BMC Chemistry

RESEARCH Open Access

Establishment and validation of HPLC methods for the determination of folic acid and parabens antimicrobial agents on folic acid oral solution

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Abstract

Background As the common antibacterial drugs in folic acid oral liquid, parabens are listed as mandatory substances in the quality standard. Both the Chinese Pharmacopoeia and the United States Pharmacopoeia use high performance liquid chromatography for the determination of folic acid, but the quantitative methods of parabens are different. Pharmacopoeias use different instruments to quantify folic acid and parabens, resulting in cumbersome and cumbersome detection methods.

Objective Without changing the type of instrument and mobile phase, two methods were established for the determination of folic acid and parabens (methyl paraben; ethyl paraben; propyl paraben) using respective wavelengths and flow comparisons Propyl benzoate) high performance liquid chromatography method.

Method Chromatographic separation was achieved on an Agilent 5 TC-C_{18} HPLC column (5 µm; 250 µm × 4.6 mm) maintained at 25 °C (column temperature). The mobile phase consisted of phosphate buffer (pH 4.0)-methanol. When the ratio is 99:1, it is used to determine the content of folic acid, and when the ratio is 79:21, it is used to determine the content of antimicrobial agents. The flow rate used was 1.2 mL/min, the injection volume of folic acid was 20 µL, and the injection volume of bacteriostatic agent was 50 µL. In addition, the blue applicability grade index (BAGI) and analytical greenness (AGREE) metric tools were used to evaluate the greenness and environmental friendliness of the developed methods.

Results The method has a good linear relationship with $R^2 \geq 0.9995$, the average recovery rate of the two methods is ≥ 95%, and the relative standard deviation (RSD%) accuracy is less than 0.21%. The BAGI tool characterizes the developed method as green. The AGREE score is around 0.5, and the method is also largely consistent with the principles of green analytical chemistry.

Conclusions The HPLC method was established for the rapid determination of folic acid and antibacterial agent of parabens in folic acid. The method has high accuracy, strong specificity, high recovery rate, good stability

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and environmental friendliness. Compared with the method in the pharmacopoeia, it has strong resistance to complex matrix interference, greatly shortens the detection time, and has little damage to the instrument and chromatographic column. It can be used for the quality standard of folic acid oral liquid.

Keywords Folic acid, Parabens, HPLC

Introduction

Folic acid, a water-soluble vitamin, is one of the B vitamins and is named because it is a biological factor found from spinach. The chemical structure of folic acid was shown in Fig. 1, a 2-amino-4-hydroxypteridine combines with the 6th carbon atom of 4-aminobenzoic acid through a methylene bridge to form pteroic acid, which then combines with glutamic acid to form folic acid. Folic acid is an essential nutrient in the human diet and involved in several metabolic pathways, mainly in carbon transfer reactions such as the interconversion of amino acids and biosynthesis of purines and pyrimidines [1, 2]. Therefore, folic acid plays an indispensable role in disease prevention and treatment, and folic acid deficiency has a significant impact on pregnant woman and newborn, which may lead to neonatal neural tube defects, placental abruption in pregnant women, gestational hypertensive syndrome, and megaloblastic anemia. In this situation, fetuses are prone to intrauterine growth retardation, preterm delivery, low birth weight, and may also lead to impaired fetal growth and mental development after birth [1, 3]. Studies in recent years have demonstrated that folic acid deficiency may lead to the development of maternal perinatal depression [4], acute leukemia [5–7], and sickle cell anemia [8–10]. Proper supplementation of folic acid can effectively improve depression, dementia, aging, and memory loss, as well as has a positive effect on the treatment and prognosis of cancer [11–17].

The folic acid oral liquid is susceptible to be contaminated by microorganisms, which leads to mold growth and fermentation. Hence, the addition of antimicrobial agents has become one of the common methods to solve the contamination of oral liquid [18]. In 1924, Sabalitschka first reported the antiseptic effect of antimicrobial agents [19], parabens are widely used in food, cosmetics and other industries because of their advantages of small additive quantity, low toxicity and high safety [20–24]. Parabens are commonly used as methyl paraben, ethyl paraben, propyl paraben, and butyl paraben, which have neutral pH and tasteless, so they won't change the color and taste of the bulk solution when added to food as antimicrobial agents. Parabens are commonly used as antimicrobial agents in oral preparations. As an additive, the Acceptable Daily Intake (ADI) of parabens is 0–10 mg/kg, exceeding this dose may cause safety hazards. Parabens is often added as a bacteriostatic agent in folic acid oral liquid, which is a mandatory substance in the quality standard. Therefore, the measurement and limits of paraben are necessary to establish quality standards for folic acid oral solution.

As a supplement to human development, growth and health, folic acid plays a very important role in the food and pharmaceutical industries. The accurate determination of folic acid in products is of great significance for the establishment of product quality standards and determination of efficacy [25]. With the development of science and technology, more and more methods for the determination of folate have been established, such as high-performance liquid chromatography [26–28], ultra high-performance liquid chromatography [29], microbiological methods [30], fluorescence analysis [31], enzyme-linked immunosorbent assay [32], spectrophotometric methods [33], electrochemical methods [34], and so on. For the quantification of folic acid in pharmaceutical products, we compared the methods for the determination of folate between the European Pharmacopoeia, the United States Pharmacopoeia and the Chinese Pharmacopoeia. The method for the quantification of folate in the European Pharmacopoeia used methanol: phosphate (containing 11.16 g/L potassium dihydrogen phosphate and 5.5 g dipotassium hydrogen phosphate) = 12:88 as the mobile phase, and the retention time for folic acid was approximately 8.5 min, but the overall run time was three times the retention time for folic acid. We applied the European Pharmacopoeia method for the determination of folate, but the phosphate content in the mobile phase of this method was very high, which was harmful to the instrument and the column. Hence, we tried the method of Chinese Pharmacopoeia 2020 version to determine folic acid: phosphate buffer (pH 5.0) was used as the mobile phase, but the detection time was so long and it took 120 min. Moreover, the folic acid peak shape appears anteriorly delayed. In response to this phenomenon, we optimized the method by changing the chromatographic conditions, which effectively solved the problems of long detection time and poor peak shape. Consequently, the retention time of folic acid was shortened to about 14 min with good peak shape, which greatly saved the detection time. The Chinese Pharmacopoeia records the application of gas chromatography for the determination of methyl paraben, ethyl paraben and propyl paraben, while the American Pharmacopoeia using the thin-layer adsorption method. After reviewing the literature, we found that these three antimicrobial agents are often determined by phosphate buffer-methanol or phosphate buffer-acetonitrile as the mobile phase

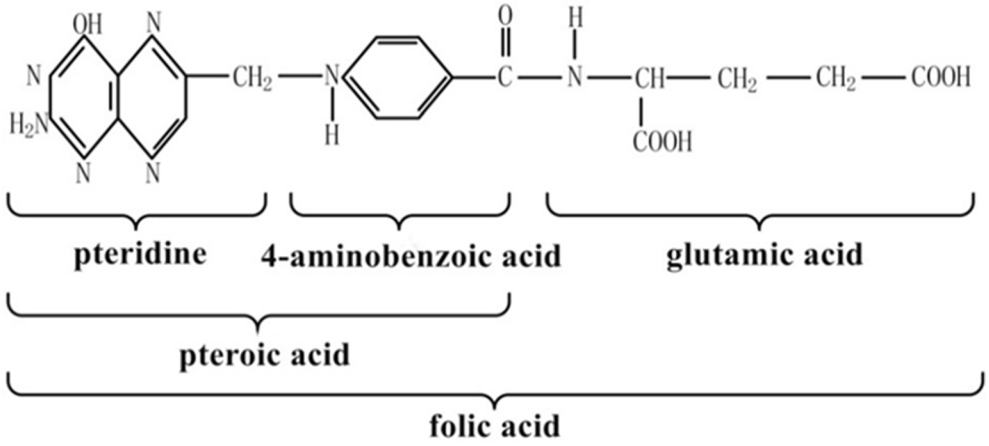

Fig. 1 Folic acid structure diagram

[23, 35], therefore we tried to optimize the determination method of folic acid. Compared with the existing methods, one of the main advantages of our method is that it saves analysis time and solvent consumption, and does not change the mobile phase and detection instruments. It can provide reference for establishing the quality standard of folic acid oral liquid. In addition, the green performance of the optimized method and its impact on the environment were evaluated using the analytical greenness (AGREE) [36] and blue applicability grade index (BAGI) [37] metric tools.

Materials and methods

Materials and reagents

Folic acid standards were purchased from the China Academy of Food and Drug Administration, and folic acid raw materials were obtained from Baoding Aihui Pharmaceutical Co., Ltd. Methyl paraben, ethyl paraben and propyl paraben were provided by Shanghai Yuanye Biotechnology Co., Ltd. and the raw materials were provided by Hubei Gedian Renfu Pharmaceutical Excipients Co., Ltd. The methanol used for HPLC was obtained from Mreda and tetrabutylammonium hydroxide was purchased from Maclean's Reagent Company, both of which were chromatographically pure. Analytically pure potassium dihydrogen phosphate and phosphoric acid were used to prepare and adjust the pH value of the mobile phase.

Apparatus, mobile phase and chromatographic conditions

HPLC separation was performed with SHIMADZU LC-2030 plus instrument equipped with a binary pump, autosampler and column oven. The UV detector waters2487 with the optimal detection wavelength of 254 nm was used for data analysis. Chromatographic separations were achieved using an Agilent 5 TC-C_{18} HPLC column (5 μm; 250 μm × 4.6 mm) and C_{18} protective column, both from Agilent technology, made in the Netherlands. The column oven temperature was set to 25 °C. An isocratic mobile phase was used, which was composed of phosphate buffer (pH 4.0)-methanol. When the ratio is 99:1, it is used to determine the content of folic acid and when the ratio is 79:21, it is used to determine the content of Bacteriostatic agent. The flow rate used was 1.2 mL/min, the injection volume was 20 μL for folic acid and 50 μL for Bacteriostatic agent.

Preparation of standard solutions

6 mL 0.5% ammonia was used to dissolve 19.81 mg folic acid standard into a 10 mL brown volumetric flask and the solution was diluted with pure water to prepare folic acid standard reserve solution; The 14 mg methyl paraben standard, 11 mg ethyl paraben standard, and 7 mg propyl paraben standard were accurately weighed in a 10 mL volumetric flask, and then diluted with methanol to prepare 1.4 mg / mL, 1.1 mg / mL and 0.7 mg / mL standard reserve solution; 5 mL of methyl paraben standard reserve solution, 4 mL of ethyl paraben standard reserve solution and 2 mL of propyl paraben standard reserve solution were taken into 20 mL volumetric flask, and then diluted with mobile phase to prepare antibacterial agent mixed standard solution. Folic acid standard solution was prepared by folic acid standard reserve solution. Methyl paraben, ethyl paraben and propyl paraben standard solution were prepared by antibacterial agent mixed standard solution. Folic acid reference solution and sample solution were prepared by taking folic acid standard and folic acid raw material 50.0 mg in 25 mL brown volumetric flask, dissolved with 15 ml 0.5% ammonia solution and diluted with pure water.

Analytical method validation

Linearity study

A series of different concentrations of folic acid standard solution (0.1188, 0.1585, 0.1981, 0.2377, and 0.2773 mg / mL), methyl paraben standard solution (4.2, 5.6, 7, 8.4, and 9.8 μg /mL), ethyl paraben standard solution (2.64, 3.52, 4.4, 5.28, and 6.16 μg /mL), propyl paraben standard solution (0.86, 1.12, 1.4, 1.68, and 1.96 μg /mL) were injected into the HPLC to obtain the chromatograms. Each concentration sample was injected twice in parallel, and the peak area was measured. Linear regression was performed with the average peak area to its concentration.

Precision

The precision of the method was investigated by repeated parallel test. The intermediate concentration of folic acid reference substance and antibacterial agent mixed standard solution was determined by repeated parallel experiments for 6 times according to the optimized method. The retention time and peak area were recorded respectively, and the RSD value was calculated.

Stability study

Continuous determination (0, 1, 2, 4, 6, 8, and 10 h) the middle concentration of folic acid reference stock solution and bacteriostatic agent mixed standard solution placed at room temperature and dark conditions. The retention time and peak area were recorded respectively, and the RSD value was calculated.

Recovery study

By comparing the measured amount with the actual weighing amount, the recovery rate was calculated. Folic acid solution (0.16, 0.20, and 0.24 mg / mL), methyl p-hydroxybenzoate (5.6 ,7.0, and 8.4 μg / mL), ethyl p-hydroxybenzoate (3.52, 4.40, and 5.28 μg / mL) and propyl p-hydroxybenzoate (1.12, 1.40, and 1.68 μg / mL) were prepared at low, medium and high concentration levels, respectively.

Limits of detection study

The folic acid reference solution, methyl p-hydroxybenzoate, ethyl p-hydroxybenzoate, and propyl p-hydroxybenzoate standard stock solution were gradually diluted until the signal-to-noise ratio (S / N) generated by the detector was 10, and the corresponding amount was its limit of quantitation.

Robustness study

An appropriate amount of folic acid raw material was used as the test sample, and the folic acid standard was used as the control sample. The folic acid test solution and folic acid reference solution under different chromatographic conditions were detected by changing the chromatographic conditions (column temperature, flow rate, wavelength, pH, mobile phase ratio, and liquid column) in a small range. The developed method was considered robust if the results were within acceptable limits.

Evaluation of environmental impact

Two greenness evaluation tools, AGREE and BAGI, were used to evaluate the green profiles of the optimization and validation methods and create corresponding pictograms.

Results and discussion

Methods development

Optimization of constant volume solvent

After the dissolution of folic acid raw material by ammonia test solution, the liquid phase diagrams obtained by pure water or mobile phase constant volume injection are shown in Fig. 2 (a) and Fig. 2 (b) respectively. It is obvious that, compared with the two samples, the folic acid peak obtained by pure water constant volume sample has a more symmetrical peak pattern and a better peak pattern. When the constant volume liquid is the mobile phase, impurities are frequently produced in the first 20 min and the separation degree is small. However, when pure water is constant volume, impurities can be separated better. Therefore, pure water is selected as the

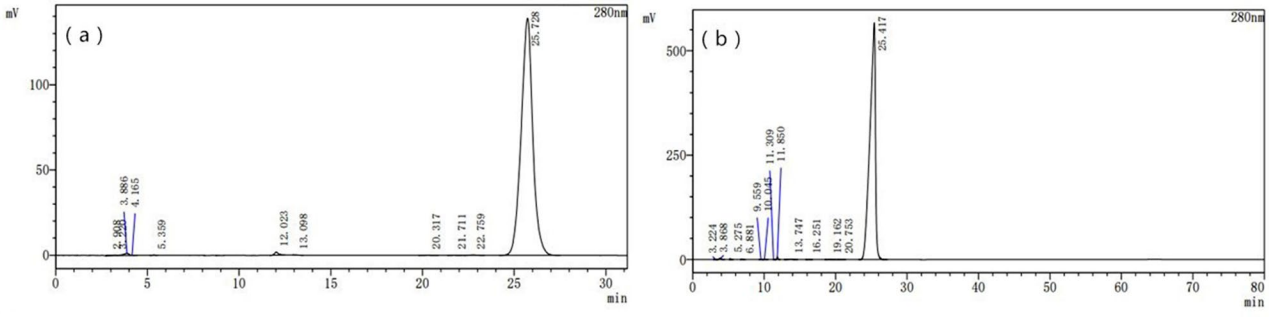

Fig. 2 HPLC-Chromatographic diagram of different constant volume solution (**a**) water; (**b**) mobile phase (Potassium dihydrogen phosphate 2.0 g was dissolved in water, then 15 mL of 0.5 mol / L tetrabutylammonium hydroxide solution, 7 mL of 1 mol / L phosphoric acid solution and 270 mL of methanol solution were added. After cooling, the pH value was adjusted to 5.0 with 1 mol / L phosphoric acid solution and diluted to 1000 mL with water.)

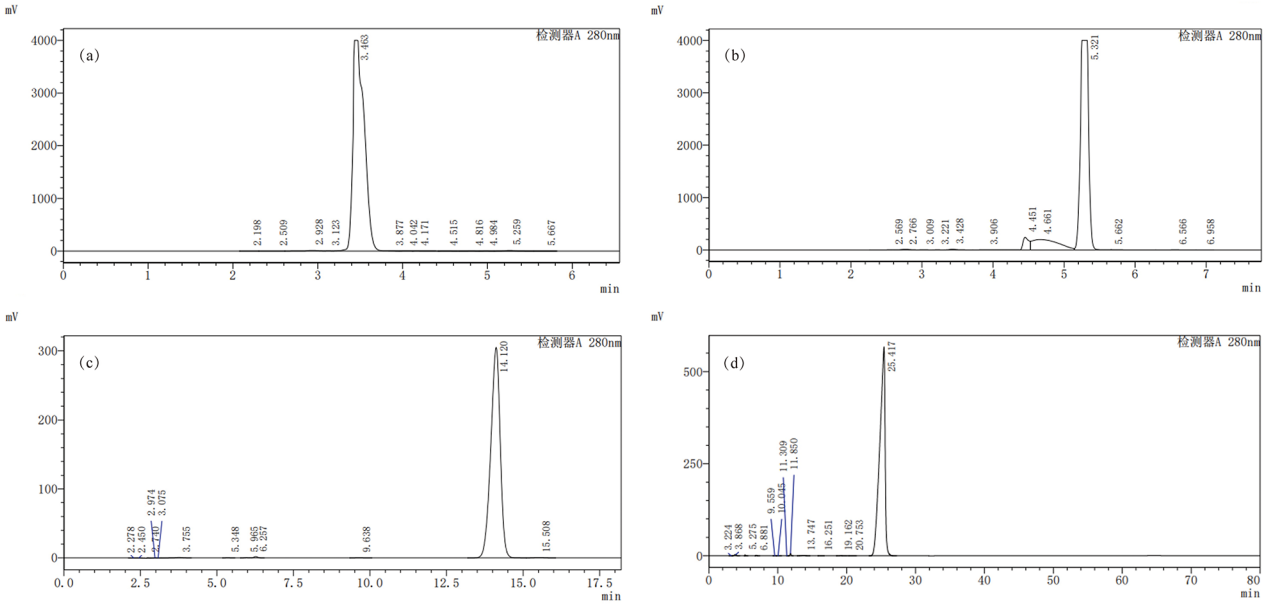

Fig. 3 HPLC-Chromatogram of different mobile phase ratio. (**a**) phosphate buffer : methanol 71:29; (**b**) phosphate buffer : methanol 80:20; (**c**) phosphate buffer : methanol 99:1; (**d**) phosphate buffer : methanol 100:0

Table 1 Gradient elution conditions

Time (min)	Phosphate buffer (%)	Methanol (%)
40	1	99
43	40	60
60	40	60
62	1	99
77	1	99

constant volume liquid in the folic acid sample configuration process.

Optimization of column temperature

For studying the effect of column temperature on the main and retention time, choosing the 25 °C and 30 °C, 35 °C, three different kinds of column temperature. The results show that the three temperatures have little difference on the main peak-peak type, separation degree and retention time. If the column temperature is 30–35 °C, the stability time of the column temperature chamber will be prolonged, so the final selection of the column temperature is 25 °C.

Optimization of mobile phase ratio

With reference to the method for the determination of folic acid in Chinese Pharmacopoeia 2020 version folic acid tablets, optimization was carried out by adjusting the ratios of phosphate buffer to methanol to 71:29, 80:20, 99:1 and 100:0, and the peak shapes and retention times of the main peaks were observed, respectively. The chromatograms under different mobile phase ratios are presented in Fig. 3, and the gradient elution conditions are presented in Table 1.With the increase of methanol ratio, the peak residence time of folic acid moved forward and the overall sampling time was greatly shortened. However, when the methanol ratio is too high, the solvent peak will be generated and the folic acid peak will be affected. Figure 3 shows the changes of folic acid peak under different mobile phase ratios. In Fig. 3 (a), the ratio of phosphate buffer to methanol is 71:29, and the peak type of folic acid is poor. In Fig. 3 (b), the ratio of phosphate buffer to methanol is 80:20, and folic acid cannot be completely separated. Therefore, the ratio of these two mobile phases should be abandoned. The flow ratio in Fig. 3 (c) and Fig. 3 (d) was 99:1 and 100:0, respectively. The peak type of folic acid was good, but the main peak residence time of folic acid was 14.120 and 25.417, respectively. Considering the problem of time efficiency, the phosphate buffer: methanol = 99:1 was selected as the best mobile phase.

In the experiment, it was also found that the peak time of excipient was longer when the sample of folic acid oral liquid was determined by isometric elution. In order to shorten the sample injection time, the gradient elution procedure was introduced after 40 min without interfering with the determination of folic acid and related substances. Experiments showed that elution according to the conditions in Table 1, with the increase of organic phase, the peak period of excipients was advanced, which effectively shortened the sampling time and accurately determined the content of folic acid and its degraded impurities in oral liquid.

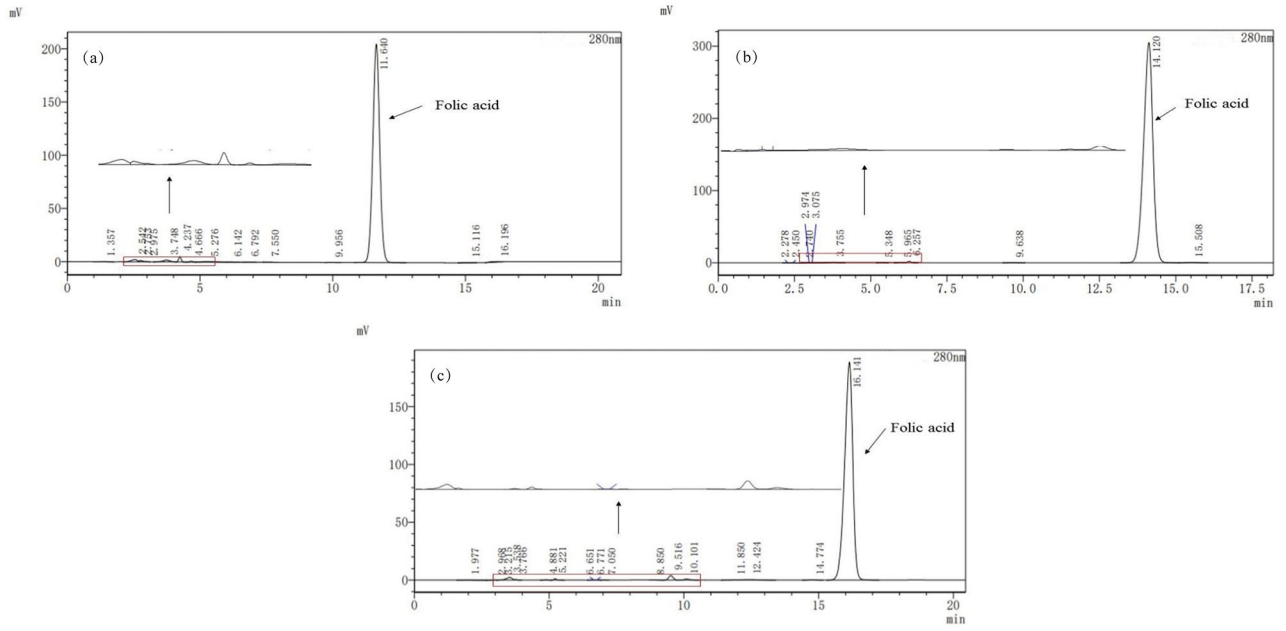

Fig. 4 HPLC-Chromatogram of different pH. (**a**) pH = 3; (**b**) pH = 4; (**c**) pH = 5

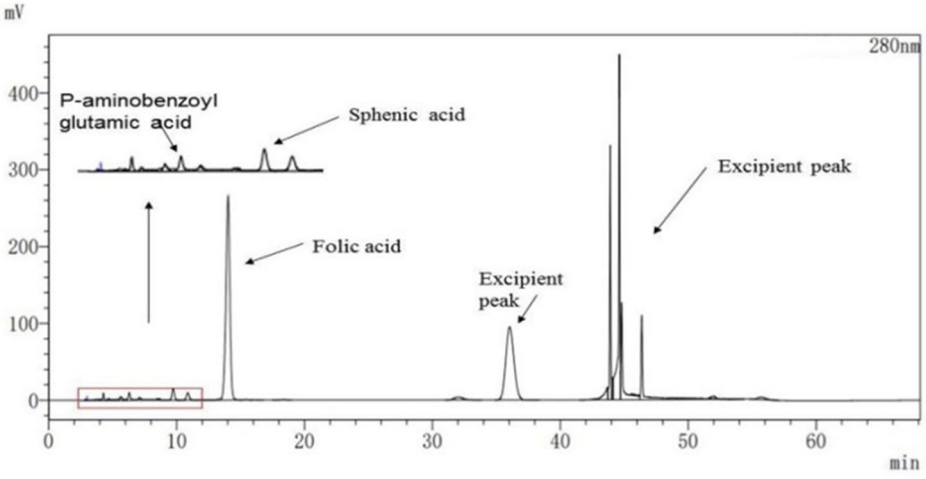

Fig. 5 Folic acid sample destruction pH = 4

Optimization of pH

Adjust the pH to 3.0, 4.0 and 5.0 with 1 mol/L phosphoric acid solution, observe the main peak type and retention time, and the liquid chromatography is shown in Fig. 4. It was observed that with the decrease of pH value, the retention time of the main peak was advanced and the detection time was shortened. When the pH of phosphate buffer is 3.0, 4.0 and 5.0, the retention time of folic acid peak is 11.640, 14.120 and 16.141 respectively. There is no significant difference between the peak and peak type of folic acid, which is in a good symmetrical state. However, it can be seen that the impurity peak is not separated in the first 5 min when the pH value is 3.0, as shown in Fig. 4 (a). When pH is 5.0 and retention time is about 2 min, the separation of impurity peak is poor, as shown in Fig. 4 (c). As shown in Fig. 5, when pH = 4.0, the sample is destroyed at high temperature to produce impurities, and then gradient elution results show that folic acid and each impurity peak can be completely separated 20 min before the sample runs, with no interference between the peaks. Therefore, pH = 4.0 is selected as the final pH of phosphate buffer.

Optimization of mobile phase ratio of bacteriostatic agent

When the established folic acid method was used for the determination of antimicrobial agents, methyl paraben, ethyl paraben and propyl paraben can be measured, and the peak type were excellent. However, the running

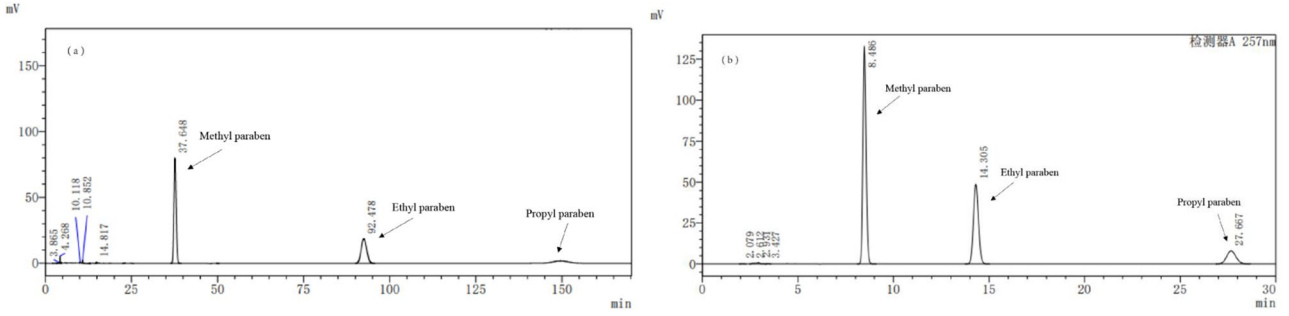

Fig. 6 HPLC-Chromatogram of different mobile phase ratio of bacteriostatic agent. (**a**)phosphate buffer : methanol 99:1; (**b**)phosphate buffer : methanol 71:29

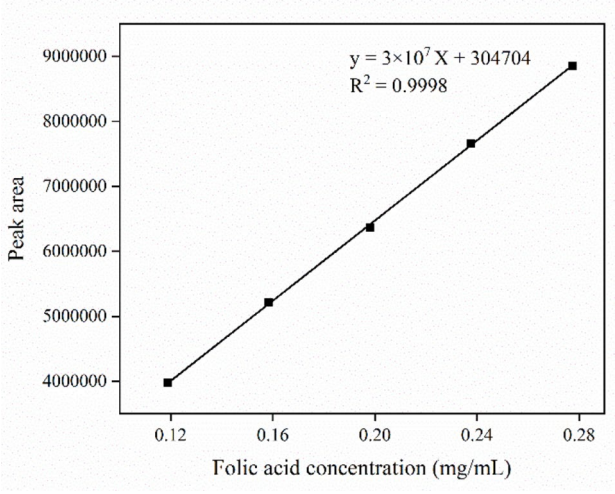

Fig. 7 Folic acid linear graph(0.1188, 0.1585, 0.1981, 0.2377, 0.2773 mg/mL)

time is too long for the determination. The peak of propyl paraben didn't appear until about 150 min. By adjusting the ratio of mobile phase, it was found that the determination could be completed within 30 min when the mobile phase ratio of phosphate buffer and methanol was 79:21, which greatly improved the detection efficiency. In addition, sharp peaks with good symmetry can be acquired in a short time. The chromatograms are presented in Fig. 6. Therefore, the mobile phase ratio was adjusted to 79:21.

Method validation

Linearity

By calculating the peak area of folic acid standard with different concentrations, the peak area ratio of folic acid was compared with the concentration of folic acid, and the linearity of the method for folic acid was established. Chromatograms of linearity are given in Fig. 7. The results showed that there was a creditable linear relationship between the concentration of folic acid over the tested concentration range ($R^2 = 0.9998$) as presented,

Table 2 The important parameters for the calibration curves in Antibacterial agent

Antibacterial agent	Concentration range (μg/mL)	Y = aX + b	R^2
Methyl paraben	4.20 ~ 9.80	Y = 284596X-42,105	0.9996
Ethyl paraben	2.64 ~ 6.16	Y = 265398X-29,921	0.9995
Propyl paraben	0.86 ~ 1.96	Y = 255841X-10,642	0.9996

and the linear regression equation of the standard curve was $y = 3 \times 10^7 x + 304{,}704$.

To test the linearity of the calibration curve of Antibacterial agent under the optimized conditions, five calibration curves for non-zero concentrations were prepared and determined. Excellent linear relationships were obtained between the concentrations of all three inhibitors and their peak area over the tester concentration range as presented in Table 2.

Precision

The intermediate sample of folic acid and bacteriostatic agent mixed standard solution was repeatedly injected for 6 times, and the retention time and peak area RSD values were shown in Table 3. The RSD of the main peak area and retention time is less than 2%, which shows that the precision of the peak area and retention time of this method is very good.

Stability

Placed the intermediate samples of folic acid control sample and bacteriostatic for 0 h, 2 h, 4 h, 6 h, 8 h and 10 h and injected them respectively. The results were shown in Table 4. For both folic acid and antibacterial agent, their RSD values were less than 2%, both in terms of peak area and retention time, which indicated that the stability of both folic acid and antibacterial agent was excellent.

Recovery

Under the conditions established in this experiment, the measured value of sample weighing was calculated according to the measured average peak area, and

Table 3 Precision and accuracy of the method for determination of folic acid and antibacterial agent

		1	2	3	4	5	6	Average	RSD (%)
Folic acid	Peak area	6,380,275	6,352,119	6,349,857	6,349,685	6,351,755	6,346,667	6,355,060	0.20
	Retention time	13.784	13.831	14.026	14.117	14.165	14.214	14.023	1.27
Methyl paraben	Peak area	2,012,762	2,012,605	2,012,343	2,012,362	2,012,713	2,012,753	2,012,590	0.01
	Retention time	8.488	8.476	8.47	8.466	8.462	8.462	8.471	0.12
Ethyl paraben	Peak area	1,171,284	1,171,030	1,170,977	1,171,348	1,171,129	1,171,505	1,171,212	0.02
	Retention time	14.308	14.28	14.265	14.259	14.247	14.248	14.268	0.16
Propyl paraben	Peak area	358,311	358,353	358,380	358,802	358,909	358,598	358,559	0.06
	Retention time	27.639	27.573	27.537	27.522	27.503	27.498	27.545	0.19

RSD%: Relative Standard Deviation = (SD/Mean) x 100

Table 4 Determination of the stability of folic acid and antimicrobial agents

		0 h	2 h	4 h	6 h	8 h	10 h	RSD (%)
Folic acid	Peak area	6,380,275	6,349,081	6,350,015	6,352,230	6,349,154	6,341,677	0.21
	Retention time	14.08	14.28	14.533	14.608	14.657	14.702	1.68
Methyl paraben	Peak area	2,012,762	2,012,639	2,010,859	2,013,172	2,014,733	2,013,903	0.05
	Retention time	8.488	8.463	8.457	8.454	8.446	8.453	0.11
Ethyl paraben	Peak area	1,171,284	1,171,128	1,170,525	1,171,281	1,172,147	1,171,810	0.11
	Retention time	14.308	14.252	14.238	14.231	14.215	14.229	0.17
Propyl paraben	Peak area	358,311	358,794	358,099	358,237	359,014	358,153	0.21
	Retention time	27.639	27.51	27.474	27.458	27.418	27.464	0.28

RSD (%): Relative Standard Deviation = (SD/Mean) x 100

compared with the actual weighing to calculate the recovery rate. The results were shown in Table 5. At the three additional levels of low, medium and high, the average recovery of folic acid was more than 95%, and the RSD was 1.08%, which was within the acceptable range, indicating that the method had good repeatability and accuracy. The theoretical values of the three antimicrobial agents are very close to the measured values. The average recovery rate of Methyl paraben, Ethyl paraben and Propyl paraben were 99.07%, 99.83% and 99.08%, respectively. The average recovery rates of the three antimicrobial agents were all above 99%, which met the established recovery acceptance criteria, indicating that the recovery rate of the analysis method was good and could meet the accurate determination of the three antimicrobial agents in folic acid nutrient supplements.

Limit of quantification

The limit of quantification (LOQ) for the investigated folic acid was experimentally determined (Fig. 8). When the injection concentration was 1.6 μg/mL, the peak height was about ten times that of noise. At this time, the injection volume was 1 μL and the quantitative limit of folic acid was about 1.6 ng/μL×1 μL = 1.6 ng. The limit of quantification (LOQ) for the investigated antibacterial agent was experimentally determined (Fig. 9). The limits of quantification for the three inhibitors were 0.7 ng, 0.88 ng and 0.14 ng, respectively.

Robustness

The robustness of the procedure was determined by changing the reaction conditions, for which the peak area and retention time of folic acid in the chromatography were recorded. The retention time and peak area for different validation parameters are shown in Tables 6 and 7. From Table 6, it could be seen that after changing the chromatographic conditions, the percentage of folic acid was about 95% on average, the RSD of the main peak area of folic acid in Table 7 was less than 2%, indicating that the chromatographic conditions of this method were robust.

Evaluation of methods greenness

To evaluate the level of environmental friendliness of methods, AGREE and BAGI tools were used. The representative pictograms of the analysis results are shown in Figs. 10 and 11.

AGREE is based on the twelve principles of green analytical chemistry, and the final score is usually expressed in the range of 0 to 1. The value with a score close to 1 indicates a greener environment, while the lower value indicates a lower green level. If the score exceeds 0.6, the method is considered green [38, 39]. The AGREE scores of folic acid and antimicrobial agents were calculated to be 0.49 and 0.53, respectively. Although the established methods may not be considered particularly green. However, the score is around 0.5, which means that the methods are largely in line with the principles of green analytical chemistry and represent a certain contribution

Table 5 The recovery test determination of folic acid and antimicrobial agents

		Actual weighing (mg)	Retention time		Peak area		Average	Actual measurement (mg)	Recovery (%)
			1	2	1	2			
Folic acid	1	37.82	14.505	14.533	4,510,035	4,503,936	4,506,986	35.14	92.91
	2	38.60	14.523	14.548	4,757,955	4,764,633	4,761,294	37.12	96.17
	3	39.10	14.561	14.562	4,834,363	4,836,470	4,835,417	37.70	96.42
	4	49.17	14.562	14.63	5,996,421	6,001,340	5,998,881	46.77	95.12
	5	49.50	14.631	14.67	6,044,620	6,031,411	6,038,016	47.07	95.10
	6	49.49	14.683	14.679	6,043,476	6,040,425	6,041,951	47.11	95.18
	7	57.60	14.721	14.697	7,022,502	7,024,135	7,023,319	54.76	95.06
	8	58.14	14.752	14.844	7,069,209	7,069,042	7,069,126	55.11	94.79
	9	58.77	14.891	14.97	7,259,623	7,204,119	7,231,871	56.38	95.94
	Average (%)								95.19
Methyl paraben	1	54.12	8.502	8.463	1,399,767	1,400,817	1,400,292	49.86	92.13
	2	56.28	8.449	8.448	1,582,447	1,581,774	1,582,111	56.34	100.10
	3	56.04	8.445	8.443	1,571,028	1,570,958	1,570,993	55.94	99.82
	4	70.59	8.442	8.444	2,011,359	2,011,138	2,011,249	71.62	101.46
	5	69.72	8.441	8.44	1,959,653	1,959,386	1,959,520	69.78	100.08
	6	70.49	8.443	8.443	1,980,077	1,981,346	1,980,712	70.53	100.06
	7	83.15	8.446	8.445	2,329,245	2,330,695	2,329,970	82.97	99.78
	8	84.03	8.442	8.445	2,358,651	2,358,530	2,358,591	83.99	99.95
	9	82.86	8.447	8.445	2,285,789	2,285,814	2,285,802	81.39	98.23
	Average (%)								99.07
Ethyl paraben	1	34.02	14.34	14.253	817,394	817,877	817,636	31.89	93.74
	2	35.85	14.214	14.211	924,092	923,607	923,850	36.03	100.51
	3	35.97	14.199	14.197	937,799	937,533	937,666	36.57	101.67
	4	45.32	14.201	14.204	1,184,285	1,184,017	1,184,151	46.18	101.91
	5	44.52	14.201	14.201	1,144,022	1,143,735	1,143,879	44.61	100.21
	6	44.98	14.208	14.205	1,155,373	1,156,175	1,155,774	45.08	100.22
	7	53.09	14.211	14.209	1,359,673	1,360,620	1,360,147	53.05	99.92
	8	53.21	14.205	14.21	1,377,458	1,377,665	1,377,562	53.73	100.97
	9	52.41	14.214	14.21	1,334,992	1,334,944	1,334,968	52.07	99.34
	Average (%)								99.83
Propyl paraben	1	10.62	27.718	27.52	244,091	244,390	244,241	9.94	93.61
	2	11.21	27.434	27.418	275,519	275,845	275,682	11.22	100.10
	3	11.15	27.384	27.389	273,543	272,928	273,236	11.12	99.75
	4	14.23	27.395	27.402	351,571	351,448	351,510	14.31	100.55
	5	14.03	27.401	27.408	343,007	342,462	342,735	13.95	99.44
	6	14.08	27.418	27.411	343,570	343,574	343,572	13.99	99.33
	7	16.65	27.425	27.414	405,737	406,081	405,909	16.52	99.23
	8	16.81	27.415	27.425	411,125	411,081	411,103	16.73	99.55
	9	16.18	27.433	27.419	398,241	398,131	398,186	16.21	100.17
	Average (%)								99.08

The low (Coding 1, 2, and 3), moderate(Coding 4, 5, and 6) and high(Coding 7, 8, and 9) concentrations were arranged in parallel at each level, and two needles were injected in parallel for each sample

Recovery (%) = Actual measurement/ Actual weighing x 100

to environmental friendliness. Due to the toxicity and flammability of methanol, it will produce certain toxic substances, endangering the safety of operators. A certain amount of waste will be produced during the configuration of the solution and the operation of the instrument.

In addition, the BAGI tool is considered to be a supplement to the perfect green indicator, based on ten parameters to describe the applicability and functionality of the analysis method. For the final score, it is recommended to be above 60 points, so that the analysis method is considered to be practical [37]. The final scores of the established folic acid and p-hydroxyethyl ester methods were 77.5 and 82.5, respectively, indicating that the method had advantages in practicality and functionality.

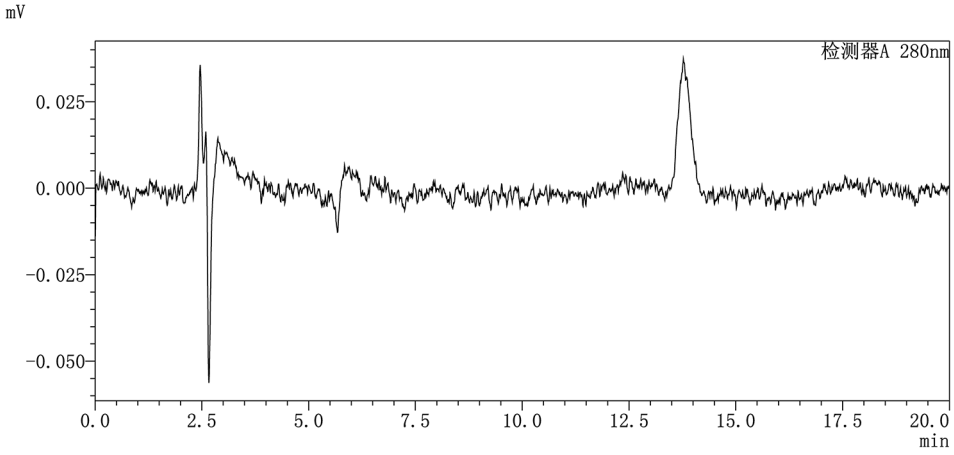

Fig. 8 The LOQ of folic acid

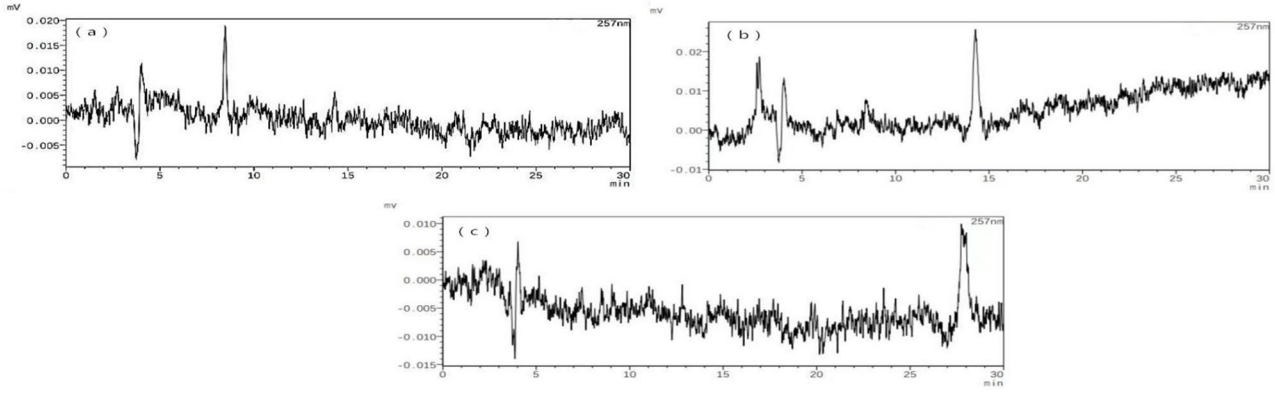

Fig. 9 The LOQ of antibacterial agent. (**a**) The LOQ of methyl paraben; (**b**) The LOQ of ethyl paraben; (**c**) The LOQ of propyl paraben

Future perspectives and study limitations

In this study, the contents of folic acid and antimicrobial agents in folic acid oral liquid were determined. However, the components of commercially available folic acid oral liquid are complex. In addition to folic acid, there were other excipients in folic acid oral liquid. These excipients may interfere with the detection of folic acid and antimicrobial agents. For example, the presence of sugars and flavorings in the oral liquid, or adsorption phenomena in the chromatographic column will affect the separation and detection effects. However, the composition and content of excipients in oral liquids of different brands and formulations are quite different. Therefore, in the later stage, the developed method will be used to determine the content of folic acid and bacteriostatic agent in the commercially available folic acid oral liquid, and the universality and practicability of the method will be further explored in order to explore the general method of folic acid and bacteriostatic agent in folic acid oral liquid.

Conclusions

In this study, we found that the antimicrobial agents were also well determined under the established method for determination of folic acid, but the detection time was long, and propyl paraben did not appear until 150 min later. Changing the ratio of mobile phase can shorten the detection time from 160 min to 30 min, which greatly improves the efficiency of detection. In addition, it was also found that compared with PDA detector, UV detector can accurately detect folic acid and antimicrobial agents of parabens, which may be due to the better sensitivity and response value of UV detector than PDA. In this study, the determination method of folic acid was optimized, and the antibacterial agent was determined in a short time. The verification results showed that the method established in this study could efficiently, accurately and sensitively determine the content of folic acid and antibacterial agent in folic acid oral liquid by changing the wavelength and flow ratio without changing the mobile phase and instrument. Compared with the detection method stipulated in the pharmacopoeia, this method saves the analysis time and solvent consumption,

Table 6 The robustness test determination of folic acid

Folic acid	Retention time		Peak area		Measurement (mg/mL)	Recovery (%)
	1	2	1	2		
Reference substance(23 ℃)	15.252	14.847	6,361,149	6,363,876	1.8848	95.16
Sample(23 ℃)	14.36	14.278	6,060,137	6,048,984		
Reference substance (27 ℃)	12.471	12.515	6,363,861	6,359,493	1.8795	94.89
Sample(27 ℃)	12.616	12.699	6,033,907	6,039,087		
Reference substance(278 nm)	13.856	13.938	6,270,745	6,270,016	1.8803	94.93
Sample (278 nm)	14.042	14.087	5,951,942	5,953,225		
Reference substance (282 nm)	14.125	14.161	6,399,222	6,398,509	1.8813	94.98
Sample (282 nm)	14.125	14.161	6,399,222	6,398,509		
Reference substance (1.0 min/mL)	16.867	7,638,304	7,614,675	7,638,304	1.8806	94.95
Sample(1.0 min/mL)	16.033	15.882	7,240,513	7,241,705		
Reference substance (1.4 min/mL)	11.261	11.226	5,466,639	5,467,894	1.8797	94.90
Sample(1.4 min/mL)	11.195	11.178	5,189,692	5,187,392		
Reference substance (0:100)	15.871	15.852	6,353,276	6,367,619	1.8801	94.92
Sample (0:100)	15.848	15.846	6,040,206	6,034,328		
Reference substance (3:97)	9.921	9.904	6,408,386	6,382,839	1.8753	94.68
Sample (3:97)	9.886	9.876	6,055,843	6,054,912		
Reference substance (pH3.8)	11.494	11.527	6,395,394	6,385,509	1.8786	94.84
Sample (pH3.8)	11.56	11.589	6,061,361	6,060,524		
Reference substance (pH4.2)	14.200	13.918	6,457,083	6,454,290	1.8785	94.84
Sample (pH4.2)	13.798	13.779	6,122,651	6,122,238		
Reference substance (1)	14.679	14.685	6,349,952	6,352,587	1.8842	95.13
Sample (1)	14.683	14.679	6,043,476	6,040,425		
Reference substance (2)	12.036	12.008	6,405,586	6,401,608	1.8793	94.88
Sample (2)	12.028	12.124	6,078,475	6,073,160		

Recovery (%) = Actual measurement/ Actual weighing x 100

Table 7 Folic acid content under different conditions

Conditions		Content (%)	Average (%)	RSD (%)
column temperatures	23℃	95.16	94.93	0.12
	27℃	94.89		
wavelength	278 nm	94.93		
	282 nm	94.98		
flow rates	1.0 mL/min	94.95		
	1.4 mL/min	94.90		
mobile phase ratio	0:100	94.92		
	3:97	94.68		
pH	3.8	94.84		
	4.2	94.84		
chromatographic columns	1	95.13		
	2	94.88		

RSD (%): Relative Standard Deviation = (SD/Mean) x 100

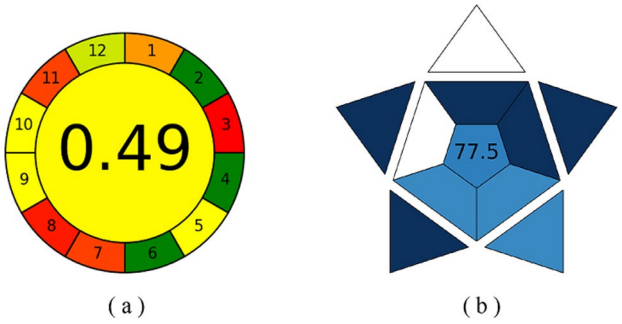

Fig. 10 Evaluation of the greenness profile of the developed folic acid method using AGREE (**a**) and BAGI (**b**) tools

reduces the workload, and has good anti-impurity interference ability. Applying AGREE and BAGI tools for green assessment, it is verified that the developed method has a certain level of greenness and environmental friendliness. In conclusion, the method developed in this work provides a method reference for the formulation of the quality standard of folic acid oral liquid.

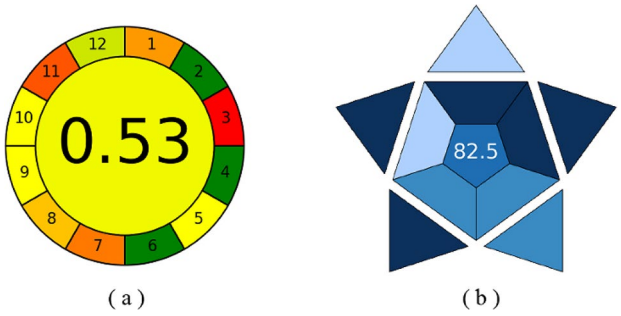

Fig. 11 Evaluation of the greenness profile of the developed antibacterial agent method using AGREE (**a**) and BAGI (**b**) tools

Acknowledgements
Not applicable.

Author contributions
Wenhong Wu is mainly responsible for data collation; Ying Liang is responsible for research ideas, research design and article writing; Renbang Zhao is responsible for the processing data and translation; Yude Shi, Jiahui Hou, and Jiumei Peng are responsible for the confirmation and verification of the follow-up methods of the research, and are gradually preparing for the follow-up related research work. Jiadi Pan is responsible for article writing, the collecting and processing data; Xiaoyi Li and Jingjing Zhou are respectively, responsible for data access.

Funding statement
This work was supported by the National Key R&D Project of China [2016YFD0401100-1]; Hebei Innovation Team of Modern Agro-industry Technology Research System of China [HBCT2018110205, HBCT2018140203]; Folic Acid and Agricultural Products Processing Projects.

Data availability
The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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Received: 29 September 2024 / Accepted: 20 December 2024
Published online: 18 January 2025

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