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Eco-Friendly and Sensitive HPLC and TLC Methods Validated for the Determination of Betahistine in the Presence of Its Process-Related Impurity

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Abstract: Reducing the amounts consumed of organic solvents while keeping good chromatographic performance has been a significant step towards the greening of analytical methodologies. When sodium dodecyl sulfate (SDS) and Brij-35 surfactants are combined in a mobile phase, they can be used as a green alternative to organic modifiers. Surfactants have numerous advantages, including low cost and toxicity, safe environmental disposal, and unique selectivity, in addition to high solubilization capabilities. In this research, two highly selective chromatographic methods were adopted for the determination of betahistine (BHS) in the presence of its pharmacopeial impurity 2-(2-hydroxyethyl)pyridine (HEP). A solvent-free HPLC method was validated, in which the mixture was separated using a C18 column (3.5 µm, 75.0 × 4.6 mm) and a mobile phase composed of 0.01 M Brij-35, 0.12 M SDS, and 0.02 M disodium hydrogen phosphate adjusted to a pH of 5.5 using phosphoric acid. The flow rate was 1.5 mL min^{-1} and the resolved peaks were detected at 260 nm. Another HPTLC-densitometric method was validated using HPTLC aluminum plates coated with silica gel 60 F254 as the stationary phase and a developing system consisting of methylene chloride/methanol/ethyl acetate/ammonia (at a ratio of 5:2:2:0.2 by volume); the separated bands were scanned at 260 nm.

Keywords: betahistine; process-related impurities; HPLC; TLC; mixed micellar chromatography

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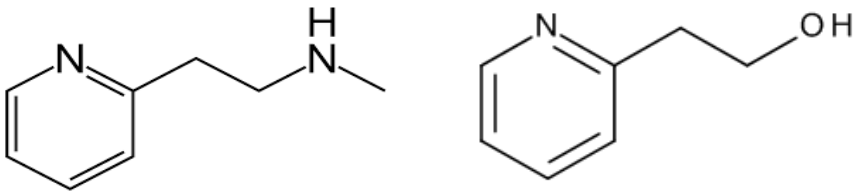
1. Introduction

Menière's disease (MD) was named after Prosper Menière, a French physician who proposed in 1861 that episodic vertigo and hearing loss could be caused by an inner ear problem [1]. Menière's disease is characterized by tinnitus, vertigo, and hearing loss. In ten percent of cases, MD reveals a genetic predisposition and a family history, with an autosomal dominant inheritance pattern [2]. There is currently no cure for Menière's disease, and the cause of the condition is still unknown. The most widely accepted theory of its etiology is endolymphatic hydrops, which is caused by a defect in the endolymph absorption in the endolymphatic sac [3]. MD is a clinical illness that affects 50 to 200 persons per 100,000, with the majority of cases occurring between the ages of 40 and 60.

Menière's medicines are expected to cost between USD 829.9 million and USD 934.2 million, or USD 5112 and USD 5748 per person each year [4]. Betahistine (BHS) is one of the most effective drugs against Menière's disease. In Europe, BHS had been prescribed as a first-line treatment for Menière's illness. In the United Kingdom, 94% of physicians prescribe BHS, according to the report by [1].

Every day, millions of analyses are performed to ensure the quality of pharmaceutical products. Because high-performance liquid chromatography (HPLC) is a successful, rapid, and reliable tool for the separation and quantification of mixed analytes, greener approaches should be followed to reduce the amount of hazardous organic waste produced by HPLC systems [5]. The high-performance thin-layer chromatography (HPTLC) technique has a number of advantages as an analysis tool. To start, the same mobile phase can be kept and used for multiple elutions (i.e., can be recycled). Secondly, the processing of the sample and standard at the same time under identical conditions enhances the analytical precision and accuracy. Furthermore, the low cost of the HPTLC technique stimulates its adoption as a cheap and economic analytical tool. The use of organic solvent-free mobile phases in HPTLC can also be accomplished through the use of aqueous salts and surfactants, which are reported to have a lower ecological impact as well as the ability to provide predictions on the lipophilic characters of newly discovered drugs [6].

BHS is chemically known as N-Methyl-2-(pyridin-2-yl) ethanamine, which is formulated as dihydrochloride salt. The British pharmacopeia (BP2017) [7] and American pharmacopeia (USP 43) [8] list an important process-related impurity known as, 2-(2-hydroxyethyl)pyridine (HEP) (chemical structures are presented in Figure 1). Pyridine and its derivatives have been linked to both acute and long-term health risks. Acute consequences include irritation of the skin, eyes, nose, and throat; long-term complications include fetal damage. Hepatotoxicity and nephrotoxicity have also been linked to pyridine and its derivatives. To show the consistency of the manufacturing process, it is critical for drug product formulators to monitor the level of predicted process-related chemicals and drug contaminants before commercial release.



(**A**) (**B**)

Figure 1. Chemical structure of (**A**) BHS, and (**B**) HEP.

Following a thorough analysis of the literature, many analytical procedures for determining BHS alone, in combination with other medications, or in the presence of impurities were found. Methods such as electrochemical [9–13], spectrophotometric [14–19], capillary electrophoresis [20], and chromatographic methods [21–26] were also reported.

In the present research, for the first time, green HPLC and HPTLC densitometric methods were developed and validated for the separation and quantification of BHS and its impurity, HEP. Each of the methods described has been successfully used to resolve the two components in a single chromatographic run. Furthermore, the proposed methods are very sensitive to accurately assess HEP (up to 0.05% for HPLC and about 0.15% for HPTLC), which is consistent with the impurity profile requirements (maximum limit of 0.2%) given by the BP2017 [7] and USP 43 [8].

2. Experimental

2.1. Materials

All reagents used were of analytical grade. Methylene chloride, methanol (MeOH), ethyl acetate, and ammonia were obtained from El NASR Pharmaceutical Chemical Company, Egypt. Brij-35 was purchased from Sigma-Aldrich, USA. Phosphoric acid, sodium hydroxide, sodium dodecyl sulfate (SDS), type-1 water for HPLC, and sodium dihydrogen phosphate were purchased from Merck, Germany.

The Egyptian International Pharmaceutical Industrial Company (EIPICo), Tenth of Ramadan City, Egypt, generously donated BHS as dihydrochloride with a purity of 99.3%. HEP was obtained from ACROS ORGANICS with a purity of 99%. A pharmaceutical tablet dosage form, Betaserc®, (labeled as containing 24 mg BHS per tablet) was purchased from a local Egyptian market (product of Abbott Pharmaceutical Company, Abbott Park, IL, USA).

2.2. Instruments and Software

HPLC separation was performed on a Waters Alliance 2695 equipped with a quaternary pump, a thermostatic column compartment, an auto sample injector, and a Waters 2996 photodiode array detector (Waters, Milford, MA, USA). Empower-3 software (Waters, Milford, MA, USA) was used to collect the data. For all calculations, we used Microsoft Excel 2013 (Microsoft Corporation, Redmond, WA, USA). Adjustment of the mobile phase pH was done using an AD1030 benchtop lab pH meter (ADWA, Romania). An analytical balance, model SA 210D (Scientech, USA), was used for the weighing procedures. The column used was a Symmetry® C18-E column (3.5 µm, 75 × 4.6 mm) from Waters, Milford, MA, USA.

A Linomat HPTLC system from CAMAG (Muttenz, Switzerland), connected to a semi-automatic sample application system and supplied with one 100 µL dosing syringe, was used for HPLC separations. All the samples' spotting was carried out in the presence of a nitrogen stream. For densitometric scanning, a CAMAG TLC Scanner 3 was utilized with HPTLC software version CATS Basic (version 1.4.4.6337). The HPTLC scanner was composed of halogen and deuterium lamps to generate the scanning radiation in the system. Merck HPTLC aluminum sheets (20 × 20 cm) pre-coated with silica gel 60 F254 were acquired from Merck (Darmstadt, Germany).

2.3. Chromatographic Conditions

The analytes were optimally separated with HPLC by isocratic elution using a mobile phase of 0.01 M of Brij-35, 0.12 M of SDS, and 0.02 M of disodium hydrogen phosphate adjusted to a pH of 5.5 with phosphoric acid. The samples were injected at a 20 µL volume. The flow rate was set to 1.5 mL min^{-1}, with the detecting wavelength set to 260 nm. The method's sustainability was improved by recycling the mobile phase in between the chromatographic runs. After each working day, the system was purged for 15 min with a mixture of water/MeOH (1:1) to remove bound surfactants from the stationary phase surface and improve the column reproducibility.

During the HPTLC, each aluminum TLC plate was split into two plates (20 × 10 cm) before usage. Elution on TLC was performed using a mobile phase composed of methylene chloride, MeOH, ethyl acetate, and 25% ammonia (50:20:20:2; v/v, respectively). Under a tnitrogen stream, samples were applied in 3 mm long bands at 5 mm intervals. Development was performed at room temperature inside a twin-trough securely closed TLC jar, which was saturated by the mobile phase for 30 min before chromatogram development. Linear ascending chromatogram development was eluted to a distance of 9 cm, where the elution time took around 10 min. After development, a dry air stream drier was applied on TLC plates to dry the sample spots. The plates were then scanned at 260 nm in the absorbance mode using a TLC Scanner 3.

2.4. Preparation of Stock Solutions and Standards

For the HPLC method validation, BHS and HEP stock solutions were prepared separately in deionized water at concentrations of 1000.0 and 50.0 μg mL^{-1}, respectively. Calibration curves were constructed using seven mixed standard solutions at concentrations of 3.0, 4.0, 10.0, 25.0, 50.0, 100.0, and 200.0 μg mL^{-1} for the BHS, and at concentrations of 0.1, 0.2, 0.4, 1.0, 2.5, 4.0, and 5.0 μg mL^{-1} for the HEP. The accuracy and precision were proven using mixed quality control (QC) standards at concentrations of 5.0 and 0.5, 100.0 and1.0, and 200.0 and 5.0 for the BHS and HEP, respectively) using the proposed HPLC method.

For the TLC analysis, stock solutions of BHS and HEP were prepared separately at concentrations of 1000.0 and 50.0 μg mL^{-1}, respectively. Linearity calibration curves were constructed by applying six different volumes of two mixed standard solutions that were prepared from the stock solutions at concentrations of 30.0 and 2.5, and 750.0 and 12.5 μg mL^{-1} for the BHS and HEP, respectively. The final spot concentration ranges were 300–15,000 ng per spot and 25–250 ng per spot for the BHS and HEP, respectively. Three other QC standards were prepared to test the accuracy and precision for the developed HPTLC method using mixtures at concentrations of 800 and 30, 4000 and 70, and 12,000 and 150 ng per spot for the BHS and HEP, respectively, prepared from the stock solutions.

2.5. Method Validation

The International Conference on Harmonisation (ICH) requirements were followed when validating the developed methods [27]. The linearity of the developed methodologies was constructed by plotting the detector response for each analyte's peak against the corresponding standard solution concentration using the linearity standards. Each standard was injected in triplicate and the average response was calculated. The limits of detection (LOD) and quantification (LOQ) were calculated from the slope (S) and standard deviation (σ) of the linearity data. The accuracy was estimated by triplicate injections of the three quality control standards; the average recovery percentages were calculated. The precision was evaluated in terms of intermediate precision and repeatability, which were assessed using the corresponding QC standards for both developed methods. Intra-day and inter-day fluctuations were computed at three different times within the same day and in three successive days in triplicate injections. The robustness of the developed methods was assessed to demonstrate the constant response against deliberate changes in the chromatographic conditions. For the HPLC, variations were induced in a mobile phase flow rate of ±0.05 mL min^{-1}, a pH of ±0.1, and a column temperature of ±2.0 °C. For the HPTLC, changes were induced in the saturation time (±5.0 min) and mobile phase composition (±0.1% methylene chloride). Then, the %RSD of the resulting peak areas (recovery percentages) of the QC standards were determined and the averages were calculated.

2.6. Preparation of Pharmaceutical Dosage Forms

Ten Betaserc® tablets were weighed and finely powdered, and then the average weight of one tablet was transferred into a 25 mL volumetric flask. The volume was made up to the mark with deionized water. The contents of the flask were sonicated for 10 min before being filtered. The filtrate was diluted (1:1) with deionized water for the HPTLC analysis. For the HPLC analysis, 1 mL of the filtrate was volumetrically diluted to 10 mL using deionized water.

3. Results and Discussion

3.1. Method Development and Optimization

The main aim of analytical chromatographers is to provide highly efficient separations at high through output. Mixed micellar chromatography has emerged lately as being a greener alternative to the use of organic solvents [28–30]. Its use offers high elution power, safe disposal, and a low ecological impact at low cost [31]. The method development of the HPLC technique was performed to provide the best possible resolution between

the analytes under study at the shortest elution time. This was accomplished using different composition ratios of the mixed surfactants and pH adjustments. The ratio of the two surfactants in a mixed-mode micellar chromatography is crucial. A total of 0.01 M of Brij-35 was mixed with SDS at four different concentrations (0.05, 0.08, 0.12, and 0.15 M). Another solution of 0.12 M of SDS was mixed separately with 0.01, 0.02, 0.03, and 0.04 M of Brij-35. Each time, the pH of the mobile phase was adjusted to 5.5 using phosphoric acid and/or 0.02 M of sodium hydroxide. SDS monomers bind to the hydrophobic part of the stationary phase, directing the sulfate group to the outside [5], and as such, the polarity of the stationary phase increases. The increase in concentration of the SDS had a negative effect on the retention time of the analytes. Meanwhile, Brij-35 binds to the stationary phase, exposing its oxyethylene chain [5], which in turn, forms H-bonding with the analytes to different strength degrees, enhancing the resolution. The optimal conditions were obtained at a ratio of 0.01:0.12 M of Brij-35/SDS. The column temperature was preserved at 30 °C and the flow rate was set at 1.5 mL min^{-1}. During the method development, the pH of the mobile phase was changed in order to improve the analytes' separation. pH values higher than 5.5 caused long retention times. pH values lower than the optimized one (5.5) did not permit base line separation between the analytes' peaks, and peak asymmetry was noticeable.

The same targeted approach was followed for the HPTLC technique, which was optimized by changing the co-solvent types and ratios in order to provide the best possible resolution between the analytes at the shortest plate development time. Being a normal phase chromatography, several organic co-solvents were tried using methanol, ethanol, 2-propanol, ethyl acetate, methylene chloride, and tetrahydrofuran. The best co-solvent that provided separation at an adequate resolution was methylene chloride/ethyl acetate/MeOH. The ratio of the chosen co-solvent mixture was then optimized to provide the best resolution. Finally, to eliminate tailing and improve the peaks' shape, a small amount of ammonia (25%) was added to the mobile phase system.

Table 1 shows the evaluation of the HPLC separation system suitability parameters, including retention time, resolution, symmetry factor, capacity, and column efficiency in terms of the number of theoretical plates. The retention factor (Rf) values for the retention of the studied analytes are also presented in Table 1.

Table 1. Chromatographic separation parameters and system suitability of the proposed methods.

	HPLC						HPTLC
	Retention Time (min)	**Selectivity (α)**	**Resolution (R_s)**	**Capacity Factor**	**Column Efficiency N [a]**	**Symmetry Factor**	**Retention Factor (R_f) [b]**
HEP	1.8	—-	——	2.47	1624	1.31	0.25 ± 0.02
BHS	3.5	2.28	4.79	5.64	2048	1.45	0.74 ± 0.02

[a] Column plate number. [b] Results = retention factor ± RSD.

3.2. Method Validation

The proposed analytical methods were found to be capable of successfully separating the investigated drugs with very good resolution (Figure 2). The linearity parameters were evaluated. Table 2 shows the obtained linearity and regression parameters. Both methods were found to be linear with very good correlation coefficients (R^2). The LODs were calculated as 3.3 σ/S, while the LOQs were calculated as 10 σ/S. The results of the LODs and LOQs for the proposed methodologies are presented in Table 2.

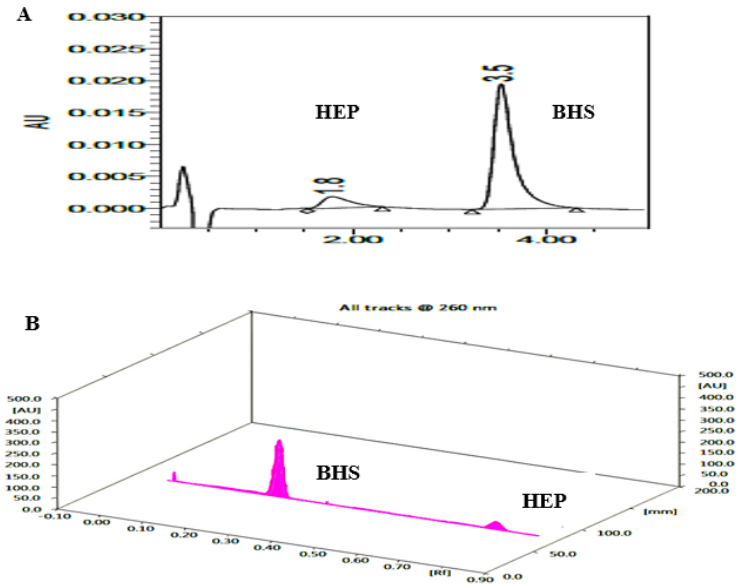


Figure 2. Separation chromatograms of the analyte mixtures under study using the proposed methods, where (**A**) HPLC (5.0:0.5 μg mL^{-1}, BHS/HEP) and (**B**) HPTLC (5000.0:50.0 ng per band, BHS/HEP).

Table 2. Linearity and regression results for the determination of BHS and HEP using the proposed methods.

Parameter	HPLC		HPTLC	
	BHS	HEP	BHS	HEP
Linearity range	3.0–200.0 [a]	0.1–5.0 [a]	300.0–15,000.0 [b]	25.0–250.0 [b]
R^2	0.999	0.999	0.999	0.999
LOD	0.96 [a]	0.03 [a]	80.0 [b]	4.0 [b]
LOQ	2.91 [a]	0.09 [a]	240.0 [b]	10.0 [b]
Accuracy [c]	99.9 ± 0.6	99.52 ± 0.8	99.0 ± 0.7	99.6 ± 0.7
Intra-day precision [d]	0.7	0.1	0.6	0.5
Inter-day precision [d]	0.4	0.1	0.6	0.8

[a] Results in μg mL^{-1}; [b] results in ng per band; [c] average recovery% ± RSD% (n = 9); [d] average RSD% for the calculated data.

The percentage recoveries of the BHS and HEP were used to assess the agreement between the actual and found results. Table 2 shows the average recoveries obtained by injecting the corresponding QC standards on each proposed methodology. Intra-day and inter-day precisions were evaluated by calculating the average percentage RSD (RSD%) obtained for replicate injections of quality control standards. Table 2 shows the precision results obtained, which indicates high precisions were acquired.

The robustness of the developed methodologies against deliberate changes in the method conditions is an important factor. The chromatographic conditions for both the HPLC and TLC techniques were changed deliberately, and then QC standards were injected to determine the percentage recoveries. Since the resolution between the two analytes was not affected greatly due to the high resolution established, an average RSD% between the recovery% results was calculated for each parameter that was changed. As shown in Table 3, good robustness results were obtained, as expressed in the low values of RSD percentages.

Table 3. Robustness of the proposed methods for the determination of BHS and HEP.

Parameter	BHS	HEP
HPLC [a]		
pH ± 0.1	0.54	0.39
Column temp. ± 2°C	0.57	1.29
Flow rate ± 0.05 mL min^{-1}	1.11	0.83
HPTLC [a]		
Methylene chloride (±0.1%)	0.64	0.82
Saturation time ± 5 min	0.81	0.83

[a] Average RSD results (n = 3).

3.3. Pharmaceutical Formulation Application

Betaserc® tablets were used to determine the ability of the methods to quantify the BHS in commercially available pharmaceutical formulations. The chromatograms presented in Figure 3 show applications of the developed methods in the determination of BHS in its marketed tablet dosage form in Betaserc® tablets. As shown, the chromatograms in both Figures 2 and 3 revealed the methods' specificity for the studied drug against its pharmacopeial impurity (HEP), as well as against excipients of the studied formulation. No additional peaks were identified at the retention times of the examined analytes. Investigations using the two developed methods revealed the absence of HEP in the Betaserc® tablets.

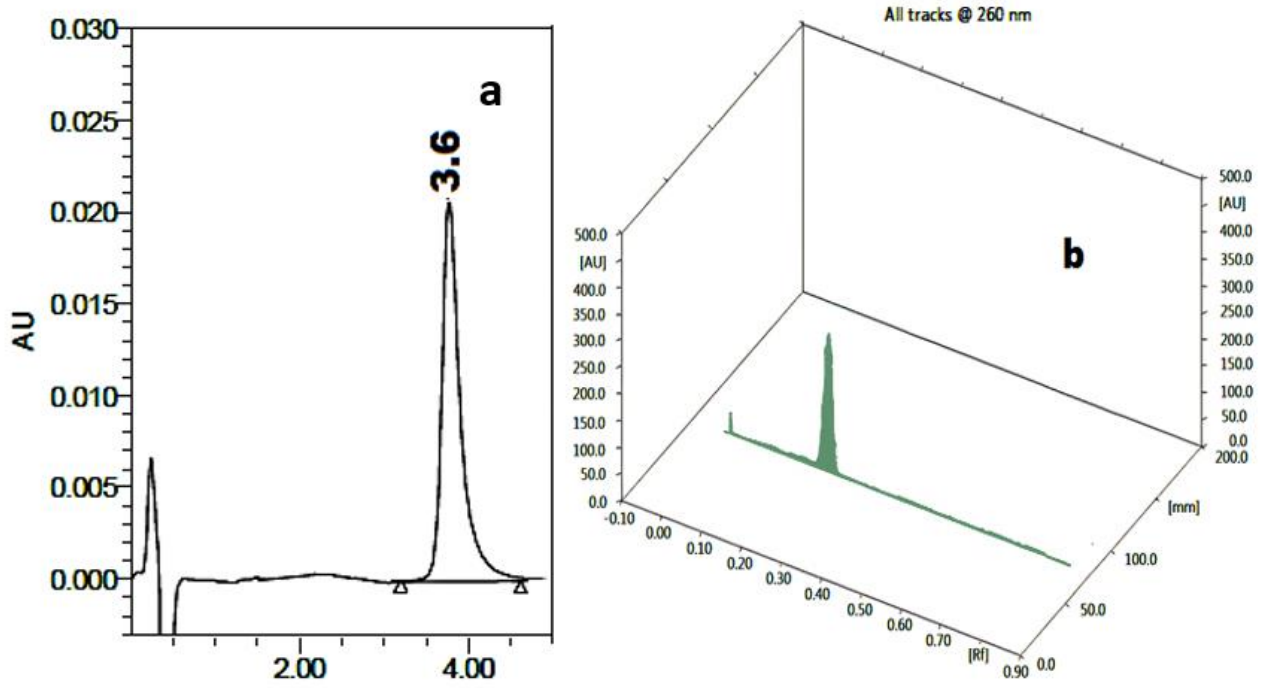


Figure 3. Chromatograms showing the application of the proposed methods for the determination of BHS in Betaserc® tablets, where (**a**) HPLC (BHS; 5.0 µg mL^{-1}) and (**b**) HPTLC (BHS; 5000.0 ng per band).

Table 4 shows the comparative results for these determinations. The results obtained by the proposed HPLC and HPTLC methods, as well as a reference HPLC method enclosed in a BP2017 monograph [7], were compared statistically. The calculated t and F values were found to be lower than the theoretical ones, as shown in Table 4, suggesting that there was no significant difference.

Table 4. Statistical comparison between the results obtained by the proposed chromatographic methods and the reported method for the determination of BHS in Betaserc® Tablets.

Parameters	HPLC	HPTLC	Reported [7]
Average BHS%	100.52	99.61	100.22
SD	0.76	1.01	1.27
Variance	0.57	1.02	1.61
n	5	5	5
Student's *t*-test (2.39) [a]	0.17	1.10	
F-test (6.39) [a]	2.79	1.58	

[a] The values between parenthesis correspond to the theoretical values of t and F (P = 0.05).

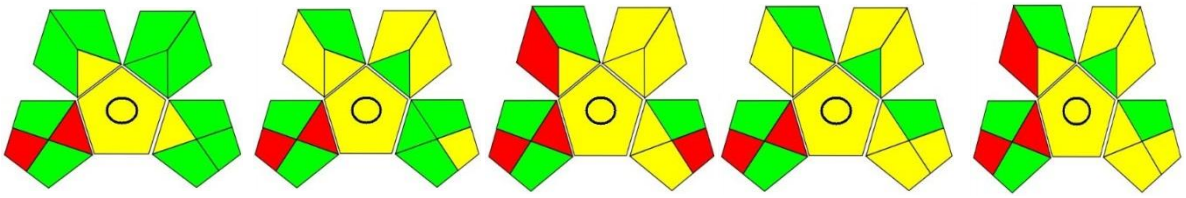
The second comparative tool was the interval plot test [7], which demonstrated that there was no meaningful difference between the results of the proposed methods and the reported methods. In the plots, the vertical lines show the confidence interval, with the center point corresponding to the interval's mean. Assume that the data group intervals of each approach overlap each other in the diagram. These plots show that there was no substantial difference between the data groups of each interval and others (Supplementary Materials, Figure S1). Another useful tool for data visualization is the boxplot [32], which depicts the distribution of the data between groups. Figure S2 in the Supplementary Materials shows HPLC and HPTLC boxplots. The middle quartile is represented by the central box, which has a line in it that indicates the data's median, upper lines that represent higher values, and whiskers that represent lower values. The boxplot shows the distribution of the data in each data category. The normal probability plot [32] is another technique for determining if the data is normally distributed (Supplementary Materials, Figure S3). The normal distribution of the data is satisfied if the straight line goes through the majority of the data points.

3.4. Assessment of Analytical Greenness

The term "green analytical chemistry" (GAC) was developed recently. GAC principles are aimed mainly at reducing environmental pollution caused by the dramatically growing waste generated by the application of analytical methodologies in various aspects of life. The use of reagents that are more benign to humans and their environment, reducing the use and generation of toxic waste as well as energy utilization became the baseline for GAC [33]. Therefore, the assessment of newly developed analytical methodologies is of great importance in order to evaluate their impacts. Several metrics were recently introduced for greenness assessment, including NEMI [34], the analytical eco-scale [35], the green analytical procedure index (GAPI) [36], and AGREE [37].

Among these metrics, GAPI still provides a simple, easy, and remarkable tool for greenness assessment, as it considers several steps involved in the process of analysis, including sampling, sample preparation, instrumentation, and used reagents, as well as generated waste. GAPI has a three-color code (red, yellow, and green), where the red color indicates a bad ecological impact, while the green color represents the best ecological tolerance. The proposed methods were assessed on GAPI and compared to three selected and reported methodologies [7,21,25] for the estimation of BHS. Table 5 shows a brief description of the parameters of the comparison methods. The proposed HPLC method does not utilize any organic solvents; however, the elution power, as expressed in the short analysis time in addition to the good separation efficiencies, as shown in Table 1, indicates the superiority of the proposed methodology over the compared methods. A GAPI pictogram of the proposed HPLC and HPTLC methods shows a greener ecological impact over the other methods as expressed by the increased number of green zones and fewer red zones.

Table 5. Comparison of the proposed analytical method with previously reported methodologies.

	Proposed Method	**Proposed Method**	**Reported Method [25]**	**Reported Method [21]**	**Reported Method [7]**
Technique	HPLC-PDA	HPTLC-UV	HPLC-FLD	HPLC-UV	HPLC-UV
Organic phase	Totally free	Methylene chloride/methanol/ethyl acetate/ammonia	Acetonitrile	Methanol	Acetonitrile
Mobile phase	0.01 M of Brij-35, 0.12 M of SDS, and 0.02 M of disodium hydrogen phosphate adjusted to a pH of 5.5 with phosphoric	Methylene chloride/methanol/ethyl acetate/ammonia (25%) (5:2:2:0.2; *v*/*v*)	30% acetonitrile and 70% sodium acetate	Acetonitrile and mixed solution (buffer ammonium acetate with sodium lauryl sulfate at a pH of 4.7)	Acetonitrile and Solution of sodium lauryl sulfate (7:13)
Flow rate	1.5 mL min^{-1}	——	1 mL min^{-1}	1 mL min^{-1}	1 mL min^{-1}
Retention time	HEP: 1.8 min BHS: 3.5 min	HEP: 0.74 Rf BHS: 0.25 Rf	HEP: Not applicable BHS: 18.4 min	HEP: Not applicable BHS: 2.6 min	HEP: 0.2 min BHS: 7.0 min
GAPI					

4. Conclusions

Selective and sensitive HPLC and HPTLC methods were established for the simultaneous separation and estimation of betahistine and its process-related impurity, 2-(2-hydroxyethyl)pyridine in their binary mixture and dosage form. The suggested methods have the value of being capable of determining betahistine in the presence of its impurity. Furthermore, the proposed methodologies were compared to previously reported ones and proved to be more environmentally friendly. With regard to applying the developed methodologies on marketed dosage forms, there were no significant differences found between the results obtained using the proposed methods when compared to those obtained using the previously reported methods.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/separations9020049/s1, Figure S1: Interval plot test for comparing the proposed (a) HPLC and (b) HPTLC methods to reported method in BP2017 monographs for determination of BHS in marketed dosage forms, Figure S2: Boxplot for comparing the proposed (a) HPLC and (b) HPTLC methods to reported method in BP2017 monographs for determination of BHS in marketed dosage forms, Figure S3: Normal probability plot for comparing the proposed (a) HPLC and (b) HPTLC methods to reported method in BP2017 monographs for determination of BHS in marketed dosage forms.

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Conflicts of Interest: The authors declare no conflict of interest.

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