Four Greenness Evaluations of Two Chromatographic Methods: Application to Fluphenazine HCl and Nortriptyline HCl Pharmaceutical Combination in Presence of Their Potential Impurities Perphenazine and Dibenzosuberone

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Abstract
Nowadays, when analysts develop a new method, they are taking into consideration the green aspects of the developed method. Two chromatographic methods were developed for the determination of antidepressant pharmaceutical combination nortriptyline hydrochloride and fluphenazine hydrochloride in presence of their potential impurities and the methods were assessed using four tools; eco-scale, analytical greenness profile (AGP), analytical greenness metric approach (AGREE), and green analytical procedure index (GAPI). Method (A) was RP-HPLC, in which the separation was carried out on C_{18} column (250 × 4.5 mm, 5 µm) by gradient elution using mobile phase consisting of a mixture of water containing 0.1% H_{3}PO_{4} (pH 2.25) and methanol, at a flow rate of 2 mL/min with DAD detection at 254 nm. Method (B) was TLC, in which the separation was carried out on silica gel TLC F_{254} plates. The mobile phase used was a mixture of methanol and acetone (9:1, v/v) with UV detection at 245 nm. The proposed methods agreed with ICH guidelines of method validation and were successfully applied for determination of the proposed components in their dosage form and the results were compared statistically to those obtained by the reported RP-HPLC method with no significant difference; which suggests the application of the developed methods for routine quality control analysis of these drugs.

Keywords Dibenzosuberone · Fluphenazine HCl · Nortriptyline HCl · Perphenazine · RP-HPLC · Thin-layer chromatography

Introduction
Sustainability, environmental effect, and little waste are key criteria that are typically connected to green analytical chemistry. It purposes to provide environmentally friendly processes for regular pharmaceutical analysis, which is of the greatest priority to analysts in the field of quality control [1]. For the assessment of the greenness of proposed analytical methods, four tools were used such as: eco-scale, analytical greenness profile (AGP), analytical greenness metric approach (AGREE), and green analytical procedure index (GAPI). It was discovered that number of parameters, such as sample preparation, the use of non-green or bio-accumulative solvents, and waste production, would alter the greenness of chromatographic techniques [2]. The analyst's biggest issue is finding a balance between his method's quality and efficiency, cost, and environmental friendliness.

Recently, the psychological and neurological complications are very common, including depression, anxiety, insomnia, schizophrenia and Alzheimer. Many patients may suffer from more than one of the previously mentioned psychological complications at the same time. Therefore, for better control of their illnesses, a combination therapy with two or more medications maybe required [3].

Nortriptyline HCl (NOR) and fluphenazine HCl (FLU), as illustrated in Fig. 1, are antidepressant agents [4]. Both are co-formulated together in Motival® tablets to treat psychotic disorders including depression and schizophrenia [5]. They were previously determined simultaneously alone or in presence of
other drugs in different matrices using spectroscopic methods [6–9] and chromatographic methods [8, 10–13]. Perphenazine (PER), as illustrated in Fig. 1, is the impurity of FLU according to the British Pharmacopeia [4]. It acts like FLU as it is a neuroleptic and used for treatment of depression [4]. It also has tranquilizer, sedative and anxiolytic effects [14, 15]. Dibenzosuberone (DBZ), as illustrated in Fig. 1, is the impurity of NOR according to the British Pharmacopeia [4]. It is a tricyclic antidepressant and serves as a starting point for the creation of a variety of physiologically active antidepressant drugs [16]. In the literature, there was only spectrophotometric and multivariate spectrophotometric methods developed for simultaneous determination of NOR, FLU, PER and DBZ [17] while there is no chromatographic methods developed for that purpose. As a result, the goal of this research is to develop, optimize, and verify RP-HPLC and TLC methods for simultaneous determination of NOR, FLU, PER and DBZ. In addition, the greenness criteria of the developed methods were assessed using the four previously mentioned greenness tools.

**Experimental**

**Instruments**

For RP-HPLC method: Waters 2695 Alliance HPLC system equipped with G 1311C quaternary pump and connected to G 1322A on-line degasser. Samples were applied using G 1329B auto-sampler. The column temperature was adjusted using G 1316A heater. All components were detected spectrophotometrically using Waters 996 photodiode array detector. Column C18 inertial ODS-3 (250 mm × 4.6 mm internal diameter, 5 µm particle size) was used. Trans instruments (BP 3001) professional bench top pH meter was used to adjust the pH of the mobile phase.

For TLC method: TLC plates made of aluminum with dimensions of (20 * 20 cm) coated with silica gel 60 F254 with thickness of 0.25 mm and particle size of 5 µm (Merck, Germany). Applications were done by 100 µL syringe using Camag Linomat IV applicator. Scanning was done using TLC scanner, model 3 S/N Camag (Muttenz, Switzerland) connected and controlled with winCATS software (version 3.15).

**Materials and Reagents**

**Pure Samples and Pharmaceutical Dosage Form**

1. NOR was purchased from Sigma Aldrich, Egypt. Its purity was found to be 99.94% [11]. FLU was kindly supplied from Al-Kahira Company for Pharmaceuticals and Chemicals, Egypt. Its purity was found to be 100.24% [11].
2. PER and DBZ were purchased from Sigma Aldrich, Egypt. Their purities were certified to be 98% for PER, and 97% for DBZ.
3. Motival® tablets (batch no. B60317) were manufactured by Glaxo Smith Kline Company, Cairo, Egypt. Each tablet is labeled to contain 0.5 mg and 10 mg of NOR and FLU, respectively.

**Solvents**

1. Water and methanol of HPLC grade were purchased from Fischer, London, UK.
2. Ortho-phosphoric acid and acetone were purchased from El-Nasr Company for Pharmaceutical Industries, Cairo, Egypt.

**Stock and Working Standard Solutions**

For RP-HPLC method: stock solutions of 1 mg/mL concentration were prepared for each of NOR, PER, FLU and DBZ, using methanol as solvent. Working solutions of 0.1 mg/mL concentration were prepared for each of NOR, PER, FLU and DBZ using their respective stock solutions, using the mobile phase as solvent.
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For TLC method: stock solutions of 1 mg/mL concentration were prepared for each of NOR, PER, FLU and DBZ, using methanol as solvent. Working solutions of 0.5 mg/mL concentration were prepared for each of NOR, PER, FLU and DBZ using their respective stock solutions, using methanol as solvent.

**Procedures**

**Chromatographic Conditions**

For RP-HPLC method: chromatographic separation was carried out on C\(_{18}\) inertsil ODS-3 (250 mm × 4.6 mm i.d, 5 μm particle size) column. Gradient elution was performed using a mixture of water containing 0.1 % H\(_3\)PO\(_4\) (pH 2.25) and methanol, the gradient sequence is given in Table 1. The used flow rate was 2 mL/min and the total run time was 14 min. The injection volume was 20 μL with UV detection at 254 nm and the temperature was adjusted at 40°C.

For TLC method: Camag Linomat IV applicator was used to apply samples on the TLC plates as bands of 3 mm width, separated from each other by 6 mm and 10 mm from the bottom edge. The mobile system composed of a mixture of methanol: acetone (9:1, v/v) was added to a glass jar and allowed to be saturated for 15 min. Then, the plates were added inside the jar, covered well, and allowed to stand until the developing system reaches the front line of the plate which lies before the end of the plate by 1 cm. Finally, the plates were scanned using UV scanner at 245 nm. The used application volume of the samples was 10 μL.

**Construction of Calibration Curves**

For RP-HPLC method: Different volumes of NOR, PER, FLU and DBZ were accurately transferred from their appropriate working solutions into four independent sets of 10 mL measuring flasks and methanol was used to complete the volume to produce concentration ranges of 2–50 μg/mL for NOR, 0.5–30 μg/mL for PER, 7–130 μg/mL for FLU, and 0.2–30 μg/mL for DBZ. Then, 20 μL of each sample were injected three times and the previous procedures in “Chromatographic Conditions” were carried out. Finally, the calibration graphs were drawn by graphing the peak area/10\(^5\) versus the corresponding concentration in μg/mL, and then the regression equations were computed.

For TLC method: Aliquot portions equivalent to 50–3000 μg of NOR, 50–1500 μg PER, 100–4500 μg FLU and 100–1200 μg DBZ were transferred from their working solution (500 μg/mL) into four separate sequences of 10 mL volumetric flasks. The volume of each flask was completed with methanol. Ten μL of each of the proposed components solutions were spotted in triplicate to TLC plates (20 *10 cm). Linear ascending development was performed under a chromatographic conditions mentioned previously in “Chromatographic Conditions” and the corresponding peak areas were recorded. The calibration curves of the proposed components were constructed by plotting the mean integrated peak areas/10\(^4\) versus the corresponding concentration in (μg/band) and then the regression equations were computed.

**Assay of Pharmaceutical Formulation**

Ten Motival® tablets were weighed, finely powdered and mixed well. An amount equivalent to 2.5 mg and 50 mg of NOR and FLU, respectively, was accurately weighed and placed into a 50-mL volumetric flask and 35 mL methanol was added. The resulting solution was sonicated for 30 min, cooled, and the volume was completed with methanol to yield a stock solution containing 0.05 mg/mL and 1 mg/mL of NOR and FLU, respectively.

For RP-HPLC method: one mL of the stock solution was transferred into 10 mL volumetric flask and the volume was completed with methanol to yield a working solution containing 5 μg/mL and 100 μg/mL of NOR and FLU, respectively. After that, concentrations of 3 and 60 μg/mL of NOR and FLU, respectively, were prepared in 10 mL volumetric flask using the working solution. Then, 20 μL of the resulting solution was injected and the concentration of each drug was calculated by substituting the resulting area and curve/10\(^5\) in its corresponding regression equation. Finally, pure powders were added to the tablet powder and mixed well to evaluate the accuracy of the proposed method.

For TLC method: two mL of the stock solution was transferred into 10 mL volumetric flask and the volume was completed with methanol to yield a working solution containing 5 μg/mL and 100 μg/mL of NOR and FLU, respectively. After that, concentrations of 3 and 60 μg/mL of NOR and FLU, respectively, were prepared in 10 mL volumetric flask using the working solution. Then, 10 μL of the resulting solution was injected as triplicates to obtain concentrations of 0.1 μg/band and 2 μg/band of NOR and FLU, respectively. The procedures detailed under

Table 1 The gradient program of the developed RP-HPLC method

| Time (min) | (％) Water (containing 0.1% phosphoric acid at pH = 2.25) | (％) Methanol |
|-----------|----------------------------------------------------------|--------------|
| 0         | 50                                                       | 50           |
| 6         | 50                                                       | 50           |
| 12        | 10                                                       | 90           |
| 13        | 50                                                       | 50           |
| 14        | 50                                                       | 50           |
“construction of calibration curve” were followed and then the concentrations of each drug were found by substituting the resulting integrated peak areas/10⁴ in its corresponding regression equation. Finally, pure powders were added to the tablet powder and mixed well to carry out standard addition technique and evaluate the accuracy of the proposed method.

Results and Discussion

The HPLC and TLC methods are popular approaches for simultaneous mixture determination because of their excellent selectivity, sensitivity, and accuracy, as well as the fact that they require little sample preparation and material composition. They have been widely utilized to determine the presence of degradation products and contaminants in pharmaceuticals [18–20]. Furthermore, by developing eco-friendly green chromatographic analytical techniques for the determination of drug combinations in various samples, the green chemistry field now addresses the environment's safety and reduces the dangers and harm to the environment's life [21–23]. Therefore, this work describes newly developed RP-HPLC and TLC techniques for determining NOR and FLU in bulk and pharmaceutical formulations, as well as their two possible and pharmacologically active impurities PER and DBZ, with high resolution and selectivity. Moreover, the greenness criteria of the two developed methods were evaluated using four greenness assessment tools were eco-scale, AGP, AGREE, and GAPI.

It was found that the chemical structures of PER and FLU were very similar to each other, as shown in Fig. 1, which made their TLC separation very difficult. In addition, their separation was easier using RP-HPLC method than using TLC method. But TLC method was greener than RP-HPLC method according to the greenness assessment evaluation.

Method Optimization

To get the best resolution and separation, many factors were tested to see how they affected the established approaches.

For RP-HPLC Method

Solvent Trials and Ratios

It was critical in this project to employ ecologically friendly solvents which are safe and have the minimum harmful impact on the environment such as water and ethanol, rather than hazardous ones such a acetonitrile, chloroform and hexane which are dangerous to the environmental and natural life [24]. Various trials of mobile phases with different types and ratios were carried out to obtain optimum separation and resolution of the proposed compounds, beginning with the mobile phase of the previously published method used for separation of NOR and FLU [11], which was composed of mixture of methanol: 25 mM KH₂PO₄ solution adjusted at pH 4.5 with H₃PO₄ (70:30, v/v), with UV detection at 254 nm and a flow rate of 5 mL/min and 40 °C. It was found that PER and FLU eluted together as a single peak, but the remaining components were separated well. Further changes of the ratio of the mobile phase components were carried out where PER and FLU still eluted together without separation.

KH₂PO₄ solution was replaced with water and the ratio was changed to (50:50, v/v) of water and methanol, where PER and FLU peaks begin to separate from each other. Different pH values were tried using H₃PO₄, 1 N NaOH, TEA, formic acid and glacial acetic acid. It was found that the acidic pH is better than the alkaline one for the separation and the shapes of the peaks. Moreover, it was observed that the lower pH the sharper peaks and the greater resolution between PER and FLU peaks and the other peaks. This was because the proposed drugs gave forked peaks at alkaline pH due to their pka values, which are in the range of (7–11). While in acidic medium, they gave symmetrical and single peaks. H₃PO₄ acid was the best choice for pH adjustment to make the peaks sharper and to remove tailing.

The gradient change of the mobile phase from 6 to 12 min, from the ratio of (50:50, v/v) to the ratio of (10:90, v/v), as shown in Table 1, was necessary to reduce the elution time of FLU and DBZ from 12 and 16 min to 8 and 11.7 min, respectively, and to keep them separated from each other at the same time, as shown in Fig. 2

Effect of pH

Different pH values of the used water were tried as mentioned previously in “Method Development”. When formic or acetic acid were used, many noises appeared in the HPLC chromatogram. While using phosphoric acid, the noises disappeared; also separation and peaks shape were improved. Therefore, the pH of 2.25 using H₃PO₄ in ratio of 0.1% in water was the best choice to make the peaks sharper and to remove tailing, as shown in Fig. 2

Effect of Temperature

Different temperatures were tried such as the room temperature (25 °C) and 40 °C. Using 40 °C, temperature was better as it gave optimum separation with sharp symmetrical peaks, as shown in Fig. 2.

Effect of Flow Rate

Various flow rates including 1, 1.5, 1.8 and 2 mL/min were tested. The flow rate of 2 mL/min resulted in the optimum
resolution and separation between the four components within a short analysis time. The lower flow rates worsen the peak shapes and increase their elution time.

**Scanning Wavelength**

Different wavelengths were tried to detect the maximum sensitivity for the four components including 230, 254, 280 and 300 nm. The best sensitivity was obtained at 254 nm with minimum noise, as shown in Fig. 2.

**For TLC Method**

**Solvent Trials and Ratios**

It was found that the chemical structures of PER and FLU were very similar to each other, as shown in Fig. 1, which made their TLC separation very difficult. Different systems’ mixtures with different ratios were tried, starting with the relatively environmentally green solvents. A mixture of methanol and ethyl acetate (1:9, v/v) was firstly tried, it was noticed that NOR, PER and FLU were not separated from each other, while DBZ appears far from them. Also, the ratio of the two previously solvents was reversed, where the four components were separated from each other, but the two peaks of PER and FLU were not completely resolved. Ethyl acetate was replaced with many other relatively green solvents such as water, ethanol, isopropyl alcohol, butanol, acetic acid, acetone, but the results did not change a lot except with acetone in ratio (1 acetone: 9 methanol, v/v), the distance between PER and FLU increased and separated well. The ratio of acetone was increased to 2 and 5 v/v, but no change was observed.

Moreover, the pH of the system was changed to study its effect on the separation using ammonia, glacial acetic acid, formic acid, phosphoric acid, TEA, NaOH and HCl, but no change was observed in the separation. Also, additional trials were performed using non-green solvents including hexane, toluene, benzene, chloroform, acetonitrile, DMS and methylene chloride, but the separation did not improve.

Therefore, the system of methanol: acetone (9:1, v/v) was the best choice, which succeeded to separate the four components well form each other with good peaks' shapes and minimum noise, as shown in Fig. 3. In addition, methanol and acetone are relatively environmentally green and harmless [24].

**Method Development**

*For RP-HPLC method:* for optimum separation and resolution of the obtained peaks, NOR, PER, FLU and DBZ mixture was determined and separated using gradient elution composed of a mixture of water containing 0.1 % H₃PO₄ (pH 2.25) and methanol, at flow rate of 2 mL/min, 40 ºC temperature, and UV detection at 254 nm.

Moreover, the pH of the system was changed to study its effect on the separation using ammonia, glacial acetic acid, formic acid, phosphoric acid, TEA, NaOH and HCl, but no change was observed in the separation. Also, additional trials were performed using non-green solvents including hexane, toluene, benzene, chloroform, acetonitrile, DMS and methylene chloride, but the separation did not improve.

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**Scanning Wavelength**

Different wavelengths were tried to detect the maximum sensitivity for the four components including 230, 245, 254, 280 and 300 nm. The best sensitivity was obtained at 245 nm with minimum noise, as shown in Fig. 3.

**For TLC Method**

**Solvent Trials and Ratios**

It was found that the chemical structures of PER and FLU were very similar to each other, as shown in Fig. 1, which made their TLC separation very difficult. Different systems’ mixtures with different ratios were tried, starting with the relatively environmentally green solvents. A mixture of methanol and ethyl acetate (1:9, v/v) was firstly tried, it was noticed that NOR, PER and FLU were not separated from each other, while DBZ appears far from them. Also, the ratio of the two previously solvents was reversed, where the four components were separated from each other, but the two peaks of PER and FLU were not completely resolved. Ethyl acetate was replaced with many other relatively green solvents such as water, ethanol, isopropyl alcohol, butanol, acetic acid, acetone, but the results did not change a lot except with acetone in ratio (1 acetone: 9 methanol, v/v), the distance between PER and FLU increased and separated well. The ratio of acetone was increased to 2 and 5 v/v, but no change was observed.

Moreover, the pH of the system was changed to study its effect on the separation using ammonia, glacial acetic acid, formic acid, phosphoric acid, TEA, NaOH and HCl, but no change was observed in the separation. Also, additional trials were performed using non-green solvents including hexane, toluene, benzene, chloroform, acetonitrile, DMS and methylene chloride, but the separation did not improve.

Therefore, the system of methanol: acetone (9:1, v/v) was the best choice, which succeeded to separate the four components well form each other with good peaks' shapes and minimum noise, as shown in Fig. 3. In addition, methanol and acetone are relatively environmentally green and harmless [24].

**Method Development**

*For RP-HPLC method:* for optimum separation and resolution of the obtained peaks, NOR, PER, FLU and DBZ mixture was determined and separated using gradient elution composed of a mixture of water containing 0.1 % H₃PO₄ (pH 2.25) and methanol (Table 1), at flow rate of 2 mL/min, 40 ºC temperature, 254 nm scanning wavelength and run time of 14 min. The obtained tᵣ values were (3.8 ± 0.01) for NOR, (5.3 ± 0.01) for PER, (7.8 ± 0.01) for FLU, and (11.7 ± 0.01) for DBZ, as shown in Fig. 2, proving the selectivity of the proposed method. Linear correlations were obtained for the proposed components and the corresponding regression equations were found to be:

\[
PA_1 = 0.0560 X_1 - 0.0403, r_1 = 0.9999 \text{ for NOR}
\]

\[
PA_2 = 0.1892 X_2 + 0.4634, r_2 = 0.9999 \text{ for PER}
\]

\[
PA_3 = 0.1671 X_3 - 0.8508, r_3 = 0.9999 \text{ for FLU}
\]

\[
PA_4 = 0.1062 X_4 + 0.4117, r_4 = 0.9998 \text{ for DBZ}
\]

where \(PA_1, PA_2, PA_3, \) and \(PA_4\) are the peak areas/10⁵, \(X_1, X_2, X_3, \) and \(X_4\) are the concentrations in μg/mL and \(r_1, r_2, r_3, \) and \(r_4\) are the correlation coefficients for NOR, PER, FLU and DBZ, respectively.

For TLC method: For optimum separation and resolution of the obtained peaks, NOR, PER, FLU and DBZ mixture was determined and separated using methanol-acetone (9:1, v/v) as a developing system and scanning wavelength at 245 nm. The obtained \(R_t\) values of NOR (0.20 ± 0.01), PER...
(0.60 ± 0.01), FLU (0.64 ± 0.01) and DBZ (0.83 ± 0.01) perform the selectivity of the proposed method, as shown in Fig. 3. Linear regressions were obtained for NOR and DBZ, while polynomial regressions for PER and FLU. The corresponding regression equations were found to be

\[
PA_1 = 0.4899 X_1 + 0.0883, \ r_1 = 0.9998 \text{ for NOR}
\]

\[
PA_2 = -0.1743 X_2^2 + 1.1223 X_2 + 0.0209, \ r_2 = 0.9999 \text{ for PER}
\]

\[
PA_3 = -0.0470 X_3^2 + 0.6192 X_3 + 0.0292, \ r_3 = 0.9999 \text{ for FLU}
\]

\[
PA_4 = 0.4696 X_4 + 0.3171, \ r_4 = 0.9999 \text{ for DBZ}
\]

Where \(PA_1, PA_2, PA_3,\) and \(PA_4\) are the integrated peak areas/10^4, \(X_1, X_2, X_3,\) and \(X_4\) are the concentrations in μg/band and \(r_1, r_2, r_3,\) and \(r_4\) are the correlation coefficients for NOR, PER, FLU and DBZ, respectively.

**Method Validation**

The suggested approaches were evaluated and validated by following the ICH standards [25]. Table 2 illustrates the linearity limits of the studied components, as well as additional factors relevant to the regression equation that have acceptable correlations. Table 2 also includes the findings of repeatability and intermediate precision, which showed accepted and satisfying results.

Additionally, as shown in Table 2, small limits of detection and quantitation levels were achieved for the examined impurities (PER and DBZ), demonstrating the suggested methodologies’ great sensitivity.

Furthermore, the method’s robustness was tested to guarantee that it would stay relatively stable and unaffected by minor fluctuation in its parameters, Table 3 summarizes these findings.

Moreover, the suggested procedures’ specificity and selectivity were verified by the 2D chromatograms given in Figs. 2, 3, which showed entire isolation of the investigated components.

The provided techniques were used to determine NOR and FLU in Motival® pills (Table 4), proving that the tablets’ matrix had no effect. Also, standard addition approach was performed to check the accuracy of the proposed methods, which yielded satisfactory results (Table 4).

Lastly, in accordance to the standard criteria [26], satisfactory system suitability results were achieved (Table 5).

**Statistical Comparison Between the Developed and the Reported Methods**

The findings of the suggested techniques and the published HPLC method [11] for the analysis of NOR and FLU in Motival® pills were compared statistically in Tables 6, 7. This is achieved by calculating of t- and F-values and performing ANOVA test. The theoretical t and F-values are higher than the observed ones, indicating that there no significant gap in precision and accuracy between the two methods.

The suggested HPLC and TLC methods have advantages over the reported HPLC including the following points: (a) the suggested HPLC method has higher sensitivity for the detection of NOR, (b) the two suggested methods are more selective as they consider the presence of PER and DBZ contaminants.
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Table 2  Assay and method validation parameters for the determination of the studied components by the proposed methods

| Parameters                          | RP-HPLC method | TLC method |
|-------------------------------------|----------------|------------|
|                                     | NOR | PER | FLU | DBZ | NOR | PER | FLU | DBZ |
| Calibration range                    | 2–50 | 0.5–30 | 7–130 | 0.2–30 | 0.05–3 | 0.05–1.5 | 0.1–4.5 | 0.1–1.2 |
| Slope                               | 0.0560 | 0.1892 | 0.1671 | 0.1062 | 0.4899 | −0.1743, 1.1223 | −0.0470, 0.6192 | 0.4696 |
| Intercept                           | −0.0403 | 0.4634 | −0.8508 | 0.4117 | 0.0883 | 0.0209 | 0.0292 | 0.3171 |
| Correlation coefficient             | 0.9999 | 0.9999 | 0.9999 | 0.9998 | 0.9998 | 0.9999 | 0.9999 | 0.9999 |
| Accuracy (mean ± SD)                | 98.94 ± 1.827 | 101.10 ± 0.754 | 100.75 ± 1.544 | 99.51 ± 1.465 | 99.74 ± 1.311 | 100.23 ± 2.170 | 100.30 ± 1.268 | 100.97 ± 1.232 |
| Precision (%RSD)b                   | 0.203 | 0.225 | 0.887 | 0.094 | 0.110 | 0.534 | 0.718 | 0.242 |
| -Repeatability & Intermediate precision | 0.491 | 0.458 | 0.291 | 0.220 | 0.200 | 0.862 | 1.044 | 0.502 |
| LODa,c                              | − | 0.122 | − | 0.037 | − | 0.014 | − | 0.029 |
| LOQa,c                              | − | 0.470 | − | 0.112 | − | 0.043 | − | 0.088 |

*aThe concentrations are in µg/mL for RP-HPLC method and in µg/band for TLC method

bAverage of three determinations. For RP-HPLC method: the intra- and inter-day RSD of concentration (5, 20 and 40 µg/mL) for NOR, (5, 15 and 25 µg/mL) for PER, (20, 60 and 100 µg/mL) for FLU, and (5, 15 and 25 µg/mL) for DBZ. For TLC method: the intra- and inter-day RSD of concentration (0.5, 1.5 and 2.5 µg/band) for NOR, (0.4, 0.8 and 1.2 µg/band) for PER, (1, 2 and 4 µg/band) for FLU, and (0.4, 0.6 and 1 µg/band) for DBZ

cLOD = (SD / slope) * 3.3, LOQ = (SD / slope) * 10

Table 3  Robustness results for the determination of the studied components by the proposed methods

| Method                              | Parameters                                      | NOR | PER | FLU | DBZ |
|-------------------------------------|------------------------------------------------|-----|-----|-----|-----|
| RP-HPLC methoda                     | Methanol (50 ± 0.2 mL)                          | 1.986 | 1.018 | 1.050 | 0.060 |
|                                    | pH (2.25 ± 0.1)                                 | 1.226 | 0.616 | 0.677 | 0.060 |
|                                    | Temperature (40 ± 1 ºC)                         | 1.482 | 0.734 | 0.959 | 0.071 |
|                                    | Flow rate (2 ± 0.05 mL/min)                     | 1.485 | 1.401 | 1.897 | 0.058 |
| TLC methoda                         | Methanol (9 ± 0.1 mL)                           | 1.502 | 0.346 | 0.317 | 0.646 |
|                                    | Acetone (1 ± 0.01 mL)                           | 0.990 | 0.569 | 0.208 | 0.671 |
|                                    | Wavelength (245 ± 1 nm)                         | 1.147 | 0.523 | 0.415 | 0.888 |
|                                    | Saturation time (15 min ± 1 min)                | 0.574 | 0.471 | 0.632 | 1.323 |

*aAverage of three determination, the %RSD was calculates for the $R_f$ values

Table 4  Determination of the fluphenazine hydrochloride and nortriptyline hydrochloride in Motivalâ® tablets by the proposed methods and application of the standard addition technique

| Pharmaceutical formulation | RP-HPLC method | TLC method |
|-----------------------------|----------------|------------|
|                             | Taken (µg/mL)  | Found % ± SDa | Pure added (µg/mL) | Recovery %b | Taken (µg/band) | Found % ± SDa | Pure added (µg/band) | Recovery %b |
| Motivalâ® tablets B.N. B60317 | NOR 3.0 | 99.61 ± 0.927 | 2 | 97.00 | 0.1 | 100.08 ± 1.225 | 0.05 | 98.00 |
|                             | 3 96.67 | 4 98.25 | Mean ± SD 97.31 ± 0.835 | 50 101.62 | 2.0 | 99.34 ± 0.968 | 1.0 | Mean ± SD 99.17 ± 1.607 | 98.40 |
|                             | 60 101.90 | 70 102.56 | Mean ± SD 102.03 ± 0.481 | Mean ± SD 102.56 | 2.5 | 96.48 | 98.51 ± 2.087 |

*aAverage of 6 determinations

bAverage of 3 determinations
Greenness Assessment of the Developed Methods

The two methods were ecologically evaluated using four different greenness assessment tools including eco-scale, AGP, AGREE, and GAPI. These tools were used to test their greenness impact on the environment and nature lifecycle.

The first assessment tool is the analytical eco-scale [1]. The eco-scale scores were calculated for the developed methods, as shown in Table 8. The results prove that both of the developed methods had an eco-scale score equal to...
80, proving the excellent green analysis for both methods according to the Globally Harmonized System of Classification and Labeling of Chemicals [1]. They are equally green as they had the same scores.

The second assessment tool is the AGP method [27, 28], where it is expressed by pentagram equally divided into five parts and can be given one of three colors: green, yellow or red. The five parts indicates the impact of five roles: health, safety, environmental, waste, and energy. The AGP colored pentagram of the suggested chromatographic methods are illustrated in Fig. 4.

The third assessment tool is the AGREE method [29], which is presented by a colored pictograms created by a software installed from a particular URL supplied in the AGREE article [29]. The AGREE figures of the suggested methods are illustrated in Fig. 5. It was found that the circular pictogram's center took the yellowish green color, proving the excellent green analysis for both methods. The overall score for the developed RP-HPLC method is 0.67, while for the developed TLC method is 0.74, indicating that the developed TLC method is greener than the developed RP-HPLC method as TLC method had higher score, as shown in Fig. 5.

Finally, the forth assessment tool is the GAPI method [30], which is also expressed by a colored pictogram divided into 15 segments, which can be colored by green, yellow and red colors according to the level of greenness of each segment. The proposed methods’ GAPI pictograms are evaluated and illustrated in Fig. 6. The GAPI pictogram of the developed RP-HPLC method shows four green segments, eight yellow segments, and three red segments. While the GAPI pictogram of the developed TLC method shows four green segments, nine yellow segments, and two red segments. These results indicate that the proposed methods are acceptably green and safe to the environment [30].

Table 8 Analytical eco-scale penalty points of the developed methods for simultaneous determination of the proposed components

|                   | RP-HPLC method |                     | TLC method |                     |
|-------------------|----------------|---------------------|------------|---------------------|
|                   | The reagents   |                     |            |                     |
| Solvents          | Amount         | Hazard<sup>a</sup> | Total penalty points<sup>b</sup> | Solvents          | Amount         | Hazard<sup>a</sup> | Total penalty points<sup>b</sup> |
| Water             | 2 (11 ~ 10–100 mL) | 0 (0 pictograms) | 0          | Methanol          | 2 (27 ~ 10–100 mL) | 6 (3 pictograms, danger) | 12 |
| Methanol          | 2 (19 ~ 10–100 mL) | 6 (3 pictograms, danger) | 12         | Acetone           | 1 (3 ~ <10 mL) | 4 (2 pictograms, danger) | 4  |
| H<sub>3</sub>PO<sub>4</sub> | 1 (0.011 ~ <10 mL) | 2 (1 pictograms, danger) | 2          |                    |                   |                         |     |
|                   | The instruments |                     |            |                     |
| Energy used       | 1 (~ 1.5 kWh/ sample) | Energy used | 1 (~ 1.5 kWh/ sample) |               |                   |                         |     |
| Occupational hazard | 0            | Occupational hazard | 0          |                     |                   |                         |     |
| Waste<sup>c</sup> | 5 (30 ~ > 10 mL) | Waste<sup>a</sup> | 3 (1.5 ~ 10 mL) |                     |                   |                         |     |
| Total penalty points | Σ 20      | Total penalty points | Σ 20       |                     |                   |                         |     |
| Eco-scale score   | 80          | Eco-scale score   | 80         |                     |                   |                         |     |

<sup>a</sup>Hazard penalty points = No. of pictograms × signal. The signal maybe warning = 1 or danger = 2

<sup>b</sup>The total penalty points = the amount penalty points × hazard penalty points

<sup>c</sup>For HPLC method: Waste = flow rate × run time. For TLC method: waste = the volume of mobile phase/ No. of spots per TLC plate
addition, the developed TLC method is relatively greener than the developed RP-HPLC method, as shown in Fig. 6.

Comparison Between the Greenness Assessment of the Proposed Methods and the Reported HPLC Method

Table 9 shows a brief comparison between the greenness behavior of the proposed RP-HPLC and TLC methods and that of the reported chromatographic one [11] for the quantitative determination of fluphenazine HCl and nortriptyline HCl. The main advantage of the proposed methods is that they could quantify higher number of analytes (four analytes: FLU, NOR, PER and DBZ), while the reported method quantify only two analytes (FLU and NOR).

Regarding the eco-scale assessment, the eco-scale scores of the three methods were above 75, indicating the excellent green analysis. While for AGP assessment, it was found that the three methods had the same figure and greenness result.

For AGREE assessment, it was found that the proposed TLC method and the reported HPLC method are almost the same. While the proposed RP-HPLC is slightly lower due to the higher amount of waste and the use of phosphoric acid.

For GAPI assessment, both the proposed TLC method and the reported HPLC method gave the same number of green, yellow and red segments. Meanwhile, the proposed RP-HPLC has one red segment more than them due to the higher amount of waste. However, the proposed RP-HPLC analyzed 4 rather than 2 analytes, so it needed longer analysis time and consumed higher amount of waste than that of the reported one.

Conclusion

The given study proposes two different liquid chromatographic methodologies for the quantitative separation of NOR and FLU in bulk and pharmaceutical formulation, as well as their two potential and pharmacologically active impurities PER and DBZ. The suggested techniques have the benefit of having higher sensitivity and selectivity than the published approaches, and they may be used for routine medication product quality control examination. Furthermore, they are straightforward, quick and cost-effective. The two methods were ecologically evaluated using four different greenness assessment tools, and the findings prove that they are environmentally friendly and less harmful and hazardous to the nature lifecycle.
Table 9 Greenness comparison between the proposed methods and the reported HPLC method for the quantitative determination of fluphenazine HCl and nortriptyline HCl

| Methods                  | Eco-scale | AGP | AGREE tool | GAPI tool |
|--------------------------|-----------|-----|------------|-----------|
| The proposed             |           |     |            |           |
| RP-HPLC method           | Water     | 0   |            |           |
|                          | Methanol  | 12  | 0.67       |           |
|                          | H₃PO₄     | 2   |            |           |
|                          | HPLC      | 1   |            |           |
|                          | Occupational hazards | 0 |          |           |
|                          | Waste     | 5   |            |           |
|                          | Total penalty points | 20 |          |           |
|                          | Analytical eco-scale | 80 |          |           |
| The proposed             | Methanol  | 12  | 0.74       |           |
| TLC method               | Acetone   | 4   |            |           |
|                          | TLC       | 1   |            |           |
|                          | Occupational hazards | 0 |          |           |
|                          | Waste     | 3   |            |           |
|                          | Total penalty points | 20 |          |           |
|                          | Analytical eco-scale | 80 |          |           |
| The reported             | Methanol  | 6   | 0.73       |           |
| HPLC method [11]         | LC        | 1   |            |           |
|                          | Occupational hazards | 0 |          |           |
|                          | Waste     | 3   |            |           |
|                          | Total penalty points | 10 |          |           |
|                          | Analytical eco-scale | 90 |          |           |

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Declarations

Conflict of interest There is no any conflict of interest occurred.

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