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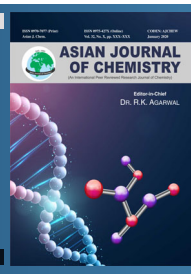

Design of Experimental Approach to Analytical Robustness Study for UHPLC Method Developed for Separation and Quantification of Spironolactone and its Impurities in Drug Substances

Y.V.S. VEERENDRA[1,2], PRADEEP KUMAR BRAHMAN[1,*], SHARAD D. MANKUMARE[2], JAYA RAJU CH[2] and J. SATISH[2]

[1]Electroanalytical Lab, Department of Chemistry, Koneru Lakshmaiah Education Foundation, Vaddeswaram-522502, India
[2]USP-India Private Limited, Hyderabad-500101, India

*Corresponding author: E-mail: veerendrayvs@gmail.com

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The present work demonstrates the development of an optimal, robust, validated UHPLC method for quantification of related impurities and assay determination of spironolactone. Design of experiment procedure, in combination with statistical evaluation of the data was used to test the robustness of developed method. A stability indicating method was established by forced degradation experiments. Analytical robustness was determined using design of experiment approach. The chromatographic separation was achieved with Agilent SB-C18 RRHD column using gradient elution with mobile phase-A consists of a mixture of 0.1 % each formic acid and ammonia in water and methanol as mobile phase-B respectively. The developed method is exhaustively validated for parameters like precision, accuracy, linearity, LOD, LOQ, ruggedness and robustness. The stability tests were also performed on drug substances as per ICH norms. Base line separation was achieved for all impurities, degradation products and the API. All impurities were eluted within 12 min, there was a remarkable 3.5-fold decrease in runtime and a clear baseline separation between all peaks in comparison with Ph.Eur monograph. A multi-dimensional design space was built to study the robustness of developed method using design expert software. Significant parameters such as effect of flow rate, buffer strength and mobile phase compositions were optimized at three levels. Plackett-Burman design was applied for screening of chromatographic conditions and factorial design was applied for optimization of essential factors in robustness studies.

Keywords: Spironolactone, Design of experiments, UHPLC, Stability indicating method, Robustness, Plackett-Burman design.

INTRODUCTION

Spironolactone (Spiro) (17-hydroxy-7α-mercapto-3-oxo-17α-pregn-4-ene-21-carboxylic acid γ-lactone acetate) is a synthetic steroid and a potassium sparing diuretic used to treat a condition in which there is a too much aldosterone in the body. Aldosterone is a hormone produced by adrenal glands to regulate the salt and water balance in body. Spironolactone also treats fluid retention (edema) in people with congestive heart failure, cirrhosis of the liver, or a kidney disorder called nephrotic syndrome. It is also used to treat or prevent hypokalemia (low potassium levels in the blood). Spironolactone is one of the essential medicines as per 19th WHO Model List of Essential Medicines (April 2015) [1]. Chemical structure of spironolactone and its impurities are given in Fig. 1.

Optimization of chromatographic parameters for the separation of impurities/compounds from a complex mixture is very difficult due to their similar chromatographic behaviours. Impurities must be monitored carefully to ensure the quality of active pharmaceutical substances. There are so many sources for impurities like synthesis related, method related, environmental related, degradation related impurities, *etc*. The presence of these impurities may affect the purity or quality of the active pharmaceutical ingredient (API) or drug substance. Control of impurities or impurity profiling is an important task while developing analytical method. The different pharmacopoeias and other regulatory authorities place several regulatory requirements to emphasize on the purity requirements and the identification of impurities in active pharmaceutical ingredient's (API's). A number of recent articles have provided guidance for the separating and identifying process-related impurities and degradation products using traditional chromatographic methods such as HPLC, GC and spectroscopic techniques, either alone or in combination with other techniques. There

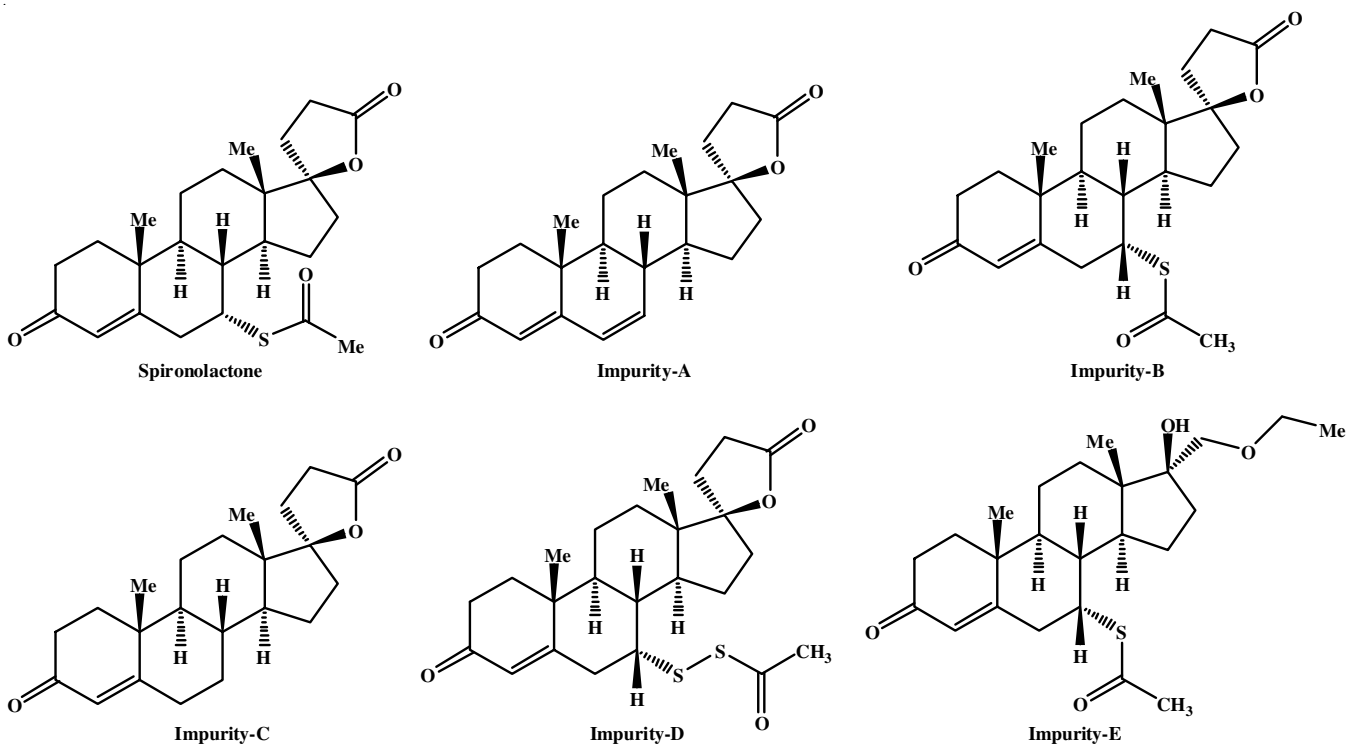

Fig. 1. Structure of spironolactone and its impurities A, B, C, D and E

are many factors, including basic parameters (*e.g.* material nature, solution stability), internal parameters (*e.g.* method parameter) and external parameters (*e.g.* environmental factors, instrument models, quality of reagents and technical skill of analysts), which should be considered when developing a robust analytical method.

Any analytical method developed tests some of the characteristics (like assay, impurities, *etc.*) of drugs (products, substances, *etc.*) against its specifications and the developed method should meet the requirements for the intended analytical applications. After development it is essential to validate the method fully considering the analytical characteristics like accuracy, precision, specificity, LOD and LOQ, linearity, range and robustness [2]. These analytical characteristics used for validation depend on intended use of the developed method. Always an intensive method validation is carried out as per the guidelines set by regulatory authorities.

Robustness is one of the important parameters of quality that we verify during the validations. In accordance with ICH/USP, robustness defines as [2,3] "the robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage". And this robustness testing will helpful to estimate the potential risk parameters in the developed method. Generally robustness testing are performed at final stages of validation during the method development and any unpredictable results at this stage will be difficult to manage and developed method will be considered as non-robust, which often led costly repetitions like redevelopment and revalidation. Number of recent articles shows that there is an increasing tendency to include thorough multifactorial robustness evaluations at an early stage of development *i.e.* all-important parameters that affect the robustness of the method should be carefully re-examined at the method development stage or at the beginning of the validation procedure [4-6]. There were different ways to evaluate the robustness testing one variable at a time (OVAT) or experimental design. During OVAT (one variable at a time) experiments, the effect of only one variable at a time will be determined while keeping others fixed. As we are verifying one variable at a time, the traditional scientific method of OVAT is a time consuming approach which doesn't provides any information about multiple factor interactions. Hence in the current study quality by design approach was used for analytical robustness assessment. A linear model was postulated and 18 full factorial designs was employed to estimate the model coefficients for intermediate precision. More specifically, Design of experiment provides a structured and organized method to determine the relationship between the factors that affecting the process and the output of that process.

A literature survey reveals that there is no stability-indicating UPLC method for determination and quantitative estimation of related substances of spironolactone in bulk drugs. However, few techniques have been reported in the literature for determination and quantification of spironolactone in human plasma samples using liquid chromatography with mass spectroscopy detection, ultraviolet (UV) absorption spectrometry, thin layer chromatography and using liquid chromatography [7-15]. Therefore, an exhaustive study on the stability of spironolactone is required. As per the current International Conference on Harmonization (ICH) guidelines that stability analysis should be done by using stability-indicating methods, developed and validated after stress testing on the drug under a variety of conditions, including hydrolysis (at various pH), oxidation, photolysis and thermal degradation [16-19]. Hence, in the present work, the chemical degradation pathways of

spironolactone were established through a forced degradation study and a selective, precise and accurate LC method for simultaneous estimation of spironolactone and its degradation products was also developed. Design of experiments (DoE) for robustness testing was done as a part of extensive method validation. The validation of the proposed method was also carried out and its applicability was evaluated in commercial form analysis.

EXPERIMENTAL

Drugs and reagents: Chromatographic grade reagents methanol, ammonia solution, formic acid, sodium hydroxide, hydrochloric acid, hydrogen peroxide manufactured by Merck (Merck KGaA, Darmstadt, Germany) were used for preparation of mobile phase, samples and standard solutions.

Instrumentation: Waters Acquity UPLC H-Class system (Model: Acquity UPLC QSM (Waters/186015018)) includes a quaternary solvent manager, sample manager with flow through needle, column manager and Photo-Diode Array detector which are controlled by empower 3 software was used for all chromatographic separations. Column in different dimensions and chemistry C18, C8 amino were used for method development and finally Zorbax SB-C18 RRHD; 100 × 2.1 mm, 1.8 µ, column was used for development and other equipment's used are as follows Xevo TQ MS from Water, Sartorius micro balance (MSA 6.6S-000-DM), Humidity Chamber (SH-242, ESPEC CORP.), Photo stability chamber (SUNTEST XLS+).

Software used: Design expert version- 8.07.1 (Stat-Ease, Inc. Minneapolis, USA) software used to verify the effect of chromatographic parameters like buffer strength, mobile phase composition and flow rate on chromatographic separations.

Chromatographic conditions: The liquid chromatograph is equipped with a 254 nm (PDA with 190-400 nm) and an Agilent ZORBAX RRHD Stable Bond 80 Å C18 column with dimensions, 2.1 × 100 mm, 1.8 µm. The flow rate and column temperature was about 0.2 mL min^{-1} and 25 °C, respectively. The chromatograph is programmed to provide variable mixtures of solution A and solution B, the percentage of solution B being 60 % at the time of injection, then increased linearly to 80 % at 17 min and decreased linearly to 60 % at 17.5 min, maintained at that percentage for 2.5 min. Compounds were identified using Xevo TQ MS from Water, the operation parameters for MS are capillary (kV) 2.50, cone voltage 5.00, extractor 3.00, source and desolvation temperatures are 150 and 400 °C, cone and desolvation gas flows are 50 LH^{-1} and 700 LH^{-1}.

Sample and solution preparations

Mobile phase and diluent preparation: Solution A: 0.1 % (v/v) formic acid and ammonia in Milli-Q-Water prepared as follows (1 mL of each formic acid and ammonia solution taken and dissolved in 1000 mL of Milli-Q-Water).

Solution B: 100 % Methanol

Diluent: Prepared a mixture of acetonitrile and water in the ratio 90:10.

Assay preparations and organic impurities preparations: For assay suitable amount spironolactone standard and spironolactone sample weighed and dissolved in diluent to get a solutions having concentration about 200 µg mL^{-1}. For organic impurities stock solution (10 µg mL^{-1}) was prepared by dissolving suitable amount of impurities in diluent and further diluted to prepare organic impurities standard solution having concentration about 0.5 µg mL^{-1} of spironolactone and its impurities. For organic impurities sample solution suitable amount of sample weighed and dissolved in diluent to get a solutions having concentration about 500 µg mL^{-1}. For Robustness standard solution prepared by dissolving 200 mg of spironolactone, added 50 µL of organic impurity stock solution and diluted up to the mark with diluent.

Degradation solutions

Acid degradation: About 100 mg of sample was dissolved in 100 mL of 0.1 M methanolic HCl solutions for 16 h and neutralized and further diluted to 500 µg mL^{-1} solution.

Alkali degradation: About 100 mg of sample was dissolved in 100 mL of 0.1 M methanolic NaOH solutions for 2 h and neutralized and further diluted to 500 mg L^{-1} solution.

Thermal degradation: About 100 mg of sample was exposed to thermal condition at 105° for 48 h and prepared dissolving 10 mg of thermal degraded sample in 20 mL diluent.

Sample humidity (exposed to 85 °C and 85 % RH for 3 days), photolytic stress (1.2 million lux hours followed by 200 watt-hours per square meter) and ultrasonic for 1 h.

Method validations: The developed method was validated for system suitability, specific, linearity, accuracy and robustness.

System suitability: As system suitability is required to ensure and verify the system and column performance. System precision was established by injecting 6 replicate injections of standard solution and assay standard. All the important parameters like % RSD, peak tailing, column efficiency, resolution were recorded. These all system suitability parameters will ensure the performance of system, method and column performance.

Specificity: Specificity studies will be performed at initial test concentration to ensure that analyte should have no interference from other extraneous components and be well resolved from them. Intentional degradations of samples were performed for forced degradation studies to provide an indication of the stability-indicating property and specificity of the proposed method. During the forced degradation studies sample solutions were exposed to acid (0.1 M methanolic HCl for 16 h), base (0.1 M methanolic NaOH for 2 h at room temperature), oxidation (6 % peroxide for 24 h at room temperature), thermal (exposed at 105 °C for 48 h), humidity (exposed to 85 °C and 85 % RH for 3 days) and photolytic stress (1.2 million lux hours followed by 200 watt-hours per square meter) and ultrasonic for 1 h.

Precision: The precision of the method was checked by injecting six individual preparations of spironolactone (500 µg L^{-1}) spiked with 0.1 % each of impurity-A, impurity-B, impurity-C, impurity-D and impurity-E. The % RSD for each impurity and content was calculated. Intermediate precision (ruggedness) was established with second analyst.

Accuracy: The accuracy of an analytical procedure expresses the closeness of agreement between the true value and the observed value. Impurity accuracy of the method was demonstrated at three different concentration levels. The analysis was carried out by spiking all the impurities on the API samples at

0.01, 0.05, 0.5 and 1.0 % level of the spironolactone concentration (500 μg mL^{-1}). For each impurity the percentage mean recoveries were calculated at each level. Assay accuracy of the method was demonstrated at three different concentration levels. The analysis was carried out assay on the API and formulation samples at 50, 100 and 150 % of the spironolactone concentration (200 mg L^{-1}). For spironolactone the percentage mean recoveries were calculated at each level.

Limit of detection (LOD) and limit of quantitation (LOQ) studies: The limit of quantitation refers to the lowest amount of an analyte in a sample that can be quantitatively determined with suitable precision and accuracy. There are different approaches to determine the LOQ and LOD. Typically the concentration level that generates a signal-to-noise ratio (S/N) of 10 is referred as the LOQ and the concentration level that generates signal-to-noise ratio (S/N) of 3 is referred as the LOD.

Linearity: Impurity linearity solutions were prepared from stock solutions at five concentration levels from 0.05 to 2.0 % of analyte concentration. The peak area *versus* concentration data were subjected to least-squares linear regression analysis. The calibration curve was drawn by plotting impurity areas against the concentration expressed in μg mL^{-1}. Assay linearity solutions were prepared from stock solution at five concentration levels from 50 to 150 % of analyte concentration. The peak area *versus* concentration data were subjected to least-squares linear regression analysis. The calibration curve was drawn by plotting BH areas against the concentration expressed in μg mL^{-1}.

Solution stability: Samples (impurities spiked at 0.1 % concentration to analyte concentration) and assay preparation was prepared in the diluent and stored the test solutions at room temperature. The spiked sample and assay preparations was injected at 0, 24, 48 h time intervals. The peak area was calculated and the consistency in the % variation at each interval was verified.

Robustness studies: To verify the robustness of developed method, parameter in experimental conditions was deliberately changed and verified the system suitability parameters. As flow rate, temperature and gradient composition have significant impact on separations for robustness study three experimental parameters were considered by simultaneously taking resolution as the responses. A multi-dimensional design space was built to study the robustness of developed method using design expert software. A 2 level factorial design used to build the mathematical models (Table-1). Resulting 2^3 (18) experiments were conducted as per designed model and in all experiments the resolution was > 2 between the impurities and tailing factor was < 1.3. To study the effect of flow rate on developed method flow rate was changed to 0.18 mL min^{-1} and 2.2 mL min^{-1} instead of 0.2 mL/min (flow rate change to ± 10 %). To verify the effect of column temperature method was studied at 30 °C instead of 25 °C, The effect of buffer and methanol ratio in the mobile phase on the developed method was studied at 38:62 and 42:58 (instead of 40:60, v/v).

RESULTS AND DISCUSSION

Method development: The purpose of this work is to develop a stability-indicating LC method for the determination of spironolactone and its related impurities and to establish the degradation pathway for spironolactone along with its five potential impurities. Spironolactone with its steroid structure shows no remarkable change in its retention times, this may be due to absence of ionizable groups in its structure; therefore at all pH ranges can be used and there will not impact of pH on its retention time [20]. We tried different reverse phase columns like C18, cyano and C8 for separation of impurities from the drug substance. But cyano column showed poor separation, almost some of the impurities are co-eluted, while in C8 very little separation observed. Therefore, it is considered only C18 column for optimization study. The UV overlay spectra indicates 254 is the optimum wavelength for detection of spironolactone and its impurities with good response and less base line noise (Fig. 2). During the method development different columns with different dimension with physical properties such as length, carbon load and different stationary phase and separation mechanism (50 mm × 4.6 mm I.D. or 100 mm × 4.6 mm I.D.) were screened. Each column that selected was screened with mobile phases containing various types and percentages of organic modifier such as acetonitrile, methanol and tetrahydrofuran (THF). Different type of buffers also tried to get better separation and stable method. The initial results of column and mobile phase screening studies provided a few conditions that could separate spironolactone and its related compound with good resolution (> 3.0), when the level of all impurities are at 0.1 % to sample concentration (*i.e.* 500 μg mL^{-1}). From the preliminary developmental studies there was 3 critical pair identified, they are impurity-B and spironolactone, impurity-D and impurity-C, impurity-C and impurity-E. When experiments were performed with acetonitrile instead of methanol as the organic solvent in the mobile phase, impurity-C, impurity-D and impurity-E were unresolved and eluting at less than 5 min. Impurity-C and impurity-E are very sensitive to acetonitrile even the selectivity of impurity-C and impurity-E were changed. As the presence of acetonitrile in mobile phase was affecting the separation and selectivity, hence we omitted the using of solvents with higher solvent strength like acetonitrile (solvent strength 3.1) and tetrahydrofuran (solvent strength 4.4). Methanol was used for mobile phase preparation. Based on these studies the increase in concentration of buffer

TABLE-1
SUMMARY OF DESIGN

File version:	8.0.7.1	Design type:	2 level factorial	Design model:	3FI	Blocks:	No blocks		
Study type:	Factorial	Center points:	2	Runs:	18	Build time (ms):	1.95594		
Factor	Name	Type	Subtype	Minimum	Maximum	Coded	Values	Mean	Std. Dev.
A	Flow rate (mL/min)	Numeric	Continuous	0.18	0.22	-1.000=0.18	1.000=0.22	0.2	0.02
B	Buffer strength (%)	Numeric	Continuous	0.08	0.12	-1.000=0.08	1.000=0.12	0.1	0.02
C	Buffer composition (%)	Numeric	Continuous	38	42	-1.000=38.00	1.000=42.00	40	1.89

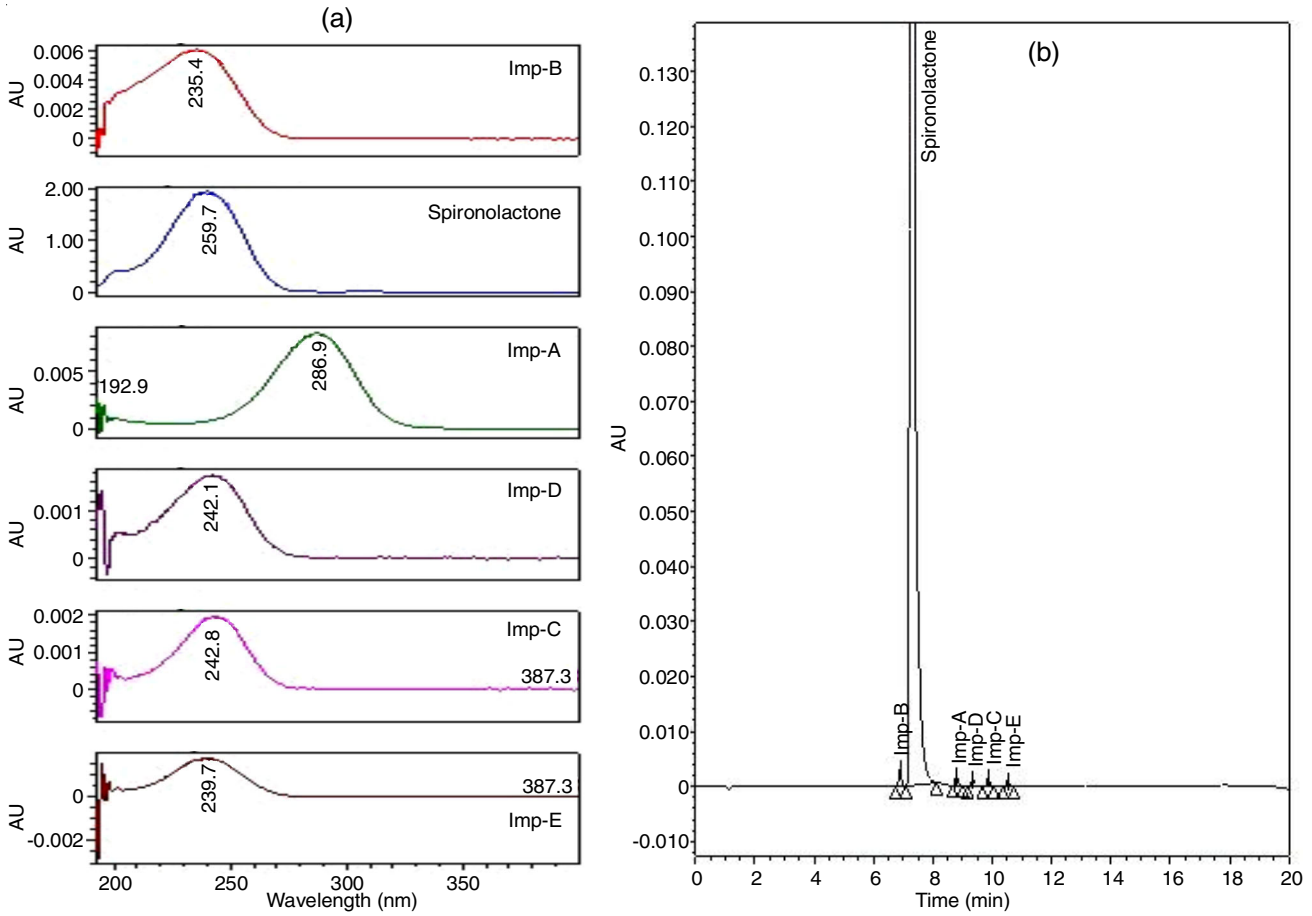

Fig. 2. (a) UV overlay spectra of spironolactone and its impurities A, B, C, D and E; (b) Chromatogram of spironolactone and its impurities A, B, C, D and E in final chromatographic conditions

(aqueous phase) results higher retention times, so concentration of buffer (aqueous phase) fixed to 40 %. When 30 mM ammonium acetate or 30 mM ammonium acetate adjusted to pH 4.5 buffers used in mobile phase broad peaks/more tailing were observed to reduce the peak tailing peak modifiers formic acid and ammonia (0.1 % ammonia and formic acid) used to get sharp peaks.

Design of experiments for robustness study: As our aim is to develop robust method a mathematical model was designed using design expert software, considering flow rate (mL min^{-1}), buffer strength and buffer composition as three variable factors. Eighteen experiments were conducted as per Full factorial (2 levels and 3 factors) experimental designs as shown in Table-2. Criteria of resolution (< 2.0) were considered as response to evaluate the influence of the factors. All the experiments were done in irregular order to minimize the errors from uncontrolled factors that may affect the responses. The effect of these variables were experimentally measured, Table-3 shows the results of experiments as a function of resolution and tailing. It was evident that in all the experiments the observed resolution was greater than 2.0, while the lowest observed resolution was 2.1 and the tailing factor was less than 1.5 in all experiments. Therefore, the method can be considered as robust, since the failure rate was 0 % in the studied design space. Fig. 3 shows the obtained resolution cubes. The designed model was validated with an application of analysis of variance (ANOVA) to examine the significance of model. Table-4 shows

TABLE-2
FULL FACTORIAL DESIGN USED IN THE PRESENT STUDY

Std	Run	Factor 1	Factor 2	Factor 3
		Flow rate (mL/min)	Buffer strength (%)	Buffer composition (%)
4	1	0.22	0.08	38
12	2	0.22	0.08	42
17	3	0.20	0.10	40
1	4	0.18	0.08	38
15	5	0.22	0.12	42
6	6	0.18	0.12	38
9	7	0.18	0.08	42
8	8	0.22	0.12	38
18	9	0.20	0.10	40
13	10	0.18	0.12	42
10	11	0.18	0.08	42
16	12	0.22	0.12	42
7	13	0.22	0.12	38
2	14	0.18	0.08	38
5	15	0.18	0.12	38
11	16	0.22	0.08	42
14	17	0.18	0.12	42
3	18	0.22	0.08	38

the ANOVA results for response (for resolutions) with model F values and model P values for all variable factors. From the results values of "Prob > F" less than 0.0500 indicate model terms are significant. Simultanesously we made few conclusions on effect of these parameters using 3D-response surface. Response suface in Fig. 4 shows intraction and effects of flow

TABLE-3
RESULTS OF EXPERIMENTS AS A FUNCTION OF RESOLUTION AND TAILING

Std	Run	Response 1	Response 2	Response 3	Response 4	Response 5	Response 7
		Resolution (Imp B* and spironolactone)	Resolution (spironolactone and Imp A*)	Resolution (Imp A* and Imp D*)	Resolution (Imp D* and Imp C*)	Resolution (Imp C* and Imp E*)	Tailing for spironolactone
4	1	2.15	7.65	2.68	2.68	3.2	1.3
12	2	2.32	8	3.38	2.26	3.3	1.3
17	3	2.33	8.1	3.1	2.5	3.3	1.3
1	4	2.17	8.12	2.59	2.88	3.2	1.3
15	5	2.3	7.9	3.2	2.3	3.3	1.3
6	6	2.2	8	2.5	2.8	3.3	1.3
9	7	2.3	8.1	3	2.5	3.3	1.3
8	8	2.2	7.9	2.7	2.6	3	1.3
18	9	2.3	8.2	3	2.6	3.3	1.3
13	10	2.33	8.1	3.1	2.5	3.3	1.3
10	11	2.3	8	3.1	2.4	3.3	1.3
16	12	2.3	7.9	3.3	2.2	3.3	1.3
7	13	2.2	8	2.5	2.8	3.2	1.3
2	14	2.2	7.9	2.6	2.8	3.3	1.2
5	15	2.2	7.7	2.7	2.7	3.2	1.3
11	16	2.3	7.9	3.3	2.2	3.3	1.3
14	17	2.4	8.1	3.1	2.5	3.3	1.3
3	18	2.1	7.8	2.8	2.7	3.3	1.3

*Imp A, Imp B, Imp C, Imp D, Imp E, means impurity A, impurity B, impurity C, impurity D, impurity E

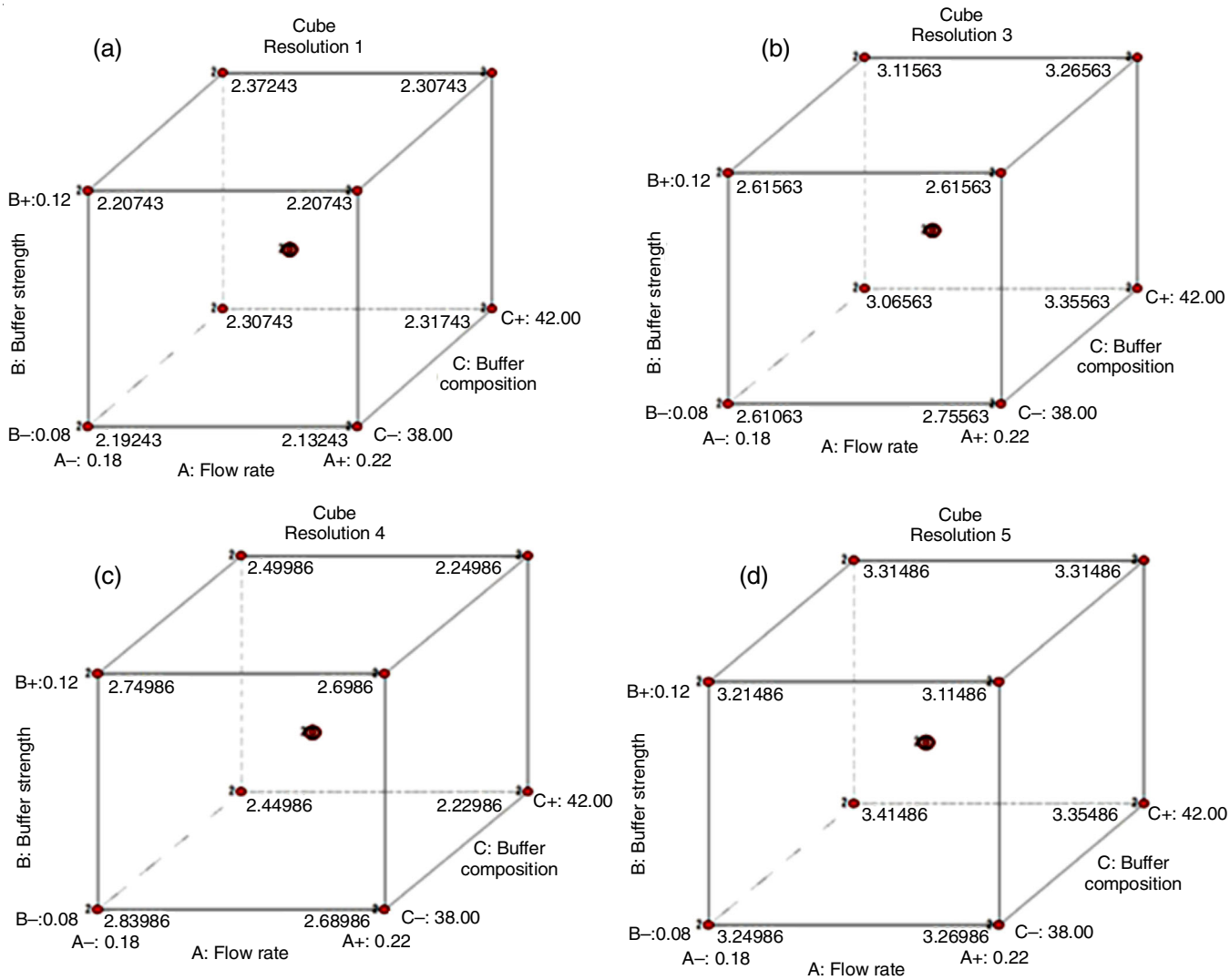

Fig. 3. (a) Design space for the resolution 1; (b) Design space for the resolution 3; (c) Design space for the resolution 4; (d) Design space for the resolution 5

TABLE-4
ANOVA RESULTS FOR RESPONSE (RESOLUTIONS) OBTAINED FROM EXPERIMENTAL DESIGN

	Source	Sum of squares	df	Mean square	Model F value	Model p-value	Prob > F
Resolution-1 (Imp B and spironolactone)	Model	0.093	7	0.013	24.99	< 0.0001	Significant
	Flow rate	0.003306	1	0.003306	6.2	0.0344	
	Buffer strength	0.005256	1	0.005256	9.86	0.0119	
	Buffer composition	0.08	1	0.08	149.64	< 0.0001	
Resolution -3 (Imp A and Imp D)	Model	1.35	7	0.19	26.58	< 0.0001	Significant
	Flow rate	0.086	1	0.086	11.76	0.0075	
	Buffer strength	0.007656	1	0.007656	1.05	0.3316	
	Buffer composition	1.22	1	1.22	167.14	< 0.0001	
Resolution -4 (Imp D and Imp C)	Model	0.74	7	0.11	21.11	< 0.0001	Significant
	Flow rate	0.11	1	0.11	22.35	0.0011	
	Buffer strength	0.000025	1	0.000025	0.004978	0.9453	
	Buffer composition	0.6	1	0.6	119.59	< 0.0001	
Resolution -5 (Imp C and Imp A)	Model	1.35	7	0.19	19.21	< 0.0001	Significant
	Flow rate	0.086	1	0.086	8.5	0.0154	
	Buffer strength	0.007656	1	0.007656	0.76	0.4035	
	Buffer composition	1.22	1	1.22	120.82	< 0.0001	

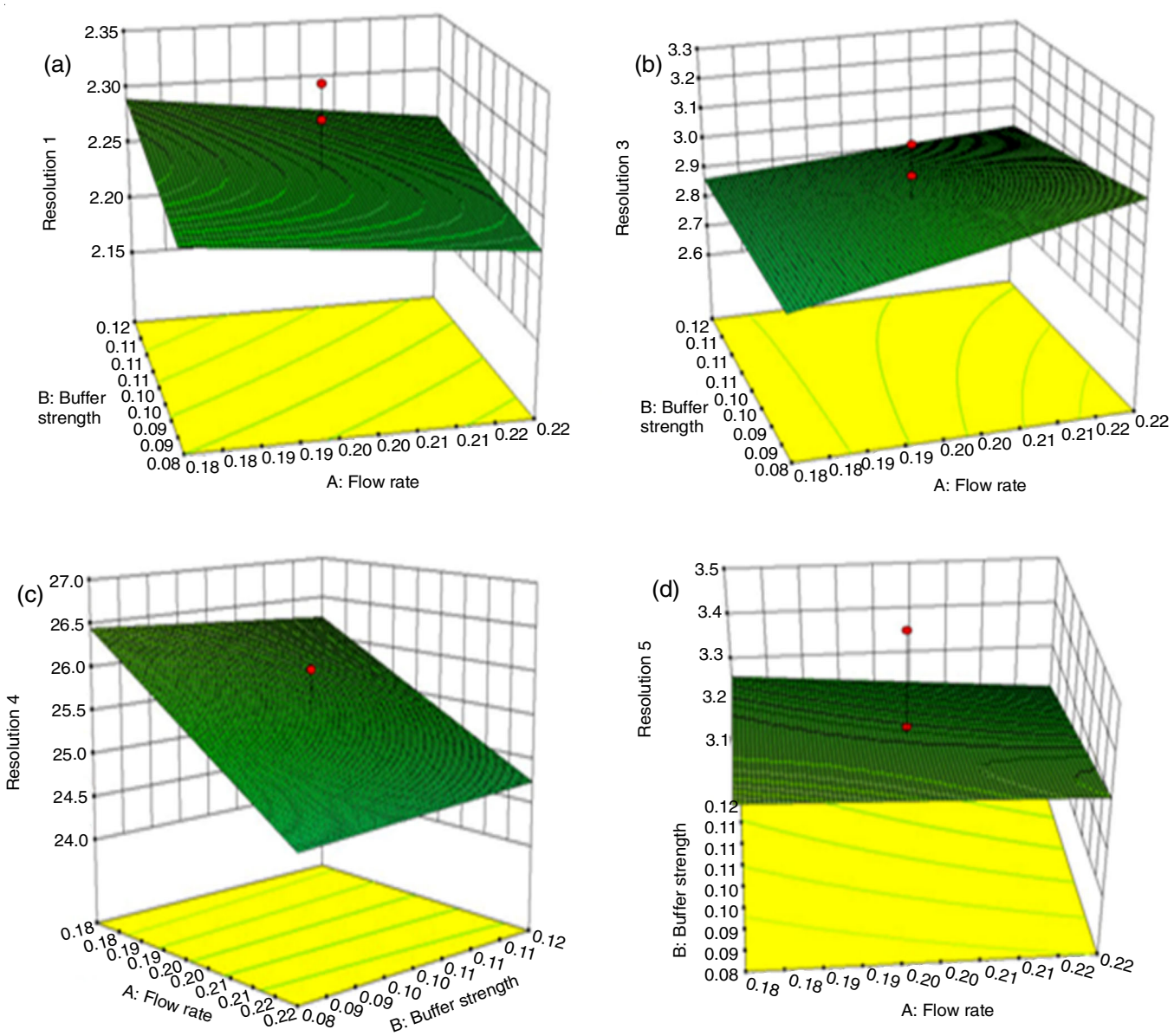

Fig. 4. (a-d): Response surfaces obtained maintaining one variables at the central level and plotting resolution against flow rate and buffer strength (resolution 1 (between impurity B and spironolactone), resolution 3 (between impurity A and impurity D), resolution 4 (between impurity D and impurity C), resolution 5 (between impurity C and impurity E))

rate and buffer strength was linear. The response surfaces formed slopes with small curvatures indicated that all factors (flow rate, buffer strength, buffer composition) contributed mostly independently towards the resolution. And all the response (resolution) was above the predicted value. Hence the developed method can considered as robust.

System suitability: System suitability test was established injecting six replicates of organic impurities standard solution (Fig. 5), resolution between all adjacent peaks, tailing factors, theoretical plates, % RSD was calculated and the results were summarized in Table-5.

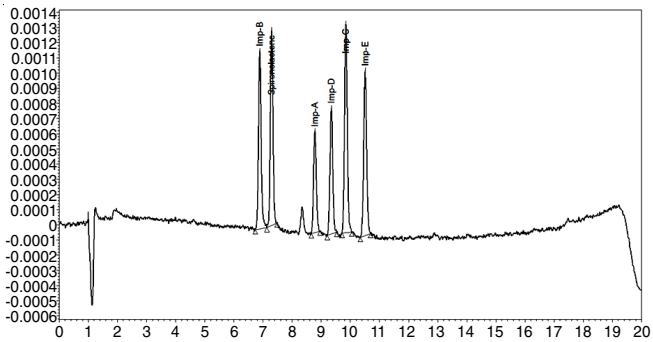

Fig. 5. Organic impurities standard solution

Specificity-stress study: The stability indicating property of the method was proved by forced degradation studies. All the forced degraded samples were injected in the developed method, no additional peaks were detected from acid degraded samples either at room temperature or after heating and also no degradation observed from peroxide, thermal, photolytic, humidity and ultrasonic stress studies. The chromatograms (Fig. 6) of the alkaline degraded samples displayed an additional peaks at RRT about 0.2, 0.7 and this is an evident that sample was sensitive to alkali. The degraded peak was well resolved from the spironolactone and its impurities. The purity threshold was found higher than angle indicates that the peak is pure. And the UV spectrum of spironolactone was compared

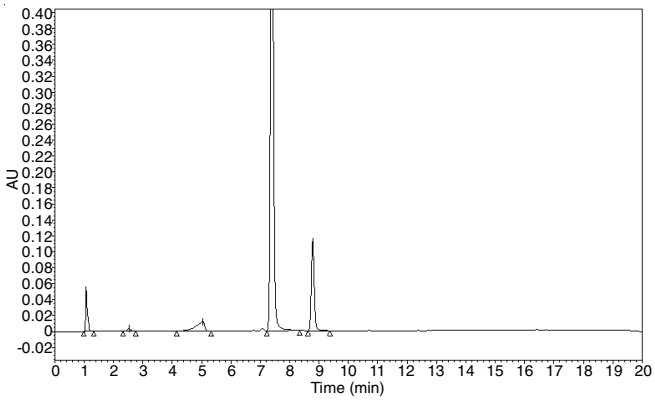

Fig. 6. Typical chromatogram of base degraded sample

with spectrum of degraded product and it was noted that there was variation in absorption pattern. It shows that the peaks due to degradation were well resolved from the peaks. The summarized results were tabulated in Table-6.

Precision and intermediate precision: Precision will expressed in terms of % RSD. During the method precision study, the % RSD obtained for spironolactone peak area was below 0.5 %. The % RSD for the peak areas of impurity-A, impurity-B, impurity-C, impurity-D and impurity-E was within 2.0 %. The % RSD obtained during the intermediate precision was within ± 0.5 % for spironolactone and % RSD for the peak areas of impurity-A, impurity-B, impurity-C, impurity-D and impurity-E were well within ± 2.0 %, These results confirm the precision of the analytical method is good.

Accuracy: The recovery percentage of spironolactone and its impurities impurity-A, impurity-B, impurity-C, impurity-D and impurity-E are ranged from 98.90 to 102.14 and impurities are ranged from 97.62 to 103.14, 96.25 to 101.23, 95.72 to 101.60, 97.44 to 100.66 and 97.86 to 104.82, respectively (Table-7).

Limit of detection and limit of quantification: The LOD and LOQ of standard solutions are approximately 0.1 and 0.25 mg L^{-1}, respectively, which is equivalent to 0.02 and 0.05 % of the analytical concentration (500 mg L^{-1}) of spironolactone.

TABLE-5
RESULTS FORM SYSTEM SUITABILITY IS AS FOLLOW

Name	Retention time	RT ratio	USP resolution	USP tailing	RSD (%)
Impurity-B	7.012	0.94		1.11	0.24
Spironolactone	7.426	1.00	2.11	1.18	0.35
Impurity-A	8.929	1.20	7.3	1.2	0.16
Impurity-D	9.513	1.28	2.79	1.14	0.51
Impurity-C	10.008	1.35	2.32	1.2	0.46
Impurity-E	10.663	1.44	3.03	1.05	0.39

TABLE-6
FORCED DEGRADATION STUDY RESULTS

Degradation conditions	Degraded (%)	Purity angle	Purity threshold	Mass balance (%)
0.1 N HCl Refluxed 1 h at 80 °C	No degradation	0.403	0.801	99.5
0.1 N NaOH bench to at 24 h at room temperature	15.4	0.037	0.232	99.1
Stressed with 6 % H_2O_2 24 h kept on bench top at room temperature	No degradation	0.796	1.326	99.3
Thermal at 105 °C for 48 h	No degradation	0.506	1.036	99.6
Exposed to visible light for about 1.2 Million Lux-hours and UV light for about 200 Watt-hours/meter square	No degradation	0.176	0.279	99.8
Humidity 85 % RH and 85 °C for 3 days	No degradation	0.525	1.000	99.7
Ultrasonic for 1 h	No degradation	0.495	0.925	99.9

TABLE-7
SUMMARY OF VALIDATION RESULTS

	Impurity-B	Spironolactone	Impurity-A	Impurity-D	Impurity-C	Impurity-E
Linearity OI	0.999974	0.999973	0.999998	0.999962	0.999955	0.999935
LOD and LOQ						
Quantitation limit (%)	0.01	0.01	0.02	0.02	0.01	0.01
Detection limit (%)	0.03	0.03	0.07	0.07	0.03	0.03
Accuracy (% recovery)						
0 %	100.61	102.14	96.25	95.72	100.66	97.86
75 %	99.11	99.31	100.18	99.28	98.44	98.29
100 %	103.14	99.39	99.90	98.20	98.83	103.42
125 %	97.62	99.59	98.48	98.49	99.68	104.80
150 %	101.05	98.90	101.23	101.60	97.44	101.20

At the selected LOQ and LOD concentrations, all the S/N for LOQ standard solutions were larger than 10 and all the S/N for LOD standard solutions were larger than 3. These results (Table-7) suggest that the developed UPLC method has good sensitivity for the estimate related compound in spironolactone.

Linearity: The obtained correlation coefficient is greater than 0.999 from the linear calibration plot for the assay method tested over a calibration range of 50 to 150 % levels. And liner calibration plot for the impurities is determined with a range of 0.05 to 2.0 % for impurity-A, impurity-B, impurity-C, impurity-D and impurity-E and the correlation coefficient obtained was greater than 0.999 (Table-7).

Solution stability: No significant changes were observed in the content of spironolactone and its impurities during solution stability and mobile phase stability experiments. The solution stability and mobile phase stability experiment data confirms that the sample solution and mobile phases used during the assay and impurity determination were stable for at least 48 h at room temperature.

Mass structural identification studies: LC studies were conducted on waters Xevo TQ instrument the results of LC-MS analysis indicated that the protonated ions $(M+H)^+$ of spironolactone, Imp-A, Imp-B, Imp-C. Imp-D and Imp-E at *m/z* 417.06 Da, 341.12 Da, 415.15 Da, 343.13 Da, 449.01 Da and 421.10 Da, respectively. Based on the mass values observed from mass spectra's confirms the structures of spironolactone and its impurities. As we used ammonia in mobile phase we observed $[M+NH_4]^+$ mass in all spectra as adduct.

Conclusion

A UHPLC method for determination of spironolactone and its impurities was successfully developed and validated as per the guidelines. The performance of the method was verified under different stress conditions of spironolactone. Experimental designs used to verify robustness of developed method. Based on the summary of validation results the method was accurate and robust. Hence the method was suitable for its intended purpose.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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