [16] [17]. As the development of a green analytical method is highly recommended, the presence of assessment tools to evaluate the greenness profile of the developed method is highly important as well. One of the assessment tools is the Analytical Eco-Scale [18] and recently a new tool known as the Green Analytical Procedure Index (GAPI) [19] was established to assess the green character of a whole analytical method. It is ideal to apply the two tools to obtain a deeper view on the greenness of a method. With virtually no development effort or investment required, LC methods utilizing fully porous column technology can easily be improved in resolution, sensitivity, and productivity by simply replacing the 5 µm or 3 µm fully porous columns with an equivalent Kinetex Core-Shell column. The Kinetex Core-Shell technology achieves higher chromatographic efficiencies in comparison to fully porous particles of similar diameters and under similar method conditions. This

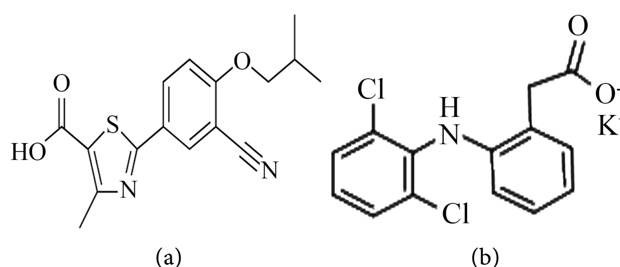


Figure 1. Chemical structures of (a) Febuxostat and (b) Diclofenac potassium.

efficiency gain allows chromatographers to maximize the performance out of their HPLC/UHPLC system by using the right particle morphology for the application. The proposed method included development for the first time of simple, rapid and green UHPLC method using Kinetex C_{18} column with UV detection for the quantitative determination of the two cited drugs in their pharmaceutical dosage form and human plasma with accepted accuracy and precision, this is allow using it in routine analysis laboratories.

2. Experimental

2.1. Instrumental

- Phenomenex Kinetex C_{18} column (4.6 × 100 mm 2.6 µm) with binary pump and UV detector (USA).
- Vortex mixer (Model: VM-300P, Gemmy Industrial Corp., Taiwan region) was used for human plasma samples preparation.
- Centrifuge (Model: 2-16P, Sigma Laborzentrifugen, Germany) was used for human plasma samples preparation.
- Sonicator (Thermo Scientific, Waltham, USA).

2.2. Chemicals and Reagents

- Pure Febuxostat: B. No. OP-FAB/06/16/001 was kindly provided by Mash Premiere with purity 100.61% according to supplier.
- Pure diclofenac potassium: B. No. DK/1808/0080B was kindly provided by The Arab Company for Gelatin & Pharmaceutical products with purity 99.85% according to supplier.
- Ethanol and methanol (Sigma - Aldrich, USA) analytical grade.

2.3. Pharmaceutical Formulation

- Xanfeb DSR® tablets, it is labelled to contain 40 mg FEB and 100 mg DIC manufactured by Indoco Remedies, India) were purchased from pharmacies.

2.4. Standard Solutions

Stock standard solutions of the drug (1.0 mg/mL) were prepared in methanol. Working solution (0.1 mg/mL) was prepared by dilution with the mobile phase.

3. Procedures

3.1. Chromatographic Conditions

- Mobile phase: consisted of water: ethanol (85:15 v/v) The mobile phase was filtered through 0.45-µm Millipore membrane filter and degassed by sonication for 30 min before use
- flow rate: 1 ml/min
- Detection: UV at 280 nm
- Temperature: ambient temperature

3.2. Construction of the Calibration Graphs

Accurately measured aliquots of the working standard solutions (100 μg/mL) were transferred into two separate sets of 10-mL volumetric flasks and adjusted to volume with the mobile phase to obtain final concentrations of (0.4 - 4.0 μg/mL) or (0.5 - 5.0 μg/mL), respectively for FEB and DIC. The standard solutions were then analyzed by injecting 10 μL of each solution under the above chromatographic conditions. The calibration graphs were constructed by plotting the peak area against the corresponding drugs concentration in μg/mL and the corresponding regression equations were computed.

3.3. Laboratory Prepared Mixtures

Mixtures of different ratios for FEB and DIC were prepared by transferring aliquots from the corresponding working solutions (100 μg/mL), mixed well and completed to volume with mobile phase to be analyzed by the proposed method.

3.4. Pharmaceutical Dosage Forms

Ten tablets of Xanfeb DSR® were accurately weighed and grounded. An amount of the powder equivalent to 80 mg FEB and 200 mg DIC was transferred to a 50-mL volumetric flask. A volume of 25 mL of methanol was added, and the flask was sonicated for 30 min, then completed to volume with methanol followed by filtration. One milliliter of this solution was diluted to 100 mL with methanol to get the final solution of 16 μg/mL of FEB and 40 μg/mL of DIC and filtered through a 0.45-μm membrane filter. Further dilution with the mobile phase was done to obtain the working standard solution to be analyzed as described above. The recovered concentration of each analyte was calculated from the corresponding regression equation.

3.5. Preparation of Plasma Samples

The calibration graphs were constructed using spiked human plasma as follows: in a stoppered centrifuge tube, an aliquot quantity of 300 μL plasma was added and spiked with 100 μL of working solutions of (1.25, 2.5, 3.75, 5 and 7.5 μg/mL) for FEB or (2, 5, 7.5, 10 and 12.5 μg/mL) DIC, 600 μL methanol was added and the samples were vortex mixed for 5 minutes and centrifugated at 3000 rpm for 15 minutes. 400 μL of the supernatant was diluted with 600 μL mobile phase to obtain final concentration in the range 0.05 - 0.3 μg/mL and 0.08 - 0.5 μg/mL for FEB and DIC; respectively, all samples were filtered through a 0.45-μm membrane filter and directly injected into the chromatographic system under the above described conditions. The linear regression equations relating the peak areas to the concentration were derived for each analyte. A blank plasma experiment was performed simultaneously.

4. Results & Discussions

The main green analytical chemistry principle utilized during the development

of the proposed method was the elimination of hazardous solvents and their replacement with green ones. Therefore, ethanol was used in the mobile phase instead of acetonitrile or methanol which is the most commonly utilized solvents. According to the US Environmental Protection Agency (EPA), acetonitrile is ranked as hazardous solvent owing to its intrinsic toxicity [20]. However, based on environment, health and safety properties (EHS) and life cycle assessment (LCA); ethanol is considered a green solvent retaining a low impact on EHS [21] owing to its derivation from renewable sources, its minor toxicity and its ease of disposal [22]. Thus, a mobile phase composed of ethanol and water was developed. Regarding the stationary phase using phenomenex kinetex C_{18} column (4.6 × 100 mm, 2.6 μm particle size) in our proposed method enabled significant reduction in retention time and solvent consumption than Agilent Zorbax SB-C_{18} column (4.6 × 250 mm, 5-μm particle size) attached to Agilent C_{18} (4.6 × 12.5 mm, 5-μm particle size) guard column which was reported by F.A .El-Yazbi *et al.* [9]. Also phenomenex kinetex C_{18} column (4.6 × 100 mm, 2.6 μm particle size) which was used in our proposed method gave better sensitivity than phenomex C_{18} column (4.6 × 250 mm, 5-μm particle size) which was reported by S. Vaibhav *et al.* [10]. Also the proposed UHPLC method was validated for the determination of FEB and DIC in their pharmaceutical dosage form and biological fluid. Moreover the greenness of the developed method is assessed using suitable analytical Eco-Scale and GAPI tools and comparison with the previously published methods has been carried out to indicate priority of the proposed method.

4.1. Optimization of the Chromatographic Performance and System Suitability

4.1.1. System Suitability Testing

System suitability test (SST) parameters were performed during the development and optimization of the method to ensure that the system is working correctly during the analysis. The test was performed by injecting the standard drug solution in triplicate and the parameters were calculated according to the BP [11] and USP [13] Guidelines. The final SST parameters including tailing factor (T), column efficiency (number of theoretical plates *N*) and Resolution (Rs) are summarized in **Table 1**.

4.1.2. Detection Wavelength

The UV absorption spectra of the methanolic solution of the studied drugs exhibited maxima at 316 for FEB and 283 nm for DIC as shown in **Figure 2**. Both drugs showed reasonable absorbance at about 280 nm. However, three wavelengths (230, 254, 280) were tried to select the most suitable one where 280 nm showed the highest sensitivity.

4.1.3. Column

Three different columns were used for performance investigations, including phenomenex kinetex C_{18} column (4.6-mm × 100-mm, 2.6 μm particle size), BDS hypersil C_{18} column (150 mm × 4.6 mm, 5 μm particle size) and Intersil C_{18}

(4.6-mm × 250-mm, 5 μm particle size). Experimental studies revealed that, the first column showed better results where the peaks of both analytes were more symmetrical and well-defined with a total run time less than 5 min. The second column was not suitable as it couldn't separate the two drugs. In addition, the third column resulted in asymmetrical peaks with delayed retention time (Table 1).

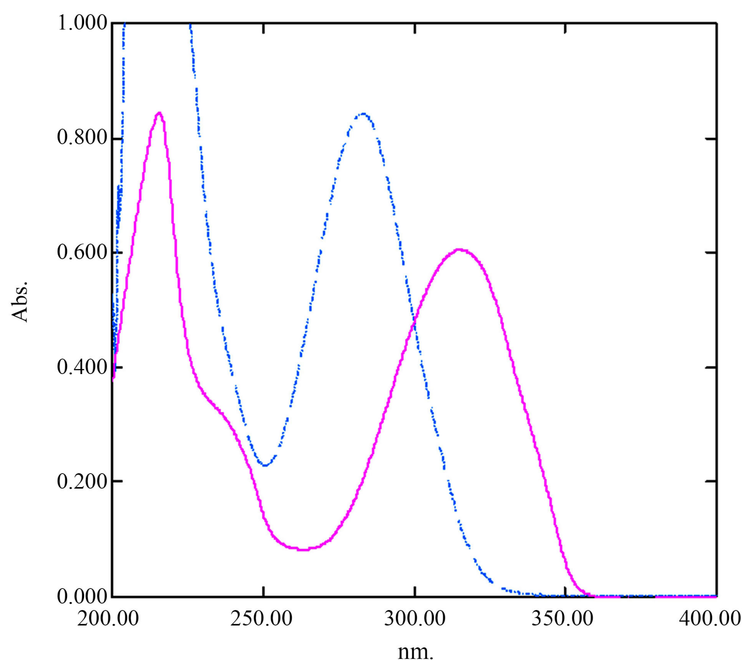


Figure 2. Zero order spectra of (8 μg/mL) of each FEB (red) & (20 μg/mL) DIC (blue) in methanol.

Table 1. Optimization of the chromatographic conditions for determination of FEB and DIC by the proposed UHPLC method.

Parameter		No. of theoretical plates		Resolution	Tailing factor	
		FEB	DIC		FEB	DIC
Column	Intersil C_{18}	-	4244	-	-	1.18
	BDS hypersil C_{18} column	3608	3916	3.41	1.32	1.22
	phenomenex kinetex C_{18} column	4558	4787	5.18	1.09	1.11
Mobile phase	ACN-buffer	4215	3112	5.99	1.24	1.73
	Ethanol-water	4558	4787	5.18	1.09	1.11
	Methanol-buffer	4459	---	---	1.16	---
Ethanol concentration (%v/v)	5%	485	572	4.61	2.05	1.97
	10%	2300	1556	4.12	1.27	1.23
	15%	4558	4787	5.18	1.09	1.11
	25%	2859	3646	3.83	1.85	1.53
	30%	3152	2957	3.29	2.04	1.81
Flow rate (mL/min)	0.6	2277	2804	3.91	1.38	1.26
	0.8	3887	3915	5.26	1.26	1.14
	1.0	4558	4787	5.18	1.09	1.11
	1.2	2486	2961	4.83	1.18	1.16

4.1.4. Mobile Phase Composition

Different mobile phases were tried as methanol: phosphate buffer, acetonitrile: phosphate buffer and ethanol:water. Initial experiments showed good separation with ethanol: water compared with the other mobile phases. Several modifications in the mobile phase composition were performed in order to study the possibilities of improving the performance of the chromatographic system which provide satisfactory, selectivity and sensitivity in a short separation time. Different % of ethanol were studied (5% - 30%) where 15.0% ethanol was found to be the optimum concentration regarding separation efficiency and resolution (Table 1).

4.1.5. Flow Rate

(Table 1) shows the effect of different flow rates (0.6 - 1.2 mL/min) on the chromatographic separation. A flow rate was optimized at 1 mL/min due to the highest efficiency in a short analysis time. Although lower flow rates showed higher resolution, but they were not selected as they led to an increase in the total run time, in addition to a decrease in the number of theoretical plates for both analytes. Optimum chromatographic conditions for the UHPLC determination of the studied drugs are summarized in Table 1. The proposed method permitted the separation of the two drugs with good resolution in a reasonable time, less than 5.0 min. Figure 3 shows typical chromatograms for laboratory prepared mixtures of FEB and DIC under the described chromatographic conditions where well-separated symmetrical peaks were observed. The retention times for FEB and DIC were 2.5 and 4.2 min, respectively.

5. Method Validation

Validation of the developed UHPLC method was performed according to the international conference on harmonization (ICH) guidelines [23].

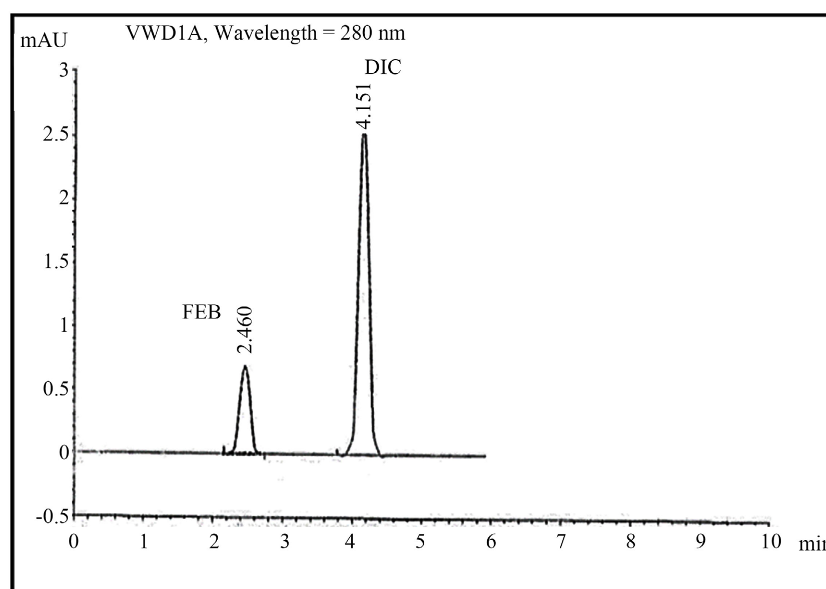


Figure 3. UHPLC chromatogram lab prepared mixture of febuxostat (0.8 μg/mL) and diclofenac potassium (2.0 μg/mL) (2:5 ratio) at 280 nm.

5.1. Linearity and Range

The linearity of the developed method was confirmed by plotting the peak area against the analyte concentration in μg/mL. The graphs were linear over the concentration range of (0.4 - 4.0 μg/mL) and (0.5 - 5.0 μg/mL) for FEB and DIC; respectively. Linear regression analysis of the obtained data was presented in Table 2.

5.2. Limits of Detection and Quantitation

LODs were determined by evaluating the lowest concentration of the analytes which could be detected but not necessarily quantitated as exact values. LOQs were determined by establishing the lowest concentration of the analytes which could be quantitatively measured with suitable precision and accuracy. The results are shown in Table 2.

5.3. Accuracy

The accuracy of the results was checked by applying the proposed method for determination of different samples of FEB and DIC. The concentrations were obtained from the corresponding regression equations of the calibration graphs. The % recoveries for the proposed method were 99.76% and 100.63% for FEB and DIC as shown in Table 2.

5.4. Precision

Three different concentrations (0.8, 2.4 and 3.6 μg/mL) of FEB and (1, 3 and 4.5 μg/mL) of DIC solution were analyzed three times each, intra-daily and on three successive days using the previous mentioned procedure described for linearity. The relative standard deviations for the two drugs were calculated using the corresponding regression equations. The results are shown in Table 2.

Table 2. The regression parameters and validation results for determination of FEB and DIC by the proposed method.

Parameter	Results	
	FEB	DIC
Wave length (nm)	280	280
Linearity range μg/mL	0.4 - 4	0.5 - 5
Slope	6.5171	8.3367
Intercept	−0.0087	−0.0145
Determination coefficient (r^2)	0.9999	0.9998
Accuracy (%R)	99.76	100.63
Inter-day precision RSD%	0.336	1.187
Intra-day precision RSD%	0.0651	1.017
LOD μg/mL	0.0159	0.0263
LOQ μg/mL	0.0481	0.0796

5.5. Robustness

The robustness of the proposed method was indicated by the constancy of the peak area with deliberate changes in the experimental parameters. These parameters included ethanol concentration (15% ± 0.5%, v/v) and UV detection (280 ± 1 nm). These minor changes did not affect the peak area of both drugs confirming robustness of the method (Table 3).

5.6. Selectivity

The selectivity of the proposed method was assured by applying it to laboratory prepared mixtures of FEB and DIC at different concentrations within the linearity range. Good recoveries of both drugs were obtained as shown in Table 4.

5.7. Sample Solution Stability and Mobile Phase Stability

Evaluation of the stability of FEB and DIC standard solutions was achieved by quantification of each drug in this solution and comparing the results obtained to that obtained from freshly prepared standard solution. Similarly, the stability of the mobile phase was checked. No significant changes were observed in standard solution or mobile phase responses, relative to freshly prepared ones. The results obtained in both cases proved that the sample solution stable up to 7 days and mobile phase used during the assay were stable up to 5 days in refrigerator.

Table 3. Robustness of the proposed UHPLC method using FEB (2.0 μg/mL) and DIC (3.0 μg/mL).

Parameter	Conc. found (μg/mL)		% Found	
	FEB	DIC	FEB	DIC
Ethanol % concentration (v/v)				
14.5	2.016	3.022	100.80	100.73
15	1.994	2.997	99.70	99.90
15.5	1.976	2.994	98.8	99.80
Mean%			99.77%	100.14%
± SD			1.002	0.511
%RSD			1.004	0.510
UV detection (nm)				
279	1.985	2.989	99.25	99.63
280	1.996	3.014	99.80	100.47
281	2.016	3.017	100.80	100.57
Mean%			99.95%	100.22%
± SD			0.642	0.516
%RSD			0.642	0.515

Table 4. Determination of FEB and DIC in laboratory prepared mixtures by the proposed UHPLC method.

Lab prepared mixtureμg/mL		FEB/DIC ratio	% Recovery	
FEB	DIC		FEB	DIC
0.8	2	2:5*	98.63	101.55
2	4	1:2	99.20	100.33
4	2	2:1	99.73	100.25
2	2	1:1	100.35	99.15
1.2	3	2:5*	100.42	99.87
Mean % ± SD			99.67% ± 0.763	100.23% ± 0.873

*Ratio of dosage form.

5.8. Application of the Proposed Method for Determination of FEB and DIC in Their Laboratory Prepared Mixtures

The proposed method was successfully applied to the simultaneous determination of FEB and DIC in laboratory prepared mixtures in different ratio and in their medicinally recommended ratios of 2:5; respectively as shown in Figure 3 and Table 4.

5.9. Application of the Proposed Method to the Determination of the Studied Drugs in Their Pharmaceutical Dosage Form

The proposed procedure was applied for the determination of FEB and DIC in their tablets. The obtained results were in good agreement with the label claim, indicating no interference from excipients and additives. The validity of the proposed method was further assessed by applying the standard addition technique, mean percentage recoveries of pure added were 100.28% ± 1.099 and 99.98% ± 1.871 for FEB and DIC; respectively (Table 5).

The obtained results were statistically compared to those obtained by the reported method [10]. No significant differences were found by applying t-test and F-test at 95% confidence level [24] indicating good accuracy and precision of the proposed method for the analysis of the studied drugs in its pharmaceutical dosage form as shown in Table 6.

5.10. Application of the Proposed Method for Determination of FEB and DIC in Spiked Human Plasma

FEB is reported to be rapidly absorbed after single or repeated doses with a maximum plasma concentration (C_{max}) of 1.9 ± 0.678 μg/mL reported for a 40 mg dose at t_{max} 1.0 - 1.5 h [25]. On the other hand, DIC is completely absorbed after oral administration. It exhibits a mean peak plasma concentration (C_{max}) 1.0 μg/ml after about two hours [26]. It is clear that UHPLC allows biological samples to be analyzed FEB and DIC a linear relationship was established by plotting the peak area against the drug concentration in μg/mL. Linear regression analysis of the data gave the following equation:

$$P = 6.665C - 0.2266\ (r = 0.9995) \text{ for FEB}$$

$$P = 5.6548C + 0.1135\ (r = 0.9993) \text{ for DIC}$$

Table 5. Determination of FEB and DIC in pharmaceutical dosage forms by the proposed UHPLC method and results obtained by standard addition technique.

Xanfeb DSR® contain 40 mg febuxostat and 100 mg diclofenac					
	Claimed taken (μg/ml)	Pure added (μg/mL)	**Mean*% ± SD**	**Pure found (μg/mL)**	R% of pure added
	0.8	1.6		1.586	99.13
FEB	0.8	2.4	100.28 ± 1.185	2.409	100.38
	0.8	2.8		2.837	101.32
	Mean % ± SD			100.28% ± 1.099	
	2	1		1.021	102.10
DIC	2	2	99.92 ± 1.036	1.971	98.55
	2	3		2.979	99.30
	Mean % ± SD			99.98% ± 1.871	

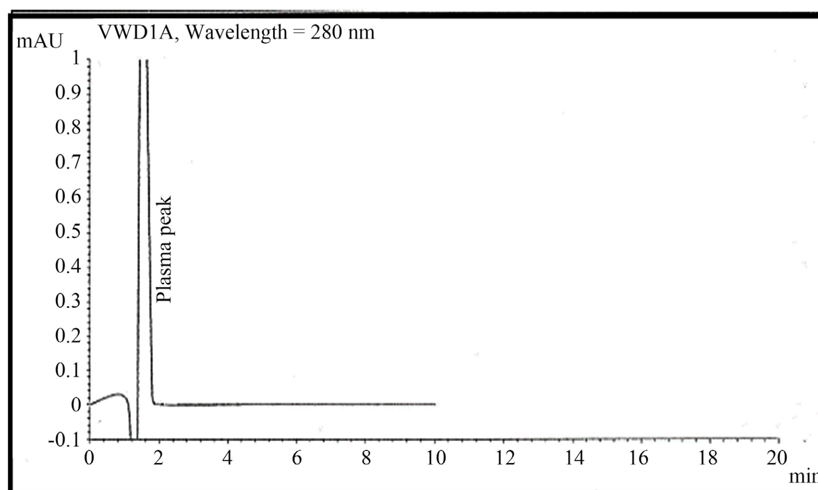
*average of five concentrations.

Table 6. Statistical comparison for the results obtained by the proposed UHPLC method and the reported method (10) for the analysis of FEB and DIC.

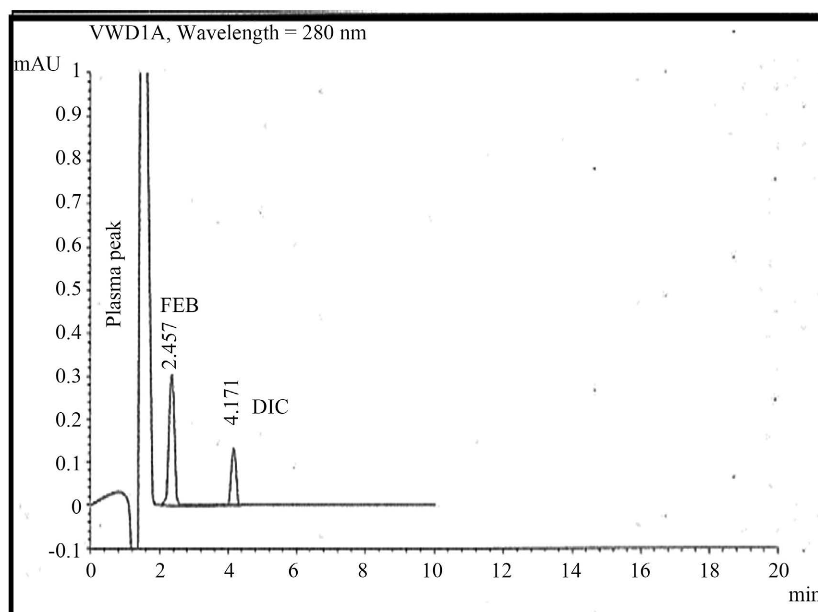
	UHPLC method			**Reported method** [10]		
	Taken (μg·mL^{-1})	**Found* (μg/mL)**	**% Recovery**	**Taken (μg/mL)**	**Found * (μg/mL)**	**% Recovery**
	0.4	0.395	98.75	5	5.06	101.19
FEB	0.8	0.813	101.63	10	10.136	101.36
	1.2	1.194	99.5	15	14.822	98.81
	1.6	1.605	100.31	20	19.812	99.06
	2	2.024	101.2	25	25.273	101.09
Mean % ± SD		100.28% ± 1.185			100.30% ± 1.255	
t-test		0.031 (2.306)			-	
F-test		1.121 (6.388)			-	
	1	1.006	100.6	20	19.99	99.95
	2	1.988	99.4	30	29.817	99.39
DIC	3	3.041	101.37	40	40.268	100.67
	4	3.975	99.38	50	50.105	100.21
	5	4.942	98.84	60	59.898	99.83
Mean % ± SD		99.92% ± 1.036			100.01% ± 0.473	
t-test		0.181 (2.306)			-	
F-test		4.792 (6.388)			-	

*Average of three separate determinations. The values between parentheses are the tabulated t and F values at $P = 0.05$([24]).

where: *P* is the peak area, *C* is the concentration of the drug in μg/mL and *r* is the correlation coefficient. The high value of the correlation coefficient (*r*) indicated the good linearity of the calibration graph constructed in human plasma. The proposed method showed satisfying results for the determination of FEB and DIC in spiked human plasma over the concentration range of 0.05 - 0.3 μg/mL and 0.08 - 0.5 μg/mL for FEB and DIC; respectively, with a lower detection limit of 0.0106 μg/mL and 0.0231 μg/mL for FEB and DIC; respectively. The assay results using the proposed method are summarized in Table 7. Figure 4(a) and Figure 4(b) show representative chromatograms for blank and spiked plasma samples.



(a)



(b)

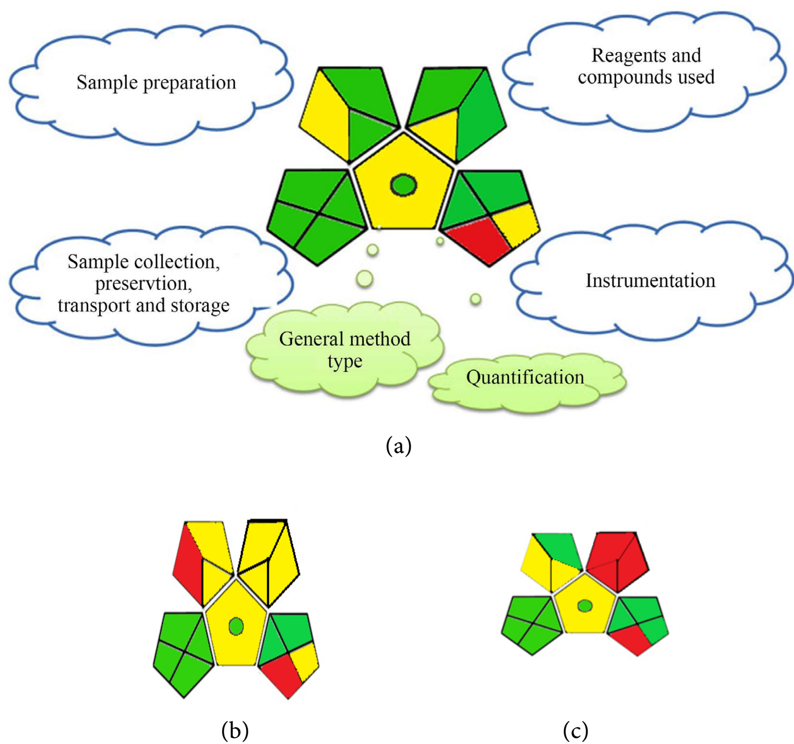
Figure 4. UHPLC chromatogram of blank plasma at 280 nm (a), UHPLC chromatogram of febuxostat (0.3 μg/mL) and diclofenac potassium (0.08 μg/mL) in spiked human plasma at 280 nm (b).

Table 7. Assay results for the determination of FEB and DIC in spiked human plasma by the proposed UHPLC method.

Parameter	Conc. taken (μg/mL)	Conc. Found (μg/mL)	% recovery	Parameter	Conc. taken (μg/mL)	Conc. Found (μg/mL)	% recovery
FEB	0.05	0.0517	103.40	DIC	0.08	0.0787	98.38
	0.1	0.0972	97.20		0.2	0.1992	99.60
	0.15	0.1479	98.60		0.3	0.3082	102.73
	0.2	0.2043	102.15		0.4	0.3916	97.90
	0.3	0.2988	99.60		0.5	0.5023	100.46
Mean %			100.19%	**Mean %**			99.81%
± SD			2.55	**± SD**			1.92
%RSD			2.545	**%RSD**			1.924

6. Assessment of Greenness of the Proposed Method

Eco-Scale was proposed and calculated by assessing penalty points obtained by each step during the whole analysis which doesn't match the ideas of greener chemistry. A green analysis is deemed ideal if it has an Eco-Scale value of 100, excellent if >75, acceptable if >50, and inadequate if <50. The penalty points are assigned for high amounts and high hazards connected with utilization of chemicals, high energy consumption, occupational hazards and generation of wastes. The analytical Eco-Scale has advantages compared to other scales due to the simplicity of calculating its score and pointing out to different aspects of the environmental impact of analytical methods in its assessment procedure [27]. The proposed method is superior over the two reported HPLC methods. The analytical Eco-Scale value of the proposed method was calculated and its score was 79 which is very close to be an excellent green analysis than the reported method [9] its score was 62 which is very close to be an acceptable green analysis and the other reported method [10] its score was 71 which is very close to be an acceptable green analysis as shown in **Table 8**. Additionally; The GAPI [19] utilizes five pentagrams to assess and quantify the environmental influence of every step of an analytical method with a color code; green, yellow and red signifying low, medium and high influence on the environment, respectively. The GAPI tool also keeps in account new criteria as the health and safety. Interpretation of the GAPI pentagrams for the proposed and the reported methods is shown in **Figures 5(a)-(c)**. Revealed that: the proposed UHPLC method has the least environmental impact as 10 fields were shaded green, 4 yellow and only 1 red as shown in **Figure 5(a)**, the reported method [9] showed 6 green, 7 yellow and 2 red fields; **Figure 5(b)** and the other reported method [10] showed 8 green, 3 yellow and 4 red fields; **Figure 5(c)**, The green evaluation for the proposed method and the reported methods using the GAPI tool indicated the greenness of the suggested method.



(a)

(b) (c)

Figure 5. GAPI assessment of the green profile of the proposed method (a) and reported methods (b and c).

Table 8. The penalty points of the proposed methods& Reported methods according to the analytical Eco-Scale.

Category	Proposed method	Reported method [9]	Reported method [10]
Reagents/Instruments	**Penalty points**		
Methanol	12	12	12
Water	0		
Phosphate buffer			2
Acetonitrile			4
Sodium hydroxide			2
Formic acid		12	
Diethyl ether		6	
Ethanol	3		
Centrifuge	1	1	1
Sonicator	1		1
HPLC		2	2
UPLC	0	0	0
Occupational hazard	0	0	0
Waste	5	5	5
Total penalty points	22	38	29
Analytical Eco-Scale total	100 − 22 = 79	100 − 38 = 62	100 − 29 = 71
	Excellent	**Acceptable**	**Acceptable**

7. Conclusion

The proposed UHPLC method provides simple, specific, rapid and green quantitative analysis of FEB and DIC in their raw materials, combined tablets and in human plasma. The proposed method is accurate, cheap and rapid enough to be applied in the quality control analysis of both drugs. Satisfactory precision and accuracy, minimum cost, quicker analysis and environmentally safe are the core features of this method.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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