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Article

# Validation of an Ion-Pair Reverse Phase High-Performance Liquid Chromatography Method for the Detection of Major Components and Related Substances in Diquafosol Sodium Eye Drops

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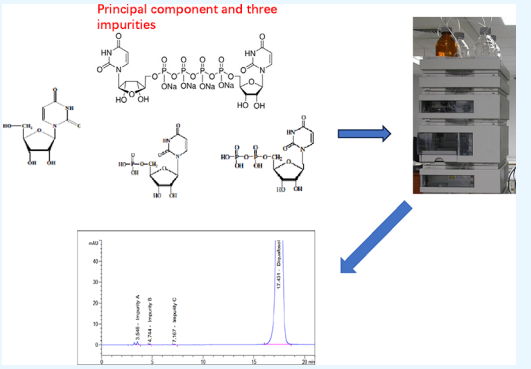
**ABSTRACT:** A simple, feasible, isocratic elution, and stable reversed-phase high performance liquid chromatography method was established and verified. The chromatographic conditions are as follows: EF-C18H, 4.6 × 250 mm, 5 $\mu$m column; column temperature 30 °C; for the mobile phase 27.2 g of $KH_2PO_4$ and 8.5 g of tetrabutylammonium hydrogen sulfate were taken, 2500 mL of water was added to dissolve, and the pH was adjusted to 6.7 with phosphoric acid:methanol solution with a ratio of 84:16 (V:V). The flow rate was 1.0 mL/min; the injection volume was 10 $\mu$L; and the wavelength was 262 nm. According to the current ICH guidelines, the developed method was verified, and the system suitability, specificity, LOD, LOQ, linearity, range, accuracy, repeatability, durability, and solution stability of the proposed method were verified. The validation results demonstrated that the LOQ for the method was 0.05% and the LOD was 0.02%. The content was detected within the concentration range of 300 to 900 $\mu$g/mL. The relationship between concentration and measurement was linear, with an $r^2$ of >0.999. The concentration of impurities ranged from 0.3 to 4.5 $\mu$g/mL. A good linear correlation was observed within the range of g/mL, with a coefficient of determination $r^2$ greater than 0.999. The accuracy and repeatability met the specified criteria.




## 1. INTRODUCTION

Dry eye is a common condition that affects 5−30% of the population worldwide.[1,2] In China, the prevalence of dry eye in ophthalmic outpatients complaining of symptoms of dry eye was 67.9%, of which the prevalence was 62.8% in men and 72.7% in women.[3] Diquafosol (3% Diquas; Santen Pharmaceu tical Co.,Osaka, Japan) is a P2Y2 purinergic receptor agonist that activates P2Y2 receptors on the ocular surface.[4] Since P2Y2 stimulates both fluid secretion from conjunctival epithelial cells and mucus secretion from conjunctival goblet cells, it acts directly on the ocular surface to normalize the tear layer both qualitatively and quantitatively, thereby improving corneoconjunctival epithelial damage.[5−7] Although diquafosol sodium eye drops have been marketed in China, Japan,[8] and Korea for many years, to our knowledge, there is no report on the quantitative determination of diquafosol sodium in its eye drops by chromatography. Therefore, it is necessary to develop a sensitive, accurate, and reliable RP-HPLC method. The objective of this study was to develop and validate a novel, simple, feasible, equal gradient, and stability indication HPLC method based on the established International Conference on Harmonization (ICH) guidelines.[9]

## 2. EXPERIMENTAL SECTION

**2.1. Chemicals and Reagents.** Diquafosol sodium eye drops was provided from Santen Pharmaceutical Co., Ltd. Placebo was provided by Fuan Pharmaceutical Group Ningbo Team Pharmaceutical Co., Ltd. Active pharmaceutical ingredients (API), impurity A, impurity B, and impurity C were bought from Hang Zhou Chemipanda Bio-Tech Co., Ltd. Potassium dihydrogen phosphate, tetrabutylammonium hydrogen sulfate, and potassium hydroxide (AR grade) were purchased from Sinopharm Chemical Reagent Co., Ltd. The HPLC grade methanol was obtained from Tedia Company, Inc. Millipore milli-Q plus water purification system was used for the preparation of high purity water. Figure 1 shows the structures of diquafosol sodium, impurity A, impurity B, and impurity C.

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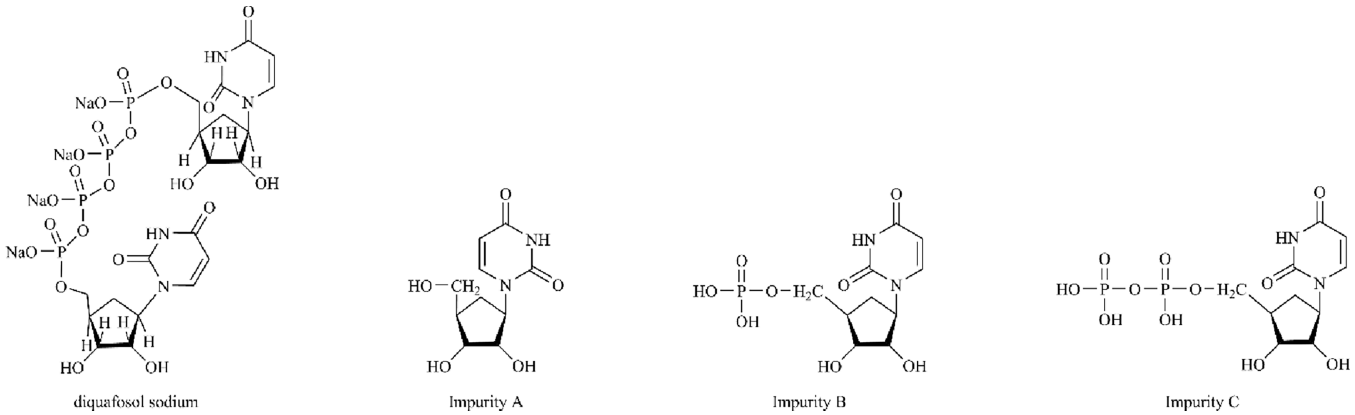


**Figure 1.** Structures of diquafosol sodium, impurity A, impurity B, and impurity C.

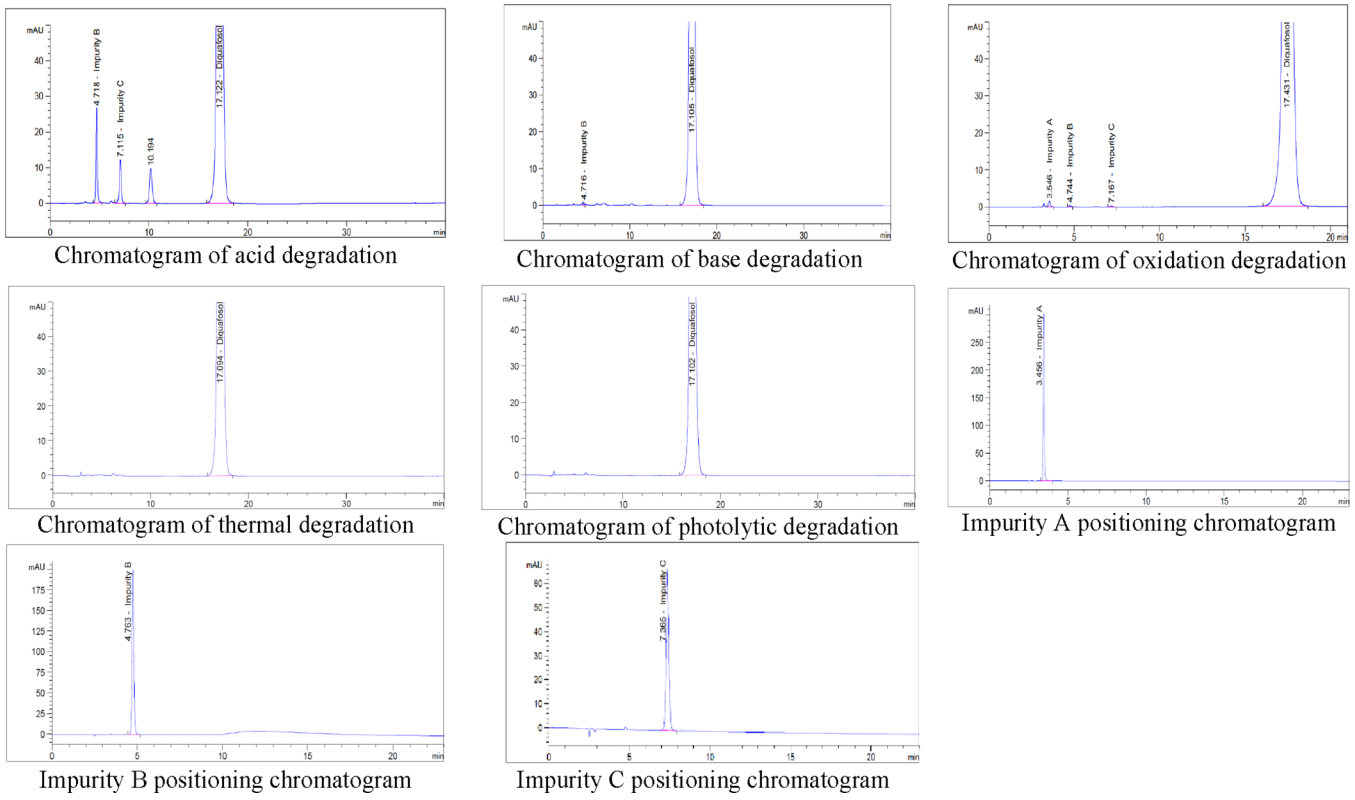


**Figure 2.** Degradation chromatogram and impurity reference locating chromatogra.m

**2.2. Instrumentation.** For the development and validation studies, the following equipment and instruments were used: water bath (HH-2 from Changzhou GuohuaTestpmart Co.,Ltd.); light stabilization chamber (SHH-100GD-2F from Yongsheng Instrument co;ltd); humidity desiccator and thermo hygrometer (CTHI-250B from stik(shanghai) Co.,Ltd.); vacuum oven (DHG-9123A from Shanghai Jing Hong Laboratory Instrument Co., Ltd.). The chromatograpic analysis was performed using an Agilent 1260 separation module with photodiode array detector equipped, degasser, a quaternary pump, and an auto sampler system.

**2.3. Chromatographic Conditions.** The following conditions were used: column: EF-C18H, 4.6 × 250 mm, 5 $\mu$m; column temperature: 30 °C; for the mobile phase, 27.2 g of $KH_2PO_4$ and 8.5 g of tetrabutylammonium hydrogen sulfate, were taken, 2500 mL of water was added to dissolve, and the pH value was adjusted to 6.7 with phosphoric acid:methanol solution with a ratio of 84:16 (V:V).The mobile phase was filtered through 0.45 $\mu$m nylon membrane filter and degassed before use. The flow rate was 1.0 mL/min and injection volume was 10 $\mu$L. The analyte was monitored at wavelength of 262 nm. The diquafosol sodium peak was eluted at about 19 min.

**2.4. Solution Preparation.** *2.4.1. Diluent.* Water was used as diluent.

*2.4.2. Preparation of Standard Solution.* Diquafosol sodium reference standard was dissolved in water with sonication to give a standard solution with a concentration of 600 $\mu$g/mL.

*2.4.3. Preparation of Sample Solution.* Six bottles of diquafosol sodium eye drops were mixed, precisely measuring 2 mL and diluting to 100 mL with diluent, and the solution was mixed uniformly as final concentration of 600 $\mu$g/mL.

*2.4.4. Preparation of Placebo Solution.* Sodium bicarbonate hydrate, sodium chloride, potassium chloride, benzalkonium chloride, dilute hydrochloric acid, and sodium hydroxide were



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**Table 1. System Suitability Test Results**

| system applicability category | parameters | specification | observed values |
|---|---|---|---|
| principal component | area (%RSD, $n = 5$) | ≤2.0% | 0.1 |
| | USP tailing | ≤2.0 | 1.2 |
| | theoretical plates | NLT 5000 | 6511 |
| principal component + impurity limit mixed | area (%RSD, $n = 5$) | ≤5.0% | impurity A: 0.1 |
| | | | impurity B: 3.4 |
| | | | impurity C: 1.4 |
| | USP tailing | ≤2.0 | impurity A: 0.9 |
| | | | impurity B: 0.9 |
| | | | impurity C: 1.0 |
| | theoretical plates | NLT 5000 | impurity A: 9266 |
| | | | impurity B: 8826 |
| | | | impurity C: 8779 |
| | resolution | 1.5 | impurity A: / |
| | | | impurity B: 6.9 |
| | | | impurity C: 9.5 |
| | | | diquafosol sodium: 19.1 |

dissolved in a certain proportion in water to form a placebo solution. Each mL contained 2 mg of sodium bicarbonate hydrate, 4 mg of sodium chloride, 1.4 mg of potassium chloride, and 0.08 mg of benzalkonium chloride as the blank control for the sample solution.

*2.4.5. Preparation of Impurity Reference Solution A.* A total of 3.34 mg of impurity A was weighed into a 50 mL volumetric flask; water was added to dissolve; and the volume was fixed to obtain 66.29 $\mu$g/mL.

*2.4.6. Preparation of Impurity Reference Solution B.* A total of 4.15 mg of impurity B was weighed into a 50 mL volumetric flask; water was added to dissolve; and the volume was fixed to obtain 81.55 $\mu$g/mL.

*2.4.7. Preparation of Impurity Reference Solution C.* A total of 3.81 mg of impurity C was weighed into a 50 mL volumetric flask; water was added to dissolve; and the volume was fixed to obtain 66.99 $\mu$g/mL.

**2.5. Optimization of Chromatographic Column Length.** If other chromatographic conditions were determined, the tailing factor and the number of theoretical plates were checked by replacing two chromatographic columns with different lengths of 100 and 150 mm.

**2.6. Forced Degradation Studies.**[10] One aspect to consider is the utilization of forced degradation testing, which serves to examine and comprehend the inherent stability properties of the drug.[11] This testing method involves subjecting the drug to a range of rigorous conditions in order to investigate its stability, degradation pathway, and resulting degradation products.[12] Another purpose of this testing is to partially validate the specificity of the analytical method employed for detecting related substances, specifically, the degradation products. It is crucial to have a comprehensive understanding of the significance of forced degradation testing in the development of impurity detection methods. This process aids in the design of scientifically sound and rational impurity analysis and detection techniques, which are essential for assuring the safety of clinical medication.[13]

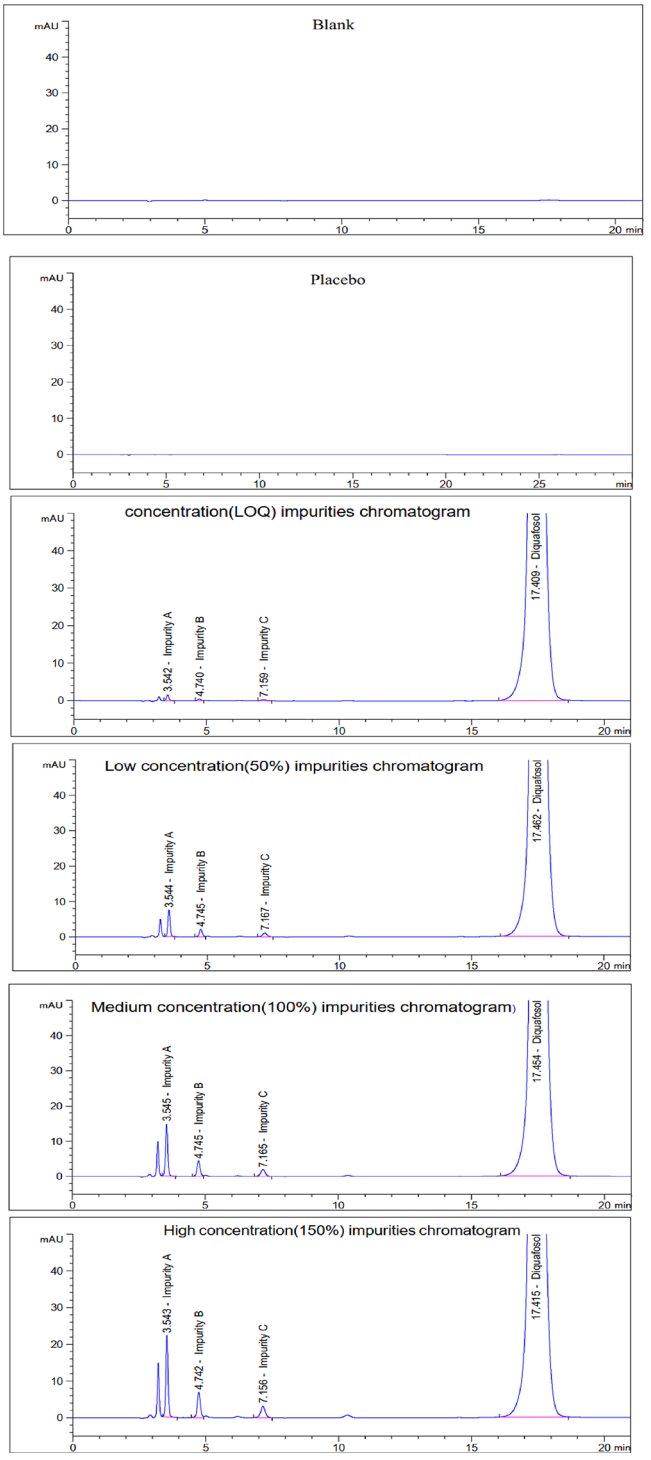


**Figure 3.** Blank solvent, placebo, low impurity, medium, and high concentration solution chromatogram.

*2.6.1. Acid Degradation.* A volume of 1 mL of diquafosol sodium eye drops was measured and subsequently combined with 2 mL of 2 N hydrochloric acid (HCl). The resulting mixture was then transferred into a volumetric flask and placed in an environment with a laboratory temperature. After a duration of 17 h, in order to achieve neutralization of the sample, a volume of 2 mL of 2N NaOH was introduced. This was followed by the addition of water to reach a final volume of 50 mL, resulting in the formation of a solution with a drug concentration of 600 $\mu$g/mL. The solution was well mixed.



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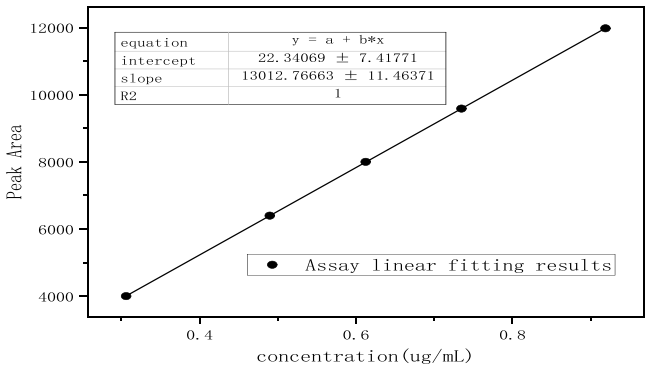


**Figure 4.** Linear results of content.

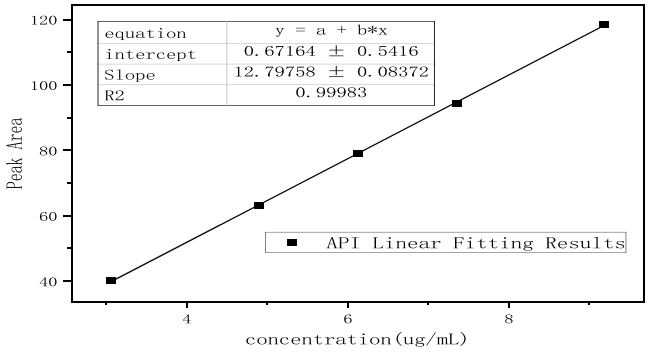


**Figure 5.** Linear results of principal component.

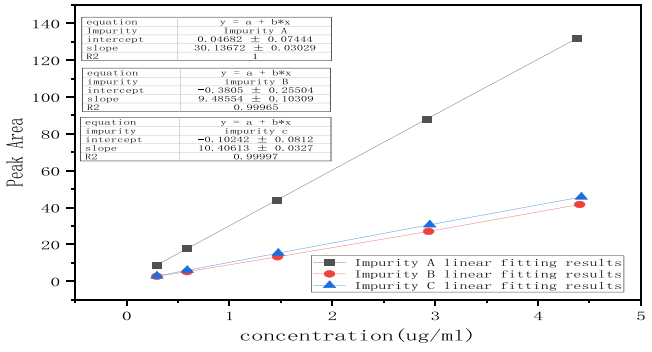


**Figure 6.** Linear results of impurities A, B, and C.

*2.6.2. Alkali Degradation.* A volume of 1 mL of diquafosol sodium eye drops was measured and subsequently combined with 2 mL of a NaOH solution. The resulting mixture was then transferred into a volumetric flask and placed in an environment with laboratory temperature. After a duration of 17 h, in order to achieve neutralization of the sample, 2 mL of 2 N hydrochloric acid (HCl) was used. The volume was then adjusted to 50 mL using water, resulting in the formation of a solution containing 600 $\mu$g/mL of the medication. The solution was thoroughly mixed.

*2.6.3. Oxidation Degradation.* A volume of 1 mL of diquafosol sodium eye drops was measured and combined with 2 mL of a 3% hydrogen peroxide solution. The resulting mixture was then left at room temperature for a duration of 3 h. The volume was afterward adjusted to 50 mL using water, resulting in a solution containing 600 $\mu$g/mL of the medicine. The solution was thoroughly mixed.

*2.6.4. Thermal Degradation.* In order to investigate the impact of heat, diquafosol sodium eye drops and a placebo were subjected to a temperature of 80 °C for a duration of 7 h. This was accomplished by placing them in a volumetric flask within a water bath. Subsequently, the samples and placebo were prepared for analysis using the methods outlined in the sample and placebo preparation section.

*2.6.5. Photolytic Degradation.* One milliliter of diquafosol sodium eye drops was transferred into a 50 mL volumetric flask. The solution was then diluted with water up to the marked scale, thoroughly mixed, and exposed to a cold white fluorescent lamp for a duration of 24 h under controlled conditions of 4500 ± 500 lux.

**2.7. Method Validation.** The verification of the system's applicability, specificity, limit of detection (LOD), limit of quantification (LOQ), linearity, range, accuracy, repeatability, durability, and solution stability of the proposed technique was conducted in accordance with the principles set out by the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH).[14]

*2.7.1. System Suitability.* Impurities A, B, and C were all degradation impurities. The specification of diquafosol sodium eye drops (5 mL: 150 mg) was stated in the manual. The maximum daily dosage of this eye drop is six drops (9 mg). According to ICH Q3B, the daily intake dose is less than 10 mg, and the impurity limit is 1.0%.

*2.7.2. Specificity.* The reference solution of impurities A−C was detected individually in order to precisely determine the location of each contaminant. An experiment was conducted to evaluate the potential impact of the placebo solution on the

**Table 2. Summary of Forced Degradation Results**

| specificity check item | placebo interference | assay | total impurities | mass balance | detected peak | peak area | resolution |
|---|---|---|---|---|---|---|---|
| initial sample | no interference | 99.4% | | 100.0% | diquafosol sodium | 8133.1 | N/A |
| sample after acid degradation | no interference | 91.0% | | | impurity B | 201.9 | N/A |
| | | | 5.9% | 96.8% | impurity C | 140.1 | 8.4 |
| | | | | | impurity RRT0.60 | 126.0 | 7.4 |
| | | | | | diquafosol sodium | 7405.0 | 10.5 |
| sample after alkali degradation | no interference | 99.8% | 0.1% | 99.9% | impurity B | 8.4 | N/A |
| | | | | | diquafosol sodium | 8116.6 | 23.7 |
| sample after oxidation degradation | no interference | 96.9% | | | impurity A | 15.2 | N/A |
| | | | | | impurity B | 6.5 | 6.9 |
| | | | 0.3% | 98.7% | impurity C | 4.9 | 9.5 |
| | | | | | diquafosol sodium | 8000.6 | 18.91 |
| sample after thermal degradation | no interference | 98.5% | 0.0% | 98.5% | diquafosol sodium | 8014.4 | N/A |
| photolytic degradation | no interference | 98.0% | 0.0% | 97.0% | diquafosol sodium | 7889.1 | N/A |



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**Table 3. Results of Assay Recovery Rate**

| standard concentration | 0.6124 mg/mL | | | | | |
|---|---|---|---|---|---|---|
| peak area | 7907.3 | 7904.9 | 7897.1 | 7893.0 | 7897.2 | |
| concentration level (%) | peak area | amount added (mg) | recovery (mg) | recovery rate (%) | mean | % RSD |
| 50% level | 3867.4 | 15.00 | 14.99 | 99.9 | 100.1 | 0.2 |
| | 3883.8 | 15.00 | 15.03 | 100.4 | | |
| | 3870.7 | 15.00 | 15.00 | 100.0 | | |
| 100% level | 7755.8 | 30.00 | 30.06 | 100.2 | 100.0 | 0.1 |
| | 7737.4 | 30.00 | 29.99 | 100.0 | | |
| | 7736.0 | 30.00 | 29.98 | 99.9 | | |
| 150% level | 11638.9 | 45.00 | 45.11 | 100.2 | 100.6 | 0.6 |
| | 11655.5 | 45.00 | 45.18 | 100.4 | | |
| | 11756.9 | 45.00 | 45.57 | 101.3 | | |
| | 129.5 | 44.74 | 44.13 | 98.6 | | |
| | 129.3 | 44.74 | 44.06 | 98.5 | | |
| | 56.1 | 44.74 | 48.48 | 108.3 | | |
| | 56.7 | 44.74 | 49.00 | 109.5 | | |
| | 38.1 | 44.74 | 42.38 | 94.7 | | |
| | 37.9 | 44.74 | 42.16 | 94.2 | | |

principal components by comparing it to a sample solution. Furthermore, approximately 0.5% concentration of established associated compounds (referred to as impurities A, B, and C) was incorporated into the sample solution for the purpose of detection. This addition serves to assess the distinction between impurities and primary peaks, evaluate the separation between impurities, and examine the potential interference of the placebo on the impurities. The approach demonstrates a high level of specificity.

*2.7.3. LOD and LOQ.* Transfered appropriate amount of the reference solution of diquafosol sodium and the reference solution of impurities A, B, and C was transferred into a volumetric bottle and the solution was prepared to be tested with 0.3 $\mu$g of diquafosol sodium and 0.3 $\mu$g of impurities A, B, and C per mL. The samples were injected until the signal-to-noise ratio (S/N) was no less than 10 (LOQ) and 3 (LOD). The limit of quantitation solution was continuously injected for 6 times, and the %RSD of retention time and peak area was calculated.

*2.7.4. Linearity.* Content detection linearity: took 1 mL of diquafosol sodium stock solution to a 10 mL volumetric flask and prepared it into a 100% (600ug/mL of diquafosol sodium) level of assay linear solution to be tested, and then an appropriate amount of diquafosol sodium sample stock solution was taken and prepared into 50, 80, 120, and 150% levels of linear solution to be tested.

Principal component: 1 mL of the diquafosol sodium standard solution was transferred to a 10 mL volumetric flask and diluted with diluent; then, 1 mL of this diluent was transferred to a 10 mL volumetric flask to prepare a 100% level linear test solution of the main component and then 50, 80, 120, and 150% water level linear test solutions of the main component.

Impurity linearity: 2.3 mL of standard solution of impurity A, 1.8 mL of standard solution of impurity B, and 2.0 mL of standard solution of impurity C were transferred into a 25 mL volumetric flask to prepare a 100% impurity limit solution (3 $\mu$g/mL), according to which 10, 20, 50, and 150% impurity linear solutions to be tested are prepared.

The calibration curve was drawn between the concentration of the analyte and the peak area. Origin was used to calculate the slope, intercept, and correlation coefficient.

*2.7.5. Accuracy.* The user obtained an adequate volume of the sample solution and thereafter prepared it to achieve concentrations of 50, 100 (600 $\mu$g/mL), and 150%. Each concentration was subjected to three parallel tests. The recovery rate and relative standard deviation (%RSD) of the given parameter were determined. The necessary volumes of impurities A, B, and C reference solutions were transferred into a volumetric flask. Subsequently, a 100% level solution was generated for testing, including 3 $\mu$g/mL of impurities A, B, and C. Additionally, 50 and 150% level solutions were prepared using the same procedure for testing purposes. The recovery rate and relative standard deviation (%RSD) were computed.

*2.7.6. Repeatability.* In order to ensure the reproducibility of the experimental results, it was recommended that six aliquots of the standard solution be utilized for testing purposes. The standard solution should include a concentration of 600 $\mu$g/mL of diquafosol sodium. The relative standard deviation (%RSD) of the recovery rate was determined for a set of six sections. To assess the repeatability of impurities, a total of six needles containing impurity mixes with a given concentration (3 $\mu$g/mL impurity A, 3 $\mu$g/mL impurity B, 3 $\mu$g/mL impurity C) were injected. Subsequently, the percent relative standard deviation (%RSD) was computed for the recovery rates obtained from these six injections.

*2.7.7. Robustness.* The study aimed to assess the robustness of the approach by modifying the instrumental and chromatographic parameters, including the detection wavelength (+2 nm), column temperature (+5 nm), the ratio of buffer salt to methanol in the mobile phase, and the flow rate (+10%).

*2.7.8. Sample and Standard Solution Stability.* The diquafosol sodium standard solution should be prepared and afterward stored at room temperature for a duration of 6 h. The mixed solution of impurity reference substance should be prepared and afterward stored at room temperature for specific time intervals, namely, 0, 4, and 14 h. Preliminary analysis and comparative study of several time intervals were conducted for the two proposed options. The ratio between the peak area at 0 h and the peak area relative standard deviation (%RSD) was determined.



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**Table 4. Results of Impurity Recovery Rate**

| impurity | | impurity A | | | impurity B | | | impurity C | | |
|---|---|---|---|---|---|---|---|---|---|---|
| standard concentration (mg/mL) | | 2.983 | | | 2.365 | | | 3.014 | | |
| peak area | | 86.3 | 88.4 | 87.9 | 27.5 | 27.4 | 27.2 | 27.1 | 27.1 | 27.1 |
| recovery rate of impurity A | | | | | | | | | | |
| concentration level (%) | peak area | amount added (mg) | | recovery (mg) | | recovery rate (%) | | mean | | % RSD |
| LOQ | 8.9 | 2.98 | | 3.03 | | 101.7 | | 101.7 | | 0.0 |
| | 8.9 | 2.98 | | 3.03 | | 101.7 | | | | |
| | 8.9 | 2.98 | | 3.03 | | 101.7 | | | | |
| 50% level | 43.6 | 14.86 | | 14.91 | | 99.6 | | 99.5 | | 0.1 |
| | 43.6 | 14.86 | | 14.91 | | 99.6 | | | | |
| | 43.5 | 14.82 | | 14.91 | | 99.4 | | | | |
| 100% level | 86.3 | 29.83 | | 29.41 | | 98.6 | | 98.6 | | 0.1 |
| | 86.3 | 29.83 | | 29.41 | | 98.6 | | | | |
| | 86.4 | 29.83 | | 29.44 | | 98.7 | | | | |
| 150% level | 129.3 | 44.74 | | 44.06 | | 98.5 | | 98.5 | | 0.1 |
| | 129.5 | 44.74 | | 44.13 | | 98.6 | | | | |
| | 129.3 | 44.74 | | 44.06 | | 98.5 | | | | |
| recovery rate of impurity B | | | | | | | | | | |
| concentration level (%) | peak area | amount added (mg) | | recovery (mg) | | recovery rate (%) | | mean | | % RSD |
| LOQ | 3.3 | 2.98 | | 2.85 | | 95.6 | | 97.5 | | 3.4 |
| | 3.3 | 2.98 | | 2.85 | | 95.6 | | | | |
| | 3.5 | 2.98 | | 3.02 | | 101.4 | | | | |
| 50% level | 17.0 | 14.91 | | 14.69 | | 98.5 | | 100.8 | | 2.3 |
| | 17.4 | 14.91 | | 15.04 | | 100.8 | | | | |
| | 17.8 | 14.91 | | 15.38 | | 103.1 | | | | |
| 100% level | 35.3 | 29.83 | | 30.50 | | 102.3 | | 104.5 | | 2.2 |
| | 36.0 | 29.83 | | 31.11 | | 104.3 | | | | |
| | 36.9 | 29.83 | | 31.89 | | 106.9 | | | | |
| 150% level | 55.0 | 44.74 | | 47.53 | | 106.2 | | 108.0 | | 1.5 |
| | 56.1 | 44.74 | | 48.48 | | 108.3 | | | | |
| | 56.7 | 44.74 | | 49.00 | | 109.5 | | | | |
| recovery rate of impurity C | | | | | | | | | | |
| concentration level (%) | peak area | amount added (mg) | | recovery (mg) | | recovery rate (%) | | mean | | % RSD |
| LOQ | 3.1 | 2.98 | | 3.45 | | 115.6 | | 114.4 | | 1.9 |
| | 3.0 | 2.98 | | 3.34 | | 111.9 | | | | |
| | 3.1 | 2.98 | | 3.45 | | 115.6 | | | | |
| 50% level | 13.5 | 14.91 | | 15.02 | | 100.7 | | 98.9 | | 2.4 |
| | 13.4 | 14.91 | | 14.91 | | 99.9 | | | | |
| | 12.9 | 14.91 | | 14.35 | | 96.2 | | | | |
| 100% level | 23.8 | 29.83 | | 26.47 | | 88.8 | | 88.9 | | 0.6 |
| | 24.0 | 29.83 | | 26.70 | | 89.5 | | | | |
| | 23.7 | 29.83 | | 26.36 | | 88.4 | | | | |
| 150% level | 38.1 | 44.74 | | 42.38 | | 94.7 | | 94.6 | | 0.3 |
| | 38.1 | 44.74 | | 42.38 | | 94.7 | | | | |
| | 37.9 | 44.74 | | 42.16 | | 94.2 | | | | |

## 3. RESULT

**3.1. Method Optimization Results.**[15] After replacing the chromatographic column, it was found that when the 250 mm chromatographic column was used for detection, its chromatographic peak pattern was good, the number of theoretical plates was more than 5000, the resolution was higher than the detection results of the other two chromatographic columns, and the tailing factor was less than 1.5. Therefore, the length of the chromatographic column was determined as 250 mm.

**3.2. Forced Degradation Behavior.** Figure 2 contains a chromatogram of the impurity localization solution and the forced degradation of the sample solution.

*3.2.1. Acid Degradation.* Under the condition of adding 2 mL of 2 mol/L HCl for 17 h, the sample was unstable, with a degradation rate of 9%, and impurities A, B, and unknown impurities were generated.

*3.2.2. Base Degradation.* The sample was stable and hardly degradable after being placed for 17 h under the condition of adding 2 mL of 2 mol/L NaOH, and only one impurity was generated.

*3.2.3. Thermal Degradation.* It can be seen that the sample solution has no impurity degradation at 80 °C for 7 h, and the sample was relatively stable

*3.2.4. Oxidation Degradation.* Under the condition of adding 2 mL of 2 mol/L H2O2 for 3 h, the degradation rate of the sample was 1.6%, and impurities A, B, and C were generated.

*3.2.5. Photocatalytic Degradation.* The sample was stable under the condition of 4500 ± 500 lx for 24 h.



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**Table 5. Repeatability of Principal Component Content**

| concentration of reference standard solution | 0.6124 mg/mL | | | | |
|---|---|---|---|---|---|
| peak area | 7907.3 | 7904.9 | 7897.1 | 7893.0 | 7897.2 |
| number | peak area | amount added (mg) | recovery (mg) | recovery rate (%) | %RSD |
| 1 | 7755.8 | 30.00 | 30.06 | 100.2 | 0.14 |
| 2 | 7737.4 | 30.00 | 29.99 | 100.0 | |
| 3 | 7736.0 | 30.00 | 29.98 | 99.9 | |
| 4 | 7731.2 | 30.00 | 29.97 | 99.9 | |
| 5 | 7727.3 | 30.00 | 29.95 | 99.8 | |
| 6 | 7724.75 | 30.00 | 29.94 | 99.8 | |

*3.2.6. Material Balance.* The material balance was between 95 and 105%.

**3.3. Method Validation Results.** *3.3.1. System Suitability.* Table 1 shows the system suitability test results. Used the HPLC workstation[15] to calculate the tailing factor of each chromatographic peak and the number of theoretical plates. The system suitability data was within the acceptable criteria (acceptance criteria: the tailing factor and the number of theoretical plates of the main peak and the chromatographic peaks of impurities A, B, and C of diquafosol sodium were not more than 1.5 and not less than 5000, respectively; the resolution of each impurity in the impurity limit solution was not less than 1.5; the %RSD of the main peak area for five consecutive injections was not more than 2.0; the %RSD of the impurity peak area was not more than 5.0). The HPLC system was applicable to analysis. Meanwhile, Figure 3 contains chromatograms of blank solution, placebo, and impurities of different concentrations. Through comparison, it can be found that both blank and placebo had no interference with impurities

**Table 7. Quantitative Limit of Principal Components**

| number | peak area (mAu s) | RT (min) | s/n of LOQ |
|---|---|---|---|
| 1 | 4.31996 | 17.083 | 19 |
| 2 | 4.21324 | 17.031 | 16 |
| 3 | 3.73521 | 17.082 | 12 |
| 4 | 4.19765 | 17.065 | 10 |
| 5 | 3.80305 | 17.082 | 20 |
| 6 | 3.71694 | 17.043 | 12 |
| % RSD | 6.9% | 0.1% | N/A |
| s/n of LOD | 3.7 | | |

*3.3.2. Specificity.* As shown in Table 2 and Figure 2, from the forced degradation data, it can be seen that the placebo has no interference with the main peak and impurity peak in sample solution; under all conditions, the resolution of impurities and main peaks was above 1.5, meeting the acceptance criteria.

*3.3.3. Linearity.* Figure 3 shows a blank solvent, placebo, low impurity, medium, and high concentration solution chromatogram. Figures 4−6 show that the assay, main components, and related substances are linear within the limited range. Draw the calibration curve between analyte concentration and peak area. Origin[16] was used to calculate the slope, intercept, and correlation coefficient (acceptance criteria: for the linearity of assay, the $r^2$ was generally required to be ≥0.998, and the intercept was within 2% of the response value at 100% of the limit; for the linearity of impurities, the $r^2$ was generally required to be ≥0.990, and the intercept was within 5% of the response value at 100% of the limit). All of the results met the criteria.

*3.3.4. Accuracy.* The results in Table 3 show that the recovery rates of samples at 50, 100, and 150% concentration levels met

**Table 6. Repeatability of Impurities A, B, and C**

| impurity | impurity A | | | impurity B | | | impurity C | | |
|---|---|---|---|---|---|---|---|---|---|
| concentration of reference standard solution (mg/mL) | 2.983 | | | 2.365 | | | 3.014 | | |
| peak area | 86.3 | 88.4 | 87.9 | 27.5 | 27.4 | 27.2 | 27.1 | 27.1 | 27.1 |
| recovery rate of impurity A | | | | | | | | | |
| number | peak area | amount added (mg) | | recovery (mg) | | recovery rate (%) | | %RSD | |
| 1 | 86.3 | 29.83 | | 29.41 | | 98.6 | | 0.05 | |
| 2 | 86.3 | 29.83 | | 29.41 | | 98.6 | | | |
| 3 | 86.4 | 29.83 | | 29.44 | | 98.7 | | | |
| 4 | 86.3 | 29.83 | | 29.41 | | 98.6 | | | |
| 5 | 86.3 | 29.83 | | 29.41 | | 98.6 | | | |
| 6 | 86.3 | 29.83 | | 29.41 | | 98.6 | | | |
| recovery rate of impurity B | | | | | | | | | |
| 1 | 35.3 | 29.83 | | 30.50 | | 102.3 | | 4.01 | |
| 2 | 36.0 | 29.83 | | 31.11 | | 104.3 | | | |
| 3 | 36.9 | 29.83 | | 31.89 | | 106.9 | | | |
| 4 | 37.6 | 29.83 | | 32.49 | | 108.9 | | | |
| 5 | 38.4 | 29.83 | | 33.18 | | 111.2 | | | |
| 6 | 39.3 | 29.83 | | 33.96 | | 113.9 | | | |
| recovery rate of impurity C | | | | | | | | | |
| 1 | 23.8 | 29.83 | | 26.47 | | 88.8 | | 1.40 | |
| 2 | 24.0 | 29.83 | | 26.70 | | 89.5 | | | |
| 3 | 23.7 | 29.83 | | 26.36 | | 88.4 | | | |
| 4 | 23.6 | 29.83 | | 26.25 | | 88.0 | | | |
| 5 | 23.1 | 29.83 | | 25.69 | | 86.1 | | | |
| 6 | 23.3 | 29.83 | | 25.92 | | 86.9 | | | |



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**Table 8. Validation Results of Impurity Limit of Quantitation and Detection Limit**

| LOQ results of impurity A | | | |
|---|---|---|---|
| number | peak area (mAU s) | RT (min) | s/n of LOQ |
| 1 | 8.75166 | 3.530 | 77.1 |
| 2 | 8.76137 | 3.530 | 116.1 |
| 3 | 8.78380 | 3.531 | 130.2 |
| 4 | 8.80032 | 3.529 | 127.9 |
| 5 | 8.77847 | 3.528 | 159.5 |
| 6 | 8.68619 | 3.528 | 96.2 |
| % RSD | 0.5% | 0.0% | N/A |
| s/n of LOD | 48.5 | | |
| LOQ results of impurity B | | | |
| 1 | 2.65865 | 4.760 | 17.8 |
| 2 | 2.66643 | 4.758 | 27.1 |
| 3 | 2.73035 | 4.758 | 30.4 |
| 4 | 2.66844 | 4.758 | 29.6 |
| 5 | 2.65011 | 4.756 | 36.8 |
| 6 | 2.64346 | 4.755 | 22.2 |
| % RSD | 1.2% | 0.0% | N/A |
| s/n of LOD | 11.5 | | |
| LOQ results of impurity C | | | |
| 1 | 2.88091 | 7.251 | 13.8 |
| 2 | 2.91819 | 7.257 | 20.4 |
| 3 | 2.91601 | 7.251 | 23.2 |
| 4 | 3.04852 | 7.243 | 23.1 |
| 5 | 2.95998 | 7.247 | 28.6 |
| 6 | 2.86585 | 7.243 | 17.1 |
| % RSD | 2.3% | 0.1% | N/A |
| s/n of LOD | 8.8 | | |

the criteria (acceptance criteria: the recovery rate was between 98.0 and 102.0%, and the %RSD was not greater than 2.0). The results in Table 4 show that the recovery rates of LOQ, 50, 100, and 150% concentration levels met the criteria (aAcceptance criteria: the recovery rate was 80−120%, and the %RSD of the recovery rate was not more than 15.0).

*3.3.5. Repeatability.* From the results in Tables 5 and 6, it can be seen that the repeatability of principal component and impurities met the criteria (acceptance criteria: recovery rate of content %RSD ≤ 2.0; recovery rate of single impurity %RSD ≤ 10.0).

*3.3.6. LOD and LOQ.* It can be seen from the results in Tables 7 and 8 that the results of LOQ and LOD met the acceptance criteria (LOQ acceptance criteria: the %RSD of retention time shall not be greater than 2.0, the peak area %RSD shall not be greater than 10.0, and the s/n shall be greater than or equal to 10; LOD: the s/n shall not be less than 3).

*3.3.7. Robustness.* Table 9 shows that under different chromatographic conditions (flow rate, column temperature, wavelength, and buffer salt ratio), the theoretical plate number of standard solution was greater than 5000, the resolution of main peak and impurity was greater than 1.5, the resolution between impurities was greater than 1.5, and the symmetry factor was between 0.8 and 1.5. The method was relatively stable under certain chromatographic conditions, and its durability meets the requirements.

*3.3.8. Solution Stability.* Table 10 results showed that after the impurity solution was placed for 4 and 14 h, its peak area was 90−110% compared with 0 h, which proved that the impurities A, B, and C reference solution was stable within 14 h, and the main component solution was stable within 6 h.

## 4. DISCUSSION

The HPLC method for optimal conditions developed in the study was validated with system suitability as required, and the sample was found to be unstable and more violently degraded under acidic and oxidizing conditions by forced degration experiments. There was no interference from placebo in the specificity validation, and impurities achieved good separation, all above 1.5. The LOQ of the method was 0.05%, and the LOD was 0.02%. The content detection was linear in the concentration range of 300−900 $\mu$g/mL and the peak area with $r^2 > 0.999$. The concentration of impurities was linear in the range of 0.3−4.5 $\mu$g/mL and the peak area with $r^2 > 0.999$. The repeatability results were good, and the %RSD met the requirements. The sample solution to be tested was stable within 14 h at room temperature. Therefore, this RP-HPLC method is suitable for the determination of diquafosol sodium and its impurities.

## 5. CONCLUSIONS

This work presents the establishment of a novel reversed-phase high-performance liquid chromatography (HPLC) approach that is characterized by its accuracy, precision, linearity, strong specificity, and exceptional durability. The method was developed specifically for the measurement of both the main components and the impurities of diquafosol sodium. The approach should be evaluated in accordance with the rules established by the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH). The findings of the method validation are deemed satisfactory. The reduced detection time can lead to cost savings and yields certain economic advantages.

**Table 9. Durability Results**

| | observed system suitability parameters (impurity A/impurity B/impurity C/principal component) | | |
|---|---|---|---|
| variation in chromatographic condition | symmetry factor (0.8−1.5) | theoretical plates >2500 | resolution >1.5 |
| column temperature 25 °C | 0.9/1.0/1.0/0.9 | 8132/7427/6834/6982 | N/A/6.0/8.8/17.6 |
| column temperature 35 °C | 0.9/0.8/0.9/0.8 | 8517/8102/8798/7737 | N/A/6.8/9.0/18.3 |
| flow rate (0.90 mL/min) | 0.9/0.9/1.1/0.8 | 9871/9097/9035/8348 | N/A/7.0/9.6/19.2 |
| flow rate (1.10 mL/min) | 0.9/0.9/1.1/0.8 | 8507/7857/7792/7395 | N/A/6.5/9.0/18.1 |
| buffer: acetonitrile (80:20) | 0.9/0.8/1.0/1.0 | 7808/7350/7213/7104 | N/A/5.5/6.2/12.7 |
| buffer: acetonitrile (90:10) | 1.0/10/1.1/10.9 | 6432/6605/8199/6616 | N/A/6.4/13.1/24.7 |
| wavelength 260 nm | 0.9/0.9/1.1/0.8 | 8375/7938/7635/7239 | N/A/6.5/8.8/17.8 |
| wavelength 264 nm | 0.9/0.9/1.1/0.8 | 8158/7717/7753/7027 | N/A/6.4/8.8/17.6 |



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Table 10. Solution Stability Results

| impurity | 0 h peak area (mAu s) | 4h peak area (mAu s) | 14h peak area (mAu s) |
|---|---|---|---|
| impurity A | 90.6979 (100.00%) | 91.1351 (100.48%) | 91.1886 (100.54%) |
| impurity B | 30.1990 (100.00%) | 30.7354 (101.78%) | 31.3355 (103.76%) |
| impurity C | 31.4542 (100.00%) | 31.6461 (100.61%) | 30.8775 (98.17%) |
| principal component | 0 h peak area (mAu s) | 6h peak area (mAu s) | |
| diquafosol sodium | 7986.34 | 8002.94 (100.21%) | |

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### Notes

The authors declare no competing financial interest.

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