High-Performance Liquid Chromatography and Liquid Chromatography/Mass Spectrometry Studies on Stress Degradation Behavior of Sulfapyridine and Development of a Validated, Specific, Stability-Indicating HPLC Assay Method

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ABSTRACT

The objective of the current investigation was to develop a simple, rapid, and stability-indicating high-performance liquid chromatography method and to study the degradation behavior of sulfapyridine (SP) under different International Conference on Harmonization (ICH)-recommended conditions. The chromatographic method was developed using C_{18} (250×4.6 mm, 5 μ) column, and mobile phase consisting of acetonitrile–0.1% formic acid (30:70 v/v) at ambient temperature, at a flow rate of 1 mL/min. The elution was monitored at 265 nm using a photodiode array detector. The developed method was subsequently validated as per ICH Q2 (R1) guidelines. The retention time of SP was observed as 4.56 min with the linearity range between 2 to 10 μ g/mL. Limit of detection and limit of quantitation for SP were 0.115 and 0.35 μ g/mL, respectively. Forced degradation studies were carried out on bulk samples of SP using prescribed acidic, basic, oxidative, thermal, and photolytic conditions. Extent of degradation in 0.1 M hydrochloric acid and under photolytic conditions was found to be 21.56% and 28.57%, respectively. The degradation products formed in stress conditions were identified by liquid chromatography-mass spectrometry (LC-MS). The utility of the method was verified by quantification of SP in different laboratory-made pharmaceutical preparations. The proposed method could be

successfully used to quantify SP in different pharmaceutical dosage forms.

Keywords: sulfapyridine, forced degradation study, stability-indicating method, HPLC, LCMS

INTRODUCTION

Sulfapyridine (4-amino-*N*-pyridin-2-ylbenzenesulfonamide, SP) is a compound derived from sulfonamide that has been used as an effective antimicrobial drug for the prevention and cure of a number of bacterial infections.^{1,2} It is used for its anti-inflammatory effect in certain ocular diseases as well as in treating inflammatory bowel disease in combination with 5-aminosalicylic acid in the form of mesalamine.³ The drug is reported to undergo photodecomposition via hydrolysis, oxidation, desulfonation, and reduction.⁴

Previous researchers have reported various methods for the analysis of SP. These include a method for assay of SP and acetyl-SP in biological fluids,⁵ analysis of SP in animal tissues,⁶ in waste water,⁷ characterization of photochemical degradation products of SP in water,^{4,8–10} analysis of adsorption characteristics of SP antibiotic in soil,¹¹ and validation of high-performance liquid chromatographic (HPLC) method for determination of sulfonamides in egg.¹²

However, none of these methods reported the stability-indicating method for SP. The aim of this study was to develop a simple, rapid, and sensitive stability-indicating HPLC method for analysis of SP in accordance with the guidelines of the International Conference on Harmonization (ICH) and to identify the various degradation products formed in different stress conditions by liquid chromatography/mass spectrometry (LC-MS)/MS.¹³ This method was also applied for assay of SP in various pharmaceutical formulations such as tablet, liposome, gel, and nanoemulsion.

MATERIALS AND METHODS

Materials

Chemicals and reagents. SP ($\geq 99\%$) and Sephadex G50 were purchased from Sigma-Aldrich (St. Louis, MO). Phospholipon 90 G was obtained as gift sample from Lipoid GmbH, Ludwigshafen, Germany. Acetonitrile (ACN; HPLC grade) and formic acid were purchased from Fisher Scientific (India). Methyl cellulose (METHOCEL™ E5LV) was received from Colorcon Asia Pvt. Ltd., Mumbai (India), as a gift sample. Labrafil® M 1944 CS and Transcutol® P were gifted by Gattefosse, Saint-Priest, France. Cholesterol, hydrochloric acid (HCl, 37%), sodium hydroxide (NaOH), hydrogen peroxide (H_2O_2 , 35% w/w), lactose monohydrate, carbopol 934, magnesium stearate, talc, and starch (potato) were purchased from Loba Chemie Pvt. Ltd. (Mumbai, India). Tween 80, chloroform, methanol (HPLC grade), sodium chloride, acetic acid (HPLC grade), and potassium phosphate monobasic (HPLC grade) were procured from Avantor Performance Materials India Limited (Mumbai, India). Purified water was obtained from Bio-Age water system (India). All other chemicals were of analytical grade and used as received without further purification.

Instruments. A Shimadzu Prominence series HPLC system consisting of a binary pump (LC20AD, Japan) with an online-degasser (DGU-20A₅) and multiple wavelength or diode array UV detector (SPD-M20A) was used for method development, method validation and analysis of degradation samples. The Shimadzu Lab Solutions chromatographic software (version 1.2) was used for data acquisition and processing.

A photostability study was performed in a photostability chamber (TP 90 S/G), from Thermolab (Vasai, India). A photostability chamber equipped with light bank comprising UV and fluorescent lamps was used to provide an overall illumination of not less than 1.2 million lux h. It also provided an integrated near ultraviolet energy of not less than 200 Wh/m² as per the option 2 of Q1B guidelines of ICH.¹⁴ Thermal degradation study was conducted in dry-air oven (LT-90; Lab-therm, India). Stability studies under accelerated conditions were carried out in a stability chamber (CHM-10S; Remi Elektrotechnik Ltd., India). Rotary tablet compression machine (Trover Pharma Mach, India) and rotary evaporator (IKA RV-8, India) were used for preparation of tablets and liposomal formulations. Centrifuge (CM-12 Plus; Remi Elektrotechnik Ltd., India), sonicator (LMUC-4; Labman Scientific Instruments Pvt. Ltd., India), analytical balance (AX 200; Shimadzu Analytical Pvt. Ltd., India), and heating mantle (CMU0250/CE; Labindia Instruments Pvt. Ltd., India) were used during the study.

LC-MS/MS studies were carried out on an ultra-fast LC system coupled with a tandem quadrupole mass spectrometer (Shimadzu 8030, Tokyo, Japan) equipped with electrospray ionization interface, LC-20AD pump, SPD-M20 PDA detector, CTO-20AC column oven, CBM-20 alite controller, and SIL-20AC autosampler. A Zorbax C18 column (50×4.6 mm, 5 μ particle size) was used for LC separation with a mobile-phase composition of 0.1% v/v formic acid (30%) and ACN (70%) at a flow rate of 1 mL/min.

Methods

Preparation of stressed/degradation samples. An initial drug concentration of 1 mg/mL was used to conduct various stress degradation studies. For carrying out hydrolysis in neutral conditions, the drug was dissolved in water and refluxed at 60°C for 48 h. The study in acidic condition was conducted in 0.1 M HCl at 60°C for 24 h. Alkaline hydrolysis was performed in 0.1 M and 1 M NaOH at 60°C for 24 h. An oxidative degradation study was carried out at room temperature in 3% and 20% H_2O_2 for 12 h. A degradation study was also conducted in solid state by exposing pure drug to dry heat at 60°C for 7 days. Pure drug was also subjected to accelerated condition of 40°C/75% relative humidity (RH) for 3 months. Photodegradation studies were performed in water and 0.1 M HCl for 5 days. Suitable samples were kept under dark. Periodic sampling was done and the collected samples were diluted suitably and subjected to HPLC analysis.^{15–17}

Development of stability-indicating HPLC method. Optimization of various HPLC parameters such as type of column, mobile-phase composition, flow rate, and detection wavelength was done to achieve proper separation between the peaks of drug and its degradation products. The details for the same have been described in the Results and Discussion section.

Method validation

Preparation of standard solution. A stock solution of pure SP was prepared at a concentration of 1,000 $\mu\text{g/mL}$ by dissolving 100 mg of SP in 100 mL of ACN.

Linearity and range. A stock solution of SP was diluted up to 100 $\mu\text{g/mL}$ using ACN, which was subsequently diluted with mobile phase to prepare working solutions of concentrations 2, 4, 6, 8, and 10 $\mu\text{g/mL}$. The dilutions were prepared in triplicate ($n=3$) and injected to the HPLC system. The linearity curve was prepared by plotting the area of peak obtained versus concentration of the solution injected.¹³

Least-squares linear regression analysis using Microsoft Excel 2010 and GraphPad Prism 5 computer programs were used to determine the slope, intercept, and regression coefficient values.

Detection and quantification limits. The detection and quantification limits were calculated from calibration curve prepared in a concentration range of 2–10 µg/mL. The limits of detection (LOD) and quantification (LOQ) were determined by using standard deviation (SD) of the response (area of peak) and slope of linearity curve [Eqs. (1) and (2)] as per ICH guidelines.¹³

$$\text{LOD} = \frac{3.3 (\text{SD})}{\text{Slope}} \quad (1)$$

$$\text{LOQ} = \frac{10 (\text{SD})}{\text{Slope}} \quad (2)$$

Precision. Values of precision (both intraday and interday) of optimized method were determined at three concentration levels, that is, low, medium, and high levels of SP (4.8, 6, and 7.2 µg/mL, respectively). Five injections ($n=5$) were injected for analysis within a day for repeatability and over the next 3 days for reproducibility. The precision of the method was measured using average concentration and % relative SD (RSD).

Accuracy. The accuracy of the HPLC method was determined by recovery studies at three concentration levels (80%, 100%, and 120%), that is, 4.8, 6, and 7.2 µg/mL. Each concentration was analyzed five times ($n=5$) under optimized chromatographic conditions and % recovery was calculated.

Robustness. The robustness of an analytical method is a measure of its capacity to remain unaffected by small, although deliberate, variations in method parameters. It provides an indication of the reliability of the method during normal usage. The robustness of the optimized method was assessed by making small changes related to flow rate (altered by ±0.2 mL/min), mobile-phase composition (±6.66% of ACN), formic acid concentration (±0.02%), detection wavelength (±2 nm), and different columns. Analyte concentration for the study was chosen as 6 µg/mL. The resulting retention time (RT) along with various system suitability parameters, that is, theoretical plate number, detector response (peak area), and tailing factor under each altered condition, were calculated.

Preparation of SP formulations. Tablets of SP were prepared by the wet granulation method. To prepare 100 tablets, SP (1 g) was accurately weighed and mixed with lactose (6 g), methyl cellulose (2 g), magnesium stearate (0.5 g), and talc (0.5 g). The mixture was homogeneously blended in a V-cone blender for 15 min at 50 rpm. The blend was converted into granules using sieve no. 25. Starch solution (3% w/v) was added to the

powdered blend to convert it into granules, which were dried for 1 h at 70°C in hot-air oven. The dried granules were further compressed into tablets using a rotary tablet compression machine having 6 mm flat circular punch with hydraulic pressure of 8 kg/cm².¹⁸

SP gel formulation was prepared by dissolving drug (5 mg) in Tween 80 (0.3 mL). Carbopol 934 (100 mg) was dissolved in purified water (100 mL) and added to the drug solution followed by mixing to obtain a one-phase solution. The gel was packed in suitable containers and stored at controlled temperature and humidity (25°C/60% RH) for further analysis.¹⁹

Nanoemulsion formulation of SP was prepared by using Labrafil M 1944 CS and Transcutol P as nonionic water-dispersible surfactant and cosurfactant, respectively. SP (5 mg) was dissolved in Tween 80 (0.6 mL), followed by addition of surfactant (0.1 mL) and cosurfactant (0.3 mL). The isotropic mixture was sonicated on an ultrasonicator bath for 15 min to dissolve the drug in the vehicle. The beaker containing clear solution of SP was stirred on a magnetic stirrer at 50 rpm using Teflon bead, and water (5 mL) was added through a glass burette slowly to achieve clear transparent nanoemulsion.²⁰

Liposomes of SP were prepared by a thin film hydration method.²¹ Briefly, SP was dissolved in a mixture of chloroform:methanol (1:1) and transferred into a suitable round-bottomed flask. The organic phase was gradually evaporated at reduced pressure by a rotary evaporator to form the thin film of components on the inner wall of the flask. After complete evaporation of organic phase, the flask was kept for 24 h in a vacuum desiccator under reduced pressure to remove traces of solvent. The hydration of dried film was carried out with 0.9% w/v saline (10 mL) at 45°C, 100 rpm, and under normal pressure. The prepared vesicles were allowed to swell overnight at room temperature.^{22,23}

Quantitative HPLC assay of SP in formulations. Prepared tablets (20) of SP were weighed accurately and average weight was calculated. These tablets were then subjected to grinding to get a powder. A quantity of powder equivalent to 100 mg of SP was transferred to a 100 mL volumetric flask, to which 75 mL of HPLC-grade ACN was added. Contents of the flask were sonicated for 15 min to dissolve SP and volume was made up to 100 mL with ACN. The resulting mixture was filtered through a 0.22 µm membrane filter. The clear solution so obtained was diluted appropriately with mobile phase and analyzed by HPLC.

For determination of SP in gel, an accurate weight formulation equivalent to 10 mg of SP was transferred to a 100 mL volumetric flask; ACN (70 mL) was added into the flask and the

Fig. 1. Representative HPLC chromatogram of SP. Key: Standard solution (10 µg/mL) (**A**), acidic degradation (**B**), alkaline degradation (**C**), neutral degradation (**D**), thermal degradation (**E**), oxidative degradation (**F**), photolytic degradation (**G**), peak purity plot of SP (**H**). HPLC, high-performance liquid chromatography; SP, sulfapyridine.

Fig. 1. (Continued).

Table 1. Optimization of Mobile Phase for High-Performance Liquid Chromatography Method Development

Trail no.	Mobile-phase composition	Column specification	Observation	Inference
1	Methanol: water (50:50 v/v)	RP C18 column (250×4.6 mm, 5 μ)	Peak tailing	Method rejected
2	ACN: water (40:60 v/v)		Peak shoulder	Method rejected
3	Methanol: 0.1% formic acid (45:55 v/v)		Broad peak	Method rejected
4	ACN: 0.1% acetic acid (25:75 v/v)		Peak impure	Method rejected
5	ACN: monobasic potassium phosphate buffer pH 3 (25:75 v/v)		Poor resolution	Method rejected
6	ACN: 0.1% formic acid (30:70 v/v)		Sharp symmetrical peak	Method selected

mixture was sonicated for 15 min to extract the drug completely from the formulation. The solution was filtered through a syringe filter (0.22 μ) and after a suitable solution, the sample was analyzed by HPLC to quantify the SP in gel formulation.

To determine SP content in nanoemulsion, an aliquot of SP nanoemulsion was diluted with ACN to obtain a theoretical concentration of 5 μg/mL. The mixture was sonicated for 15 min to extract SP and filtered through a 0.22 μ syringe filter before injecting into an HPLC column.

Entrapment efficiency of liposomes was determined using the Sephadex G-50 minicolumn centrifugation method. Sephadex G-50, which was allowed to swell overnight in water at room temperature, was filled in 1 mL syringe and then centrifuged at 2,000 rpm for 3 min. Two hundred microliters of liposomal formulation was added at the top of minicolumn and centrifuged again at the same rpm for the same time. The eluent having liposomes separated from free drug was diluted with methanol (2 mL for 10 μL formulation) to disrupt the liposomes for release of drug. The sample was filtered through a 0.22 μ syringe filter and analyzed by HPLC. The percentage entrapment efficiency was calculated by Equation (3) as follows:

Entrapment efficiency (%) =

$$\frac{\text{Amount of drug released from liposomes}}{\text{Amount of drug used in preparing formulation}} \times 100 \quad (3)$$

The assay of SP was carried out in triplicate and mean value of peak area of all the determinations was used to quantify the drug in various formulations.

RESULTS AND DISCUSSION

Development of Stability-Indicating HPLC Method

A fast stability-indicating HPLC method was developed for the analysis of SP. The RT of SP was found to be 4.56 min and chromatogram has been depicted in *Figure 1A*. By the measurement of the response of the eluent under optimized conditions, a standard well-defined symmetrical peak for SP was obtained (*Table 1*).

The optimized method was developed on the Nucleodur® C₁₈ (250×4.6 mm, 5 μ) column (Macherey-Nagel, Germany). Mobile phase consisted of ACN and 0.1% formic acid in Millipore water (30:70 v/v). The injection volume was 25 μL and the analysis was carried out at a detection wavelength (λ_{max}) of 265 nm.

Forced Degradation Study of SP

SP was found to be quite labile to acidic and photolytic conditions. SP exhibited 28.57% degradation in photolytic conditions, and degradation products were observed at RTs 1.941, 2.553, and 5.136 min (*Fig. 1G*). On heating the drug in 0.1 M HCl at 60°C for 24 h, 21.56% degradation has been seen and the degradation products appeared at RTs 1.941 and 2.730 min (*Fig. 1B*). SP was found to be stable in oxidative condition (3% and 20% H₂O₂), and the peak observed in the chromatogram at RT 2.5 min was attributed to the impurity present in the solvent, hence can be regarded as blank peak (*Fig. 1F*). In alkali condition (1 M NaOH) as well as in water, no degradation was observed (*Fig. 1C, D*). In solid-state

Table 2. Percentage Degradation and Retention Time of Degradation Products Observed During Forced Degradation Study

Degradation type	% Degradation	RTs of degradation products
Acidic degradation	21.56	1.94 and 2.73
Alkaline degradation	No degradation	—
Oxidative degradation	No degradation	—
Thermal degradation	No degradation	—
Photolytic degradation	28.57	1.94, 2.55, and 5.13

RT, retention time.

Fig. 2. Total ion chromatogram of SP subjected to photolytic degradation (A), mass spectra of SP (B), mass spectra of various degradation products appeared at RT 1.211 (C), 1.654 (D), and 5.383 (E) in total ion chromatogram; MS/MS spectra of SP (F) and degradation products/fragments with m/z 95 (G), 186 (H), 115 (I), and 263.95 (J). MS, mass spectrometry; RT, retention time.

Fig. 2. (Continued).

Fig. 2. (Continued).

studies, SP was found to be stable to the effect of temperature, and also the combined effect of temperature and humidity. On subjecting the drug to either dry heat at 60°C or 40°C/75% RH, no degradation was observed for 3 months (Fig. 1E).

The percentage degradation of SP in various stress conditions has been given in Table 2.

HPLC chromatograms of SP in various stress conditions have been shown in Figure 1.

Intermediate/Product Identification by LC-MS/MS

Three degradation products have been identified on photodecomposition of SP, with relevant information, including total ion chromatogram and mass spectra (Fig. 2). On the basis of degradation product identification, SP has been found to undergo three reactions, including photo-induced hydrolysis, photo-oxidation, and desulfuration, which are also common reaction types for photodecomposition of other compounds.

The same degradation products have been reported earlier by Xu *et al.* in 2014, which corroborates our observations.⁴ In total ion chromatogram, the various photolytic degradation products appeared at RTs 1.211, 1.654, and 5.383 min, with a molecular ion peak of m/z 186.00, 115.05, and 263.95, respectively (Fig. 2).

The MS/MS analysis of SP and degradation products confirmed the chemical structures of various intermediate and degradation products. The proposed degradation pathways have been illustrated in Figure 3.

The amino sulfonamides typically produce ions with m/z 156, 108, and 92 and a similar profile has been observed for SP also.^{24,25} Additional fragment ions were observed consistent with the loss of 66 and 155 Da. The base peak in the MS spectra at m/z 156 is due to the cleavage of sulfonamide bond, further undergoing loss of SO_2 to give an ion at m/z 92. Fragment ion that appeared at m/z 108 is formed *via*

a rearrangement, while the loss of 66 Da suggests the loss of H_2SO_2 . Complementary ion to m/z 156 is observed due to loss of 155 Da, which appeared at m/z 95 (Fig. 3A).

One of the degradation products of SP, that is, *N*-(pyridin-2-yl)benzene-1,4-diamine appeared at m/z 186, which was formed by loss of SO_2 , that is, desulfuration reaction.^{26,27} Fragmentation of this molecules yielded daughter ions at m/z 169, 95, and 108 due to cleavage of the amino group, anilino group, and pyridine moiety, respectively.²⁸ Further fragmentation of ion having m/z 95 gave 1,2-didehydropyridinium ion (m/z 78), which subsequently loses HCN to give a fragment C_4H_3^+ at m/z 51.²⁹ The fragment (m/z 108) gave another fragment by loss of 15 Da, which on further fragmentation formed two daughter fragments at m/z 66 and 65 (Fig. 3C).³⁰

The second degradation product of SP observed was 2-(dihydroxyamino)1*H*-pyrrol-1-ium ion, with an m/z of 115 and an elemental composition of $\text{C}_4\text{H}_7\text{N}_2\text{O}_2^+$. Fragmentation of this ion yielded two daughter ions, *N*-oxo-*N*-

Fig. 3. MS/MS fragmentation of SP (A), proposed degradation products of SP (B), fragmentation of degradation products appeared at RT 1.211 min (C), 1.654 min (D), and 5.383 min (E) in total ion chromatogram.

Fig. 3. (Continued).

vinylhydroxylammonium ion (m/z 74, $\text{C}_2\text{H}_4\text{NO}_2^+$) and 2-iminoethan-1-ium ion (m/z 42, $\text{C}_2\text{H}_4\text{N}^+$) (Fig. 3D).

The third degradation product of SP with m/z of 264 and an elemental composition of $\text{C}_{11}\text{H}_{10}\text{N}_3\text{O}_3\text{S}^+$ was formed due to photohydroxylation, which is one of the common degradation pathways for sulfa drugs.^{31,32} Loss of H_2SO_2 resulted in the formation of another fragment having m/z of 198. A base peak in the mass spectrum was observed at m/z 170 due to loss of 94 Da. Another SO_2 extrusion product with m/z of 200 and an elemental composition of $\text{C}_{11}\text{H}_{10}\text{N}_3\text{O}^+$ was observed, which on subsequent oxidation gave daughter with m/z of 216.³³ Cleavage of phenyl-sulfonyl bond resulted in formation of fragment (m/z 92), which on oxidation gave another fragment with m/z 106.

Method Validation

Linearity and range. The response of the drug was found to be linear in the investigated concentration range, that is, 2–10 $\mu\text{g/mL}$, calculated by linear regression analysis. The correlation coefficient, slope, and intercept in the calibration curve were observed at 0.9995, 113,372, and 10,242, respectively.

LOD and LOQ. LOD and LOQ were observed as 0.115 and 0.35 $\mu\text{g/mL}$, respectively, which reflect the sensitivity of the developed method.

Precision. The precision of the method was evaluated by taking into account its repeatability and intermediate

Table 3. Precision, Accuracy, and Robustness Results for Sulfapyridine**Intraday and interday precision**

Concentration ($\mu\text{g/mL}$)	Intraday precision		Interday precision					
	Observed concentration $\pm$ SD ($\mu\text{g/mL}$)	% RSD	Day 1		Day 2		Day 3	
			Observed concentration $\pm$ SD ($\mu\text{g/mL}$)	% RSD	Observed concentration $\pm$ SD ($\mu\text{g/mL}$)	% RSD	Observed concentration $\pm$ SD ($\mu\text{g/mL}$)	% RSD
4.8	4.86 $\pm$ 0.06	1.33	4.86 $\pm$ 0.04	0.98	4.89 $\pm$ 0.06	1.34	4.85 $\pm$ 0.05	1.12
6	5.95 $\pm$ 0.09	1.59	5.88 $\pm$ 0.10	1.71	5.83 $\pm$ 0.03	0.51	5.81 $\pm$ 0.08	1.52
7.2	7.19 $\pm$ 0.01	0.21	7.12 $\pm$ 0.07	1.11	7.09 $\pm$ 0.12	1.79	7.16 $\pm$ 0.09	1.39

Recovery data for sulfapyridine

	Levels		% Recovery $\pm$ SD		% RSD		
	80		101.63 $\pm$ 0.05		1.12		
	100		98.24 $\pm$ 0.11		1.91		
	120		98.43 $\pm$ 0.10		1.51		

Influence of changes in experimental parameters on the performance of chromatographic system

Parameter	Modification	RT (min)	Peak area	Tailing factor	Theoretical plate number
Mobile-phase ratio (v/v) ACN: 0.1% formic acid	28:72	4.62	710179.8	0.766	2250
	30:70	4.52	663442.2	0.787	2379
	32:68	3.96	775654.8	0.784	2428
Flow rate (mL/min)	0.8	5.24	860379.0	0.788	2543
	1	4.51	663442.2	0.787	2395
	1.2	3.50	576444.2	0.815	2314
Concentration of formic acid (%)	0.08	4.53	642952.1	0.779	2224
	0.1	4.51	652985.2	0.784	2394
	0.12	4.55	651278.2	0.781	2310
Column (250 $\times$ 4.6 mm, 5 μ)	Nucleodur [®] C18	4.50	661845.1	0.782	2351
	Kinetex C18	4.23	631782.4	0.713	2102
Wavelength (nm)	263	4.56	657389.4	0.786	2396
	265	4.56	663442.2	0.787	2379
	267	4.56	659348.0	0.787	2379

The results were reported as an average of five replicate injections.
ACN, acetonitrile; SD, standard deviation; RSD, relative SD.

Fig. 4. HPLC chromatogram of formulations: (A) tablet, (B) gel, (C) nanoemulsion, (D) liposomes. Placebo formulation chromatogram (*inset*) as the control.

precision aspects. Values of %RSD (<2%) which were within the acceptance criteria of ICH Q2(R1) guidelines indicated the developed method to be precise (Table 3).

Accuracy. The results of accuracy studies showed percentage recovery at all three levels in the range of 98.24%–101.63%, and % RSD values that are well below 2% as shown in Table 3. The results of percentage recovery and RSD are also within the prescribed limits as per Q2 (R1) guideline.¹³

Robustness. The results of robustness revealed that under various deliberately changed HPLC conditions, the method was able to demonstrate sufficient ruggedness for analysis of SP. The results of robustness study have been summarized in Table 3.

Specificity. The developed method was found to be specific for SP. As evidenced from the purity plot in Figure 1H, the purity index for the drug peak in a mixture of stressed samples was found to be 0.999. This indicates that the drug peak was free from any coeluting peak. Also, the resolution factor for the SP peak (Fig. 1B) was 7.32 from the nearest resolving peak.

Application of the Developed Method to Quantify SP in Pharmaceutical Formulations

The proposed validated HPLC method was used to determine the amount of SP in tablet, gel, nanoemulsion, and liposomes. The peak areas of triplicate samples were analyzed by regression equation obtained from calibration plot to get the content of SP in various formulations. There was no interference from excipients in all the formulations, indicating the specificity of the developed method. Both the placebo and SP chromatograms of various formulations have been shown in Figure 4. The % recovery of SP in tablet, gel, nanoemulsion, and liposomes was found to be $97.8\% \pm 1.48\%$, $82.4\% \pm 1.18\%$, $85.2\% \pm 1.67\%$, and $59.6\% \pm 2.24\%$, respectively.

CONCLUSION

The developed method presents a simple and validated stability-indicating HPLC method for estimation of SP in the presence of degradation products. The drug was found to undergo degradation in acidic and photolytic conditions and was stable in alkali, oxidative, and thermal conditions.

The statistical analyses proved the validated HPLC method to be accurate, precise, robust, reproducible, and specific and may be applied for routine quality control, assay, *in vitro* drug release, and stability studies. The method has been applied with success to the analysis of SP in various formulations, as no interference was observed due to excipients or other components present.

DISCLOSURE STATEMENT

No competing financial interests exist.

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Abbreviations Used

ACN	=	acetonitrile
H ₂ O ₂	=	hydrogen peroxide
HCl	=	hydrochloric acid
HPLC	=	high-performance liquid chromatography
ICH	=	International Conference on Harmonization
LC-MS	=	liquid chromatography-mass spectrometry
LOD	=	limit of detection
LOQ	=	limit of quantification
NaOH	=	sodium hydroxide
PDA	=	photodiode array
RH	=	relative humidity
RSD	=	relative SD
RT	=	retention time
SD	=	standard deviation
SP	=	sulfapyridine