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Development of a gradient method for sulfamethoxazole, trimethoprim, isoniazid, and pyridoxine hydrochloride in rabbit plasma through QbD-driven investigation

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The current study developed a method for quantifying four drugs—Sulfamethoxazole, Trimethoprim, Isoniazid, and Pyridoxine—in rabbit plasma. The method uses gradient liquid chromatography based on analytical quality by design. To achieve separation, a Eclip Plus C18 (250 mm × 5 mm, 4.6 µm) column with L1 packing was used, and analytes were detected at 254 nm at ambient temperature. The optimized mobile phase consisted of 50 mM potassium dihydrogen phosphate buffer (pH 6.5) and Methanol. The concentration of Methanol was 3% (0–5 min), 15% (5–15 min), 55% (15–27 min), and 3% Methanol until the end of the 30-min runtime, and the flow rate was set at 0.95 mL/min. Control Noise Experimentation was used to screen studies, revealing that flow rate, pH, and Methanol concentration significantly affected the analytical attributes. The study identified critical attributes (resolution and asymmetric factor) and developed a quality target method profile. A central composition design was used to optimize the essential parameters. The method developed for the drugs showed peaks at retention times of 6.990 min for Isoniazid, 7.880 min for Pyridoxine, 15.530 min for Sulfamethoxazole, and 26.890 min for Trimethoprim, respectively. The method was validated with linearity in the range of 10–640 ng ml^{-1}, with R^2 of 0.9993, 0.9987, 0.9993, and 0.9992 for Sulfamethoxazole, Trimethoprim, Isoniazid, and Pyridoxine, respectively.

Keywords Bioanalytical quality by design, Gradient elution, Sulfamethoxazole, Trimethoprim, Isoniazid, Pyridoxine

Abbreviations

SUL	Sulfamethoxazole
Trim	Trimethoprim
INH	Isoniazid
B6	Pyridoxine
UFLC	Ultra flow liquid chromatography
BQbD	Bioanalytical quality by design
CAA	Critical analytical attributes
QTMP	Quality target method profile
CNX	Control noise experimentation

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CMP	Critical method parameters
RPN	Risk priority number
C&E	Cause-and-effect
CCD	Central composition design
BBD	Box-Behnken design
RSM	Response surface methodology
DS	Design space
API	Active pharmaceutical ingredient
IAEC	Institutional Animal Ethical Committee
QC Level	Quality control level
CV	Coefficient of variation
SD	Standard deviation
LLOQ	Lower limit of quantification
LQC	Low quality control
MQC	Mid quality control
HQC	High quality control
%RE	Percentage of relative error
CV	Coefficient of variation
Factor A	Flow rate of the mobile phase
Factor B	Mobile phase pH
C	Organic modifier
R1	Response1
Rs	Resolution
R2	Response 2
As	Asymmetric factor
R1(SUL)	Resolution of sulphonamide
R1(TRIM)	Resolution of trimethoprim
R1(INH)	Resolution of isoniazid
R1(B6)	Resolution of pyridoxine
R2(SUL)	Asymmetric factor of sulphonamide
R2(TRIM)	Asymmetric factor of trimethoprim
R2(INH)	Asymmetric factor of isoniazid
R2(B6)	Asymmetric factor of pyridoxine

The HIV virus, which causes AIDS, is one of the world's most dangerous public health problems. Efforts are being made to stop the spread of HIV and guarantee treatment for all those living with the virus globally[1]. Recent statistics from UNAIDS indicate that there are more than 39 million HIV-positive people worldwide. Despite significant efforts, there is currently no cure for HIV/AIDS, and some individuals are still unaware of prevention, care, and treatment[2].

HIV targets CD4 + T cells and decreases their count, making individuals more susceptible to opportunistic infections[3]. Patients with low CD4 + levels or AIDS-defining illness are at higher risk of lethal infections caused by various pathogens, including Herpes simplex viruses, Cryptococcus neoformans, Pneumocystis jirovecii, and others[4]. High HIV prevalence has been linked to a wide range of opportunistic infections globally[5,6]. In India, TB is the most common opportunistic illness among HIV-positive individuals. Oral candidiasis, herpes zoster, cryptococcal meningitis, cerebral toxoplasmosis, and cytomegalovirus retinitis are some opportunistic infections that have been documented[7,8].

Patients with HIV/AIDS are treated with a combination of sulfamethoxazole (Sul), trimethoprim (Trim), isoniazid (INH), and pyridoxine (B6) to prevent opportunistic infections[9–11]. This treatment is highly effective in preventing tuberculosis, isosporiasis, pneumonia, and toxoplasmosis and has reduced mortality and hospitalizations among HIV/AIDS patients. It is an essential tool in the management of HIV/AIDS and plays a critical role in improving the quality of life for those suffering from this disease[12,13].

Analyzing four drugs-Sul/Trim/INH/B6—requires accounting for their varying polarities to develop a successful assessment method. The Ultra Flow Liquid Chromatography (UFLC)-UV method with gradient elution is necessary to ensure the retention of individual analytes with optimal peak shape. The technique plays a critical role in retaining variables and the peak shape of the analytes[14]. The composition of the mobile phase impacts the solute properties of the analytes, making it challenging to manage all variables through trial and error. Therefore, a structured process becomes crucial to attaining the desired outcomes. Applying the bioanalytical Quality by Design (BQbD) approach assists in identifying, understanding, and controlling Critical Method Parameters (CMP) that influence the quality of outcomes[15,16]. By integrating the gradient principle and BQbD in bioanalytical development through UFLC, a robust method capable of eliminating the need for revalidation and enhancing separation can be achieved, further improving the effectiveness of the bioanalytical method and making it a state-of-the-art process[17].

Using gradient elution in chromatography enhances the separation of four different polarities (Sul/Trim/INH/B6) with better peak resolution. Additionally, applying Quality by Design (QbD) analysis ensures the quality and safety of the bio analytical method.

Critical method parameter	Critical method attributes			Initial risk assessment scores	C,N,X	Experimental strategy
	Retention time	Asymmetric factor	Resolution			
Isocratic Binary Parameter	2	2	2	40	C	Calibrated
Flow Rate	10	10	10	300	X	DOE
Stationary Phase	5	5	5	100	C	New Column
Particle size	2	2	2	40	C	Optimum
Dimension	2	2	2	40	C	Standard
Column Temp	5	5	5	100	N	Ambient
Buffer pH	10	10	10	300	X	DOE
% organic Modifier	10	10	10	300	X	DOE
Solvent Grade	5	5	5	100	N	UFLC grade
Injection Vol	2	2	2	40	C	20µL
Flow Cell temp	5	5	5	100	C	40^0 C
PDA	5	5	5	100	N	Standard

Table 1. Control-noise-experimentation (CNX) approach. C-Control, N-Noise & X-Experiment Score Low Risk-2, Medium Risk-5 & High Risk- 10. Total Score = (Risk level of First CMA × 10) + (Risk level of Second CMA × 10).

Run	Factor :A	Factor :B	Factor :C	R1: Response1: Resolution (Rs)				R2: Response2 : Asymmetric factor (As)			
				R1(SUL)	R1(TRIM)	R1(INH)	R1(B6)	R2(SUL)	R2(TRIM)	R2(INH)	R2(B6)
1	0.95	6.5	13.0454	15.001	28.782	6.322	1.954	1.068	0.954	0.962	1.052
2	0.95	6.5	8	13.111	26.254	7.254	1.641	0.992	0.837	0.730	1.007
3	1.2	7.5	5	13.011	39.329	6.800	1.574	0.965	1.134	0.990	1.307
4	0.95	6.5	8	13.145	26.254	7.254	1.641	0.992	0.835	0.730	1.270
5	0.95	6.5	8	13.185	26.254	6.904	1.522	0.992	0.839	0.730	1.270
6	0.95	6.5	8	13.122	26.254	7.254	1.614	0.992	0.838	0.731	1.270
7	0.95	6.5	2.95462	12.233	29.053	8.52	1.769	0.882	1.086	1.111	1.097
8	1.37045	6.5	8	16.156	35.551	6.176	1.193	1.184	0.822	0.711	1.118
9	0.95	6.5	8	13.155	26.254	7.254	1.854	0.992	0.833	0.732	1.270
10	0.7	7.5	5	12.235	32.060	8.350	1.733	0.993	0.988	0.882	1.076
11	0.7	5.5	5	15.089	17.045	7.556	1.764	0.989	0.921	0.824	1.202
12	0.95	6.5	8	13.185	26.254	7.254	1.726	0.992	0.837	0.737	1.270
13	0.529552	6.5	8	13.322	25.296	7.198	1.936	1.075	0.988	0.884	1.065
14	0.7	7.5	11	15.025	27.083	7.556	1.550	1.302	0.938	0.831	0.892
15	0.95	8.18179	8	10.025	24.43	6.36	1.625	2.079	0.896	0.794	1.020
16	1.2	5.5	11	15.798	28.581	6.045	1.081	0.98	0.901	0.801	1.173
17	0.95	4.81821	8	11.232	19.421	6.34	1.745	1.973	1.019	0.964	0.780
18	1.2	5.5	5	12.252	23.176	6.547	1.543	0.993	0.964	0.867	1.173
19	0.7	5.5	11	14.755	17.845	6.629	1.149	0.865	0.971	0.857	1.108
20	1.2	7.5	11	16.555	33.022	6.557	0.928	1.114	0.926	0.82	1.190

Table 2. Experimental design: central composite design: 3 factors with 2 responses for SUL/TRIM/INH/B6. Factor A, Flow Rate of the mobile Phase; Factor B, Mobile phase pH; C, Organic Modifier; R1, Response1, Resolution (Rs), R2, Response2, Asymmetric factor (As); R1(SUL), Resolution of Sulphonamide; R1(TRIM), Resolution of Trimethoprim; R1(INH), Resolution of Isoniazid;R1(B6), Resolution of pyridoxine; R2(SUL), Asymmetric factor of Sulphonamide; R2(TRIM), Asymmetric factor of Trimethoprim; R2(INH), Asymmetric factor of Isoniazid; R2(B6), Asymmetric factor of pyridoxine.

Result

Bio analytical method Risk assessment by QbD

A Control-Noise-Experimentation (CNX) approach was utilized for SUL/TRIM/INH/B6 to identify the significant variables that impact the method's attributes[18–20]. This approach used a Cause-and-Effect (C&E) Risk Assessment matrix (Table 1).

Design of experiment

The Central Composite design (CCD) was used to enhance the quality and separation using Design Expert 13, as shown in Table 2[21]. All experiments were conducted randomly to minimize the impact of uncontrolled

	R1 (SUL)	p-Value	R1 (TRIM)	p-Value	R1 (INH)	p-Value	R1 (B6)	p-Value
Intercept	+13.05		+26.25		+7.18		+1.20	
A	+0.4180	0.1744	+3.4700	0.0005	−0.4029	0.0022	−0.0171	0.0619
B	−0.1970	0.5064	+3.9052	0.0002	+0.1557	0.1465	−0.1988	< 0.0001
C	+1.1001	0.0033	−0.4053	0.5649	−0.4020	0.0023	−0.2256	< 0.0001
AB	+0.5596	0.1650	−0.4574	0.6183	−0.0770	0.5644	+0.0641	0.0006
AC	+0.5814	0.1507	+0.4094	0.6552	+0.0770	0.5644	+0.0411	0.0069
BC	+0.3881	0.3233	−2.1900	0.0338	+0.0657	0.6218	+0.0554	0.0014
A^2	+0.8136	0.0152	+1.5022	0.0470	−0.0930	0.3566	+0.0152	0.0710
B^2	−0.6139	0.0519	−1.5014	0.0467	−0.3359	0.0058	−0.0869	0.0005
C^2	+0.4424	0.1430	+0.9687	0.1745	+0.0176	0.8583	+0.0571	< 0.0001

Table 3. ANOVA coefficients with p-values for response 1(Resolution). Factor A: Flow Rate of the mobile Phase; Factor B: Mobile phase pH; C: Organic Modifier; R1: Response1: Resolution (Rs), R2: Response2: Asymmetric factor (As); R1(SUL): Resolution of Sulphanilamide; R1(TRIM): Resolution of Trimethoprim; R1(INH): Resolution of Isoniazid;R1(B6): Resolution of pyridoxine.

	R2 (SUL)	p-Value	R2 (TRIM)	p-Value	R2 (INH)	p-Value	R2 (B6)	p-Value
Intercept	+1.00		+0.8300		+0.7318		+1.27	
A	+0.0063	0.9164	−0.0120	0.4057	−0.0143	0.4312	−0.0072	0.8534
B	+0.0531	0.3867	+0.0014	0.9212	−0.0070	0.6964	+0.1951	0.0077
C	+0.0464	0.4474	−0.0359	0.0266	−0.0367	0.0625	+0.2832	0.0001
AB	−0.0419	0.5969	+0.0197	0.2994	+0.0138	0.5607	+0.0176	0.7485
AC	−0.0061	0.9379	−0.0335	0.0931	−0.0262	0.2773	−0.0739	0.2049
BC	+0.0744	0.3549	−0.0300	0.1274	−0.0238	0.3231	+0.1931	0.0081
A^2	−0.0305	0.6047	+0.0251	0.0917	+0.0111	0.5295	−0.0631	0.1117
B^2	+0.2864	0.0005	+0.0451	0.0074	+0.0394	0.0433	+0.0415	0.5678
C^2	−0.0852	0.1669	+0.0672	0.0005	+0.0965	0.0002	+0.1607	0.0024

Table 4. ANOVA coefficients with p-values for Response 2 (Asymmetric Factor). Factor A, Flow Rate of the mobile Phase; Factor B, Mobile phase pH; C, Organic Modifier; R1, Response1, Resolution (Rs), R2, Response2, Asymmetric factor (As); R2(SUL), Asymmetric factor of Sulphanilamide; R2(TRIM), Asymmetric factor of Trimethoprim; R2(INH), Asymmetric factor of Isoniazid; R2(B6), Asymmetric factor of pyridoxine.

variables, as per the standard practice. This design facilitated optimizing and assessing main, interactions, and quadratic effects[22–24]. The impact of the factors on the dependent variables was examined using ANOVA. Tables 3 and 4 depicts the ANOVA coefficients with p-values for the SUL/TRIM/INH/B6 responses (Resolution and Asymmetric factor) and interprets the relationship between factors and responses[25,26].

Method validation[27]

Specificity

The technique employed successfully distinguished the peaks of plasma and interference from the peak of SUL/TRIM/INH/B6 gradually. This verified the specificity of the method, enabling the selective identification of four drugs in rabbit plasma[28]. Figure 1 is the representative chromatograms of specificity studies. As per the FDA bioanalytical guidelines, the acceptance criteria encompass blank and zero calibrators, which must be devoid of interference at the retention times of the analyte(s)[29].

Accuracy and precision

The developed method's accuracy and intra- and inter-day precision results are shown in Tables 5 and 6. The LLOQ, LQC, MQC, and HQC values meet the acceptable limit (RSD ≤ 15%) according to the international guideline, indicating that the established method is reliable and precise for quantifying each drug in rabbit plasma[30,31].

Linearity

This study aimed to establish a calibration curve standard for optimized chromatographic conditions using a 20 µL injection volume. The correlation between the peak area and the respective concentration created the calibration curve[32]. The data was analyzed using both unweighted and weighted linear regression, and the optimal weighting variables were selected based on Correlation Coefficient (R^2) and percentage relative error (% ΣRE), as shown in Table 7. The regression models were statistically significant, as evidenced by the SUL/TRIM/INH/B6 F-values, which were not disclosed in the text. The p-values for SUL/TRIM/INH/B6 are below

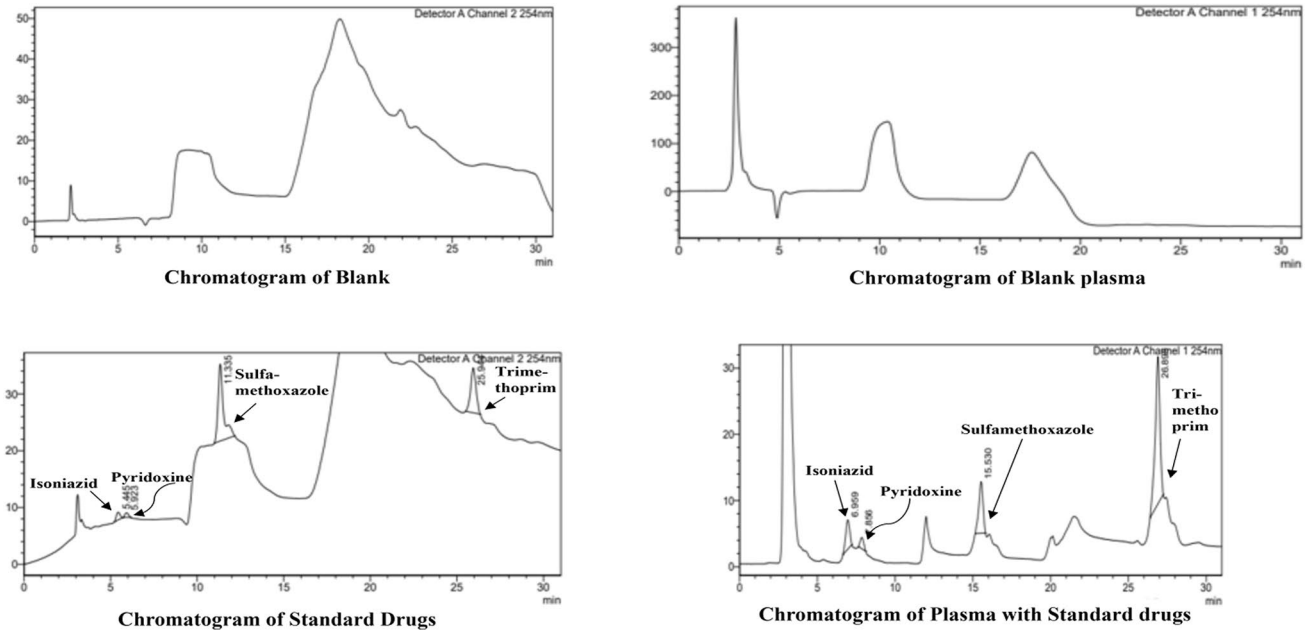


Fig. 1. Specificity study of SUL/TRIM/INH/B6.

0.05, respectively[33]. This indicates statistical significance at a 95% confidence level. These findings suggest that the current model is reliable for analyzing the calibration curve standard data. Acceptance Criteria include Non-zero calibrators should be ± 15% of nominal (theoretical) concentrations, except at LLOQ, where the calibrator should be ± 20% of the nominal concentrations in each validation run[34,35].

Recovery

Table 8 displays the percentage recoveries for the LQC, MQ, and HQC samples. A diluent (methanol: water, 7:3) was chosen as the extraction solvent to optimise recovery[36]. The percentage recovery achieved for each drug was sufficient to ensure an accurate and precise determination within the defined detection range[37].

Stability studies

Four investigational candidates' stability studies were conducted under various conditions. According to the acceptance criteria, the accuracy at each level should be within ± 15% of the nominal value[38]. The results are presented in Table 9.

Discussion

The method aimed to determine the levels of SUL/TRIM/INH/B6 present in rabbit plasma. The reverse phase chromatographic conditions, such as the flow rate, mobile phase composition, and mobile phase pH, were optimized to separate the eluted compounds adequately in gradient mode. Several peak parameters were optimized to obtain an appropriate separation of eluted compounds, including height, capacity, theoretical plates, tailing factor, and resolution.

The ChemAxon Log-D predictor (demo version) was used to generate the Log-D curve based on which column selection and appropriate mobile phase buffer pH range were established. The flat slope of the Log-D plot in the pH range of 9–13 for SUL revealed that the retention time of SUL was constant in this range. The curved Log-D plot value for TRIM in the given pH range of 2–4 and 9–11 demonstrated that the retention values of the peak were sensitive to even a modest pH change. The Log-D plot for INH was found to be a flat slope in the pH range of 5–11, revealing that the retention time for INH was constant in this range. Similarly, the Log-D plot value for B6 was curved in the given pH range of 3–6 and 8–11, demonstrating that the retention values of the peak were sensitive to even a modest pH change.

Furthermore, the pKa values were established to be 6.16 (strongly acidic) and 1.97 (strongly basic) for SUL, 17.33 (strongly acidic) and 7.16 (strongly basic) for TRIM, 13.61 (strongly acidic), 3.35 (strongly basic), 9.4 (strongly acidic), and 5.58 (strongly basic) for INH and B6, respectively. During method development, a pH range of 4–6.5 was studied for the choice of buffer and column, considering the physiochemical properties of the APIs. After analyzing the drugs with UV, it was found that the optimal wavelength for detection is 254 nm. This wavelength resulted in a better response for all four drugs.

After exploring various mobile phase compositions to elute title ingredients, the mobile phase system containing 50 mm Potassium dihydrogen Orthophosphate pH 6.5: Methanol with 1 ml/min flow rate in gradient mode was chosen. The Eclip Plus C18 column was considered suitable for chromatographic separation at ambient temperature with an injection volume of 20 µl and runtime of 30 min for all the solutions. The pH value 6.5 of the buffer was chosen for improved selectivity and sensitivity of the APIS. The isocratic mode could

QC Levels	Nominal conc	SUL (ng mL^{-1})			TRIM (ng mL^{-1})			INH (ng mL^{-1})			B6 (ng mL^{-1})		
		Mean conc ± SD	%CV	%RE	Mean conc ± SD	%CV	%RE	Mean conc ± SD	%CV	%RE	Mean conc ± SD	%CV	%RE
LLOQ	10	8.590 ± 0.259	3.020	−14.1	8.535 ± 0.422	4.952	−14.65	8.516 ± 0.5128	6.021	−14.833	8.596 ± 0.270	3.147	−14.033
LQC	30	27.236 ± 0.498	1.828	−9.21	27.645 ± 1.176	4.257	−7.85	27.583 ± 1.183	4.291	−8.055	27.243 ± 0.491	1.804	−9.188
MQC	200	186.990 ± 2.858	1.528	−6.505	191.023 ± 1.785	0.934	−4.486	184.2 ± 2.163	1.174	−7.900	186.960 ± 2.822	1.509	−6.520
HQC	600	578.813 ± 3.240	0.559	−3.531	581.673 ± 2.938	0.505	−3.0545	582.260 ± 4.028	0.691	−2.956	575.480 ± 5.474	0.951	−4.086

Table 5. Accuracy data of SUL/Trim/INH/B6 (n = 6). QC Level, Quality Control Level; CV, coefficient of variation; SD, Standard Deviation; LLOQ, Lower limit of quantification; LQC, Low Quality control; MQC, Mid Quality control; HQC, High Quality control; %RE, Percentage of Relative Error; CV, coefficient of variation; SD, Standard Deviation.

Analyte	QC Level	Nominal concentration (ng mL^{-1})	Intraday precision			Interday precision		
			Measured concentration (Mean ± SD)	% CV	% RE	Measured concentration (Mean ± SD)	% CV	% RE
SUL (ng mL^{-1})	LLOQ	10	8.596 ± 0.533	6.200	−14.033	8.616 ± 0.613	7.117	−13.833
	QC	30	26.600 ± 0.538	2.024	−11.333	26.666 ± 0.480	1.800	−11.111
	MQC	200	190.766 ± 2.970	1.557	−4.616	190.200 ± 2.163	1.137	−4.900
	HQC	600	583.800 ± 3.504	0.600	−2.700	583.133 ± 3.115	0.352	−2.811
TRIM (ng mL^{-1})	LLOQ	10	8.557 ± 0.444	5.194	−14.430	8.551 ± 0.434	5.076	−14.490
	LQC	30	27.637 ± 1.181	4.276	−7.874	27.647 ± 1.189	4.303	−7.842
	MQC	200	191.006 ± 1.813	0.949	−4.497	191.005 ± 1.803	0.944	−4.497
	HQC	600	588.664 ± 2.959	0.508	−3.055	581.611 ± 3.006	0.5168	−3.064
INH (ng mL^{-1})	LLOQ	10	8.520 ± 0.563	6.609	−14.800	8.563 ± 0.510	5.957	−14.336
	LQC	30	27.266 ± 1.549	5.682	−9.111	27.556 ± 1.296	4.703	−8.144
	MQC	200	184.200 ± 2.116	1.149	−7.900	184.003 ± 1.890	1.027	−7.998
	HQC	600	581.843 ± 3.430	0.589	−3.026	579.790 ± 3.499	0.603	−3.368
B6 (ng mL^{-1})	LLOQ	10	8.570 ± 0.468	5.469	−14.300	8.576 ± 0.378	4.414	−14.233
	LQC	30	27.276 ± 0.840	3.079	−9.077	27.273 ± 0.597	2.190	−9.088
	MQC	200	186.933 ± 3.453	1.847	−6.533	186.893 ± 3.490	1.867	−6.553
	HQC	600	575.446 ± 5.558	0.965	−4.092	575.220 ± 5.558	0.965	−4.070

Table 6. Intra and inter- day precision of SUL/Trim/INH/B6 (n = 6). QC Level, Quality Control Level; CV, coefficient of variation; SD, Standard Deviation; LLOQ, Lower limit of quantification; LQC, Low Quality control; MQC, Mid Quality control; HQC, High Quality control; %RE, Percentage of Relative Error; CV, coefficient of variation; SD, Standard Deviation.

Drug	Weight factor(w)	weighting least square linear regression			
		X	1/X	$1/\sqrt{x}$	$1/x^2$
SUL	R^2	0.9993	0.4705	0.6408	0.2750
	% RE	13.7850	5056.8000	67152.6140	30026.7900
TRIM	R^2	0.9987	0.5164	0.6859	0.3164
	% RE	18.8281	1606.5640	50.062	23746.0200
INH	R^2	0.9993	0.4682	0.6374	0.2751
	% RE	15.4101	751.3880	47.4311	25972.0800
B6	R^2	0.9992	0.4875	0.6556	0.2917
	% RE	1.6090	721.7670	46.2760	28023.1100

Table 7. Linearity data of SUL/TRIM/INH/B6 (n = 6). R^2, Correlation Coefficient; %RE, Percentage of Relative Error; QC Level, Quality Control Level; CV, coefficient of variation; SD, Standard Deviation; LLOQ, Lower limit of quantification; LQC, Low Quality control; MQC, Mid Quality control; HQC, High Quality control %RE, Percentage of Relative Error CV, coefficient of variation SD, Standard Deviation.

not solve peaks of the four drugs with proper Resolution (Rs) and Retention time (Rt); therefore, further method development was done using gradient mode.

This study employed the protein precipitation method with methanol as the best approach for extracting four drugs from rabbit plasma. The method is relatively simple and provides quick sample clean-up in plasma.

The CNX experiment has implemented a Cause and Effect metric approach for risk assessment to identify the Critical Method Parameters (CMPs) that facilitate quality separation and elution. Among the CMPs that significantly impact the method attributes are the Flow Rate of the mobile Phase (Factor A), pH of Mobile phase (Factor B) and the percentage of organic modifier at (0.01 to 5 min) (Factor C). The Risk Assessment has determined that the Critical Method Attributes are R1, Resolution (Rs), and R2, Asymmetric factor (As). To develop an optimized and efficient process, an investigation was conducted on the interaction between these essential attributes of the method and parameters, creating a design space.

In DOE, the resolution of sulfamethoxazole was significantly influenced by model terms C and A^2, with both terms having synergistic effects. Similarly, model terms A, B, BC, A^2, and B^2 significantly impacted Trimethoprim Resolution. The experimental design suggested that Trimethoprim Resolution could be affected by the antagonist effect of factors BC and B^2, while factors A, B, and A^2 had a synergistic impact. The resolution of Isoniazid was potentially affected by all three aspects, namely A, C, and B^2, with an antagonistic effect. Model terms B, C, AB, AC, BC, B^2, and C^2 significantly impacted Pyridoxine resolution. The experimental design

Analyte	QC Level	Nominal Concentration (ng mL^{-1})	% Recovery	
			Mean ± SD	%CV
SUL	LLOQ	10	8.526 ± 0.775	9.090
	LQC	30	26.433 ± 0.508	1.923
	MQC	200	186.570 ± 2.077	1.113
	HQC	600	577.603 ± 2.143	0.371
TRIM	LLOQ	10	8.515 ± 0.391	4.598
	LQC	30	27.568 ± 1.070	3.882
	MQC	200	190.972 ± 1.841	0.964
	HQC	600	581.306 ± 2.650	0.455
INH	LLOQ	10	8.566 ± 0.512	5.986
	LQC	30	27.483 ± 1.186	4.318
	MQC	200	184.080 ± 2.131	1.157
	HQC	600	580.526 ± 2.399	0.413
B6	LLOQ	10	8.506 ± 0.455	5.354
	LQC	30	27.196 ± 0.619	2.278
	MQC	200	186.833 ± 2.487	1.331
	HQC	600	575.050 ± 5.940	1.032

Table 8. Percentage recovery studies of SUL/TRIM/INH/B6 (n = 3). QC Level, Quality Control Level; CV, coefficient of variation; SD, Standard Deviation; LLOQ, Lower limit of quantification; LQC, Low Quality control; MQC, Mid Quality control; HQC, High Quality control; CV, coefficient of variation SD, Standard Deviation.

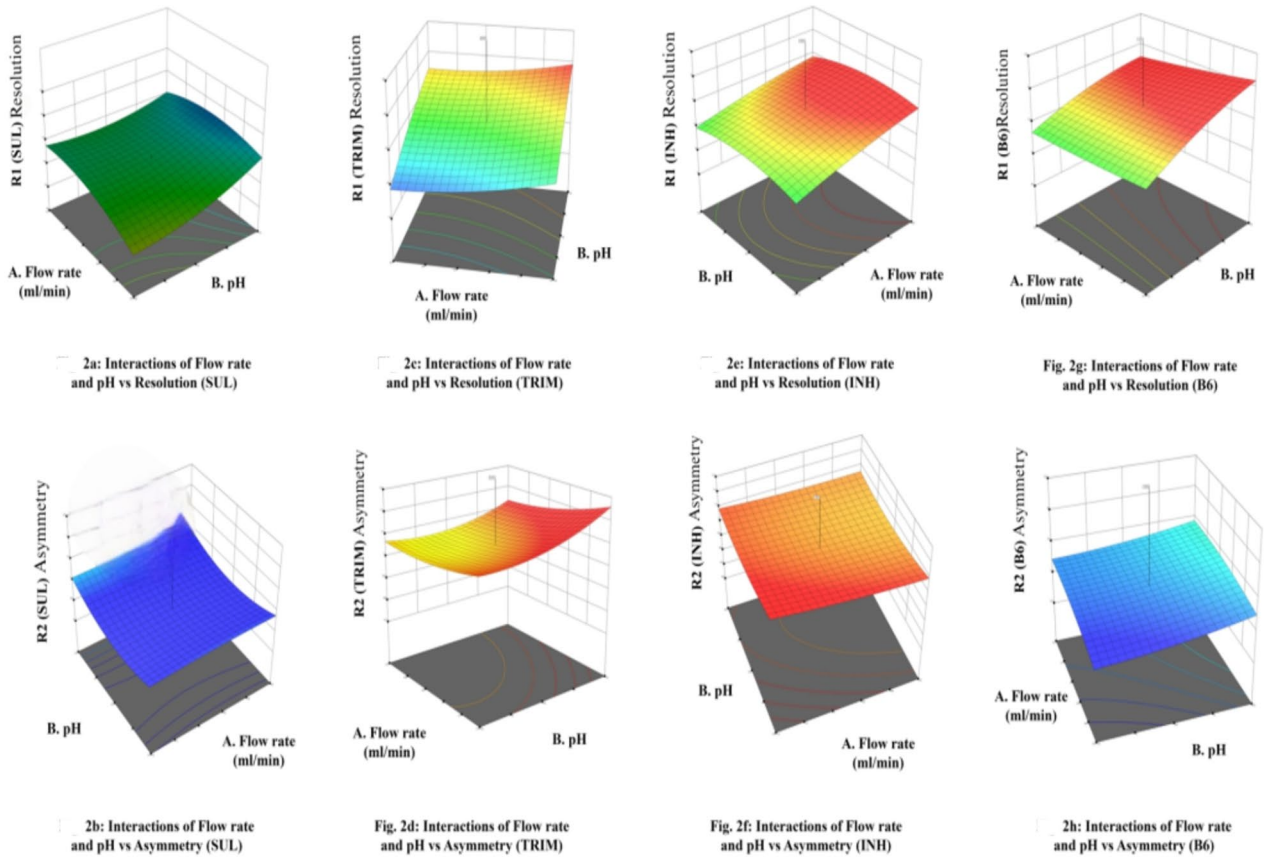
indicated that Pyridoxine resolution could be substantially influenced by the antagonist effect of factors B, C, and B^2. In contrast, the synergistic effect of factors AB, AC, BC, and C^2 was not more significant than 0.1000. The significant model term in the sulfamethoxazole Asymmetric factor was B^2, which had a synergistic effect. Model terms C, B^2, and C^2 substantially impacted the Trimethoprim Asymmetric Factor. The experimental design suggested that Isoniazid Resolution could be affected by the antagonist effect of factor C and the synergistic effect of polynomial terms B and C. For the Isoniazid Asymmetric factor, model terms B^2 and C^2 had a synergistic effect. Similarly, model terms B, C, BC, and C^2 significantly impacted the Asymmetric factor of pyridoxine. The experimental design indicated that the pyridoxine Asymmetric factor could be substantially influenced by the antagonist effect of all four factors. Concerning R^2 and Adjusted R^2 values, the present study found that sulfamethoxazole, Trimethoprim, Isoniazid, and pyridoxine had R^2 and Adjusted R^2 values of 0.9010, 0.9879, 0.9319, 0.9952 and 0.9220, 0.9869, 0.9807, 0.9890, respectively. For the Asymmetric factor, the Coefficient of determination R^2 and Adjusted R^2 values were found to be 0.9651, 0.9315, 0.9120, 0.9397 and 0.9538, 0.9799, 0.9429, 0.9622 for Sulfamethoxazole, Trimethoprim, Isoniazid and pyridoxine, respectively. An adjusted R2 value of ≥ 0.80 showed a strong correlation between the experimental data and the fitted model[39]. The adequate precision was 7.436 (SUL), 11.739 (Trim), 7.777 (INH), 46.510 (B6) for resolution and 7.945 (SUL), 6.934 (Trim), 7.320 (INH), 12.554 (B6) for asymmetric factor. These values indicated an adequate signal. The Coefficient of variance (C.V.), which measures the model reproducibility, was found to be 3.830 (SUL), 5.350 (TRIM), 5.290 (INH), 2.990 (B6) for resolution and 4.350 (SUL), 5.530 (Trim), 7.770 (INH), 7.130 (B6) for Asymmetric factor. A Coefficient of variance (C.V.) value of < 10% is considered reasonably reproducible[40]. Therefore, the model was considered significant for undergoing the separation.

Response Surface Methodology (RSM) was employed to study the influence of individual variables on responses[41]. Figure 2 depicts the relationship between selected responses and variables in 3D contour plots for Sulfamethoxazole, Trimethoprim, Isoniazid, and Pyridoxine. The independent variables were analyzed for their impact on the individual responses using RSM The 3D-response surface plot of Sulfamethoxazole showed a linear increase in resolution with an increase in flow rate and a decrease in the pH of the mobile phase. The asymmetric factor is maximum when the flow rate is minimum and the mobile phase pH is lowest. The 3D-response surface plot of Trimethoprim demonstrated that Trimethoprim resolution increases with a decrease in flow rate and an increase in pH of the mobile phase. The asymmetric factor for Trimethoprim will be at its maximum if the flow rate and mobile phase PH are increased. The 3D-response surface plot of Isoniazid depicted that resolution increases with a decrease in the flow rate and pH of the mobile phase. The response plot showed that the asymmetric factor of Isoniazid increases with a reduction in flow rate and mobile phase pH. The 3D-response surface plot of Pyridoxine showed a linear increase in resolution with an increase in flow rate and pH of the mobile phase. The plot showed a linear decline in the asymmetric factor with decreased flow rate and mobile phase pH.

The software "Design Expert" visualizes each factor's impact on the separation process through multi-dimensional plots. This approach entails establishing and verifying acceptable ranges for different parameters of the analytical process under defined conditions. As a result, a dependable and robust analytical process that consistently provides accurate results is obtained[42]. Using three factors, namely flow rate, mobile phase pH, methanol concentration up to five minutes during a 30-min run time, and three responses, namely retention

Stability	Measured concentration (Mean ± SD)					% CV				%RE			
	Nominal Concentration (ng mL^{-1})	SUL	TRIM	INH	B6	SUL	TRIM	INH	B6	SUL	TRIM	INH	B6
0 h	10	8.563 ± 0.639	8.562 ± 0.445	8.620 ± 0.556	8.593 ± 0.272	7.468	5.197	6.455	3.168	−14.360	−14.370	−13.800	−14.060
	30	26.566 ± 0.523	27.704 ± 1.124	27.556 ± 1.296	27.276 ± 0.840	1.938	4.058	4.703	3.079	−11.440	−7.650	−8.140	−9.077
	200	190.27 ± 2.510	191.02 ± 1.786	184.546 ± 2.120	186.966 ± 3.426	1.319	0.935	1.149	1.832	−4.865	−4.486	−7.720	−6.516
	600	583.436 ± 2.79	583.436 ± 2.95	582.293 ± 4.110	575.616 ± 5.546	0.477	0.508	0.705	0.980	−2.706	−3.055	−2.950	−4.063
Bench Top (24 h) Rt	10	8.560 ± 0.871	8.559 ± 0.443	8.596 ± 0.505	8.580 ± 0.383	10.18	5.176	5.876	4.465	−14.400	−14.40	−14.030	−14.200
	30	26.433 ± 0.515	27.637 ± 1.198	27.516 ± 1.241	27.240 ± 0.623	1.981	4.337	4.511	2.289	−11.800	−7.870	−8.270	−9.200
	200	190.04 ± 2.612	190.975 ± 1.830	184.443 ± 1.946	186.770 ± 2.339	1.374	0.958	1.055	1.252	−4.980	−4.510	−7.770	−6.615
	600	583.436 ± 3.18	581.56 ± 3.024	581.626 ± 2.089	574.810 ± 5.474	0.545	0.520	0.359	0.952	−2.760	−3.072	−3.060	−4.198
Freeze and Thaw (− 80 °C for 3 three cycles)	10	8.530 ± 0.416	8.522 ± 0.394	8.559 ± 0.580	8.553 ± 0.502	4.885	4.633	6.785	5.874	−14.700	−14.770	−14.400	−14.46
	30	26.363 ± 0.560	27.618 ± 1.218	27.446 ± 1.141	27.130 ± 0.588	2.124	4.411	4.159	2.170	−12.120	−7.940	−8.511	−9.566
	200	189.79 ± 1.978	190.77 ± 1.830	184.336 ± 1.977	186.373 ± 3.694	1.042	0.959	1.072	1.982	−5.101	−4.610	−7.831	−6.813
	600	582.43 ± 2.242	581.263 ± 2.67	581.056 ± 3.766	574.770 ± 5.686	0.385	0.460	0.648	0.989	−2.927	−3.122	−3.157	−4.205
Long term (−80° 45 days)	10	8.51 ± 0.434	8.517 ± 0.390	8.516 ± 0.512	8.503 ± 0.489	5.109	4.589	6.021	5.756	−14.900	−14.826	−14.830	−14.96
	30	26.03 ± 0.020	27.564 ± 1.081	27.346 ± 1.360	27.030 ± 0.588	0.076	3.924	4.975	2.178	−13.230	−8.117	−8.844	−9.900
	200	188.796 ± 1.978	190.506 ± 1.64	184.110 ± 2.191	186.373 ± 3.786	1.047	0.861	1.190	2.031	−5.601	−4.747	−7.945	−6.813
	600	581.77 ± 1.955	580.863 ± 2.42	580.526 ± 2.399	574.043 ± 6.058	0.336	0.416	0.413	1.055	−3.038	−3.189	−3.245	−4.326

Table 9. Stability of analytes in rabbit plasma in four QC level (n = 6). QC Level, Quality Control Level; CV, coefficient of variation; SD, Standard Deviation; LLOQ, Lower limit of quantification; LQC, Low Quality control; MQC, Mid Quality control; HQC, High Quality control; %RE, Percentage of Relative Error; CV, coefficient of variation SD, Standard Deviation.



2a: Interactions of Flow rate and pH vs Resolution (SUL)

2c: Interactions of Flow rate and pH vs Resolution (TRIM)

2e: Interactions of Flow rate and pH vs Resolution (INH)

Fig. 2g: Interactions of Flow rate and pH vs Resolution (B6)

2b: Interactions of Flow rate and pH vs Asymmetry (SUL)

Fig. 2d: Interactions of Flow rate and pH vs Asymmetry (TRIM)

Fig. 2f: Interactions of Flow rate and pH vs Asymmetry (INH)

2h: Interactions of Flow rate and pH vs Asymmetry (B6)

Fig. 2. Interference study of SUL/Trim/INH/B6.

time, resolution, and asymmetric factor, a two-dimensional design space (DS) has been created[43], as shown in Fig. 3. The 2D contour plots in this space highlight the design space for resolution and the asymmetric factor, represented by the shaded red region. This region indicates the robustness of the method and the results that meet the predefined criteria[44]. The primary objective was to minimize asymmetric factors and maximize the resolution of the symmetrical peak[45]. Derringer's desirability function (D) is thus an appropriate strategy where several responses are available to optimize with various targets[46]. The maximum desirability function's response surface plot (D = 1) is shown in Fig. 3, demonstrating the mathematical model's effectiveness. The coordinates result in a maximum desirability value at a mobile phase flow rate of 0.95 ml min^{-1}, a mobile phase of pH 6.5, a concentration of 3% V/V methanol from initial to five minutes, and an exact concentration of methanol from twenty-seven to thirty minutes.

The final chromatographic conditions employed for the separation of analytes. A Eclip Plus C18 column (250 mm × 5 mm × 4.6 µm, L1 packing) was utilized at a 0.95 ml/min flow rate. The optimized mobile phase consisted of a 50 mM potassium dihydrogen phosphate buffer mixture with pH 6.5 and methanol. The methanol concentration was set to 3% from the start to five minutes of runtime, 15% from five to fifteen minutes, and 55% from fifteen to twenty-seven minutes. The column was re-equilibrated with 3% methanol concentration until 30 min of runtime. The analytes were detected at 254 nm at ambient temperature for 30 min. The chromatogram showed the peaks of the drugs at retention times of 5.309 min for Isoniazid, 5.787 min for Pyridoxine, 11.317 min for Sulfamethoxazole, and 25.150 min for Trimethoprim, respectively.

Method

Material

The SUL/TRIM/INH/B6 compound (purity ≥ 99.00%) was acquired from Yarrow Chem Products at Swastik disa corporate park, opp. Shreyas Talkies, lbs Road, Ghatkopar (west), Mumbai-India. The solvents utilized in the experiment, including Acetonitrile (UFLC grade), Methanol (UFLC grade), Ortho phosphoric acid (UFLC grade), and Formic acid (UFLC grade), were obtained from s d fine chem limited at 1502, marathon Icon, Marathon Nextgen veer santaji marg, Lower Parel, Mumbai India. Potassium dihydrogen orthophosphate, NaOH (AR grade), was procured from Thermo Fisher Scientific India Pvt. Ltd. Navi Mumbai, India. Type-I water employed in the study was obtained from the Millipore Ultrapure Water Purification System (AnaMatrix, Chamrajpet, Bengaluru – 560018, Karnataka, India).

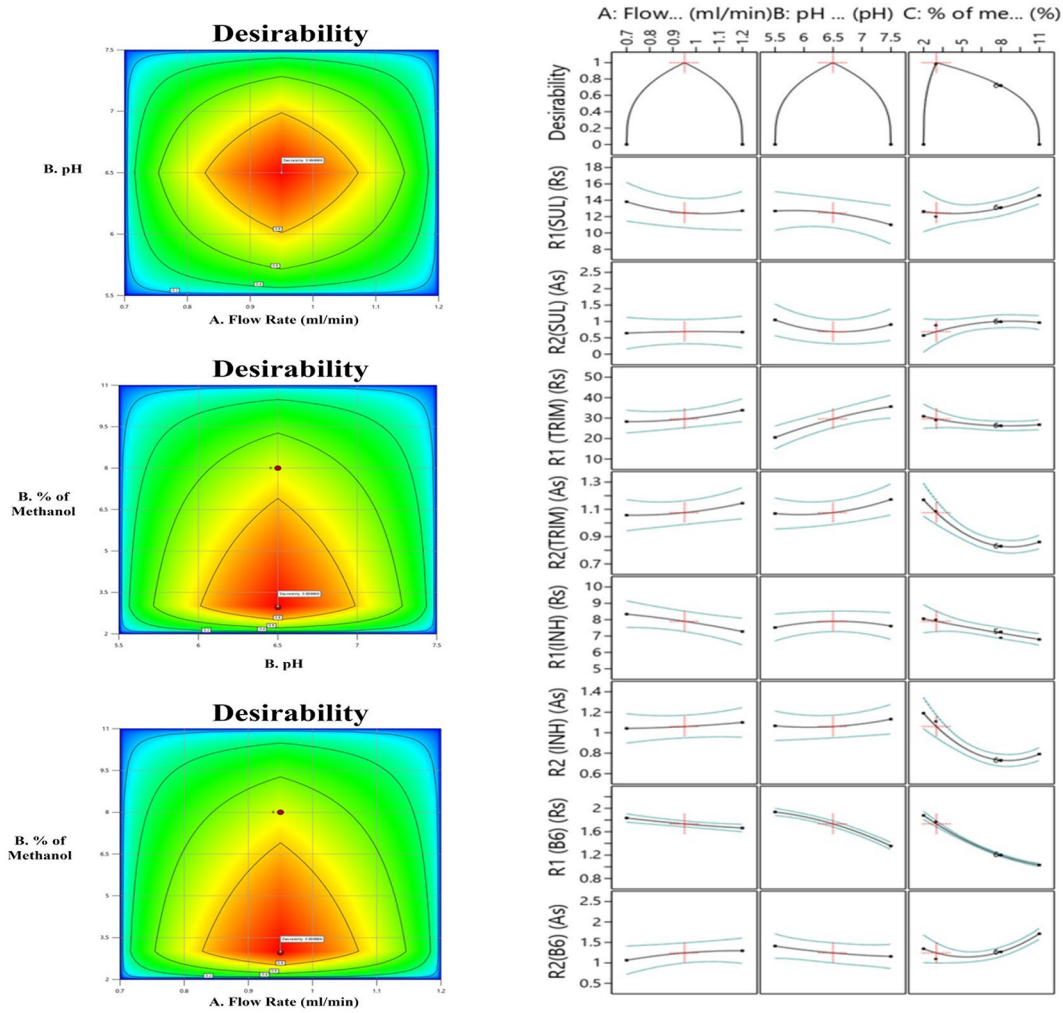


Fig. 3. Design Space and Derringer's desirability function of SUL/Trim/INH/B6.

Instrument and software

The chromatographic separation process has been performed using Prominence LC-20A, Quaternary Gradient UFLC from Shimadzu, Japan. The UFLC integrated software (LC Realtime Analysis) was employed for integration and data processing purpose. The development of the chromatographic method was aided by a weighing balance (ACCULAB, Sartorius, Bangalore, India) and digital pH meters (MK VI, Systronics, Ahmedabad, Gujarat, India).

Experimental animal

The study used New Zealand White rabbits weighing between 1.5 and 2.5 kg. These animals were kept in the central animal facility at Sri Adichunchanagiri College of Pharmacy, B G Nagara, and were provided with standard laboratory conditions, that is, 22 ± 2 °C temperature and 12 h cycle of light and dark. They were also given a standard pellet diet and unrestricted water access. The Institutional Animal Ethics Committee (IAEC) at Sri Adichunchanagiri College of Pharmacy, B G Nagara, approved the experimental protocols (Approval Number: SACCP-IAEC/2020/01/28). Blood samples were obtained from the ear veins of rabbits (which require no anaesthetising) and subjected to centrifugation to extract Drug-free plasma. The animals used in this study were not euthanized and sacrificed at any point. Instead, a single blood sample was collected from each animal for the express purpose of isolating plasma. All procedures were implemented according to the CPCSEA guidelines for laboratory animal facilities, and the ARRIVE guidelines reported the study.

Statistical design

The Design of Experiments (DOE) uses statistical principles to control and estimate the association between factors and the output. It involves randomization, replication, and blocking techniques. "Central Composite Designs" (CCD) and "Box-Behnken" design (BBD) are famous for optimizing processes in experimental design under the Response Surface Methodology (RSM)[47]. RSM helps understand the relationship between factors and responses. The CCD is employed to estimate a quadratic model. This design is derived from a two-level factorial design, incorporating centre and axial points. It provides exceptional predictive capability within the design space's central region, often referred to as the bullseye. When comparing BBD and CCD, it is essential

to note that BBD involves fewer design points than the axial points of CCD, resulting in fewer experiments. Additionally, CCD tests are conducted under extreme conditions, making them better suited for quadratic models[25]. The Central Composite Design (CCD) was used to generate and test the data with the help of the trial version of Design-Expert® 13 software.

Risk assessment

The primary goal of the risk assessment (RA) is to identify the factors influencing critical quality attributes (CQA). Risk can be expressed quantitatively as a risk priority number (RPN) or qualitatively as 'high', 'medium', and 'low'. In addition, 'risk scores' further describe the descriptors for ranking. The assessment of CQAs is crucial for evaluating the quality of the developed method[48]. The risk assessment tools utilized in quality by design (QBD) play a vital role in assessing and managing potential risks associated with pharmaceutical development and manufacturing processes. These tools assist in identifying and prioritizing risks, determining the level of risk for each identified factor, and implementing appropriate controls and mitigation strategies. The methods for determining risks include: (1) Control-noise-experimentation, or cause-effect approach, (2) Failure mode effect analysis, (3) Failure mode effect and criticality analysis, (4) Fault tree analysis, risk ranking and filtering, and ishakaba diagramatic tool. This study employed the control-noise-experimentation (CNX) tools to screen the risk factors in method development.

Stock solution preparation

A solution containing SUL/TRIM/INH/B6 was prepared by dissolving approximately 10 mg of the compound in methanol: water (7:3) to create a stock solution at 1000 µg mL^{-1} concentration. Working solutions were then prepared by diluting the stock solution with the same solvent system. The working and stock solutions were stored in a cold environment, maintained at a temperature range of 2–8 °C[49].

Preparation quality control sample

A solution was prepared by combining a 200 µL working standard, 200 µL plasma, and 600 µL methanol as a protein precipitating agent. This solution contains four analytes (SUL/TRIM/INH/B6). The concentrations of the combination were set at 10, 20, 40, 80, 120, 160, 240, 320, and 640 ng mL^{-1}, respectively. Quality control solutions for Lower limit of quantitation (LLOQ, 10 ng mL^{-1}), Low Quality Control sample (LQC, 30 ng mL^{-1}), Medium Quality control sample (MQC, 200 ng mL^{-1}), High Quality Control sample (HQC, 600 ng mL^{-1}) of SUL/TRIM/INH/B6 in combination were prepared using the working standard solution. All solutions were vortex-mixed for one minute and then centrifuged at 5000 rpm at 4 °C for five minutes. The clear supernatant underwent filtration through a 0.22 µm pore size and was subsequently sonicated for five minutes before the analysis.

Preparation of plasma sample

The bioanalytical samples were prepared by adding 200 µL of drug solution and 600 µL of methanol to 200 µL plasma (blank) in 1.5 ml Eppendorf tubes. The solutions were vigorously mixed for a minute and then centrifuged at 5000 rpm for five minutes at 4 °C[50]. Then, clear supernatant liquid went through filtration by a 0.22 µm membrane filter[51,52].

Initial chromatographic condition

Chromatographic separation process of four investigational candidates was executed on a C18 Eclipse column (250 mm × 5 mm × 4.6 µm) through a mobile phase flow rate of 1.00 mL/min. The optimal composition for symmetrical peaks with a peak purity index of 0.999 in gradient elution was obtained by incorporating methanol into the mobile phase containing a mixer 50 m M Phosphate Buffer (pH 6.5). The concentration of methanol was 8% from the initial to five minutes. From six to fifteen minutes, the percentage of methanol in the mobile phase was fifteen cents, steeply increasing to thirty-five cents up to twenty-seven minutes. Finally, the mobile phase was re-equilibrated with eight per cent up to thirty minutes. Both analytes were detected at 254 nm with a 30-min runtime at ambient temperature.

Method validation

The proposed method has been evaluated for specificity, linearity, accuracy, precision, recovery and stability according to the US FDA Bioanalytical Technique Validation guideline[27,53].

Conclusion

The QbD approach has been implemented successfully to develop a robust method for estimating Sulfamethoxazole, trimethoprim, Isoniazid, and Pyridoxine in rabbit plasma. The physicochemical properties of these drugs were considered while selecting input variables for the Design of the Experiment using a Central Composite Design. The concentration of Sulfamethoxazole, trimethoprim, Isoniazid, and Pyridoxine was not considered a quantitative variable in this design due to their narrow concentration range. Qualitative variables such as mobile phase pH, % organic modifier mobile phase & flow rate were considered and controlled. Each step of the Bioanalytical QbD process has been studied to determine the Design Space. Response surface plots graphically illustrated the significant effects of mobile phase pH, % organic modifier mobile phase, and flow rate on the separation. Using the QbD approach, the method's robustness was established even before validation. The process was validated for accuracy and precision, and the results were highly satisfactory. The technique is cost-effective but also linear, precise, and accurate. The entire process of method development was rationalized by the BQbD approach through an empirical approach. Various experimental designs were applied to the screening and optimization of factor values, helping to understand the variability in responses. The selection of optimum

conditions was justified, leaving no scope for uncertainty. The BQbD approach aided in upholding the method's quality and performance by establishing a design space. It demonstrated method conditions having flexibility and high reliability. Validation further added to the reliability of the process for the intended purpose. Thus, the BQbD approach is proposed to be rational, safe, and reliable over regular method development techniques. Using the QbD approach has resulted in a highly reliable and effective method for estimating the four drugs in rabbit plasma.

Data availability

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

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Competing interests

The authors declare no competing interests.

Additional information

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