Experimentally designed chromatographic method for the simultaneous analysis of dimenhydrinate, cinnarizine and their toxic impurities†

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Recently, experimental design has beaten the traditional optimization approach (one variable at a time) by providing better quality for chromatographic separation using minimal effort and resources. Benzophenone (BZP) and [1-(diphenylmethyl)piperazine] (DPP) were reported to be the most toxic impurities for dimenhydrinate (DMH) and cinnarizine (CIN), respectively. Additionally, there is no reported HPLC method for the simultaneous determination of DMH, CIN and their toxic impurities. A custom experimental design was adopted to estimate the optimum conditions that achieved the most acceptable resolution with adequate peak symmetry within the shortest run time. Desirability function was used to define the optimum chromatographic conditions and the optimum separation was achieved using XBridge® HPLC RP-C18 (4.6 × 250 mm, 5 µm), acetonitrile: 0.1% sodium lauryl sulphate (SLS) in water (90 : 10, v/v) as a mobile phase at flow rate 2 mL min⁻¹ and UV detection at 215 nm. Method validation was carried out according to ICH guidelines and linearity was achieved in the ranges of 2–25, 1–25, 1–12.5, and 1–12.5 µg mL⁻¹ for DMH, CIN, BZP and DPP, respectively. By application of the proposed method to the market dosage form, no interference from excipients was observed. Moreover, the greenness of the method was evaluated using the National Environmental Method Index (NEMI), Analytical Eco-Scale and Green Analytical Procedure Index (GAPI) metrics and the results revealed the green environmental impact of the developed method.

1. Introduction

Dimenhydrinate (DMH), is chemically defined as 1-[(2-(benzhydryloxy)ethyl)(methyl)amino)-8-chloro-3-methyl-3,7-dihydro-1H-purine-2,6-dione.¹ It consists of a 1 : 1 mixture salt of diphenhydramine (DIP) and 8-chlorotheophylline (CHP) as reported by the United States Pharmacopeia (USP),² British Pharmacopeia (BP)³ and European Pharmacopeia (EP).⁴

Cinnarizine (CIN), is a piperazine derivative that is chemically named 1-(diphenylmethyl)-4-(3-phenyl-2-propenyl)piperazine.² It has an antihistaminic and a calcium channel blocking activity. Dimenhydrinate and cinnarizine are either prescribed separately or in combination for the treatment of nausea and vomiting, motion sickness and vertigo from vestibular or non-vestibular sources.⁵–⁷

Dimenhydrinate has several official impurities.⁵ In the last published work of our team, we reported that the precursor of DMH, benzophenone (BZP) is the most toxic DMH impurity.⁶ p-Hydroxybenzophenone is the carcinogenic, estrogenic and hepatocellular necrotic metabolite of BZP.⁶–¹¹ The in silico toxicity studies of CIN official impurities revealed that 1-(diphenylmethyl)piperazine (DPP) had higher toxicity than the other CIN impurities.⁸

The USP,² BP³ and EP⁴ and reports potentiometric, argentometric, and HPLC methods for the assay of DMH. Additionally, the BP³ and EP⁴ developed a potentiometric titration for the assay of CIN. On the other hand, the DMH and CIN combination is not officially included in any Pharmacopeia. The literature review revealed a spectrophotometric,¹⁴ electrochemical¹⁵ and chromatographic¹⁶–¹⁸ methods for determination of DMH and its impurities. CIN was assayed spectrophotometrically,¹⁹,²⁰ electrochemically²¹,²² and by chromatographic methods²¹–²⁸
either alone or in combination with other drugs and/or impurities. Moreover, CIN and DMH were simultaneously determined by TLC-densitometry,29,30 HPLC,30,31 and UV-spectrophotometry.12,33 The binary mixture of DMH and CIN was analyzed in presence of DPP a spectrophotometric,14 TLC-densitometric35 and an HPLC35 methods. A single TLC-densitometric method was developed in our laboratory for the simultaneous assay of DMH, CIN and their toxic impurities; BZP and DPP.8

HPLC separation includes a wide range of variables that control the separation process; mobile phase pH, buffer concentration, flow rate, column temperature, detecting wavelength, etc. For several decades, HPLC method development strategies were based on trial and error supported by the experience and knowledge of the analyst. This conventional one factor at a time (OFAT) strategy consumes time, effort and resources to produce incompletely justified outcomes.36 On the contrary, the design of experiment approach (DOE) uses the minimum number of experiments to evaluate the effect of the variables and their interactions on the measured responses to achieve the desired output.37 Custom design is a flexible experimental design tailored to meet the available budget and desired output. It allows analysts to define factor constraints and assess the main effects and interaction through the minimum number of experiments.38,39

On the other way, environmental awareness has grown over the last few decades to introduce the greenness concepts to analytical chemistry. Analysts aim to reduce or avoid the consumption and emission of hazardous substances throughout the analytical procedure.40,41 Different green chemistry metrics have been developed for the evaluation of greenness standards in such procedures. The National Environmental Method Index (NEMI) is among the oldest techniques built to provide a clear representation of the greenness of the analytical method.42 Another green chemistry metric is the Analytical Eco-Scale,43 which is a semi-quantitative tool involving additional environmental impact investigating parameters.44 The Green Analytical Procedure Index (GAPI) is a recent approach that utilizes additional safety and health criteria to provide a more comprehensive evaluation for each step during the analytical procedures.45

The literature failed to reveal any HPLC method for simultaneous determination of DMH, CIN and their toxic impurities; BZP and DPP. The current work aims to implement a custom experimental design to evaluate the critical main effects and interactions controlling the separation process. The developed method was optimized to quantify the toxic impurities along with their active drugs with high sensitivity within short analysis time. It was validated according to the ICH validation guidelines.46 Analyst’s considered the greenness concepts throughout the method development process to minimize the environmental burdens. The ability of the NEMI, Analytical Eco-Scale and GAPI greenness assessment metrics were compared to evaluate the overall method greenness. The method was validated as per ICH guidelines.

2. Experimental

2.1. Materials and reagents

Dimenhydrinate sample was kindly supplied by Kahira Pharmaceuticals and Chemical Industries Company (Shoubra, Cairo, Egypt) with a purity of 99.10% according to the reported method.39 Cinnarizine pure sample was obtained as a gift from Amoun Pharmaceuticals (El Obour City, Cairo, Egypt) with a purity of 100.26% according to the reported method.35 Benzophenone and DPP were bought from Sigma Aldrich, Chemie GmbH (Darmstadt, Germany) with a purity of 97% and 99%, respectively according to the manufacturing certificates of analysis. Methanol and acetonitrile were of HPLC grade (Fisher, Loughborough, UK) and were purchased from Sigma Aldrich Chemie GmbH (Darmstadt, Germany). Orthophosphoric acid, sodium lauryl sulphate (SLS) and potassium dihydrogen phosphate were purchased from EL-Nasr Pharmaceutical, Chemicals Co., Abu Zabaal, Cairo, Egypt and were of analytical grade. Amocerebral® tablets (batch no. 6221025030658) were manufactured by Amoun Pharmaceutical Company (Cairo, Egypt), each tablet was labeled to contain 20 mg of DMH and 10 mg of CIN.

2.2. Instruments

Thermo Scientific Dionex Ultimate S 3000 HPLC, equipped with Ultimate quaternary solvent delivery pump, ACC-3000 autosampler and DAD-3000 diode array detector (Germany) was used. The stationary phases tested were Hypersil Gold® C18 (2.1 × 50 mm, 1.9 µm), Hypersil Gold® C18 (4.6 × 150 mm, 3 µm), XBridge® HPLC RP-C18 (4.6 × 250 mm, 5 µm), Xterra® CN (4.6 × 150 mm, 3 µm) and Xterra® RP-C8 (2.1 × 50 mm, 1.9 µm). The output signal was monitored and processed using Chromel® chromatography data system software.

Digital balance (Sartorius, Germany) and Sonix TVSS-series ultrasonicator (CA, USA) were used.

2.3. Software programs

Custom experimental design and data analysis calculations were performed using the JMP® software version 15.2.0 Copyright© 2019, SAS Institute Inc., Cary, North Carolina, USA software.47

2.4. Solutions

(a) Stock standard solutions of DMH, BZP, CIN, and DPP were prepared by accurate weighing of 0.1 g of each and dissolving in acetonitrile to obtain a 100 mL stock solution of 1 mg mL⁻¹ each.

(b) Working standard solutions (100 µg mL⁻¹) of the studied components were prepared by transferring 1 mL of each component from its respective stock standard solution (1 mg mL⁻¹) into four separate 10 mL volumetric flasks, then the volume was completed to the mark with acetonitrile.
2.5. Experimental design
The JMP® software version 15.2.0 (466311) Copyright© 2019, SAS Institute Inc., Cary, North Carolina, USA software was used to build two levels-three factors custom experimental with one center point. An initial screening study was carried out to select the critical factors affecting the chromatographic separation. The flow rate, SLS concentration and acetonitrile percentage in the mobile phase were selected as the independent variables. The run time, tailing factors of CIN and DPP, and resolution of BZP, CIN, and DIP were recorded for each run and used as the dependent variables (responses) (Table 1). Sixteen experiments were conducted in random order. The second-order polynomial equations characterizing the quadratic models and indicated the interaction between the factors were computed using the standard least square method. The results were statistically analyzed and the final optimization was achieved using the desirability function. The run time and tailing factors were set to minimize while the resolution was set to maximize.

2.6. Chromatographic conditions
A Thermo Scientific Dionex Ultimate S 3000 HPLC (Germany) instrument and XBridge® HPLC RP-C18 (4.6 × 250 mm, 5 µm) columns were used. The injection volume was 30 µL and the separation was carried out using isocratic elution with a mobile phase mixture of acetonitrile: 0.1% SLS (90 : 10, v/v) flowed at a rate of 2 mL min⁻¹. The overall run time was 15 min and the UV detection was carried out at 215 nm while the temperature was adjusted to 25 °C.

2.7. Construction of calibration curves
Sample solutions with different concentrations 2–25, 1–12.5, 1–25, and 1–12.5 µg mL⁻¹ for CHP, BZP, CIN, and DPP, respectively were prepared by dilution of their respective working solutions with the mobile phase in 5 mL volumetric flasks. A volume of 30 µL of each solution was injected in triplicates and the above chromatographic instructions were then performed. Calibration plots relating the area under the peak against the corresponding analyte concentration were plotted and the regression equations were computed.

2.8. Application to the pharmaceutical formulation
Ten Amocerebral® tablets were ground to a uniform mixture, and an amount equivalent to 50 mg DMH and 25 mg CIN was accurately weighed and transferred to a 25 mL volumetric flask, ultrasonicated with 10 mL acetonitrile for 30 min, cooled to room temperature and the volume was completed with acetonitrile and finally filtered using a 0.22 µm nylon membrane filter.

The previously prepared solution was diluted using the mobile phase to reach the concentration of 2 and 1 mg mL⁻¹ for DMH and CIN, respectively, and a volume of 30 µL was injected in triplicates and chromatographed under the optimum chromatographic conditions.

3. Results and discussion
Optimization of an HPLC method is a complex process since several variables must be adjusted simultaneously to achieve the desired separation. Multiple advantages can be acquired by using an experimental design during the optimization process, among these advantages are enhancing the quality of separation, reducing the number of experiments, and minimizing the demand of laboratory resources and trained labor. The custom design is of choice when the targeted design has specific parameters like time, budget and resources. Its flexibility allows the building of smart validated designs with minimum effort, time and resources.

### Table 1 Levels, factors and responses in the custom experimental design

| Experiment no. | Flow rate | SLS conc. | % acetonitrile | Run time | TF<sub>CIN</sub> | TF<sub>DPP</sub> | RS<sub>BZP</sub> | RS<sub>CIN</sub> | RS<sub>DIP</sub> |
|---------------|-----------|-----------|---------------|----------|----------------|----------------|---------------|---------------|---------------|
| 1             | 1.5       | 0.05      | 95            | 25       | 1.1            | 1.5            | 6.61          | 6.29          | 6.1           |
| 2             | 1.5       | 0.1       | 85            | 13.5     | 1.25           | 1.2            | 4.29          | 5.09          | 2.37          |
| 3             | 2         | 0.05      | 90            | 13.38    | 0.98           | 1.38           | 3.68          | 4.64          | 3.95          |
| 4             | 1.5       | 0.05      | 85            | 14.5     | 1.29           | 1.3            | 4.12          | 4.98          | 2.17          |
| 5             | 2         | 0.1       | 95            | 25       | 0.99           | 1.24           | 7.23          | 7.3           | 4.94          |
| 6             | 2         | 0.05      | 85            | 14       | 1.22           | 1.25           | 3.75          | 4.32          | 2.07          |
| 7             | 1.75      | 0.075     | 90            | 13.06    | 0.98           | 1.42           | 3.92          | 4.16          | 3.66          |
| 8             | 1.75      | 0.05      | 95            | 25       | 0.98           | 1.31           | 6.7           | 6.59          | 6.21          |
| 9             | 1.5       | 0.075     | 90            | 14.3     | 1.14           | 1.3            | 4.21          | 4.5           | 3.99          |
| 10            | 1.5       | 0.05      | 85            | 14.5     | 1.34           | 1.3            | 4.12          | 4.98          | 2.17          |
| 11            | 2         | 0.1       | 85            | 12.5     | 1.17           | 1.19           | 2.85          | 4.7           | 2.55          |
| 12            | 1.75      | 0.1       | 90            | 14.52    | 1.02           | 1.3            | 3.95          | 4.74          | 2.19          |
| 13            | 2         | 0.075     | 95            | 25       | 0.99           | 1.4            | 7.21          | 7.29          | 4.9           |
| 14            | 1.75      | 0.075     | 85            | 13.45    | 1.22           | 1.25           | 3.85          | 4.64          | 2.09          |
| 15            | 1.5       | 0.1       | 95            | 25       | 1.1            | 1.32           | 6.8           | 6.7           | 4.6           |
| 16            | 2         | 0.05      | 95            | 25       | 0.99           | 1.25           | 7.12          | 7.25          | 6.8           |
3.1. Preliminary separation and screening of the critical chromatographic variables

Different stationary phases with different packing materials, lengths, and particle sizes were tested, including Hypersil Gold® C18 (2.1 × 50 mm, 1.9 μm), C18 (4.6 × 150 mm, 3 μm), XBridge® HPLC RP-C18 (4.6 × 250 mm, 5 μm), C8 (2.1 × 50 mm, 1.9 μm) and CN (4.6 × 150 mm, 3 μm) columns. Preliminary results showed that the XBridge® HPLC RP-C18 (4.6 × 250 mm, 5 μm) column produced acceptable retention and separation for the studied compounds.

Different mobile phase mixtures of different compositions were tested as trails to achieve the most efficient separation and the least peak tailing within suitable analysis time. Mobile phases containing different composition of water, 0.05 M potassium dihydrogen phosphate buffer, SLS solution, acetonitrile or methanol were tried. All mobile phases containing methanol resulted in asymmetric peaks as well as strong retention of CIN on the stationary phase while acetonitrile containing mobile phases improved the peak shape and resulted in a better resolution except for DPP which was best eluted using of SLS solution and acetonitrile mobile phase mixture.

The four analytes demonstrated UV absorption at the wavelength range of 200–400 nm. However, the limited conjugation in the chromophore of DMH and DPP forced the UV detection at

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### Table 2

| Term       | Run time | TF<sub>CIN</sub> | P-Value | TF<sub>DPP</sub> | P-Value | Rs<sub>BP</sub> | P-Value | Rs<sub>CIN</sub> | P-Value | Rs<sub>DPP</sub> | P-Value |
|------------|----------|------------------|---------|------------------|---------|----------------|---------|------------------|---------|-----------------|---------|
| I          | 13.5939  | <0.0001          | 1.0153  | <0.0001          | 1.2510  | <0.0001        | 3.9337  | <0.0001          | 4.3554  | <0.0001         | 3.2752  |
| A          | -0.2147  | 0.2119           | -0.0554 | 0.0023           | -0.0412 | 0.0006         | -0.1322 | 0.0148           | 0.0871  | 0.0751          | 0.0239  |
| B          | -0.1679  | 0.3099           | -0.0076 | 0.4643           | -0.0561 | 0.0001         | -0.0083 | 0.8261           | 0.1267  | 0.0216          | -0.4136 |
| C          | 5.7151   | <0.0001          | -0.0993 | 0.0002           | -0.0426 | 0.0005         | -0.1261 | 0.0262           | 1.0442  | <0.0001         | 1.5920  |
| AB         | -0.0176  | 0.9206           | 0.0039  | 0.7349           | 0.0197  | 0.0216         | -0.1261 | 0.0262           | -0.0250 | 0.5883          | -0.0125 |
| AC         | 0.2296   | 0.2247           | -0.0069 | 0.5484           | 0.0042  | 0.3145         | 0.3418  | 0.0004           | 0.3288  | 0.0006          | 0.0221  |
| BC         | 0.3313   | 0.1048           | 0.0148  | 0.2319           | 0.0028  | 0.6619         | 0.1406  | 0.0177           | 0.0090  | 0.8435          | -0.5300 |
| ABC        | 0.0610   | 0.7376           | -0.0016 | 0.8946           | 0.0074  | 0.2846         | -0.0154 | 0.8337           | -0.0699 | 0.1776          | -0.0724 |
| A<sup>2</sup> | -0.2483  | 0.4826           | 0.0499  | 0.0654           | 0.0294  | 0.0537         | -0.0154 | 0.8337           | 0.3417  | 0.1556          | 0.3461  |
| B<sup>2</sup> | 0.4116   | 0.2582           | -0.0150 | 0.5061           | 0.0207  | 0.1326         | -0.0972 | 0.2674           | 0.1178  | 0.2176          | 0.0588  |
| C<sup>2</sup> | 5.5814   | <0.0001          | 0.0917  | 0.0077           | -0.0113 | 0.3797         | 1.5101  | <0.0001          | 1.2410  | <0.0001         | 0.3404  |

* Highlighted values (bold) indicate significant effect on the corresponding response. Coef. = coefficient (slope), I = intercept, A = flow rate, B = SLS conc., C = % acetonitrile, TF = tailing factor and Rs = resolution.
shorter wavelengths. The four analytes demonstrated adequate sensitivity at 215 nm (Fig. 1S†).

3.2. Experimental design

The preliminary separation study results showed that the tailing factor for CHP and BZP as well as resolution between DPP and DIP were not critical. Besides, DMH was determined as CHP due to its higher response in comparison to DIP, hence, DIP peak shape was not targeted during the optimization process. The optimization study assigned the mobile phase shape was not targeted during the optimization process. The

The JMP® software† version 15.2.0 was used to build a custom design of sixteen experiments. The design included four experiments at the center point (zero levels of the three factors) to evaluate the curvature. The experiments were performed, and the assigned responses were measured to feed the model (Table 1). The prediction models were constructed for each critical response. The models compute a coefficient for each main effect, interaction, and quadratic term. The latter defined the non-linear (polynomial) relationships, whereas, the coefficients defined the direction and strength of each effect on the respective response. ANOVA statistical analyses were performed, and P-values were calculated to indicate the significance of each term at significance level α = 0.05. Significant terms are highlighted in Table 2.

It was observed that the SLS concentration and acetonitrile percentage significantly affected the resolution of DIP peak. Besides, the run-time prediction model indicated a significant, non-linear, and directly proportional effect of acetonitrile concentration and its quadratic term on the run time (Table 2) (Fig. 1 and 2S†). On the other hand, the tailing factor of CIN was significantly affected by the flow rate, acetonitrile percent and its quadratic term. It decreased at high flow rates and higher acetonitrile percentages. The three factors had a significant inversely proportional effect on the tailing factor of DPP. Lower tailing factors could be achieved at high levels of each factor. However, the though lower yet significant directly proportional effect of the interaction between the flow rate and SLS concentration necessitates a prudent selection of the working levels from each factor (Table 2) (Fig. 1 and 2S†). The resolution of BZP and CIN was affected by certain main effects, interactions, and the quadratic term of acetonitrile percentage. The relatively high coefficient for acetonitrile and its quadratic term indicated its pronounced effect on the resolution of the two compounds (Table 2) and (Fig. 1 and 2S†).

The constructed model we used to predict the responses. The latter were plotted against the experimentally found responses. The calculated R² for the six responses approached unity indicating the perfect fit of the predicted and experimental responses. Additionally, the small gap between R² and R² adjusted confirmed good fitting of the model. The root mean square error of predictions (RMSEP) was negligible revealing the accuracy of the prediction model respectively. ANOVA tests were carried out for each of the six prediction models. The calculated low P-values (<0.05) proved the significance of the six models for the prediction of their respective responses (Table 3). On the other hand, the calculated residuals presented in Fig. 3S† appeared to be approximately normally distributed (with a mean of zero), and be independent of one another over time. Consequently, residual analysis confirmed that the models appropriately represent the data.

In the presence of multiple responses, the desirability function approach provides a simple, easy, and accurate tool for their optimization. Optimum settings for separation were determined using the response optimizer tool (Fig. 2). Also, the “maximize desirability” option was selected for achieving maximum desirability. Acetonitrile: 0.1% SLS in water (90 : 10, v/v) flowed at 2 mL min⁻¹ was the optimum chromatographic conditions since appropriate resolution, symmetric peaks and suitable run time were obtained, Fig. 3.

3.3. Methods validation

Method validation was carried out according to guidelines for method validation from the International Conference on Harmonization (2005).66

3.3.1. Linearity of the calibration curves. Linearity was evaluated by analyzing seven concentrations of each drug and six concentrations for each impurity in triplicates. Calibration curves were linear over the concentration ranges of 2–25, 1–12.5, 1–25 and 1–12.5 μg mL⁻¹ for CHP, BZP, CIN and DPP, respectively with correlation coefficients (r) of 0.9999, 0.9999, 1.0000 and 0.9992, respectively. The calculated regression parameters including the slopes and intercepts are presented in Table 4.

3.3.2. Accuracy and precision. Accuracy of the developed method was assessed by analyzing different concentrations of the

| Term                  | Run time | TF_CIN | TF_DPP | Rs_BZP | Rs_CIN | Rs_DIP |
|-----------------------|----------|--------|--------|--------|--------|--------|
| R²                    | 0.9973   | 0.9775 | 0.9841 | 0.9980 | 0.9958 | 0.9658 |
| R-Adjusted            | 0.9920   | 0.9324 | 0.9609 | 0.9941 | 0.9874 | 0.8975 |
| Predicted root mean square error | 0.5034 | 0.0326 | 0.0180 | 0.1215 | 0.1303 | 0.5277 |
| P-Value                | <0.0001  | 0.0017 | 0.007  | <0.0001 | <0.0001 | 0.0046 |

P-Value define the model significance for prediction of the corresponding response.
analytes in their linearity ranges following the instructions of the established method and calculated as percentage recovery. Good recoveries have been obtained and are given in Table 4. Additionally, the standard addition technique was applied to Amocerebral® tablets to evaluate the accuracy of the proposed method, the good results obtained confirmed that no interference was found from additives (Table 5).

Fig. 2  Prediction profiler of the experimental design.
The precision was assessed by calculating the intra and interday variations. Intraday variation (repeatability) was calculated by separately analyzing three concentration of CIN and CHP (5, 10, and 15 μg mL⁻¹), DPP and BZP (7.5, 10, and 12.5 μg mL⁻¹). Samples were analyzed three times on the same day following the instructions of the method and results were expressed as RSD (%). Interday variations (intermediate precision) were assessed by analyzing the same concentrations on three consecutive days. Acceptable results and low RSD were obtained (Table 4).

3.3.3. Limits of detection and quantitation. Calculation of the limits of detection (LOD) and quantitation (LOQ) has proven the sensitivity of the established system. The following equations were used to calculate them: LOD = 3.3 × SD/slope and LOQ = 10 × SD/slope. The standard deviation (SD) was measured from the lower part of the calibration curve, while the slope for each analyte was referred to be the calibration curve slope. The developed method showed high sensitivity as demonstrated by LOD and LOQ values given in (Table 4).

3.3.4. Selectivity. The selectivity of the proposed method was confirmed by the complete separation between the four analytes shown in Fig. 3. Additionally, values of resolution and selectivity factors were within the acceptable limits ensuring complete separation among the five components (Table 1S†).

3.3.5. Robustness. Robustness is defined as the ability of the method to withstand slight changes in chromatographic conditions such as the alteration of SLS concentration (±0.01%) and (±1%) percent of acetonitrile. The impact of these changes was measured as the SD on the $t_R$ value. Changes in the studied

| Parameters | CHP | BZP | CIN | DPP |
|------------|-----|-----|-----|-----|
| Calibration range (μg mL⁻¹) | 2–25 | 1–12.5 | 1–25 | 1–12.5 |
| Slope | 38.6891 | 52.4460 | 65.2321 | 39.9060 |
| Intercept | 0.9905 | 26.4510 | 1.4030 | –23.9060 |
| Correlation coefficient | 0.9999 | 0.9999 | 1.0000 | 0.9992 |
| Accuracy | 100.88 | 99.66 | 99.74 | 100.33 |
| Repeatability (% RSD)⁵ | 0.39 | 0.73 | 2.00 | 1.81 |
| Intermediate precision (% RSD)⁶ | 0.62 | 2.19 | 2.18 | 2.60 |
| LOD⁷ | 0.89 | 0.13 | 0.05 | 0.15 |
| LOQ⁸ | 1.24 | 0.38 | 0.15 | 0.46 |

⁵ The intra-day precision ($n = 9$), average SD of three different concentrations repeated three times within one day. ⁶ The inter-day precision ($n = 9$), average SD of three different concentrations repeated three times on three successive days. ⁷ Limit of detection. ⁸ Limit of quantification.
parameters were found to have no significant effect on the $t_R$ values of the separated peaks (Table 2S†).

3.3.6. **System suitability testing parameters.** System suitability was evaluated by calculating the tailing factor (TF), column efficiency (number of theoretical plates), resolution ($R_s$), selectivity ($\alpha$), capacity factor ($k'$) and height equivalent of a theoretical plate (HETP). All measured parameters (Table 1S†) were within acceptable ranges, indicating the reproducibility, selectivity and resolution were adequate for the developed method.

3.3.7. **Application of the method.** The developed method was successfully applied to the available dosage form; Amocerebral® tablet. The results presented in Table 5 showed that the method developed was suitable for the simultaneous determination of DMH and CIN in their available dosage form without interfering from tablets excipients. Furthermore, the application of standard addition technique confirmed the accuracy of the method.

3.4. **Greenness assessment**

Green analytical chemistry is proposed to eliminate or minimize the amount of hazardous solvents that are globally produced and consumed every day. The NEMI tool offers a clear visualization and simple comparison of the results. However, the NEMI pictogram preparation is a merely qualitative method that consumes a lot of time and effort in excessive searches for every reagent and chemical in multiple lists and does not present any information about the quantity of hazards produced.

The developed HPLC and the reported TLC-densitometric methods were used non-PBT noncorrosive solvents and

| Parameter                        | Proposed method | Reported HPLC method† |
|----------------------------------|-----------------|-----------------------|
| Mean                             | 100.61          | 100.21                |
| SD                               | 1.52            | 0.87                  |
| $t$-Test (2.228)$^a$             | 0.07            | 1.12                  |
| $F$-Value (5.050)$^a$            | 2.27            | 1.02                  |
| Standard addition (mean ± SD)$^b$| 99.00 ± 1.34    | 99.35 ± 1.81          |
| DMH                             | 99.70           | 100.26                |
| CIN                             | 2.29            | 0.88                  |

$^a$ The values between parentheses correspond to the theoretical values of $t$ and $F$ ($\alpha = 0.05$). $^b$ Standard addition was performed on three different levels and each was repeated three time.

Fig. 4  Assessment of the greenness profile of (a) the developed HPLC and (b) reported methods using NEMI and GAPI tools.
produced waste less than 50 g. However, they failed to satisfy the NEMI requirements by using of the hazardous solvents, acetonitrile and hexane, respectively (Fig. 4).

Analytical Eco-Scale provides a semi-quantitative tool that is superior to other qualitative approaches (NEMI, Efactor) for evaluating the greenness of an analytical process, taking into account the environmental impacts and energy use over the life cycle of the analytical method. Analytical Eco-Scale was used to assess and easily compare the proposed and reported methods greenness, the calculated Analytical Eco-Scale scores of both were >75 meaning excellent green analysis.43 (Table 3S†).

Green Analytical Procedure Index (GAPI) is considered as a well-organized and efficient alternative to NEMI as it employs additional criteria to assess each stage of the analytical procedure. In GAPI, to classify the green character of each stage, a particular pictogram utilizes ranked from green to yellow to red for the low, medium and high environmental impacts, respectively.44 Interpretation of the GAPI pentagram for the proposed and the reported method4 (Table 4S†) (Fig. 4) revealed a comparable green impact of the proposed method by using of greener solvents.

Using the three-assessment metrics: NEMI, Analytical Eco-Scale and GAPI, a clear image of the greenness profile of the proposed and reported methods and was given. There was a parallel consensus between the three metrics that there is an environmental impact of the proposed approach equal to or greener than the developed one.

3.5. Statistical analysis
The results obtained by the proposed HPLC method for Amocerebral® tablets were statistically compared to the reported method.35 using Student’s t-test and variance ratio F-test at a 95% confidence level and the values of the calculated t and F were less than the tabulated ones (Table 5), revealing no significant difference concentration accuracy and precision between the proposed method and the reported one.

3.6. Comparison to the reported HPLC methods
The developed method showed higher selectivity due to its ability to simultaneously determine DMH, CIN, BZP and DPP. In addition to its lower detection limit and wider linearity range relative to the reported ones.36,31,35 Moreover, the developed method is greener when compared with the reported method.30 On the other hand, using of the experimental design during the optimization of the developed method saved time, effort and money.

4. Conclusion
In the present work, a worthwhile custom experimental design was adopted for the optimization of an RP-HPLC method for the determination of the combined therapy DMH and CIN simultaneously with their toxic impurities; BZP and DPP. Interpretation of the regression and ANOVA test results allowed the estimation of main, quadratic and interaction effects of the studied factors on each response. Besides, the created response surface plots were statistically validated and used for response prediction. Optimum separation with adequate peak shape and suitable run time was acquired with a minimum number of experiments. The developed method was successfully applied for the determination of the studied compounds in their combined dosage form. Evaluation of the greenness profile of the proposed method proved that it is an excellent green analytical procedure. The developed method has advantages over the published chromatographic method, of being time and cost effective. Additionally, method greenness assessment was firstly performed during the analysis of DMH and CIN combined therapy.

Conflicts of interest
There are no conflicts to declare.

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