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Research Article

A novel, green and affordable semi-quantitative limit test for 2-aminobutanol impurity in ethambutol hydrochloride bulk and tablets

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Abstract

The pharmacopoeial limit test for the impurity 2-aminobutanol (AB) in ethambutol hydrochloride (ETB) relies on florescence measurement using a fluorometer. However, this instrument is often unavailable in many laboratories due to its cost. A novel colorimetric method was therefore developed using genipin, an amine-reactive reagent, to react with AB, producing a blue color. The color intensity was subsequently measured using a more affordable UV-vis spectrophotometer. To overcome matrix effects, the standard addition technique was incorporated. The test effectively detected AB at concentrations as low as 0.125% in ETB, below the compendial limit of 1.0%. The analysis of ETB bulk and tablets showed consistent semi-quantitative results with the USP method. Furthermore, it aligned with green chemistry by eliminating the use of organic solvents and employing safe, naturally derived reagent. In summary, the proposed method provides a reliable, green, and cost-effective alternative for analyzing AB in ETB, ensuring compliance with pharmacopeial standards.

Keywords

limit test, 2-aminobutanol, ethambutol hydrochloride, UV-vis spectrophotometry, genipin

Introduction

A limit test, as defined by pharmacopoeias, is an analytical procedure used to assess the presence of impurities in pharmaceutical active ingredients or products. The primary objective of this type of test is to determine whether the level of a particular impurity within a drug substance or product falls within an acceptable range established by regulatory authorities or pharmacopoeial standards. Accordingly, limit tests play an essential role in pharmaceutical quality control, as it helps to ensure the safety, efficacy, and purity of drug products.

Ethambutol (ETB), an antibiotic drug used for the treatment of tuberculosis, may contain 2-aminobutanol (AB) as a known impurity originating from the synthesis process. To this end, pharmacopoeias establish limits for AB in ETB bulk drug and formulations, typically set at 1.0% (The European Pharmacopoeia Commission 2007; The United States Pharmacopeia Convention 2020; The British Pharmacopoeia Commission 2022), meaning that AB of not more than 1 g is allowed in the sample containing 100 g of ETB. In both the United States Pharmacopeia (USP) and British Pharmacopoeia (BP), the test for AB in ETB is a semi-quantitative analysis. It is carried out by preparing two

solutions: one containing only the ETB sample and another comprising the ETB sample added with a known amount of AB standard (equivalent to 1.0% AB in ETB). Subsequently, fluorescamine is introduced to both solutions to react with amines. After a precisely timed incubation for 1 min, the fluorescence intensities of both solutions are measured using a fluorometer. Based on the standard addition technique, the acceptance criteria dictate that the fluorescence intensity of the sample solution should not exceed the difference between the intensities of the two solutions.

While the procedure of the pharmacopeial AB limit test is not too complicated, in practice, performing this test can be problematic for many laboratories due to the lack of a fluorometer since this instrument is relatively expensive. In the pursuit of other previously reported methods used for analyzing AB in various sample types, non-aqueous titration is found to be as an option (Sigma-Aldrich 2024); however, it exhibits inadequate sensitivity and specificity for accurately detecting subtle amounts of AB impurity in the ETB matrix. Apart from that, the analysis of AB using thin layer chromatography (World Health Organization 2023) and gas chromatography (Sigma-Aldrich 2024) have been reported, but they come with drawbacks, such as laborious operation and the requirement for more sophisticated and expensive instrument, respectively. Since the test relying on the absorbance measurement using common and affordable UV-vis spectrophotometers is simpler and more practical, the objective of this work was to develop a new alternative limit test by changing from fluorometry to colorimetry. For this purpose, a naturally derived reagent, namely genipin, was tested for serving as a chromogenic reagent. From literature, this compound is employed as a crosslinking agent in tissue engineering (Cassimjee et al. 2022), enzyme immobilization (Tacias-Pascacio et al. 2019; Phadungcharoen et al. 2019) and drug delivery systems (Yu et al. 2021). Besides, it can be used as a food colorant (Neri-Numa et al. 2017) and colorimetric reagent for certain analytes (Lee et al. 2003; Winotapun et al. 2012), by producing a blue color upon reaction with primary amines. However, its use for the analysis of AB has not been found yet. Notably, genipin is also biocompatible and safe, thus generally regarded as a greener alternative to other reagents e.g. glutaraldehyde and ninhydrin (Levinton-Shamuilov et al. 2005). Once the suitable conditions for the reaction between genipin and AB were obtained from the optimization studies, the protocol for a semi-quantitative limit test was established. The method was subsequently validated and applied for analysis of AB in ETB samples, compared to the USP method. Furthermore, the practical and green aspects of the test were discussed.

Experimental

Chemicals and instrumentation

AB (purity of 99.7%), ETB (purity of 100%) and fluorescamine were purchased from Sigma (MO, USA). Genipin was obtained from Challenge Bioproducts Co., Ltd. (Taichung, Taiwan). ETB tablets (400 mg per tablet) were obtained from a drugstore in Thailand. Other ingredients in the tablets included microcrystalline cellulose, corn starch, anhydrous lactose, colloidal silicon dioxide and talc. UV-vis spectrophotometer (Cary 60 UV-Vis Spectrophotometer, Agilent Technologies, Germany) was used for the absorbance measurement in the proposed limit-test, and spectrofluorophotometer (RF-6000, Shimadzu, Japan) was used for measuring the fluorescence intensity following the USP method.

Procedure for the proposed limit test of AB in ETB using genipin

Initially, a stock standard solution of AB at a concentration of 0.1 mg/mL was prepared in water, while a 10 mM genipin solution was made by dissolving genipin in 0.1 M potassium phosphate buffer, pH 8.0. The ETB sample solution was prepared by dissolving either ETB bulk or ground ETB tablets in water to have an ETB concentration of 4 mg/mL. In the case of tablet-derived solutions, they underwent filtration through a 0.45 µm filter to eliminate insoluble excipients before proceeding with the reaction.

To perform the analysis, 500 µL of sample solution was dispensed into two separate 1.5 mL microcentrifuge tubes using a micropipette. In the first tube, referred to as NS (no standard AB), 500 µL of water was added, resulting in the solution containing only 2 mg/mL ETB. In the second tube, labeled as SA (standard addition), 200 µL of 0.1 mg/mL standard AB solution and 300 µL of water were added, yielding a solution with 2 mg/mL ETB and 0.02 mg/mL AB. To each tube, 200 µL of 10 mM genipin solution was added. The solutions were thoroughly mixed and subsequently heated in a water bath at 80 °C for 15 min. Following cooling on ice for 1 min, the solutions were subjected to absorbance measurements at 590 nm utilizing a UV-vis spectrophotometer. The acceptance criterion for the limit test was that the absorbance of the NS solution was not greater than the difference between the absorbances of the SA solution and NS solution (NS < SA-NS).

Selection of chromogenic reagents

Before the above-mentioned protocol had been established, an investigation was conducted to establish whether a chromogenic reagent, genipin, could be used as an alternative for the fluorogenic fluorescamine. To this end, genipin was tested for its capability to generate blue colored products with AB both in the absence and presence of ETB. In these experiments, excess amounts of the colorimetric reagent, specifically 7.5 mM genipin, was used to react with 0.01 mg/ml AB solution, 1 mg/mL ETB solution, and a mixture solution of 0.01 mg/ml AB and 1 mg/mL ETB (equal to 1% AB in ETB). Subsequent to the reactions, the absorbance of the solutions was measured at the wavelength of 590 nm.

Optimization of the chromogenic reaction between genipin and AB

The effects of the concentration of genipin stock solution (5, 10, 15 mM), reaction's pH (7, 8, 9), and heating duration (10, 15, 20 min) on the development of blue color were examined using AB in the range of 0.01–0.05 mg/mL, alongside the presence of 2 mg/mL ETB. The optimal condition, which yielded a standard curve of AB with a steeper slope and enabled a faster and more reagent-saving test compared to the others, was selected for establishing the protocol.

Method validation

The proposed method was validated according to the guidelines outlined in USP 43⟨1225⟩ VALIDATION OF COMPENDIAL PROCEDURES for a semi-quantitative limit test (Category II) (The United States Pharmacopoeia Convention 2020). The specificity of the test was confirmed by investigating the interference effects of ETB drug and the tablet excipients, i.e., microcrystalline cellulose, corn starch, anhydrous lactose, colloidal silicon dioxide and talc. The linear relationship between the concentration of AB (X), ranging from 0.01–0.05 mg/mL in the presence of 2 mg/mL ETB (to simulate the real samples), and the absorbance at 590 nm (Y) was examined by plotting a standard curve. Subsequently, the regression equation and the coefficient of determination (r^2) were determined. The detection limit was calculated based on 3.3 times the standard deviation of the response divided by the slope of the standard curve.

Applicability to analysis of real samples

The proposed method was applied for semi-quantitative analysis of AB in the real samples, i.e., ETB chemical bulks and commercial tablets (400 mg/tablet). Since AB levels in these samples were normally below 1% as they had undergone quality control checks prior to their release and marketing, synthetic samples were additionally made and tested to verify the reliability of the test. This involved adding varying amounts of AB (both exceeding and falling below 1%) to the commercial samples and subsequently conducting the analysis. Finally, the results obtained from the proposed method were compared with those from the USP method.

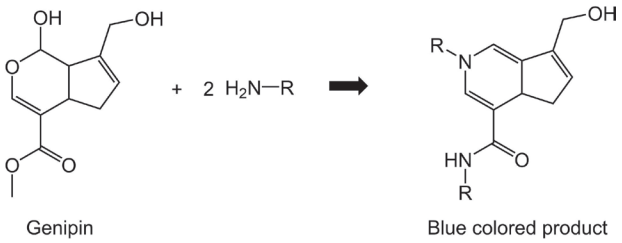

Figure 1. The chromogenic reaction of amine and genipin.

Results and discussion

Selection of genipin as an effective chromogenic reagent

In light of AB containing a primary amine whereas ETB does not, a chromogenic reagent specifically reacting with this functional group was explored for the development of a new colorimetric method. Additionally, since the greenness of the method was concerned, with a particular emphasis on the safety of the reagent and the avoidance of organic solvents, a test enabled in an aqueous medium was preferred. Consequently, ninhydrin, a well-known chromogenic chemical was excluded from the study due to its relatively unsafe nature and its typical use in organic solvents such as acetone, ethanol, or N, N-dimethylformamide. Considering genipin's ability to react with primary amines (as illustrated in Fig. 1), it emerged as the preferred option for this study.

From the experiments, it was revealed that the reaction of genipin and AB in water produced an intense blue color, evidenced by the high absorbance at 590 nm (Fig. 2). When genipin was introduced to ETB, only faint blue color was

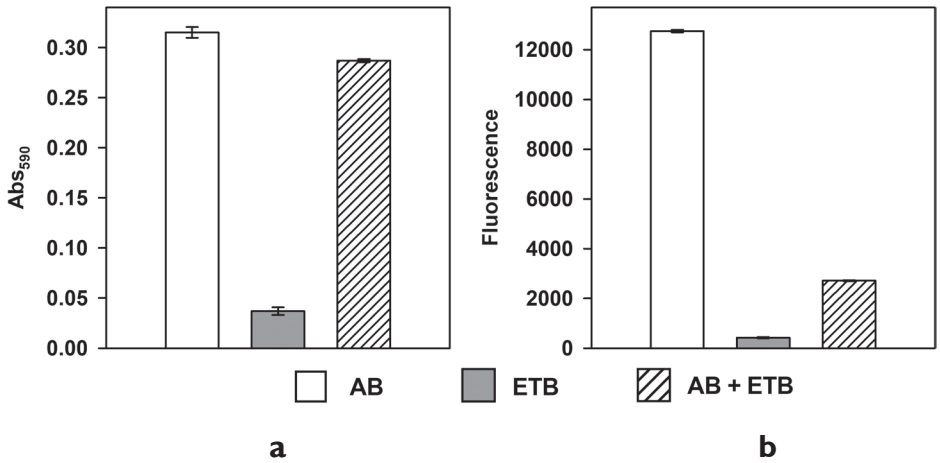

Figure 2. The absorbance and fluorescence intensity of the solutions (n = 3) resulting from the reactions between genipin (**a**) or fluorescamine (**b**), respectively, with 0.01 mg/ml 2-aminobutanol (AB) solution, 1 mg/mL ethambutol hydrochloride (ETB) solution, and a mixture solution of 0.01 mg/ml AB and 1 mg/mL ETB (AB+ETB).

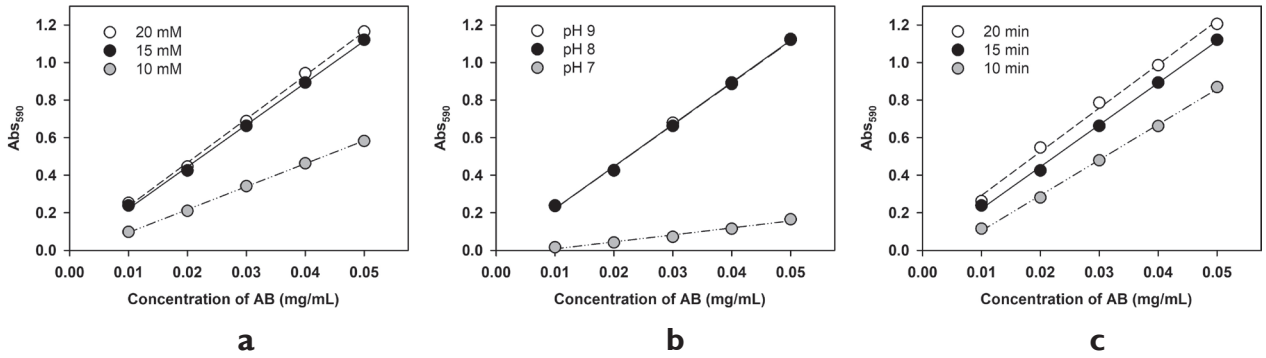

Figure 3. The effects of the concentration of the genipin stock solution (**a**), pH of the reaction (**b**) and heating time (**c**) on the color formation reaction between 2-aminobutanol (AB) and genipin.

produced. Moreover, even in the presence of ETB, genipin reacted with AB to produce the blue color, albeit with a slightly lower intensity compared to the reaction without ETB. In comparison to fluorescamine, genipin demonstrated superior suitability for detecting AB, especially in situations where this impurity coexisted in an abundance of ETB. This was evident as the presence of ETB (1 mg/mL) caused only a 10% reduction in the absorbance of the genipin reaction, while the fluorescence intensity in fluorescamine reactions decreased by approximately 80%. Based on these observations, genipin was chosen for the alternative colorimetric limit test. Furthermore, the results suggested the existence of some degree of matrix effect due to ETB, necessitating the application of the standard addition technique to overcome this effect in the test.

Optimal reaction conditions

As illustrated in Fig. 3, increasing the concentration of the genipin stock solution, pH of the reaction and heating time from 10 mM, pH 7, and 10 min to 15 mM, pH 8, and 15 min led to an increase in the slopes of the standard curves, representing the higher sensitivity. However, neither the slope nor the sensitivity was further enhanced when these factors were raised to 20 mM, pH 9, and 20 min. Therefore, the optimal condition for the test was found to be using 15 mM genipin stock solution, pH 8, and a heating time of 15 min, since it was generally preferred to conduct the reaction close to a neutral pH within a shorter time and saving the reagent.

Method validation results

The validation results (studied in the presence of 2 mg/mL ETB) demonstrated a good linear relationship between the concentration of AB and the absorbance, with a regression equation $y = 22.331x - 0.0019$ and r^2 of 0.9985. The detection limit for AB was determined to be 0.0025 mg/mL, indicating that the method could effectively detect the impurity at a level as low as 0.125% (0.125 mg AB in 100 mg ETB). This detection limit is below the pharmacopoeial limit (1.0%) set for both ETB bulk and tablets. Regarding specificity, the method was unaffected by the tablet excipients tested, as these ingredients lack amine groups to react with genipin or produce the blue color. However, despite ETB lacking amine groups, it interfered with the test by lowering the intensity of the blue color. This interference was seen by the reduction of slope of the standard curve obtained from samples containing ETB compared to those without ETB (Fig. 4). Nevertheless, as this interference manifested as a rotational or proportional type of matrix effect (i.e. altering the slope of the standard curve but not its intercept), this issue could be addressed by implementing standard addition technique in the test (Ellison et al. 2008; Rojanarata et al. 2022).

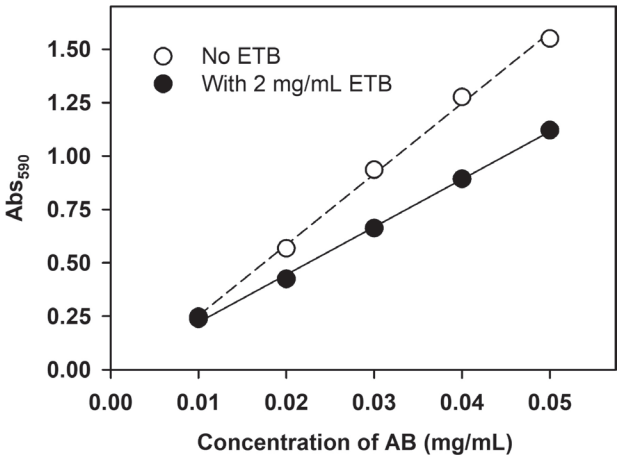

Figure 4. Standard curves of 2-aminobutanol (AB) in the absence and presence (2 mg/mL) of ethambutol hydrochloride (ETB).

Applicability of the method and comparison to the USP method

The analysis results of AB in both the commercial samples and the synthetic samples (commercial samples supplemented with additional AB) are presented in Table 1. It is evident that in all instances, the proposed method provided the conclusions based on semi-quantitative determination, i.e., either pass (AB < 1.0%) or reject (AB > 1.0%), consistent with the USP method. Notably, for the synthetic bulk sample with 0.95% AB added, both methods reported the same "reject" conclusion, likely due to the total amount of AB exceeding 1.0%. These findings affirmed the reliability of the proposed method, as it consistently gave results aligned with the USP limit test, rendering it suitable for alternative test method.

Table 1. Results of semi-quantitative limit test for 2-aminobutanol (AB) in ethambutol (ETB) samples.

ETB sample	Proposed method (genipin)				USP method (fluorescamine)			
	Absorbance			Conclusion[a]	Fluorescence intensity			Conclusion[b]
	NS solution	SA solution	Difference		NS solution	SA solution	Difference	
Bulk								
Original sample	0.098	0.478	0.379	Pass	1462	3115	1654	Pass
Synthetic samples								
Bulk + 0.9% AB added	0.350	0.730	0.381	Pass	1719	3451	1732	Pass
Bulk + 0.95% AB added	0.383	0.762	0.380	Reject	1827	3526	1698	Reject
Bulk + 1.0% AB added	0.400	0.790	0.390	Reject	1900	3583	1683	Reject
Bulk + 1.05% AB added	0.428	0.810	0.382	Reject	1979	3676	1698	Reject
Bulk + 1.1% AB added	0.449	0.831	0.382	Reject	2044	3769	1725	Reject
Tablets								
Original sample	0.036	0.435	0.399	Pass	389.052	2246.128	1857	Pass
Synthetic samples								
Tablets + 0.9% AB added	0.386	0.816	0.430	Pass	1718.971	3549.880	1831	Pass
Tablets + 0.95% AB added	0.406	0.842	0.435	Pass	1848.690	3712.807	1864	Pass
Tablets + 1.0% AB added	0.435	0.866	0.431	Reject	2024.844	3868.151	1843	Reject
Tablets + 1.05% AB added	0.460	0.890	0.430	Reject	2193.672	4014.129	1820	Reject
Tablets + 1.1% AB added	0.483	0.915	0.432	Reject	2356.443	4209.773	1853	Reject

[a] Pass if the absorbance of NS solution was not greater than the difference;
[b] Pass if the fluorescence intensity of NS solution was not greater than the difference.

Practical aspects and greenness of the proposed method

The limit test using genipin developed in this work presented numerous advantages over the USP method, particularly in terms of affordable and practical operations as well as alignment with green chemistry. By switching from fluorometry to colorimetry, laboratories can use UV-spectrophotometers, which are more commonly available and cost-effective instruments, instead of fluorometers. Besides, while it is essential to promptly determine the fluorescence intensity after a timely 1-min incubation of the fluorescamine reaction, as indicated in the USP test instructions, the absorbance measurement in the proposed method allows for greater flexibility, as the intensity of the blue color remains stable and unchanged for at least 30 min after complete formation (Fig. 5).

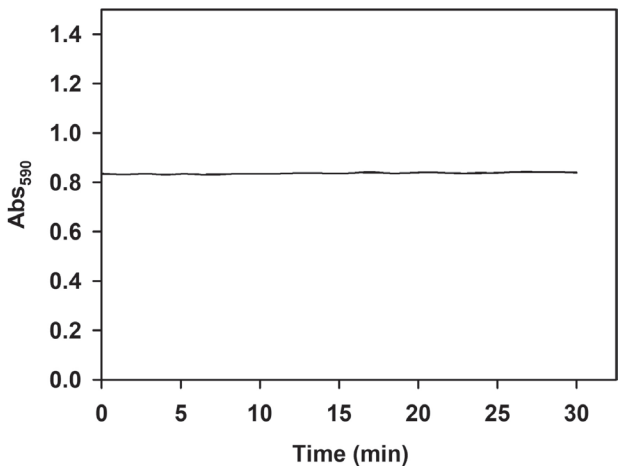

Figure 5. Stability of the blue color formed in the proposed limit test using genipin. The time zero was counted after the reaction was heated for 5 min and then cooled on ice for 1 min.

In regards to greenness, the test was conducted in water without the need for organic solvents like acetone, which is used as a solvent for fluorescamine in the USP method. Additionally, genipin is favored for green chemistry as it is derived from natural sources e.g. gardenia fruit and is considered a safe and environmentally friendly substance (Ahmed et al. 2024). The sole drawback of the proposed test was its need for a 15-min heating step, which could potentially consume more energy and time compared to the USP method. Nonetheless, the capability to analyze multiple samples in a single run can reduce both the energy cost per sample and the overall runtime. The greenness evaluation conducted using the Analytical Greenness Metric (AGREE) (Pena-Pereira et al. 2020) revealed a score of 0.74, exceeding 0.7, which is considered environmentally friendly (Fig. 6a). Furthermore, the Green Analytical Procedure Index (GAPI) (Płotka-Wasylka 2018) illustrated 6 green sections, 5 yellow sections, and only 3 red sections, indicating the method's overall satisfactory greenness (Fig. 6b). These results conclusively confirmed that the method proposed in this study aligns with green analytical chemistry principles.

Conclusion

This study presents a novel semi-quantitative limit test for AB in ETB bulk and tablets. The method relied on a colorimetric reaction between genipin and primary amines of AB, with the intensity of the resulting blue color being measured spectrophotometrically at 590 nm. To address the matrix effects, especially those attributed to ETB, the standard addition technique was incorporated. Despite changing from fluorometry to colorimetry, the method exhibited adequate sensitivity, capable of detecting AB at levels eight times lower than the pharmacopeial limit of 1%, and it gave the consistent analysis results with those obtained from the USP method.

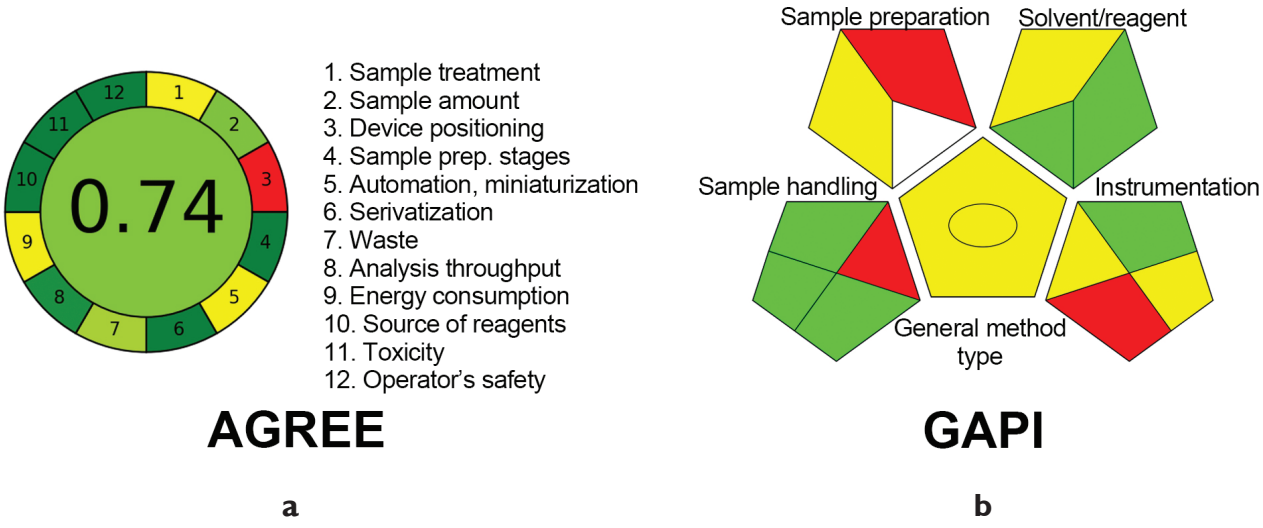

Figure 6. Greenness of the proposed limit test assessed by (**a**) Analytical GREEnness (AGREE) and (**b**) Green Analytical Procedure Index (GAPI).

The method provided enhanced flexibility for conducting the reaction and subsequent absorbance measurement since the blue color formed was remarkably stable. Additionally, a greener reagent and required less expensive instrumentation were used in the test. Although the test required a 15-min heating step, the simultaneous analysis of multiple samples could decrease energy expenses and overall runtime. In summary, the proposed method provided a reliable alternative method for the analysis of AB in ETB samples, ensuring compliance with pharmacopeial standards.

Conflict of interest

The authors declare that there is no conflict of interest.

Acknowledgements

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