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# Development and validation of a stability-indicating RP-HPLC method for the determination of fifteen impurities in rivaroxaban

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ABSTRACT

A simple and stability-indicating reverse phase high-performance liquid chromatographic (RP-HPLC) method for the determination of rivaroxaban (RIX) and its related substances was developed. Fifteen impurities of RIX, including three unreported isomers, were identified, synthesized, purified, and confirmed using MS, $^{1}$H NMR, $^{13}$C NMR, and HSQC spectral methods. This new method offered baseline separation for all monitored impurities, and was fast and reliable when compared to the European Pharmacopoeia method. Optimum separation for RIX and its related impurities was achieved on an octyldecyl silica column (YMC Core C18, 4.6 ×100 mm, 2.7 μm) by using a gradient HPLC method in 38 min. The final method was validated with respect to precision, LOD and LOQ, linearity, accuracy, and robustness. This developed method was suitable for routine quality control and drug analysis of RIX active substance.

## 1. Introduction

Anticoagulants are commonly used to prevent blood clots or to stop existing clots from growing larger, which can cause heart attacks or strokes by blocking blood flow to the heart or brain. Rivaroxaban (RIX) has been approved by the US FDA and the European Commission as an oral anticoagulant for preventing venous thromboembolism in adult patients following total hip replacement or total knee replacement surgery [1,2]. As a critical component, quality control usually encounters challenges in the pharmaceutical industry, as it did in the case of us exploring appropriate analytical methods to ensure the quality of RIX during the process development stage. Among them, high-performance liquid chromatography (HPLC), known for its sensitivity, reliability, and widespread use, is generally regarded as an ideal technique for ensuring the quality and consistency of both active pharmaceutical ingredients (APIs) and finished dosage forms.

Numerous HPLC-UV methods have been documented in the literature for detecting RIX API and its finished dosage forms, notably for tablets [3–8]. Additionally, "high-performance thin-layer chromatography (HPTLC)" has been used for testing RIX in tablet formulations [9–12]. Furthermore, some techniques have been proposed for analyzing RIX in plasma [13,14]. Among these methods, the most widely accepted approach is included in the 10th edition of the European Pharmacopoeia (Ph. Eur. 10th) [15]. During our study, in addition to the eight impurities (Imp B, D, E, F, G, H, I, and J) known and listed in the RIX monograph, we successfully discovered and synthesized seven new impurities, consisting of precursors, degradants [8], and positional isomers. Upon a re-evaluation of the EP method, it was found that the HPLC method outlined in the monograph was not capable of accurately quantifying all fifteen impurities that might be present in RIX and potentially affect the quality of the API. This deficiency is most likely due to the similar physicochemical properties shared by these impurities. Furthermore, it is necessary to enhance the robustness of the current monograph method to accommodate variations in parameters or equipment, without compromising the quality of the analytical results [16]. Therefore, the development of a selective, efficient, and robust analytical method is still highly desirable to achieve better quality control of RIX.

In this study, fifteen impurities of RIX were identified, synthesized, purified, and confirmed using MS, $^{1}$H NMR, $^{13}$C NMR, and HSQC spectral methods. The chemical structures of the impurities are displayed in Fig. 1, and the partition coefficients (log P) and dissociation constants (pKa) were estimated by ACD/Labs and are presented in Table 1. Except for the eight Pharmacopoeia impurities, Imp M and Q were identified as

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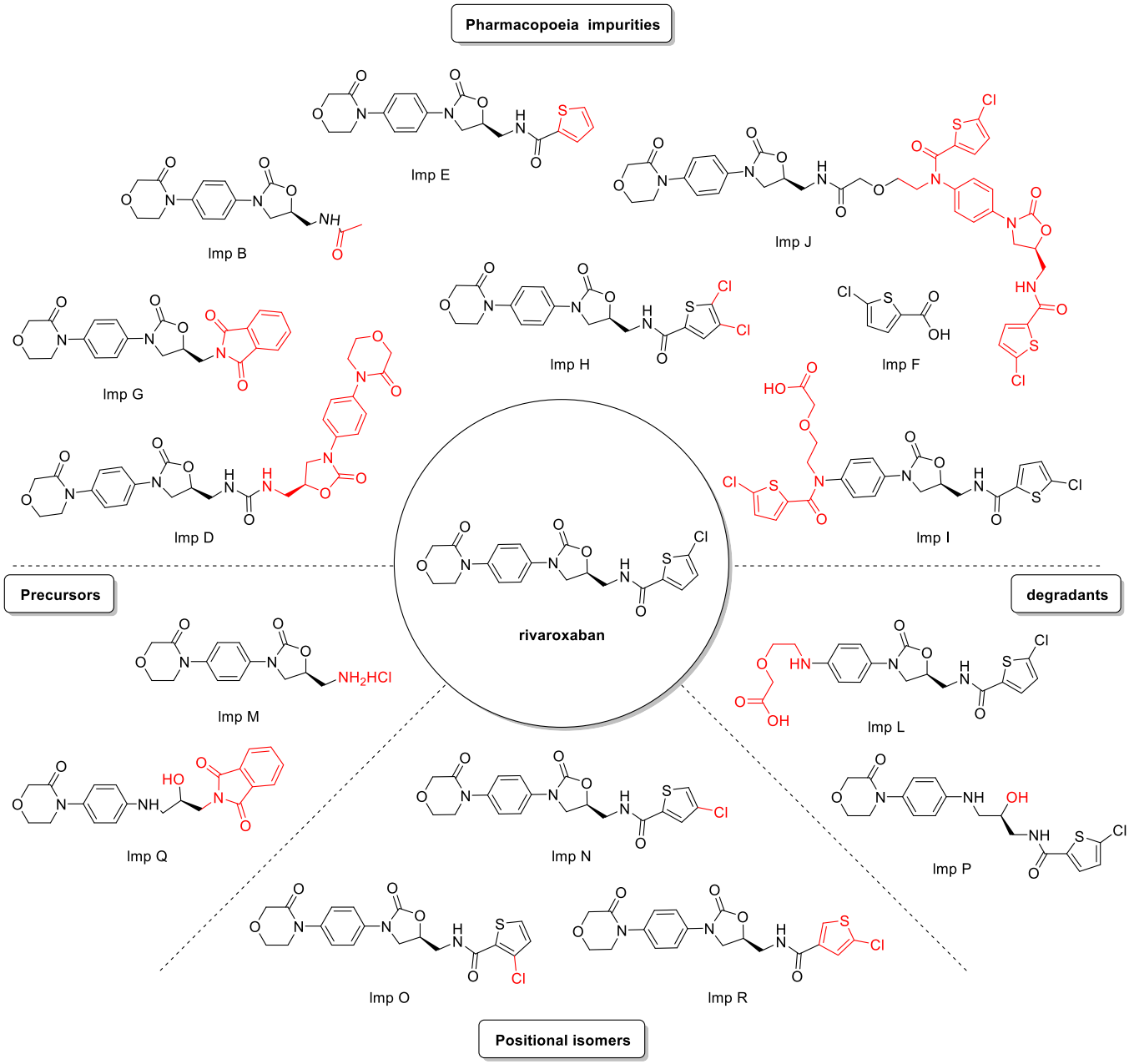


**Fig. 1.** The chemical structures of the analytes.

**Table 1**
The estimated log P and pKa of the fifteen analytes.

| | log P | pKa |
|---|---|---|
| Imp B | -0.8 | - |
| Imp D | -0.52 | - |
| Imp E | 0.95 | - |
| Imp F | 2.63 | 3.18 |
| Imp G | 1.8 | - |
| Imp H | 2.62 | - |
| Imp I | 3.47 | 3.39 |
| Imp J | 3.47 | - |
| Imp L | 1.76 | 4.58, 3.25 |
| Imp M | -0.42 | 8.96 |
| Imp N | 1.85 | - |
| Imp O | 1.10 | - |
| Imp P | 1.22 | 3.9 |
| Imp Q | 1.05 | 3.88 |
| Imp R | 1.11 | - |

precursors of RIX, while Imp P and L were identified as degradation products, observed in forced degradation studies under base condition [8]. Imp N, O, and R are positional isomers of RIX that have not been reported in the current literature and were found to originate from the isomeric starting materials. After identifying and preparing all the impurities in this study, a new RP-HPLC method was developed and validated for RIX in accordance with ICH principles [17]. The method provides better solutions to ensure drug quality and patient safety.

## 2. Experimental

### 2.1. Reagents and materials

HPLC purity water was prepared by Millipore Milli-Q system (Millipore synergy, France). The AR grade potassium dihydrogen phosphate (with purity ≥ 99.5%) used for the preparation of buffer solution was purchased from Xilong Scientific Co., Ltd. (Sichuan, China), and the

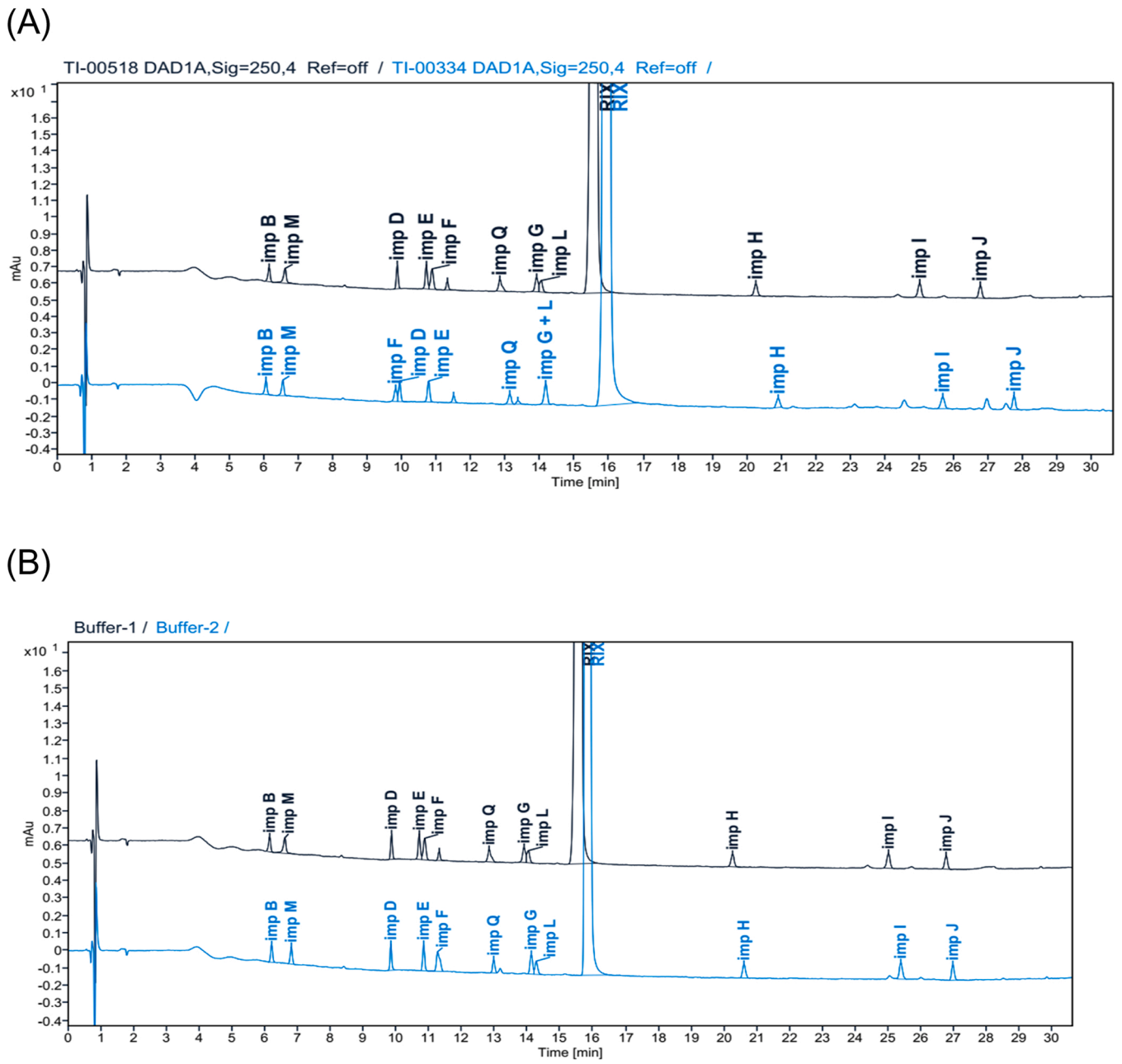


**Fig. 2.** A) Typical chromatogram for 10th EP method on different apparatuses using the same mobile phase; B) Typical chromatogram for 10th EP method on mobile phases prepared by different operators.

HPLC grade phosphoric acid (with purity ≥ 85%) used for pH adjustment of the buffer solutions was ordered from Aladdin (Shanghai, China). Gradient grade acetonitrile (ACN) was purchased from Futun Science and Technology Co., Ltd (Hubei, China). Methyl Sulphoxide-D6 (DMSO-$D_6$) used for the NMR was purchased from Beijing InnoChem Science & Technology Co., Ltd. (Beijing, China). 5-chlorothiophene-2-carboxylic acid (Imp F, 99.6%) was purchased from Zhejiang Regen Chemical Co., Ltd. (Zhejiang, China). Related substances (Imp B, D, E, G, H, I, J, O, P, Q, L, M, N, and R) and three batches of RIX bulk drug samples (Lot: LFS-201705101, LFS-201706101, LFS-201706102) were provided by HEC Research and Development Center (Guangdong, China). Imp B, D, E, G, H, I, J, O, Q, M, N, and R were synthesized and purified by recrystallization or chromatography. Imp P and L were isolated from forced degradation samples via chromatography (Imp P) or recrystallization (Imp L). All buffer solutions were filtered with 0.22 μm pore size membrane filters, which were purchased from Tianjin Jinteng Experimental Equipment Co., Ltd (Tianjin, China) with the aid of a vacuum pump, and then degassed in an ultrasonic bath.

### 2.2. Equipment and software

The analytical balance and pH meter used were from Mettler-Toledo (Greifensee, Switzerland), while the ultrasonic bath and vacuum pump were from ILMVAC GmbH (Ilmenau, Germany). ACD/Labs software (Ontario, Canada) was used for estimating the physical-chemical parameters, and HPLC experiments were conducted on an Agilent 1260 chromatographic system with Open LAB CDS software (Santa Clara, California, USA) for data acquisition. 1 H NMR, 13 C NMR, and HSQC were performed on a Bruker AVANCE III HD 600 (Billerica, Germany), while molecular mass measurements were conducted on a 6530 Q-TOF from Agilent (Santa Clara, California, USA). Minitab V17.1.0 (State College, PA, USA) was used for defining the Plackett-Burman

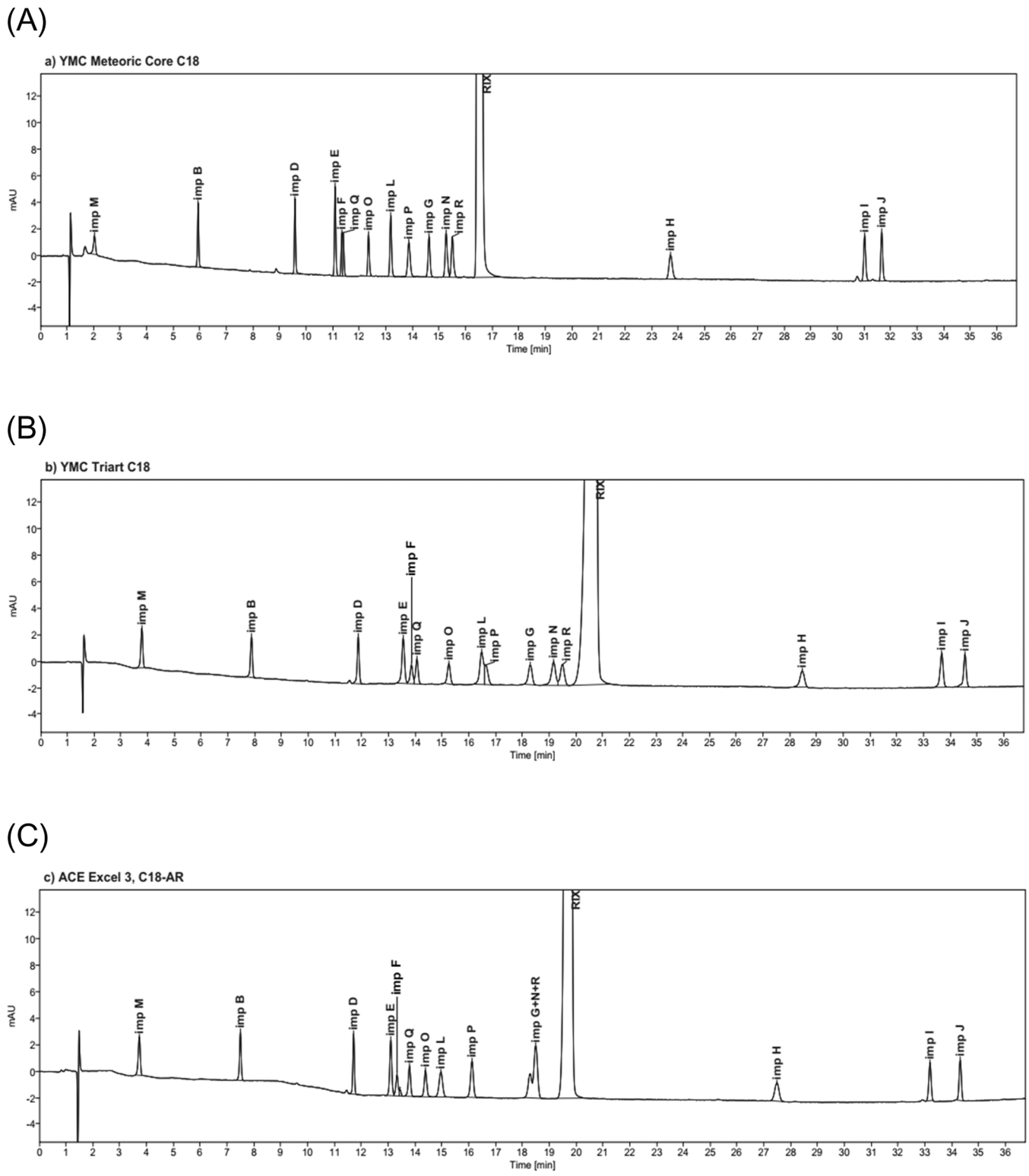


**Fig. 3.** A) Typical chromatogram on YMC Meteoric Core C18; B) Typical chromatogram on YMC Triart C18; C) Typical chromatogram on ACE Excel 3, C18-AR.

experimental plan.

### 2.3. HPLC columns

Three different columns with similar dimensions were used during the method development: (1) ACE Excel 3, C18-AR (4.6 × 100 mm, 3 μm) with highly inertial silica particles (ACE. Co., LTD., Aberdeen, Scotland), (2) YMC Triart C18 (4.6 × 100 mm, 3 μm) with monolithic silica particles (YMC. Co., LTD., Japan), (3) YMC Meteoric Core C18 (4.6 × 100 mm, 2.7 μm) with core-shell type silica particles (YMC. Co., LTD., Japan). The method described in the Ph. Eur. 10th monograph [15] specifies a 150 × 3.0 mm C18 column with 3.5 μm average diameter particles, therefore the initial method was tested at a Zorbax Eclipse XDB C18 (150 × 3.0 mm, 3.5 μm) column (Agilent, Santa Clara,

**Table 2**
Comparisons of pH under optimized condition[a].

| pH 2.5 | | | pH 3.0 | | | pH 3.6 | | | pH 4.4 | | | pH 6.2 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Compounds | Rs | As | Compounds | Rs | As | Compounds | Rs | As | Compounds | Rs | As | Compounds | Rs | As |
| Imp M | - | 0.95 | Imp M | - | 0.93 | Imp M | - | 1.03 | Imp M | - | 1.04 | Imp M | - | 1.69 |
| Imp B | 33.2 | 1.03 | Imp B | 36.6 | 1.02 | Imp B | 32.8 | 1.09 | Imp F | 23.3 | 0.72 | Imp F | 13.2 | 0.77 |
| Imp D | 5.81 | 1.02 | Imp D | 7.36 | 1.08 | Imp D | 10.6 | 0.85 | Imp B | 0.42 | 1.34 | Imp B | 4.41 | 1.04 |
| Imp E | 16.4 | 0.97 | Imp E | 16.1 | 0.98 | Imp F | 0.30 | 2.64 | Imp D | 29.2 | 1.05 | Imp D | 39.6 | 1.00 |
| Imp Q | 11.2 | 1.02 | Imp F | 5.88 | 1.11 | Imp E | 7.51 | 1.05 | Imp E | 15.8 | 1.02 | Imp E | 15.9 | 1.09 |
| Imp F | 3.75 | 1.01 | Imp Q | 4.45 | 1.03 | Imp Q | 16.2 | 1.06 | Imp L | 9.95 | 1.01 | Imp L | 1.43 | 0.89 |
| Imp O | 5.01 | 1.08 | Imp O | 5.27 | 1.00 | Imp O | 3.92 | 1.09 | Imp Q | 6.90 | 0.97 | Imp Q | 15.1 | 1.04 |
| Imp L | 0.77 | 0.71 | Imp L | 3.14 | 1.14 | Imp L | 3.39 | 1.05 | Imp O | 3.41 | 1.01 | Imp O | 3.46 | 1.01 |
| Imp P | 0.34 | 1.37 | Imp P | 3.69 | 1.02 | Imp P | 5.07 | 1.00 | Imp P | 9.14 | 1.07 | Imp P | 9.28 | 1.07 |
| Imp G | 7.81 | 1.07 | Imp G | 4.50 | 1.09 | Imp G | 2.91 | 0.97 | Imp G | 2.28 | 0.98 | Imp G | 2.32 | 1.08 |
| Imp N | 4.59 | 1.01 | Imp N | 4.63 | 1.09 | Imp N | 4.58 | 1.01 | Imp N | 2.09 | 1.04 | Imp N | 4.58 | 0.78 |
| Imp R | 1.61 | 1.04 | Imp R | 1.67 | 0.99 | Imp R | 1.64 | 1.00 | Imp R | 1.69 | 1.07 | Imp R | 1.65 | 1.05 |
| RIX | 6.58 | 1.00 | RIX | 6.55 | 1.01 | RIX | 6.58 | 0.99 | RIX | 6.52 | 1.00 | RIX | 6.61 | 0.99 |
| Imp H | 37.7 | 1.04 | Imp H | 37.8 | 1.05 | Imp H | 37.7 | 1.05 | Imp H | 37.5 | 1.46 | Imp I | 26.9 | 0.69 |
| Imp I | 40.6 | 1.03 | Imp I | 37.8 | 1.04 | Imp I | 32.6 | 1.00 | Imp I | 3.61 | 0.95 | Imp H | 12.9 | 1.04 |
| Imp J | 5.87 | 1.09 | Imp J | 7.74 | 1.09 | Imp J | 14.3 | 1.08 | Imp J | 20.81 | 1.07 | Imp J | 46.5 | 1.09 |

Note：
[a] Rs, resolution; As, tailing factor.

California, USA).

### 2.4. Sample solutions

Standard stock solutions of 15 impurities and RIX were prepared at 25 μg•ml$^{-1}$ in a diluent solvent (ACN: water, 40:60, v/v). Working standard solutions of 0.5 mg•ml$^{-1}$ RIX and 2.5 μg•ml$^{-1}$ of each impurity (at 0.5% concentration relative to RIX) were prepared by diluting the stock solutions in a 50 ml volumetric flask with the diluent. Commercial batch samples (25 mg) were dissolved and diluted directly in 50 ml volumetric flasks using the same diluent.

### 2.5. Characteristic data for RIX impurities

**(*S*)− 4-chloro-N-((2-oxo-3-(4-(3-oxomorpholino)phenyl)oxazolidin-5-yl)methyl)thiophene-2-carboxamide (Imp N):** *m/z* [M+ 1]$^+$ 436.0758; $^1$H NMR (400 MHz, DMSO-d6), δ8.99 (t, *J*=5.6 Hz, 1 H), 7.83 (s, 1 H), 7.81 (s, 1 H), 7.57 (d, *J*=8.9 Hz, 2 H), 7.41 (d, *J*=8.9 Hz, 2 H), 4.85 (m, 1 H), 4.20 (s, 2 H), 4.19 (m, 1 H), 3.97 (m, 2 H), 3.87 (dd, *J*=9.0, 6.2 Hz, 1 H), 3.71 (m, 2 H), 3.64 (m, 2 H); $^{13}$C NMR (100 MHz, DMSO-d6), δ166.5, 161.2, 154.6, 140.3, 137.6, 137.0, 128.6, 126.8, 126.4, 124.1, 118.9, 71.8, 68.2, 64.0, 49.5, 48.0, 42.7.

**(*S*)− 3-chloro-N-((2-oxo-3-(4-(3-oxomorpholino)phenyl)oxazolidin-5-yl)methyl)thiophene-2-carboxamide (Imp O):** *m/z* [M+ 1]$^+$ 436.0736; $^1$H NMR (600 MHz, DMSO-d6), δ8.44 (t, *J*=5.8 Hz, 1 H), 7.84 (d, *J*=5.3 Hz, 1 H), 7.57 (d, *J*=8.9 Hz, 2 H), 7.41 (d, *J*=8.9 Hz, 2 H), 7.14 (d, *J*=5.2 Hz, 1 H), 4.89 (m, 1 H), 4.20 (m, 1 H), 4.19 (s, 2 H), 3.97 (m, 2 H), 3.89 (dd, *J*=9.0, 5.9 Hz, 1 H), 3.71 (m, 2 H), 3.65 (m, 2 H); $^{13}$C NMR (151 MHz, DMSO-d6), δ154.0, 136.9, 136.4, 131.3, 129.6, 129.0, 125.8, 123.9, 118.2, 71.0, 67.6, 63.4, 48.9, 47.2, 42.0.

**(*S*)− 5-chloro-N-((2-oxo-3-(4-(3-oxomorpholino)phenyl)oxazolidin-5-yl)methyl)thiophene-3-carboxamide (Imp R):** *m/z* [M+ 1]$^+$ 436.0725; $^1$H NMR (600 MHz, DMSO-d6), δ8.73 (t, *J*=5.7 Hz, 1 H), 8.06 (d, *J*=1.6 Hz, 1 H), 7.56 (d, *J*=9.0 Hz, 2 H), 7.50 (d, *J*=1.6 Hz, 1 H), 7.40 (d, *J*=8.9 Hz, 2 H), 4.83 (m, 1 H), 4.19 (s, 2 H), 4.18 (t, *J*=8.9 Hz, 1 H), 3.97 (m, 2 H), 3.86 (dd, *J*=9.0, 6.1 Hz, 1 H), 3.71 (m, 2 H); $^{13}$C NMR (151 MHz, DMSO-d6), δ165.9, 161.5, 154.0, 137.0, 136.4, 136.2, 128.7, 125.9, 118.2, 71.2, 67.6, 63.4, 48.9, 47.4.

The spectroscopic data for the other twelve impurities (Imp B, D, E, F, H, Q, G, P, L, M, I and J) were listed in the Supplementary material.

## 3. Results and discussion

### 3.1. The assessment of the ruggedness of the EP method

Impurities M and Q are two precursors in the synthetic process of RIX, which may remain in RIX. Impurities P and L are reported as degradation products, and are observed in forced degradation studies under base condition. Impurity L is notably the major impurity found in HEC commercial samples. Besides, EP Impurities E and H originate from process impurities in the starting material 5-chlorothiophene-2-carboxylic acid (Impurity F). Additionally, three other potential positional isomers of the said starting material were also identified in batches from some of the starting material suppliers during earlier HEC research. These potential impurities in the starting material could transform into Impurities N, O, and R, which were present in earlier RIX samples. Given the likelihood of the presence of these seven impurities in actual RIX samples, we included them in the assessment of the EP method.

Upon strict adherence to the official method from the Ph. Eur. 10th for determining RIX, various analysts in the HEC laboratory observed several minor defects during the method's recurrence [15]. The first issue was the inability to achieve baseline separation between Imp G and degradant Imp L (Fig. 2A). The second issue was the significant influence of apparatuses, even when using the same mobile phase, on the separation between Imp D, Imp E, and Imp F. Additionally, the separation was also affected by the mobile phase prepared by different operators (Fig. 2A, B). The inadequate tolerance of the official method could be ascribed to the presence of an ion-pair reagent in the mobile phase, which could significantly impact the ionization of the analytes and the chromatographic retention of compounds with different pKa values. Given these considerations, the development of a new, improved, and robust HPLC method for the simultaneous determination of the process substances and degradation impurities in RIX API is imperative.

### 3.2. Method optimization

Simultaneous determination of RIX and its fifteen impurities in a single HPLC-UV analysis requires high selectivity and robustness. To achieve this goal in an even shorter run time, method optimization was performed sequentially by column selection, pH screening, column temperature screening, gradient procedure optimization, injection volume adjustment, and "ghost peak" mitigation. Detailed discussion are as follows.

**Table 3**
The test results of gradient procedure[a].

| Gradient | R1 | R2 | R3 |
|---|---|---|---|
| 1 | 2.67 | 4.70 | 1.71 |
| 2 | 2.79 | 4.77 | 1.79 |
| 3 | 2.74 | 5.14 | 1.95 |

Note:
Gradient1: 5–24% B for 0–10 min, 24–33% B holds for 10–25 min, 33–52% B for 25–35 min, 52–77% B for 35–40 min, and 5% B holds for 40.1–43 min for re-equilibration;
Gradient2:5–24% B for 0–10 min, 24–29% B holds for 10–20 min, 29–52% B for 20–30 min, 52–77% B for 30–35 min, and 5% B holds for 35.1–40 min for re-equilibration;
Gradient3:5–24% B for 0–10 min, 24% B holds for 10–20 min, 24–52% B for 20–28 min, 52–77% B for 28–33 min, and 5% B holds for 33.1–38 min for re-equilibration.
[a] R1, resolution of Impurity E and F; R2, resolution of Impurity P and G; R3, resolution of Impurity N and R;

**Table 4**
Optimal chromatic condition for the analysis of RIX.

| Parameter | Value |
|---|---|
| Column | YMC Core C18, 4.6 × 100 mm, 2.7 μm |
| Flowrate | 1.0 ml/min |
| Wavelength | 250 nm |
| Injection volume | 2 μL |
| Column temperature | 42 °C |
| Mobile phase A | pH 3.0 potassium dihydrogen phosphate (10 mM) |
| Mobile phase B | Acetonitrile |
| Gradient | 0 min (95% A-5% B) |
| | 10 min (76% A-24% B) |
| | 20 min (76% A-24% B) |
| | 28 min (48% A-52% B) |
| | 33 min (23% A-77% B) |
| | 33.1 min (95% A-5% B) |
| | 38 min (95% A-5% B) |

#### 3.2.1. Column selection

The column-screening experiments were conducted based on an initial gradient elution of line A (0.1% phosphoric acid) and line B (ACN) at a flow rate of 1.0 ml·min$^{-1}$. The column temperature was set at 40ºC, and the detection wavelength was set at 250 nm as per the EP official method. Three octadecyl silica HPLC columns with similar dimensions from three different suppliers were included in the test series: (1) YMC Meteoric Core C18 (4.6 ×100 mm, 2.7 μm); (2) YMC Triart C18 (4.6 ×100 mm, 3.0 μm); (3) ACE Excel 3 C18-AR (4.6 ×100 mm, 3.0 μm). In this case, the critical peak pair selected was the Imp N and Imp R. The best achieved experimental resolution value was 1.5 on YMC Core C18 column (Fig. 3A), and the tailing factors of RIX and fifteen impurities obtained were between 0.9 and 1.2. Back pressure was significantly lower on this column due to the core-shell particle, which could be an advantage in routine use. Resolutions for Imp F, Q, E, L and P on YMC Triart C18 column were all less than 1.5, and the resolution of the critical peak pair (Rs, crit) value was also lower (Resolution = 1.3) than the former column. The resolution between the critical peak pair on ACE Excel 3 C18-AR column was much less than the former two columns, and three peaks (Imp G, Imp N, and Imp R) were even co-eluted under the selected experimental conditions (Fig. 3C). Despite that baseline separation was not achieved for Imp F and Imp Q on the YMC Meteoric Core C18 column, this column was still selected for further studies for the better separation of the other impurities and the better Rs, crit.

#### 3.2.2. Screening experiments on pH and column temperatures

Systematic pH screening experiments were performed for a better separation on Imp F and Q. A series of buffers with pH values in the range of 2.5–6.2 were prepared by using potassium dihydrogen phosphate and ammonium acetate (Table 2). As suggested by the test results, Imp F, G, I, L, M, P, and Q were significantly affected by the mobile phase pH. In addition, the relative position of Imp F, Q and L shifted considerably along with pH changes, and the resolutions of Imp F, Q, P and L were also largely influenced. Fortunately, the resolutions of Imp M, G, Q and I were all satisfactory (Resolutions > 2.2) at every investigated pH. In conclusion, the best experimental results were obtained at pH 3.0 for satisfactory separations between Imp F and Imp Q (Resolution = 4.45). Besides, baseline separations were all achieved for Imp M, G, I, P, and L (Resolutions > 3.0).

Based on the selected column and pH, Imp N and Imp R, Imp E and Imp F were selected as the critical peak pairs. Interestingly, the separation of the two critical peak pairs was also sensitive to the column temperature. As a result, an elaborate screening on column temperatures (ie. 35 °C, 38 °C, 40 °C, 42 °C, 45 °C) were performed to get the optimum separations for the two critical peak pairs. The separation between Imp N and R gradually improved as column temperature increased. On the contrary, the separation between Imp E and Imp F gradually decreased. Finally, the optimal column temperature was chosen at 42 °C with consideration of both the two critical pairs getting the most suitable separation.

#### 3.2.3. The selection of the gradient procedure

Subsequently, optimization on gradient procedure was conducted in an attempt of getting even better resolutions in a shorter run time. However, as depicted in Table 3, different gradient only had a slight influence on the resolution of the critical peak pairs. Therefore, the optimal procedure was selected to achieve a shortest rum time as satisfying Rs, crit. values (≥ 1.5) for Imp N/R were observed under all tested gradients. The chosen gradient is listed as follows: 5–24% B for 0–10 min, 24% B holds for 10–20 min, 24–52% B for 20–28 min, 52–77% B for 28–33 min, and 5% B holds for 33.1–38 min for re-equilibration (Table 4).

#### 3.2.4. Sample preparation and injection volume

Although these samples were diluted with the diluent mixture (ACN: $H_2O$, 40: 60, v/v %) according to the Ph. Eur. 10th, peak distortion of the newly investigated Imp M was still observed at a sample volume of 3 μL under developed chromatographic conditions. To improve column performance, decreasing solvent strength or injection volume are both possible measures. Nevertheless, RIX could not be fully solubilized at a concentration of 0.5 mg·ml$^{-1}$ in diluent mixture with decreased strength. Under this circumstance, reducing injection volume to 2 μL might be more practical to get good peak shape for Imp M. Although the injection volume was lower than 3 μL as per the official method, the preliminary result of the test on the sensitivity and repeatability by injecting standards solution was satisfactory for the quantitative analysis of all aimed impurities.

#### 3.2.5. The study of two main "ghost peaks"

During method development, it was observed that two primary "ghost peaks" appeared in addition to a decrease in peak area for Imp Q and Imp G. The two peaks only emerged in new glass HPLC vials, while sample solutions stored in plastic HPLC vials or glass HPLC vials soaked in diluted hydrochloric acid did not display the two peaks above the detection limit. The literature suggests that solution degradation frequently occurs due to the leaching of alkaline impurities from glass HPLC vials [18,19]. Subsequent research aimed to identify the two degradants' structure using high liquid chromatography-tandem mass spectrometry (LC-MS/MS). The TOF MS ES+ spectrum of the two ghost peaks showed protonated ions at *m/z* 414.1662 and 440.1453, respectively. Based on the MS/MS findings and the partial hydrolysis reaction of compounds containing isoindoline-1,3-dione group, the two primary "ghost peaks" were identified as Imp S and Imp T (Fig. 4). These were solution degradants of Imp Q and Imp G under alkaline conditions in new glass HPLC vials. A typical chromatogram of RIX (0.5 mg•ml$^{-1}$)

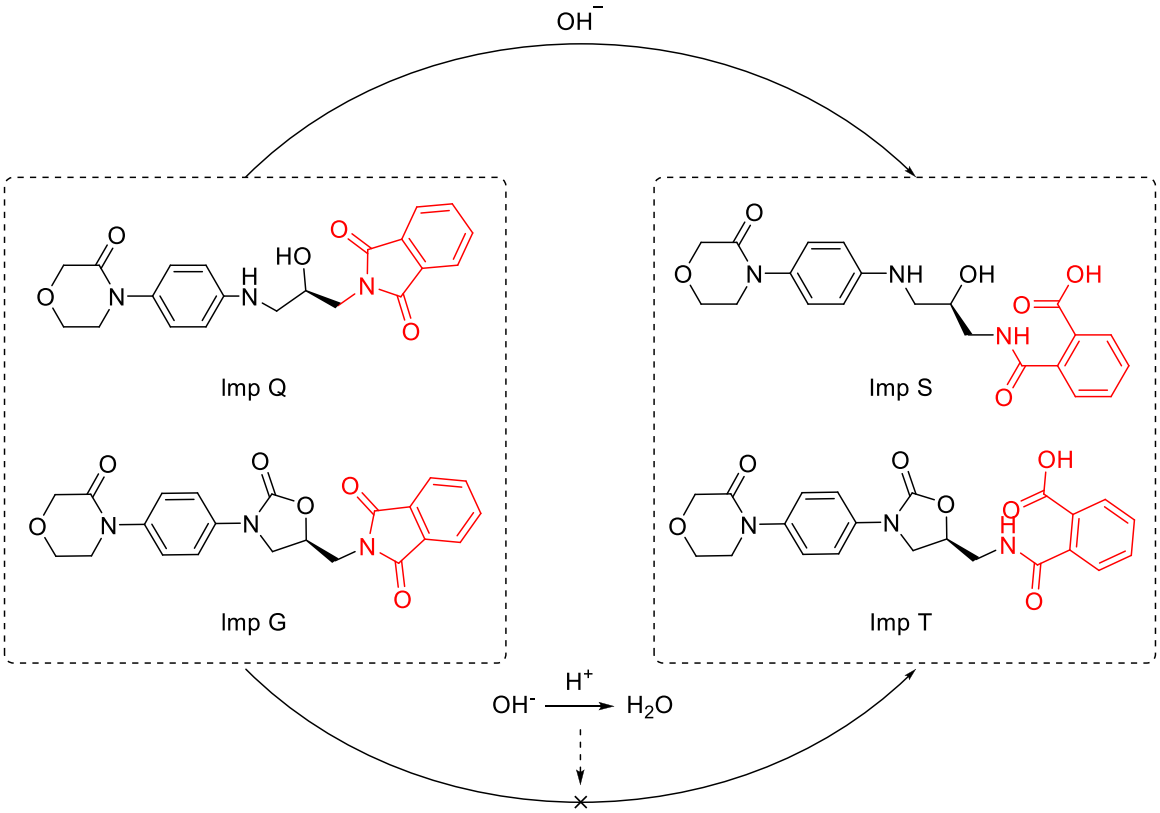


**Fig. 4.** Degradation mechanism of Imp S and Imp T from Imp Q and Imp G.

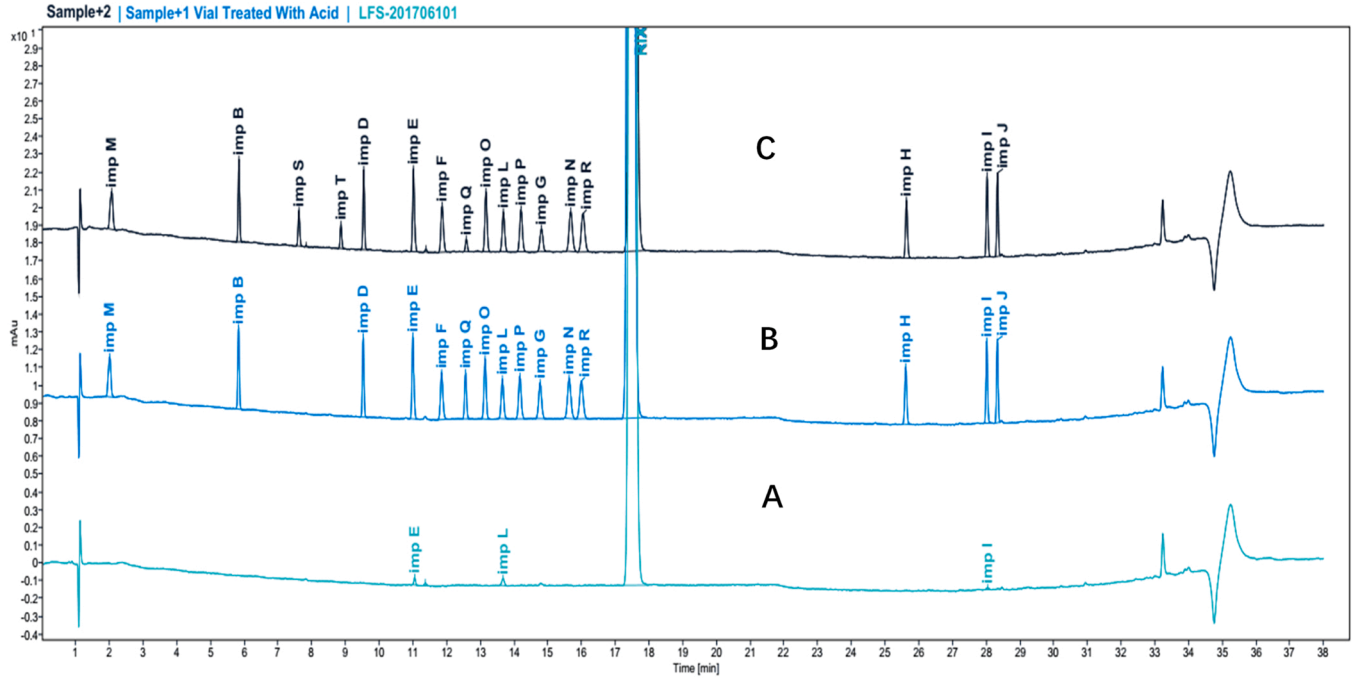


**Fig. 5.** Chromatograms obtained on commercial samples during method application: (A) Sample solution, (B) Sample solution spiked with all impurities, (C) A typical chromatogram of RIX spiked with 0.5% impurities containing Imp-S and Imp-T.

spiked with 0.5% w/w of each of fifteen potential impurities (2.5 μg•ml$^{-1}$) and degradants Imp S and Imp T was shown in Fig. 5C. Lastly, an acid pretreatment of the glass HPLC vials avoid the emergence of Imp S and Imp T, and these impurities were not required to be investigated during the analytical method development.

### 3.3. Method validation

The devised method underwent validation in accordance with the present ICH guidelines Q2 (R1), encompassing a range of parameters such as precision, Limit of Detection (LOD) and Limit of Quantitation

**Table 5**
Results of Precision, LOD and LOQ.

| Compounds | Precision | | LOQ[a] | | LLOQ[a] | | LOD[a] | |
|---|---|---|---|---|---|---|---|---|
| | Inter-day , RSD%, n = 6 | intra-day , RSD%, n = 6 | Concentration ( μg.ml$^{-1}$ ) | S/N range | Concentration ( μg.ml$^{-1}$ ) | S/N range | Concentration ( μg.ml$^{-1}$ ) | S/N range |
| **Imp B** | 2.14 | 2.06 | 0.277 | 26.0–27.4 | 0.111 | 11.2–11.6 | 0.055 | 3.7–3.9 |
| **Imp D** | 2.00 | 1.94 | 0.275 | 26.7–27.8 | 0.110 | 11.2–11.6 | 0.055 | 3.6–4.0 |
| **Imp E** | 0.58 | 1.23 | 0.278 | 29.5–30.9 | 0.111 | 12.6–12.9 | 0.056 | 4.2–4.5 |
| **Imp F** | 1.35 | 1.56 | 0.275 | 14.4–15.0 | 0.165 | 10.1–10.8 | 0.055 | 2.9–3.4 |
| **Imp G** | 1.61 | 1.98 | 0.276 | 17.4–20.5 | 0.165 | 10.9–12.3 | 0.055 | 2.9–3.2 |
| **Imp H** | 1.11 | 2.18 | 0.279 | 21.1–22.0 | 0.112 | 10.4–11.2 | 0.056 | 3.0–3.2 |
| **Imp I** | 0.21 | 1.24 | 0.260 | 24.0–25.2 | 0.104 | 11.3–11.7 | 0.052 | 3.1–5.5 |
| **Imp J** | 1.28 | 1.52 | 0.275 | 27.1–28.3 | 0.110 | 11.5–11.7 | 0.055 | 3.7–3.9 |
| **Imp L** | 0.94 | 1.07 | 0.261 | 17.8–20.6 | 0.157 | 11.5–12.9 | 0.052 | 3.4–3.9 |
| **Imp M** | 1.17 | 1.33 | 0.265 | 13.4–13.7 | 0.159 | 9.0–10.2 | 0.053 | 2.8–3.2 |
| **Imp N** | 1.94 | 2.27 | 0.262 | 20.0–23.0 | 0.157 | 12.4–14.2 | 0.052 | 3.3–3.9 |
| **Imp O** | 1.01 | 1.64 | 0.288 | 20.7–21.5 | 0.115 | 10.0–10.7 | 0.058 | 2.9–3.2 |
| **Imp P** | 1.13 | 2.15 | 0.256 | 19.3–22.2 | 0.153 | 11.8–14.0 | 0.051 | 3.6–4.0 |
| **Imp Q** | 1.61 | 1.24 | 0.265 | 13.2–14.1 | 0.159 | 10.2–11.0 | 0.053 | 3.0–3.5 |
| **Imp R** | 1.23 | 1.93 | 0.275 | 18.5–21.0 | 0.165 | 11.4–13.0 | 0.055 | 3.1–3.3 |
| **RIX** | 0.82 | 1.21 | 0.282 | 18.7–19.6 | 0.169 | 10.1–12.4 | 0.056 | 3.3–3.9 |

Note:
[a] Results of three determinations.

**Table 6**
Linearity data [a,b].

| Compounds | Range (μg. ml$^{-1}$) | Linear equation[a] | r$^{2}$[a] | RRF[b] | Residual sum of squares | P-value |
|---|---|---|---|---|---|---|
| **Imp B** | 0.17–4.6 | y = 4.81x + 0.15 | 0.9988 | 0.75 | 0.554 | 0.363 |
| **Imp D** | 0.16–4.2 | y = 5.29x - 0.01 | 0.9993 | 0.82 | 0.297 | 0.926 |
| **Imp E** | 0.15–4.1 | y = 6.36x + 0.01 | 0.9991 | 0.99 | 0.550 | 0.942 |
| **Imp F** | 0.15–4.1 | y = 5.14x - 0.11 | 0.9987 | 0.80 | 0.502 | 0.486 |
| **Imp G** | 0.15–4.0 | y = 4.36x - 0.10 | 0.9983 | 0.68 | 2.424 | 0.577 |
| **Imp H** | 0.15–4.1 | y = 4.88x - 0.07 | 0.9990 | 0.76 | 0.355 | 0.613 |
| **Imp I** | 0.26–2.6 | y = 5.71x + 0.65 | 0.9986 | 0.89 | 0.401 | 0.057 |
| **Imp J** | 0.16–4.2 | y = 5.53x + 0.16 | 0.9995 | 0.86 | 0.224 | 0.145 |
| **Imp L** | 0.12–3.3 | y = 3.90x - 0.12 | 0.9969 | 0.60 | 2.485 | 0.336 |
| **Imp M** | 0.16–4.3 | y = 4.46x + 0.03 | 0.9965 | 0.69 | 1.154 | 0.883 |
| **Imp N** | 0.16–4.3 | y = 5.73x - 0.11 | 0.9991 | 0.89 | 0.488 | 0.469 |
| **Imp O** | 0.15–4.0 | y = 6.02x + 0.001 | 0.9992 | 0.93 | 0.427 | 0.994 |
| **Imp P** | 0.16–4.2 | y = 4.88x - 0.07 | 0.9987 | 0.76 | 0.468 | 0.630 |
| **Imp Q** | 0.15–4.1 | y = 3.90x + 0.14 | 0.9977 | 0.61 | 0.528 | 0.399 |
| **Imp R** | 0.17–4.5 | y = 5.31x + 0.16 | 0.9990 | 0.83 | 0.507 | 0.309 |
| **RIX** | 200–800 | y = 6.08x − 74.87 | 0.9998 | - | 1322 | 0.142 |

Note:
[a] Average of two determinations;
[b] RRF means relative response factor;

(LOQ), linearity, accuracy, and robustness. A typical chromatogram of RIX (0.5 mg•ml$^{-1}$) was depicted in Fig. 5B after being spiked with 0.5% w/w of each of fifteen potential impurities (2.5 μg•ml$^{-1}$). In this chromatogram, all the spiked impurities, as well as RIX itself, exhibited excellent peak symmetries and resolutions, thereby achieving validation criteria, all within 38 min.

### 3.3.1. Precision

The method's precision was evaluated by conducting six repeated analyses of spiked RIX solutions (0.5 mg·ml$^{-1}$) containing each impurity at a concentration of 0.05% (0.25 μg·ml$^{-1}$). The samples were analyzed six times on the same day (intra-day precision) and consecutive days (inter-day precision) by a different analyst. The results, summarized in Table 5, revealed that the obtained values for relative standard deviation (RSD) and bias of intra- and inter-day studies were indicative of satisfactory precision. The developed method demonstrated RSD% values that ranged from 0.21% to 2.27%. Furthermore, the robustness of the proposed method was confirmed by applying the same procedures on different days, with two different operators yielding consistent results.

### 3.3.2. LOD and LOQ

Initially, LOQ of the new method was set at 0.05%, which was also the reported threshold of the EP method. Standard stock solutions containing RIX and impurities were gradually diluted to suitable concentrations for determination of LOD and Lower Limit of Quantitation (LLOQ). The peak height of each component was recorded after three repeated analyses. The LOD and LOQ for RIX and fifteen impurities were calculated by normalization of the signal/noise ratio (S/N) to 3 for LOD and S/N to 10 for LLOQ, and the results were summarized in Table 5. The obtained results demonstrated that this method was sensitive enough and suitable for the analysis of RIX and impurities.

### 3.3.3. Linearity

The method linearity was evaluated from 0.2 mg·ml$^{-1}$ to 0.8 mg·ml$^{-1}$ for RIX and from 0.15 μg·ml$^{-1}$ to 4.0 μg·ml$^{-1}$ (nine different standard solutions within the linear range containing 0.15, 0.25, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 and 4.0 μg·ml$^{-1}$) for each impurity. The linearity was evaluated by linear regression analysis, and the regression equations were calculated from the calibration graphs. The relative response factors (RRF) of each impurity were determined by the ratios of the slopes of calibration curves for each impurity with respect to the slope of the calibration curve for RIX. The results of the method linearity study were summarized in Table 6 and demonstrated good linearity for each impurity component ($r^2 > 0.9965$) and RIX ($r^2 = 0.9999$).

### 3.3.4. Accuracy

In order to know whether the bulk interfere with the analysis, the recovery tests were performed by standard addition technique. The accuracy samples were prepared by adding known amounts of the impurities to the RIX API and the results were evaluated based on the

**Table 7**
The accuracy and recovery results under optimal condition [a].

| Compounds | Accuracy (%) | | |
|---|---|---|---|
| | 0.05% level | 0.10% level | 0.15% level |
| Imp B | 102.6 | 99.2 | 98.1 |
| Imp D | 102.3 | 99.8 | 102.0 |
| Imp E | 98.6 | 101.3 | 102.7 |
| Imp F | 98.9 | 99.0 | 103.0 |
| Imp G | 99.2 | 100.5 | 101.6 |
| Imp H | 99.6 | 101.1 | 101.0 |
| Imp I | 96.1 | 97.1 | 98.7 |
| Imp J | 99.7 | 100.4 | 98.3 |
| Imp L | 101.2 | 98.6 | 98.6 |
| Imp M | 96.5 | 100.5 | 101.5 |
| Imp N | 102.2 | 99.7 | 102.5 |
| Imp O | 100.8 | 97.0 | 97.2 |
| Imp P | 99.5 | 102.4 | 100.9 |
| Imp Q | 99.6 | 103.9 | 101.9 |
| Imp R | 97.1 | 99.8 | 97.9 |

Note:
[a] Average of three determinations.

**Table 8**
Robustness results of the method[a,b].

| Run | A | B | C | D | E | F | Kn[b] | Kr[b] | Rs, crit.[b] |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 7% | 2.8 | 47 | 1.1 | a | y | 13.95 | 14.34 | 2.10 |
| 2 | 7% | 3.2 | 47 | 0.9 | b | y | 15.79 | 16.25 | 1.91 |
| 3 | 3% | 2.8 | 37 | 0.9 | a | y | 15.17 | 15.59 | 2.08 |
| 4 | 7% | 2.8 | 47 | 0.9 | a | y | 15.71 | 16.18 | 2.04 |
| 5 | 3% | 3.2 | 37 | 0.9 | a | y | 16.80 | 17.15 | 1.53 |
| 6 | 7% | 3.2 | 37 | 1.1 | a | x | 14.19 | 14.49 | 1.55 |
| 7 | 3% | 2.8 | 47 | 1.1 | b | x | 14.67 | 15.07 | 1.96 |
| 8 | 7% | 2.8 | 37 | 0.9 | b | y | 17.28 | 17.68 | 1.52 |
| 9 | 3% | 2.8 | 37 | 1.1 | b | y | 15.88 | 16.22 | 1.59 |
| 10 | 3% | 3.2 | 47 | 1.1 | a | y | 13.91 | 14.25 | 1.99 |
| 11 | 7% | 3.2 | 37 | 1.1 | b | x | 15.35 | 15.70 | 1.56 |
| 12 | 7% | 3.2 | 47 | 0.9 | b | x | 16.39 | 16.85 | 1.89 |

Note:
[a]A: percent of Mobile phase B; B: pH of the mobile phase; C: column temperature (°C); D: flowrate (ml/min); E: two HPLC instruments; F: two columns;
[b]Kn: the retention factor of Imp N; Kr: the retention factor of Imp R; Rs, crit: the resolution of Imp N and R.

recovery values. As the limit for every impurity in the EP RIX monograph was not more than 0.10%, the accuracy of each impurity was evaluated at three different levels 0.05%, 0.10% and 0.15%, each level with 3 samples. The obtained recoveries for each impurity ranged from 96.1% to 103.9%, as shown in Table 7. The accuracy and recovery results were satisfactory for the quantitative HPLC method.

### 3.3.5. *Robustness*

We conducted a comprehensive evaluation of the robustness of our previously developed LC method for determining RIX and its fifteen impurities. Seven factors, namely the acetonitrile content in the mobile phase, pH of the water phase, column temperature, flow rate, column type, and different HPLC instruments, were included in the Plackett–Burman experimental plan. The retention factors of Imp N and R ($k_N$ and $k_R$, respectively), and the critical resolution of for Imp N and R were selected as the evaluation criteria. The results of the experimental plan are presented in Table 8. The analysis showed that there was no significant difference among the results, indicating that the proposed method was robust.

### 3.3.6. *Sample test*

The method's applicability to real samples was demonstrated on commercial batches of RIX with a concentration of 0.5 mg•ml$^{-1}$. Representative chromatograms for the sample solution spiking with or without impurities were presented in Fig. 5A and B, respectively, and the contents of each impurity were summarized in Table 9. Among the monitored related substances, only Imp L was present in quantifiable amounts in the analyzed samples, while the other impurities were below the LOD or LOQ limits. The total impurity content of the commercial samples was below 0.071%, which is below the maximum admitted level (0.3%) of the Ph. Eur. 10th. Based on these results, the method was deemed highly suitable for the analysis of real commercial samples.

**Table 9**
Results obtained during method application on commercial samples.

| Impurity | RRT | LFS-201705101 | LFS-201706101 | LFS-201706102 |
|---|---|---|---|---|
| Impurity M | 0.11 | <LOD | <LOD | <LOD |
| Impurity B | 0.33 | <LOD | <LOD | <LOD |
| Impurity D | 0.54 | <LOD | <LOD | <LOD |
| Impurity E | 0.63 | <LOQ | <LOQ | <LOQ |
| unknown Impurity-1 | 0.65 | <LOQ | <LOQ | <LOQ |
| Impurity F | 0.68 | <LOD | <LOD | <LOD |
| Impurity Q | 0.72 | <LOD | <LOD | <LOD |
| Impurity O | 0.75 | <LOD | <LOD | <LOD |
| Impurity L | 0.78 | 0.062 | 0.071 | 0.064 |
| Impurity P | 0.81 | <LOD | <LOD | <LOD |
| Impurity G | 0.85 | <LOD | <LOD | <LOD |
| Impurity N | 0.90 | <LOD | <LOD | <LOD |
| Impurity R | 0.92 | <LOD | <LOD | <LOD |
| Impurity H | 1.47 | <LOD | <LOD | <LOD |
| Impurity I | 1.60 | <LOD | <LOD | <LOD |
| Impurity J | 1.62 | <LOD | <LOD | <LOD |
| unknown Impurity-2 | 1.64 | <LOQ | <LOD | <LOD |
| **Total Impurity** | - | 0.062 | 0.071 | 0.064 |

## 4. Conclusions

In summary, a streamlined and effective gradient RP-HPLC technique has been developed to examine RIX and its fifteen associated impurities, including three newly discovered ones, which were comprehensively characterized by MS, $^1$HNMR, $^{13}$C NMR, and HSQC. The ensuing validation affirmed the method's selectivity (Resolution > 1.5), precision, accuracy, sensitivity, and robustness. This validated technique was successfully employed to assess impurities related to both the manufacturing and degradation of RIX API in three different batches. The method is highly suitable for process optimization and quality control of RIX API and has been demonstrated to be swift, robust, and dependable when compared to the European Pharmacopoeia method.

## CRediT authorship contribution statement

**Wanbing Rao**: Conceptualization, Writing – original draft, Project administration. **Lijun Li**: Resources. **Chenxia Zhang**: Investigation, Formal analysis, Validation. **Jinfu Zheng**: Resources. **Xiaomei Fan**: Methodology. **Baolei Luan**: Methodology. **Jiaxiang Sun**: Formal analysis. **Meiyan Qiu**: Validation, Investigation. **Shuming Wu**: Writing – review & editing. **Yanhua Li**: Supervision. **Zhongqing Wang**: Supervision, Writing – review & editing.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jpba.2023.115325.

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