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Optimization of the experimental conditions for determination of roxithromycin in bulk and dosage forms

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Abstract: Roxithromycin (RXT), which is an antibiotic used to treat respiratory tract and urinary infections, is official in Korean Pharmacopoeia (KP) and is marketed in various dosage forms including tablet, granule, suspension, and tablet for suspension in Korea. This study presents how a universal and reliable method to quantify RXT in bulk drug and formulations was developed. Effects of factors including column type, buffer concentration, type and concentration of organic solvent, buffer pH, and type and concentration of mobile phase additive, were examined, and some categorical or crucial factors including the types of column, organic solvent, mobile phase additive and the buffer pH were optimized by one-factor-at-a-time approach. Subsequently, concentrations of the buffer and additive and column temperature were optimized by response surface methodology using Box-Behnken design aiming to acquire the RXT peak of good shape. The optimized method employed a Phenomenex Gemini 5μ C18 110A (150 × 4.60 mm, 5 μm) maintained at 30 °C with the mobile phase consisting of 25 mM phosphate buffer (pH 6.0) with 0.3 % tetrabutylammonium hydroxide and methanol at a ratio of 37:63 (v/v). Method validation results showed that the developed method was linear, precise, and accurate. Compared to the compendial methods in KP 10 that exhibited a significant tailing of the RXT peak despite using unfavorably high buffer concentrations and were not harmonized among bulk drug and formulations, this method could be universally applied to RXT bulk drug and marketed products in various dosage forms and thus was adopted in KP 11.

Key words: roxithromycin, response surface methodology, official compendium, compendial method, asymmetry factor

1. Introduction

Antibiotics can be classified into tetracyclins, macrolides, aminoglycosides, and aminophenols according to their chemical structures and mechanisms of actions.[1,2] Roxithromycin (RXT), which is derived from erythromycin, is a semi-synthetic macrolide antibiotic containing a 14-membered lactone ring. It is used to treat respiratory tract and urinary infections. Compared to erythromycin, plasma concentrations

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of RXT are higher, and its half-life time is longer due to its fast absorption and slow metabolism.[3]

Numerous analytical methods have been reported to quantify RXT in bulk, dosage forms, and biological fluids, and the methods were usually based on high performance liquid chromatography (HPLC) coupled with ultraviolet (UV),[4-6] fluorescence,[7] electrochemical detection,[8-10] or mass spectrometry.[11,12] RXT is listed in a number of official compendia including European Pharmacopoeia (EP), Japanese Pharmacopoeia (JP), and Korean Pharmacopoeia (KP), and all the compendial methods for RXT employ HPLC-UV-based analytical methods.

In Korea, RXT is marketed in various dosage forms, including granule, tablet, tablet for suspension, and suspension. Careful examination of the RXT compendial methods in KP 10 revealed that the experimental conditions were not harmonized among the bulk drug and different formulations; mobile phase conditions, flow rates, detection wavelengths, and sample concentrations somewhat differed. In addition, the aqueous buffer concentrations in the mobile phase were unfavorably high (≥ 410 mM) in all methods. Therefore, it was aimed develop an optimized assay method that could be universally applicable to bulk drug and various dosage forms. To this aim, effects of a number of factors on the chromatographic performance were systematically investigated, and a number of influential factors were optimized using response surface methodology (RSM). The optimized method was validated and successfully applied to monitor commercial products in Korean market.

2. Materials and Methods

2.1. Chemicals

RXT (EP grade) was purchased from Glentham Life Sciences Ltd. (Corsham, UK). Roxithromycin bulk drug was kindly provided by Kolon Pharma (Seoul, Korea). Roxithromycin products available in various dosage forms were acquired directly from the producing pharmaceutical companies. Granules were from Dong Hwa Pharm (Seoul, Korea), Sama Pharm (Seoul, Korea), and Kolon Pharma (Seoul, Korea), and all of the products were labeled to claim 50 mg per 1 g. Tablets were obtained from Dong Hwa Pharm (Seoul, Korea; label claim, 150 mg per tablet) and Ildong Pharmaceutical (Seoul, Korea; 150 mg per tablet), while tablets for suspension were from Handok (Seoul, Korea; 50 mg per tablet). Suspensions were purchased from Daewon Pharm (Seoul, Korea; 1,000 mg per 100 mL). Ammonium phosphate monobasic (99.0 %) and phosphoric acid (85 %) were obtained from Sigma-Aldrich (St, Louis, MO, USA). Tetrabutylammonium hydroxide (TBA-OH), dodecyltrimethylammonium chloride (DTMA-Cl), sodium 1-octanesulfonate, trimethylamine (TEA), and diethylamine (DEA) were purchased from Tokyo Chemical Industry (Tokyo, Japan). HPLC-grade water, methanol, and acetonitrile were from J. T. BAKER (Philipsburg, MT, USA). All other chemicals were of analytical grade or higher and purchased from Sigma-Aldrich unless stated elsewhere.

2.2. Preparation of stock and standard solutions

A stock solution of RXT was prepared in the mobile phase at a concentration of 2 mg mL^{-1}. A series of standard solutions (400-600 μg mL^{-1}) were prepared by diluting the stock solution in the mobile phase.

2.3. Sample preparation of bulk and formulation solutions

RXT bulk and solid dosage forms were finely pulverized using a mortar and a pestle before weighing, while suspension was directly used for dilution. An appropriate portion of powder or liquid from each sample type was taken and dissolved in the mobile phase to make a sample solution containing 500 μg mL^{-1} of RXT. The content in the flask was sonicated for 10 min or until no solid residues were observed. The solutions were filtered using a membrane filter (Whatman; Piscataway, NJ, USA) prior to injection for the HPLC analysis.

2.4. Analytical instruments and the chromatographic conditions

The HPLC analysis was conducted using a Perkin

Elmer HPLC system (Norwalk, CT, USA), equipped with a Perkin Elmer Pump (Series 275), a photodiode array detector (Series 275), an autosampler (Series 275), and a column oven (Series 200). Analytical columns tested were Agilent Extend C18 (150 × 4.60 mm, 5 μm), Agilent Eclipse Plus C18 (150 × 4.60 mm, 5 μm), Agilent Zorbax 80 Å Extend C18 (150 × 4.60 mm, 5 μm), Phenomenex Luna C18 (150 × 4.60 mm, 5 μm), and Phenomenex Gemini 5 μ C18 110A (150 × 4.60 mm, 5 μm). The column temperature was maintained at 30 °C.

The optimized mobile phase was prepared by mixing solvent A (25 mM phosphate buffer with 0.3 % TBA-OH, pH 6.0 adjusted with 2 M phosphoric acid) and solvent B (methanol) at a ratio of 37:63 (v/v). Flow rate, injection volume, and detection wavelength were 1.0 mL min^{-1}, 20 μL, and 205 nm, respectively.

2.5. Method validation

The established method was validated in terms of linearity, precision, accuracy, and system suitability according to the KFDA guideline.[13] For linearity, the RXT standard solutions at five different concentrations (400, 450, 500, 550, 600 mg mL^{-1}) were analyzed in triplicate, and the nominal standard concentrations were plotted against the corresponding RXT peak areas to establish a linear regression curve. Intraday and interday precisions were estimated within one day (n=3) and three separate days (n=3 × 3), respectively, at the concentrations of 400, 500, 600 mg mL^{-1}.

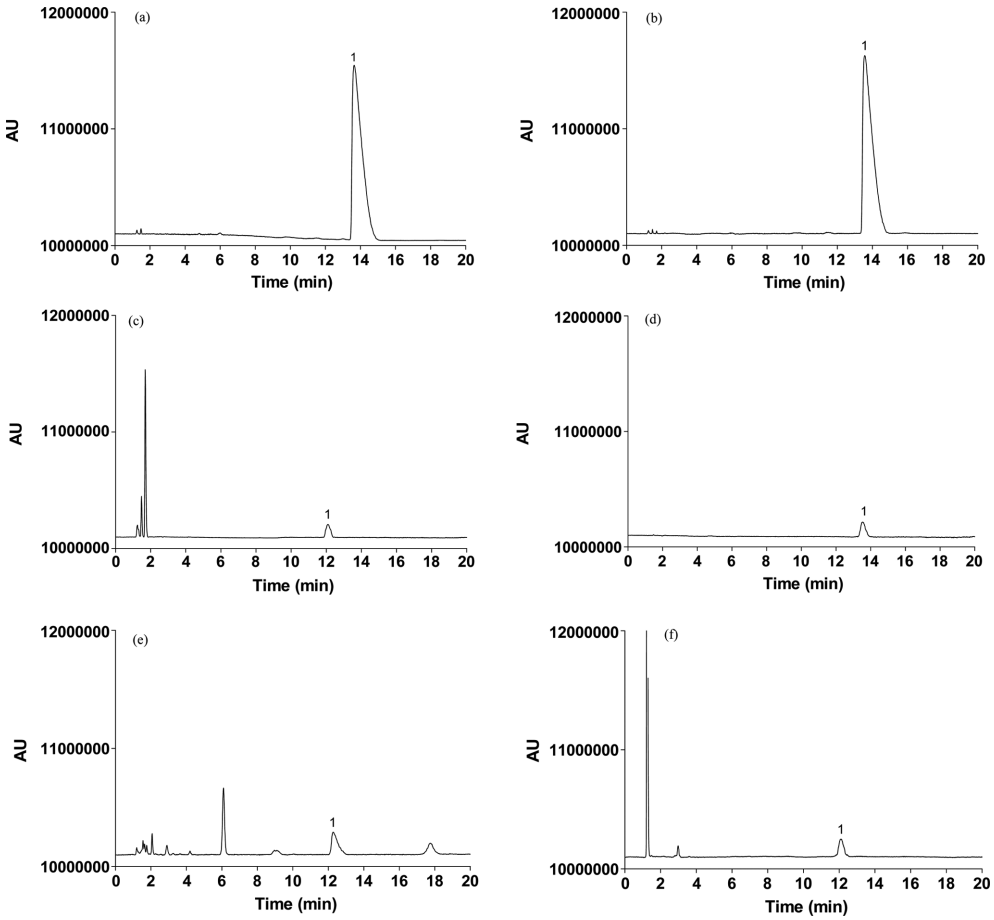

Fig. 1. HPLC-UV chromatograms of RXT standard (a), RXT in bulk drug (b), granule (c), tablet (d), suspension (e), and tablet for suspension (f). Experimental conditions were as indicated in KP 10. The stationary phase used was a Phenomenex Gemini C18 (4.6 × 150 mm, 5 μm). Peak identification: 1, RXT

Accuracy was measured as recovery at low, middle, and high concentrations (500, 550, and 600 mg mL^{-1}, respectively) in triplicate. System suitability was evaluated as repeatability by analyzing a standard solution of 500 mg mL^{-1} in sextuplicate.

2.6. Software used for response surface methodology

Design-Expert Ver. 8.0 (Statease Inc., Minneapolis, MN, USA) was used to design experiments and statistically analyze the obtained results[16]

3. Results and Discussion

3.1. Examination of the compendial methods

The compendial methods for the RXT quantification in KP 10 were similar, but not in complete agreement among the bulk drug and dosage forms, employing harsh chromatographic conditions. Application of these methods to the analysis of real samples presented a number of issues to address. As displayed in *Fig.* 1, the chromatographic analysis time was unnecessarily long, which somehow appeared to have caused inconsistent retention times for RXT among the different formulations. Moreover, the peak shapes of RXT differed depending on the sample type and concentration, and the peak tailing became severe as the sample concentrations increased. For example, the asymmetry factor (AF) was larger than 3 at the concentration of 2000 μg mL^{-1} in bulk drug, indicating that the high buffer concentrations did not help improve the peak shape. Therefore, we attempted to investigate various factors that could affect the chromatographic performance with a focus on the peak shape and retention time.

3.2. Selection of the detection wavelength and the stationary phase

RXT lacks a chromophore. The compendial methods adopted two detection wavelengths, 205 nm (bulk drug, granule, tablet, and tablet for suspension) and 225 nm (suspension), and other different wavelengths were used in previous reports on the HPLC-UV analysis of RXT, such as 202 nm[14] and 215 nm.[4,5]

Table 1. Chromatographic performance depending on column, methanol content in the mobile phase, and buffer pH

Factor tested	Condition	t_R (min)	AF
Column	Extend C18	7.1	3.606
	Eclipse Plus C18	9.4	1.828
	Luna C18	12.1	2.368
	Zorbax 80Å Extend C18	7.5	2.659
	Gemini C18	12.0	2.107
Methanol content (% v/v)	60	10.56	2.485
	65	6.21	2.094
	70	4.27	1.859
Buffer pH	2.0	6.55	2.325
	3.0	6.50	2.698
	4.0	6.64	2.279
	5.0	7.02	2.319
	6.0	8.43	1.651
	7.0	15.81	1.837
	8.0	22.44	1.939
	9.0	30.08	2.276

Based on the UV spectrum of RXT measured and the cut-off values of common solvents for mobile phase, the detection wavelength was fixed at 205 nm, which was employed in all the subsequent analyses regardless of sample types.

Then, five different C18 columns of the same dimension were tested in order to select a stationary phase that could result in the elution of RXT with selectivity and least tailing. Instead of 250 mm, columns of 150 mm were tested to reduce analysis time and solvent consumption. As shown in *Table* 1, Eclipse Plus C18 and Gemini C18 presented less tailing than the other three, however, the former yielded split peaks for RXT. As a result, Gemini C18 was selected as the suitable column and used in the subsequent studies.

3.3. Assessment of effects of various factors in the mobile phase conditions

3.3.1. Buffer concentration

While the type of aqueous buffer was ammonium phosphate (pH 5.3) in common in the compendial methods, their concentrations were different depending on the sample type and no lower than 410 mM. Three different mobile phases composed of acetonitrile (30 % v/v) and ammonium phosphate buffer (70 %

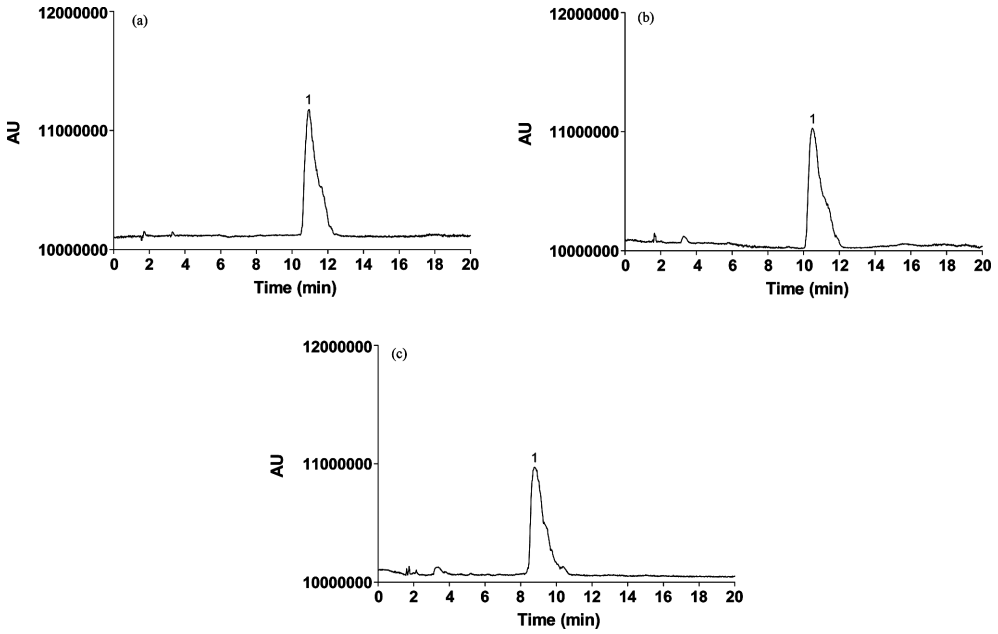

Fig. 2. HPLC-UV chromatograms of RXT standard analyzed using the mobile phase containing different concentrations of ammonium phosphate buffer (pH 5.3). (a) 250 mM; (b) 125 mM; (c) 25 mM buffer solution. Experimental conditions: stationary phase, Phenomenex Gemini C18 (4.6 × 150 mm, 5 μm); mobile phase, a mixture of $NH_4H_2PO_4$ buffer and methanol at 40:60 (v/v); isocratic elution at a flow rate of 1.0 mL min^{-1}. Peak identification: 1, RXT.

v/v) of which concentrations varied at 25 mM, 125 mM, and 250 mM were compared. As displayed in *Fig.* 2, the retention times of RXT decreased, but the peak shapes deteriorated with tailing exacerbated as the buffer concentrations were lowered. These results suggest that the buffer concentration should be optimized carefully. Accordingly, the buffer concentration was selected as the influential factor to optimize using RSM.

3.3.2. Organic solvent type and concentration

Although acetonitrile was included in the mobile phase in the compendial methods, methanol was more effective in reducing peak tailing[4,15] and is economically favorable compared to acetonitrile. Accordingly, methanol was selected as the organic modifier, and its contents were varied between 60-70 % v/v, while maintaining the ammonium phosphate buffer concentration at 25 mM (pH 5.3). As the methanol content decreased, the retention times and the AF values increased (*Table* 1). In order to prevent potential buffer solubility problems, a relatively low methanol content, 63 % v/v was selected and used thereafter.

3.3.3. pH of the aqueous buffer

pH values of the aqueous buffer solutions differed among the compendial methods and literature. In this study, a wide range of pH (2.0-9.0) was tested to identify the most suitable pH, with the methanol content fixed at 63 % v/v. As seen in *Table* 1, the smallest degree of tailing was observed at pH 6.0 (AF=1.651), which was therefore fixed in the next experiments.

3.3.4 Type and concentration of mobile phase additive

The peak tailing problem was not resolved completely by altering a number of factors above, including the concentration and pH of buffer, and the organic modifier content. Thus, a number of common additives were tested to improve the peak shapes. Five additives, DEA, TEA, DTMA-Cl, TBA-OH, and sodium 1-octanesulfonate, were assessed at the

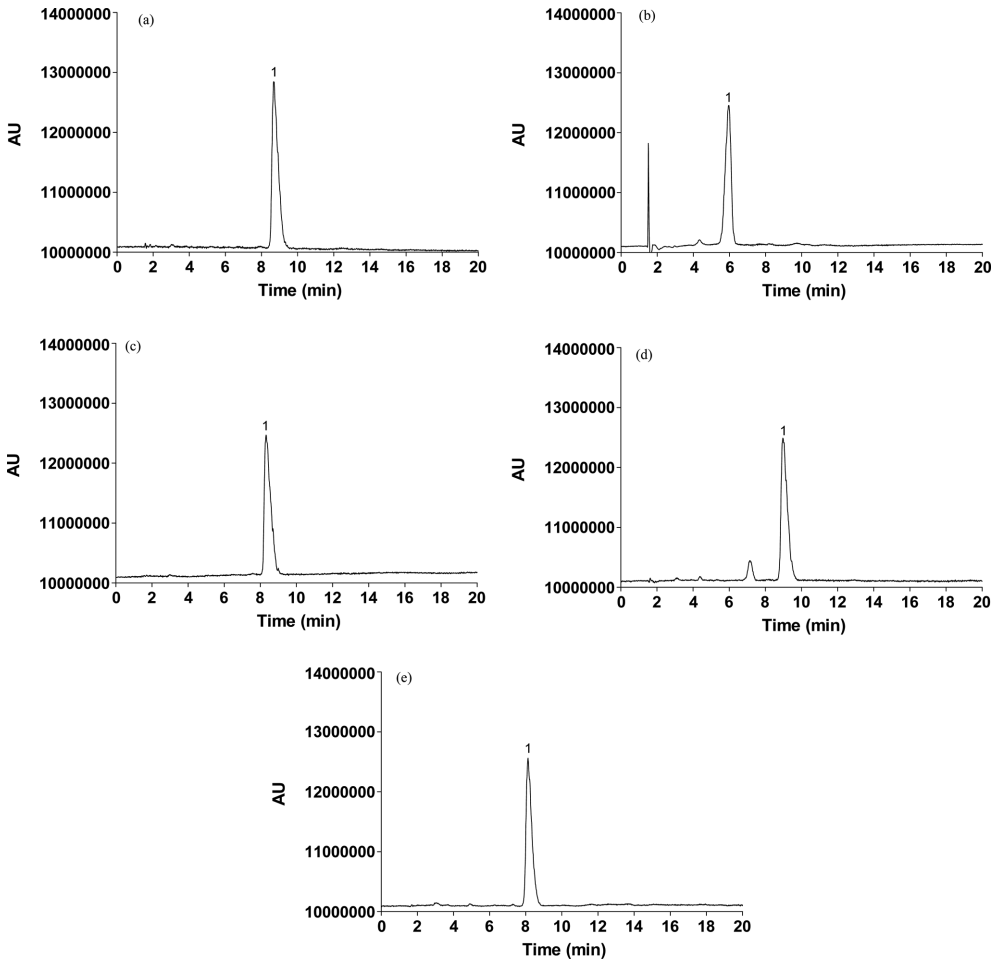

Fig. 3. HPLC-UV chromatograms of RXT standard analyzed using the mobile phase containing different types of additive. (a) 0.1% DEA; (b) 5mM DTMA-Cl; (c) 5 mM sodium 1-octanesulfonate; (d) 0.2% TEA; (e) 0.1% TBA-OH in the 25 mM $NH_4H_2PO_4$ buffer (pH 6.0). Experimental conditions: stationary phase, Phenomenex Gemini C18 (4.6 x 150 mm, 5 μm); mobile phase, a mixture of $NH_4H_2PO_4$ buffer and methanol at 37:63 (v/v); isocratic elution at a flow rate of 1.0 mL min^{-1}. Peak identification: 1, RXT.

lowest concentrations recommended by the suppliers, and the results are shown in *Fig.* 3. All of the additives significantly affected the retention behavior of RXT. Specifically, the retention times varied moderately, and the peak shapes were generally improved, although tailing resisted (*Fig.* 3(a), (c), (d), and (e)) or even fronting occurred (*Fig.* 3(b)). TBA-OH (0.1 % v/v) exhibited the lowest AF value (1.609) with the retention time at 8.12 min. Elevation of its concentration to 0.15 % lowered the AF value to 1.505 and slightly increased the retention time (8.78 min). These results suggest that the concentration of TBA-OH in the mobile phase could be an influential factor that needed further optimization.

3.4. Optimization of the important factors by RSM

Investigation of several numerical factors based on one-factor-at-a-time approach above led us to carefully optimize the two factors, the ammonium phosphate concentration and the TBA-OH concentration in the aqueous buffer solution. Besides these two factors, column temperature was also included as an additional factor to optimize based on our research experiences.

RSM is an efficient technique in experimental design to optimize multiple variables by conducting a minimum number of experiments. In this study, three-level Box-Behnken design (BBD) was adopted, which is considered an efficient alternative to central composite design (CCD) with a less number of experiments for three or more factors than CCD. As a result, three factors, buffer concentration (*A*), additive concentration (*B*), and temperature (*C*), with the AF value as the response, were applied to BBD, which resulted in 15 experiments including three replicates of the center point. The factors that varied between -1 to +1 coded levels were as follows: *A*, 25-75 mM; *B*, 0.0-0.6 %; *C*, 30-50 ℃. The resulting design matrix and the observed data are displayed in *Table* 2.

Table 2. Design matrix and the observed data performed according to Box-Behnken design

Buffer concentration (*A*, mM)	TBA-OH (*B*, %)	Column temperature (*C*, °C)	AF (observed response)
25 (-1)	0.3 (0)	30 (-1)	1.087
75 (+1)	0.3 (0)	50 (+1)	1.387
50 (0)	0.0 (-1)	50 (+1)	1.686
50 (0)	0.6 (+1)	30 (-1)	1.309
50 (0)	0.6 (+1)	50 (+1)	1.093
50 (0)	0.3 (0)	40 (0)	1.297
75 (+1)	0.3 (0)	30 (-1)	1.500
75 (+1)	0.6 (+1)	40 (0)	1.388
50 (0)	0.3 (0)	40 (0)	1.311
25 (-1)	0.6 (+1)	40 (0)	0.924
25 (-1)	0.3 (0)	50 (+1)	0.772
50 (0)	0.0 (-1)	30 (-1)	2.293
75 (+1)	0.0 (-1)	40 (0)	1.925
25 (-1)	0.0 (-1)	40 (0)	1.368
50 (0)	0.3 (0)	40 (0)	1.291

A polynomial quadratic equation was obtained to fit the established model (p=0.0031) and is given as follows:

$$\hat{y}(AF) = 1.30 + 0.26A - 0.32B - 0.16C - 0.023AB + 0.051AC + 0.098BC - 0.15A^2 + 0.26B^2 + 0.040C^2$$

According to ANOVA results of the model, all the three factors were significant ($p < 0.05$), and no significant interactions between the factors were detected. As can be inferred by the coefficients in the model equation and the response surface plots (*Fig.*

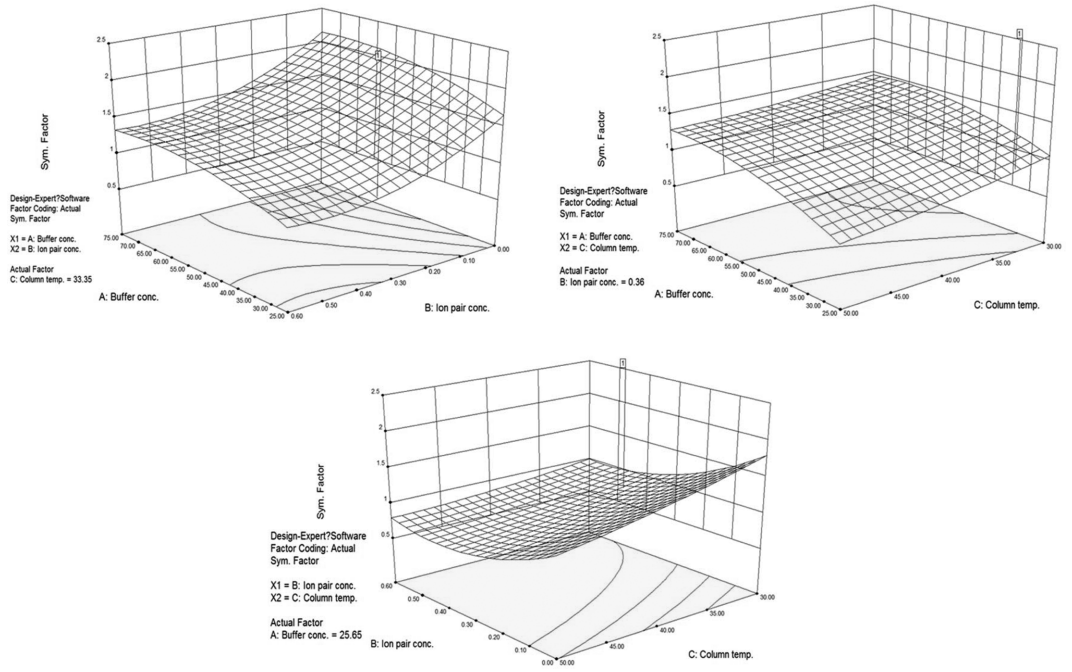

Fig. 4. Three dimensional response surfaces obtained from BBD. Factors: *A*, ammonium phosphate concentration (mM); *B*, TBA-OH concentration (%); *C*, column temperature (°C).

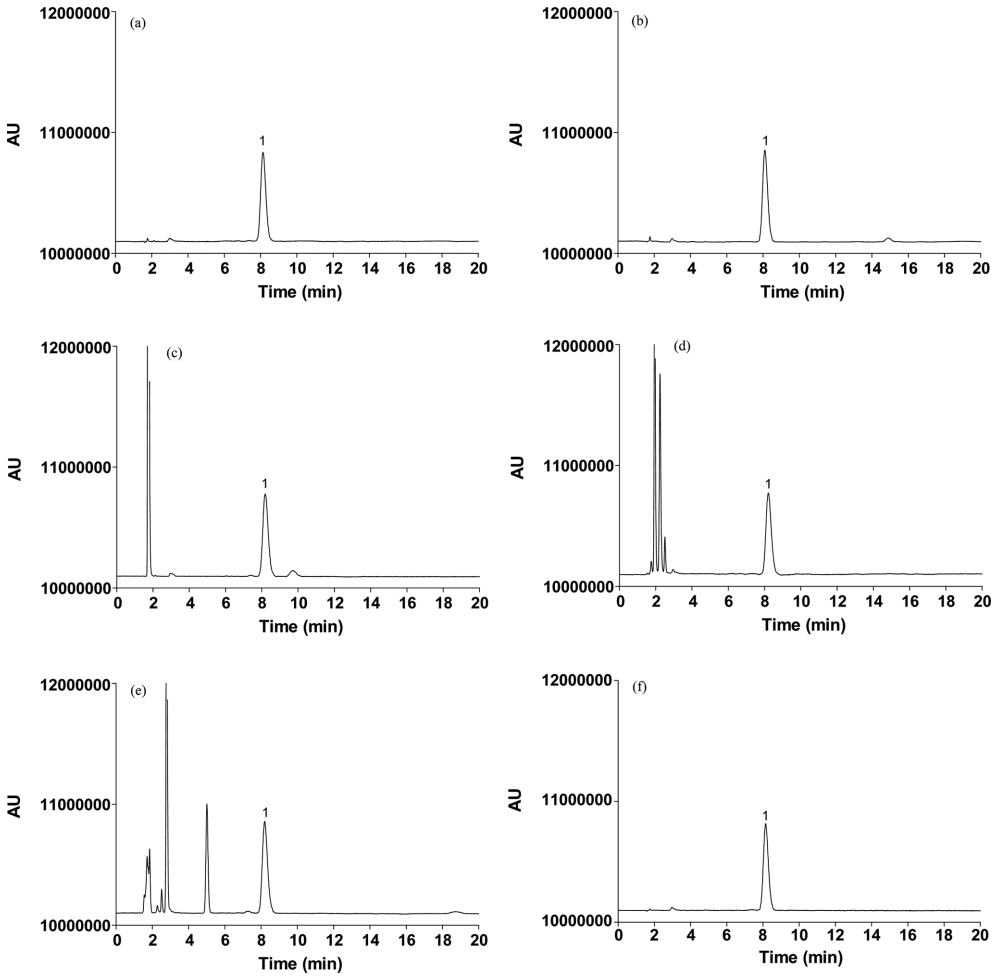

Fig. 5. HPLC-UV chromatograms of RXT analyzed using the optimized condition. (a) RXT standard, (b) bulk drug, (c) tablet for suspension, (d) granule, (e) suspension, (f) tablet. Peak identification: 1, RXT.

4), the TBA-OH concentration (*B*) was the most influential, followed by the buffer concentration (*A*). Increased TBA-OH concentration and decreased buffer concentration generally drove the AF values close to 1.0, and lower column temperatures were also favorable for suppressing the peak tailing. Optimized values determined from the established model were *A*, 25.95 mM; *B*, 0.36 %; and *C*, 33.32 °C. For convenience, practical experiments were conducted using an aqueous buffer solution (solvent A) containing 25 mM ammonium phosphate buffer and 0.3 % TBA-OH at the column temperature set at 30 °C. A representative chromatogram obtained under these conditions is displayed in *Fig.* 5(a), of which the AF value was measured 1.153. These results showed that the BBD-based RSM approach allowed us to efficiently optimize the factors and that the optimized conditions were effective and reproducible. The resulting chromatographic conditions are mild and practical, and the method could be universally applicable to all forms of RXT formulations.

3.5. Method validation-linearity, precision, accuracy, and system suitability

A calibration curve was established in the range of 400-600 mg mL^{-1} with the linear regression equation, y = 14926x – 3648 (n=3). The r^2 values were equal to or larger than 0.9990, supporting the linearity of the

Table 3. Assay precision and accuracy

	Sample type	Concentration[a]	Intraday (n=3)	Interday (n=3×3)
Precision (% RSD)	RXT standard	Low	0.63	0.72
		Middle	0.59	0.74
		High	0.34	0.56
		Concentration[b]	Recovery (%)	% RSD
Accuracy	Bulk drug	Low	97.72	0.45
		Middle	96.30	0.71
		High	98.26	0.92
	Granule	Low	97.06	0.54
		Middle	96.32	0.29
		High	97.39	0.46
	Tablet	Low	97.58	0.63
		Middle	98.08	0.47
		High	95.19	0.08
	Suspension	Low	103.49	0.37
		Middle	100.28	0.50
		High	99.58	0.34
	Tablet for suspension	Low	98.61	0.88
		Middle	97.47	0.16
		High	96.57	0.14

[a]400, 500, and 600 mg mL^{-1} for low, middle, and high concentrations, respectively.
[b]500, 550, and 600 mg mL^{-1} for low, middle, and high concentrations, respectively.

developed method. Intraday and interday precisions that were evaluated at low, middle, and high concentrations were lower than 0.63 % RSD and 0.74 % RSD, respectively (*Table* 3).

Recovery was used to evaluate the method accuracy, which was measured in the range of 96.30-98.26 %, 96.32-97.39 %, 95.19-98.08 %, 99.58-103.49 %, and 96.57-98.61 % for bulk drug, granule, tablet, suspension, and tablet for suspension formulations, respectively (*Table* 3). These results show that the current method is reasonably precise and accurate. Repeatability was measured 0.51 % RSD, which supports that the system suitability was acceptable.

3.6. Real sample monitoring

RXT bulk drug and marketed formulations were analyzed using the developed method. As shown in *Fig.* 5, the method yielded chromatograms displaying the RXT peak that was resolved well from interferences nearby, consistently eluting at the same retention time with similar intensities regardless of sample types. The RXT contents were determined to be 96.14-105.47 % of the label claimed (data not shown), all of which were within the required range for formulations by KP.

4. Conclusions

In this study, numerous factors that could affect the chromatographic performance for the RXT quantification, including column type, buffer concentration, type and concentration of organic solvent, buffer pH, and type and concentration of mobile phase additive, were systematically investigated. Then, three significant factors, buffer concentration, additive concentration, and column temperature were efficiently optimized by RSM with AF set as response. The resulting optimized conditions yielded the chromatographic analysis on a Phenomenex Gemini 5 μ C18 110A (150 × 4.60 mm, 5 μm) at 30 °C using the mobile phase consisting of 25 mM phosphate buffer (pH 6.0) with 0.3% TBA-OH and

methanol at a ratio of 37:63 (v/v). The validity of the developed method was demonstrated by linearity, precision, and accuracy, all of which met the requirement criteria, and could be universally applied to monitor marketed RXT products in various dosage forms. Based on these results, the developed method was adopted as the compendial methods in KP 11 by replacing the previous methods in KP 10 that involve the use of harsh and unharmonized chromatographic conditions among bulk drug and formulations.

Acknowledgements

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