https://doi.org/10.48048/tis.2023.5209

Development and Validation of RP-HPLC Method for Quantitative Determination of 4-Amino Benzene Sulphonamide in Sulphonamide Hydrochloride

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Received: 14 July 2022, Revised: 19 August 2022, Accepted: 26 August 2022, Published: 15 March 2023

Abstract

A simple, reliable and sensitive analytical method was developed and validated for the quantitative determination of organic impurity 4-amino benzene sulphonamide in sulphonamide hydrochloride by high performance liquid chromatography (HPLC) with UV-visible and photo-diode array (PDA) detector. It is challenging to develop and validate method on gradient elution with shortest run time and the lesser elution. The RP-HPLC method was developed by using YMC-Triart C8 (250×4.6 mm^2) 5µ. The mobile phase was used with flow rate is 1.0 mL/min for 40 min run time and 5µL of injection volume. With respect to gradient program, 25 °C column oven temperature 265 nm detection wavelengths, respectively. An analyte is well resolved and selective in view of analytical validation.The method is accurate and precise as well as linear in the range of LOQ to 200 % level with respect to limit concentration of sulphonamide hydrochloride and observed correlation coefficient is 0.999. Recovery at each level for all known impurities were found in range between 85 and 115 %. The method is sensitive and rapid; also accomplish all the criteria of stability, as well as robustness. The propose method would be useful for rapid screening, control impurity and routine analysis of quantitative determination of 4-amino benzene sulphonamide in sulphonamide hydrochloride.

Keywords: Sulphonamide hydrochloride, Sensitive, PDA, 4-amino benzene sulphonamide, Determination, Analysis, Development and validation

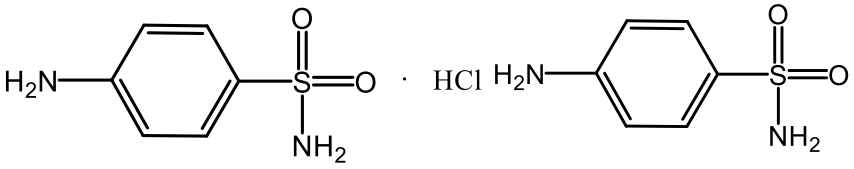
Introduction

Pharmaceutical drug discovery, development, and production depend on the development and validation of analytical methods. Quality control laboratories employ the official test technique that emerges from these procedures to guarantee the identity, purity, potency, and performance of drug product "quality" which is crucial for drug safety and efficacy [1,2]. Analytical methods are used to useful resource in the process of drug synthesis, screen potential drug candidates, assist formulation studies, monitor the stability of bulk prescription drugs and formulated products, and test final products for release. Over current years the Regulatory Authorities have emerge as increasingly aware about the need of making sure that the information supplied them to in applications for marketing (and perhaps clinical) authorization were obtained the usage of confirmed analytical methodology [3,4]. This has resulted in the publication of a sequence of necessities and guidelines through numerous authorities [5,6]. Validation trials seek to characterize the effectiveness of a method, analyze the likelihood of errors, and give the data required to assess whether a method can be used to gauge conformity with particular standards. A few protozoa, including coccidian, and a few gram-positive and gram-negative bacteria are

all inhibited by the broad-spectrum antimicrobials known as sulfonamides. They are widely used in veterinary medicine to prevent and treat infectious illnesses [7,8].

Sulfonamides are derivatives of sulfanilamide (p-amino benzene sulfonamide) and, relying at the substitution of the amido or aromatic group, they may be generally described as N1- or N4-substituted sulfonamides, respectively. Substitutions on the amido group with heterocyclic aromatic nuclei bring about N1 compounds that modify in degrees of antimicrobial activity. Moreover, numerous sulfonamides are administered through feed (premix) and additionally the cross-contamination phenomenon needs to be controlled [9,10].

The structure and chemical name of sulphonamide hydrochloride and 4-amino benzene sulphonamideare shown in **Figure 1**.



Sulphonamide HCl Salt 4-amino benzene sulphonamide

Figure 1 Structure and chemical name of sulphonamide hydrochloride and 4-amino benzene sulphonamide.

Most specifically of carbonic anhydrase inhibitors known as aromatic, heterocyclic sulfonamides has received the most attention from researchers. Because it forms additional interactions with the hydrophobic and/or the hydrophilic portion of the active site, the substituent at the benzene sulfonamide ring contributes favorably to the binding affinity and selectivity [11,12].

The "tail" strategy is commonly used in the novel design of pharmaceuticals employing benzene sulfonamide scaffolds. This technique entails adding moieties to the sulfonamides bearing free amino groups that provide the desired physicochemical qualities. When it comes to inhibiting enzyme carbonic anhydrase isoforms, the orientation of the tail moiety, the nature of the spacers between the top and the tail moieties, and the presence of polar groups in the tail all have a significant bearing on the efficacy and selectivity of benzene sulfonamides. Sulfanilamide was used as a starting chemical in the synthesis of a large number of benzene sulfonamides, which resulted in the formation of N-4-mono-substituted or 4-N, N-disubstituted derivatives [13,14].

The screening strategies have a growing achievement way to their capacity of choosing the suspect samples at significantly decreased expenses and times, in order that properly timed decision may be taken. Making decisions is crucial when managing the analysis for official residue control of pharmacologically active drugs in animals that generate food. Fast and reasonably-priced screening strategies are specifically demanded for sulfonamides because the EU monitoring programmes contain each year a few lots of analyses of those antibiotics in food and feed [15,16].

However, the current sulfonamide screening techniques are primarily based totally on chromatographic techniques or microbiological growth inhibition. Chromatographic techniques (HPLC) are very appropriate for affirmation however now no longer for screening of huge numbers of check samples being costly such as the maximum used compounds in the veterinary practice [17]. As N-derivatives of 4-amino benzene sulphonamide, sulfonamides are made up of a large group of man-made antibacterial compounds. They were used in human medicine to treat a wide range of microorganisms, and they are still mostly used to treat urinary tract infections. They are also used a lot as veterinary drugs for both prevention and treatment in farm animal feed and fish farming. Also, sulfonamides are growth-promoting chemicals, and their left over in food are a problem because they could cause cancer and could make people more resistant to antibiotics. They could also cause severe allergic reactions [18,19].

Method leads one to the conclusion that the procedure will prove useful for both speedy screening and routine analysis. For the routine analysis of bulk drugs containing sulphonamide hydrochloride as active components, this HPLC method is rapid, faster, more cost effective, and requires less time than other methods which is been existed [20,21].

Materials and methods

The sulphonamide hydrochloride and impurity 4-amino benzene sulphonamide (Purchased from the Sudharshan Chemicals, Pune, India) sample, reference standards) (see **Figure 1**) were used for method

optimization and validation. Analytical grade acetonitrile, HPLC grade water and Di-potassium hydrogen phosphate buffer (Purchased from the Sudharshan Chemicals, Pune, India) as an analytical grade was used throughout the experiment. The HPLC system (Waters Alliance) with YMC-Triart C8 (250×4.6 mm^2) 5 µm column (HPLC with UV-Visible and PDA detector including auto sampler). Before the development and validation of analytical method, all the instruments were calibrated as per the norms of validated standard operating procedure. (Details mentioned in General analytical method parameter).

General analytical method

The different HPLC chromatographic conditions were optimized for best selective, precise and rugged method for quantitative determination of organic impurity 4-amino benzene sulphonamide in sulphonamide hydrochloride. During the early trials, several factors such as mobile phase composition, column type, and diluents were optimized and modified in order to achieve best suitable method. Different mobile phase and columns were used for the optimization of sharp peak shape and therefore select gradient mobile phase for better resolution of 4-amino benzene sulphonamide. Finally, in gradient elution (shown in **Table 1**) mobile phase- A prepared with 1.74 g of dipotassium hydrogen phosphate in to 500 mL of water, filtered and degassed while 500 mL of acetonitrile as mobile phase-B with flow rate of 1.0 mL/min. Using this optimized mobile phase the YMC-Triart C8 (250×4.6 mm^2) 5µ column, 265 nm detection wavelength, 25 °C column temperature, 5 µL/mL injection volume and 40.0 min of run time was selected.The sharp peak shape was observed and eluted with lesser elution time. The established method was validated in accordance with ICH guideline. **Figure 2** depicts the optimized chromatogram. The optimized chromatogram was obtained with Sulphonamide hydrochloride salt standard solution.

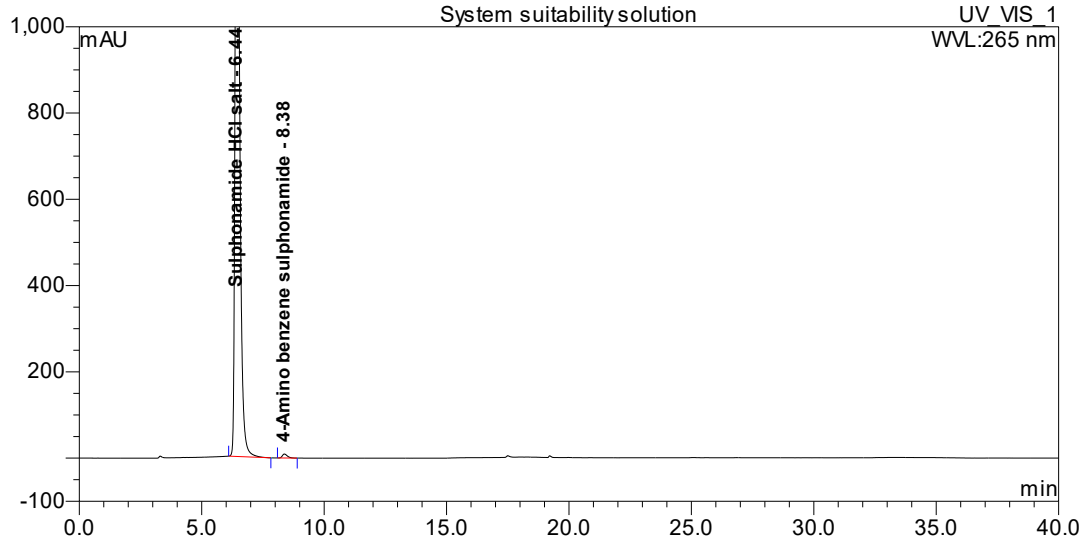


Figure 2 Optimized chromatogram of sulphonamide hydrochloride and 4-amino benzene sulphonamide impurity.

Sample preparations for specificity, linearity, accuracy, LOQ and LOD

Sample solution prepared upto 1000 ppm concentration, with respect to sample concentration 5.0 ppm impurity solution spiked in system suitability solution and in test (sample) spike solution. Solutions preparation for specificity is shown below.

1) Preparation of Diluent: HPLC grade water used as diluent.

2) 4-Amino benzene sulphonamide stock preparation (Preparation-1): Accurately weighed and transferred 5.048 mg of 4-Amino benzene sulphonamide impurity standard in 10.0 mL volumetric flask, added 5mL diluent sonicated to dissolve and made volume up to mark (500 µg/mL).

3) Sulphonamide hydrochloride salt standard solution: Accurately weighed and transferred 25.025 mg of Sulphonamide hydrochloride salt standard in to 25.0 mL volumetric flask, added 0.25 mL preparation-1 and 15 mL diluent, sonicated to dissolve and made the volume up to mark with diluent (1000 µg/mL).

4) 4-Amino benzene sulphonamide at spec level: Transferred 0.1 mL of preparation-1 in to 10.0 mL of volumetric flask and made up volume with diluent (5 µg/mL).

5) Sample preparation: Weighed and transferred 25.07 mg sample into 25.0 mL volumetric flask, added about 10 mL diluent, sonicated to dissolve and made up volume with diluent (1000 µg/mL).

6) Sample preparation with spike impurity: Weighed and transferred 25.025 mg sample into 25.0 mL volumetric flask and added about 10 mL diluent, sonicated to dissolved, added 0.25 mL of preparation-1 made up volume with diluent (1000 µg/mL sample and spiked impurity at 5 µg/mL concentration).

Procedure

Inject 5 µL of each of blank preparation and sample solution into the chromatograph. Run the chromatograph for 40 min for blank and test solution and record the chromatogram disregards the peak due to blank.Retention time of 4-Amino benzene sulphonamide is about 8.4 min.

Table 1 Gradient program.

Time (min)	Mobile phase A	Mobile phase B
0	95	5
10	95	5
20	30	70
30	30	70
32	95	5
40	95	5

Results and discussion

Analytical validation

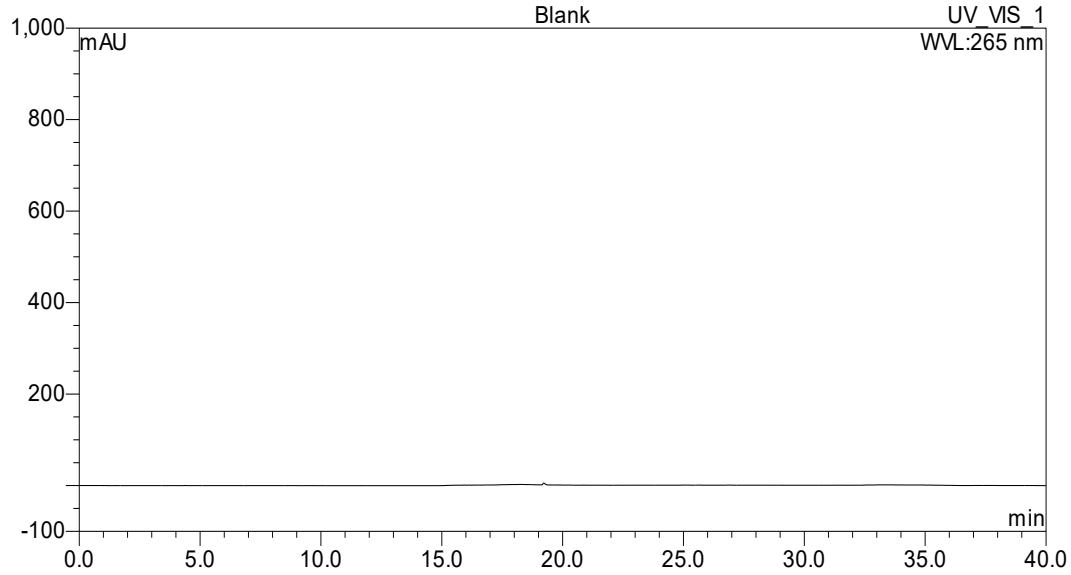
Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically, those may contain contaminants, degradants, matrix, etc.Lack of specificity of an individual analytical approach can be remedied via way of means of different supporting analytical procedure(s). In purity test, to make sure that everyone the analytical procedures done permit an accurate declaration of the content material of impurities of an analyte, i.e. related materials test, heavy metals, residual solvents content material, etc. [21]. Specificity research is performed by injecting the blank and sample solution evaluating the interference and spectral homogeneity of peak correspond to sulphonamide hydrochloride salt. Selectivity chromatogram for blank, impurities standard, un-spiked and spiked test solution is shown in **Figure 3**.

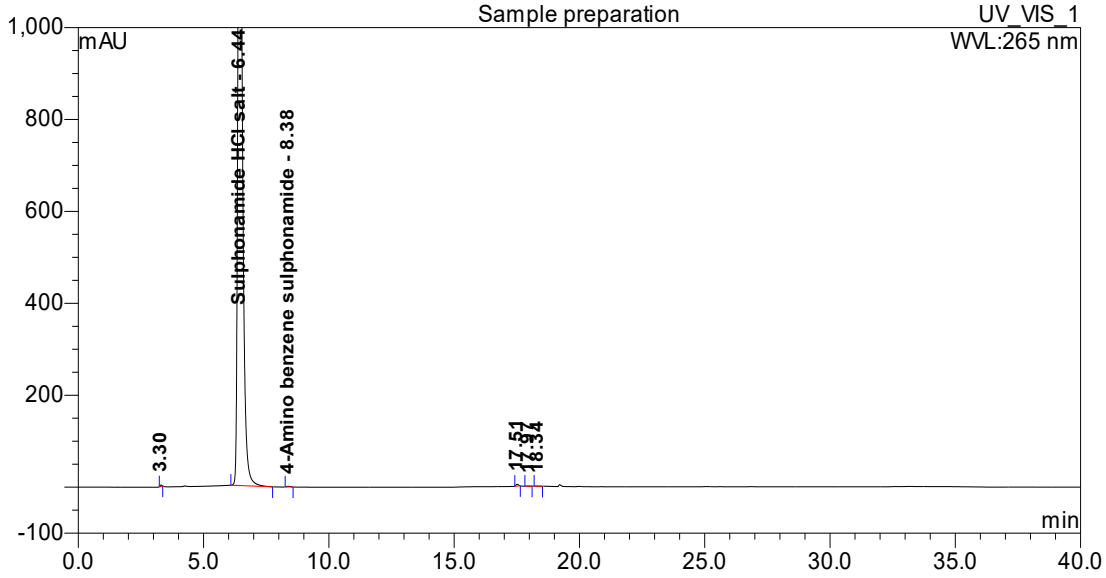
Table 2 Peak retention time, peak area and relative retention time.

Sr. No	Sample Name	Peak Name	R.T. (min)	Peak Area (mAU*min)	RRT
1	Blank (diluent)	NA	NA	NA	NA
2	4-Amino benzeneSulphonamide at spec level	4-Amino benzene sulphonamide	8.34	2.054	NA
3	Sample preparation	Sulphonamide hydrochloride salt	6.44	351.237	1.00
		4-Amino benzene sulphonamide	8.38	0.219	1.30
4	Spike sample preparation	Sulphonamide hydrochloride salt	6.44	378.075	1.00
		4-Amino benzene sulphonamide	8.38	2.237	1.30

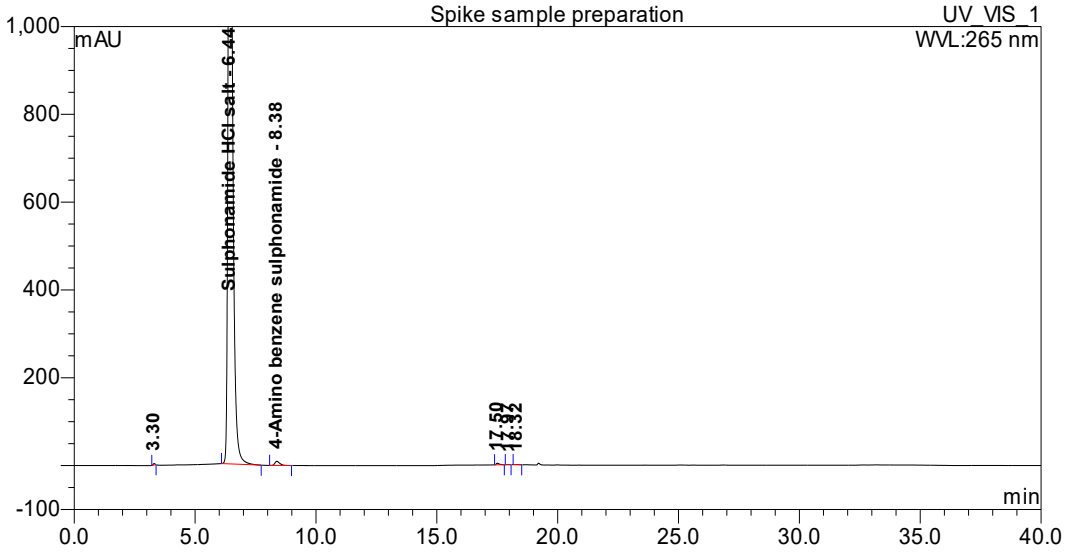
Note: The purity angle of sulphonamide hydrochloride is 0.172 and Purity threshold observed 1.022 and therefore it concludes that there is not interference of any peak.



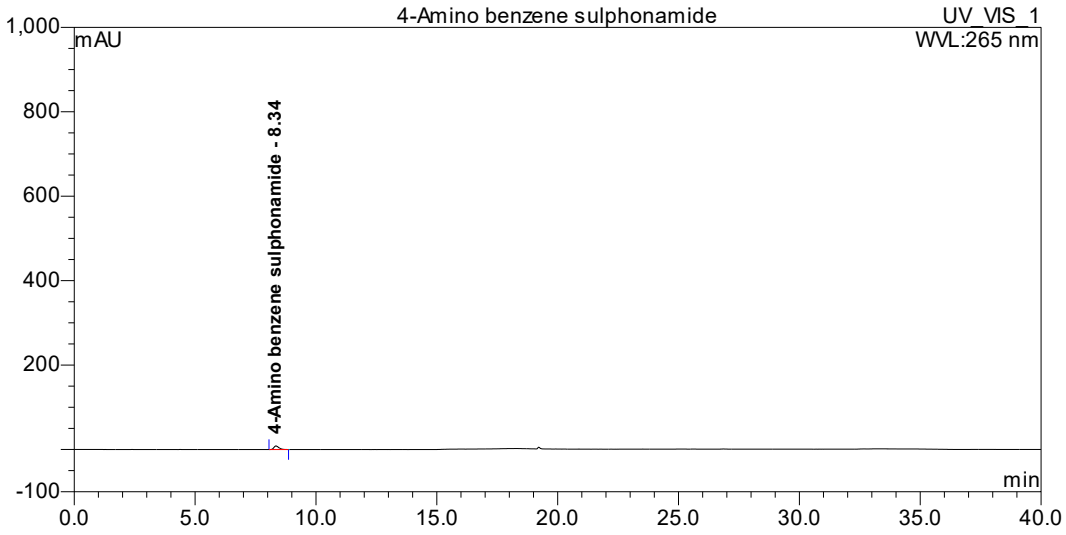
(a)Chromatogram for blank sample



(b) Chromatogram for sample preparation



(c) Chromatogram for spiked impurity



(d) Chromatogram for 4-amino benzene sulphonamide impurity

Figure 3 Chromatograms specificity study: (a) Chromatogram for Blank sample, (b) Chromatogram for Sample Preparation, (c) Chromatogram for Spiked impurity, and (d) Chromatogram for 4-amino benzene sulphonamide impurity.

LOD and LOQ

The quantitation limit of a single analytical process is the smallest amount of analyte in a sample that can be decided with enough precision and accuracy using quantitative methods. The quantitation limit is a parameter of quantitative assays for low levels of compounds in sample matrices. It is mostly used to find impurities and/or degradation products. The detection limit of a particular analytical process is the smallest amount of analyte in a sample that can be found, but isn't always quantified [21]. The signal-to-noise ratio of 3:1 and 10:1 tells us what the LOD and LOQ are (shown in **Table 3**). LOD and LOQ that have been seen are between 0.066 and 0.067µg/mL and between 0.200 and 0.202 µg/mL, respectively. Limit of detection and quantitation study is based on signal to noise ratio which is mention in **Table 3**.

Table 3 Observation table for LOD and LOQ.

Name of Compound	LOD			LOQ		
	Actual Conc. in µg/mL	In % w.r.t. Nominal sample preparation	s/n	Actual Conc.in µg/mL	In % w.r.t. Nominal sample preparation	s/n
Sulphonamide hydrochloride salt	0.066	0.0066	6	0.200	0.020	14
4-amino benzene sulphonamide	0.067	0.0067	7	0.202	0.020	15

Linearity

Linearity is the ability of an analytical method to produce test results that are directly related to the concentration (amount) of analyte in a sample (within a given range) [21]. The linearity of sulphonamide hydrochloride and impurity 4-amino benzene sulphonamide is achieved by using concentrations that range from the LOQ level to 200 µg/mL of the specification limit concentration. All injected concentration solutions in duplicate into the liquid chromatograph and recorded the chromatograms. The Y-intercept, the slope of the regression line, the correlation coefficient, and the regression coefficient value were all calculated.

By injecting different concentrations of sulphonamide Hydrochloride salt and 4-amino benzene sulphonamide standard preparation and recording the peak areas, the proposed method checked the linearity of Sulphonamide Hydrochloride salt and 4-amino benzene sulphonamide salt. Linearity plot of peak area Vs concentration was plotted and correlation co-efficient, y-intercept and regression coefficientvalue were determined as well shown in **Table 4** and **Figure 4**.

Table 4 Observation table for linearity.

Linearity Level	Concentration in (µg/mL)		Peak area(mAU*min)	
	4-amino benzene sulphonamide	Sulphonamide hydrochloride	Mean peak area for 4-amino benzenesulphonamide	Mean peak area for sulphonamide HCL salt
LOQ	0.202	0.200	0.103	0.069
50%	2.519	2.500	1.018	0.683
75%	3.778	3.750	1.568	1.081
100%	5.038	5.000	2.088	1.430
150%	7.557	7.500	3.132	2.169
200%	10.076	9.999	4.276	2.978
y-intercept			**−0.022**	**−0.0318**
Slope			**−0.42225**	**−0.2971**
R^2 (Regression coefficient)			**0.9995**	**0.999**
Correlation coefficient			**0.99975**	**0.99951**

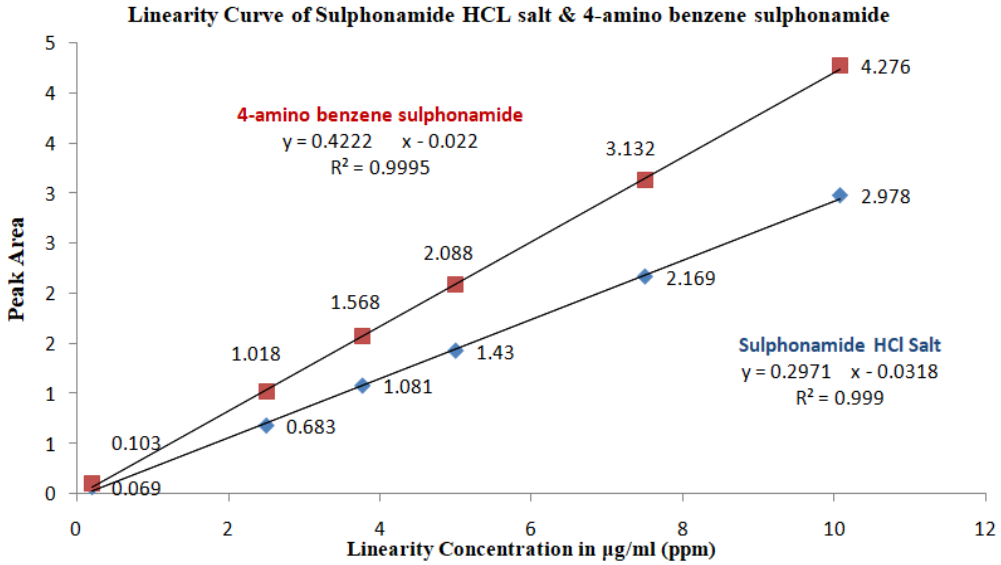


Figure 4 Linearity curve.

Accuracy

The accuracy of an analytical technique expresses the closeness of agreement among the value that is established both as a traditional real fee or an accepted reference value and the value found. This is sometimes termed trueness [21]. To demonstrate accuracy of test method by preparing accuracy samples in triplicate at LOQ, 50, 100 and 150 % range of specification limits for impurities. Perform the accuracy by injecting triplicate of injections of every individual accuracy level and calculated accuracy of 4-amino benzene sulphonamide shown in **Table 5**. Recovery at each level for all known impurities should be between 85 and 115 %.

Table 5 Recovery calculation for 4-Amino benzene sulphonamide.

Impurity	LOQ level % Recovery	50% level % Recovery	100% level % Recovery	150% level % Recovery
4-amino benzene Sulphonamide	115%	101.9%	103.4%	108.2%
SD	0.00000	2.54034	3.14325	2.87460
%RSD	0.000	2.490	3.042	2.66

% Relative standard deviation for each level should not be more than 10.0.
Overall %relative standard deviation for all the levels should not be more than 10.0.

Range

For linearity the rangeis from LOQ to 200 % of nominal concentration of specification limit and for accuracy it's from 50 to 150 % of nominal concentration of specification limit.

Method precision and intermediate precision

The closeness of agreement (degree of scatter) among a sequence of measurements that were taken from more than 1 sampling of the same homogenous sample under the stipulated conditions is what constitutes the precision of an analytical procedure. Repeatability, intermediate precision, and reproducibility are the 3 different levels of precision that can be taken into consideration. The utilization of consistent, genuine samples ought to be researched in order to study precision. However, if it is not possible to obtain a sample that is homogeneous, it is still possible to analyze the issue by making use of samples that have been artificially prepared or a sample solution. In most cases, the precision of an analytical technique is stated as a function of the variance, preferred deviation, or coefficient of variability of a series of observations [21]. The table that follows offers suggestions for both the method precision and the intermediate precision. Standard solutions of sulphonamide hydrochloride and 4-amino benzene

sulphonamide were replicated six times in order to evaluate the system's degree of precision. In order to determine the precision of the approach, 6 identical samples of the test solution were injected while simultaneously adding 4 contaminants at the maximum level allowed by the specification. While at the same time, an intermediate precision was measured on a different day using a different analyst, equipment, and column. Different preparations, totaling 6 spiking test sets, are made in the exact same way as the preparation that was discussed in the specificity research. The following table provides an observation table for both the method's precision and the intermediate precision.

Method precision and intermediate precision was performed on different days in different analytical instruments and by different analyst without affecting method parameters. In this study, 6 sets of individual samples were prepared for both the parameters and run that on respective HPLC system as per above mentioned criteria. Preparation of samples mentioned below.

Sample preparation with spike impurity Set-1

Accurately weighed and transferred 100.11 mg Sulphonamide hydrochloride salt sample into 100.0mL volumetric flask and added about 40mL diluent. Sonicated to dissolved, added 1.0 mL of stock solution-A and made up the volume up to the mark with diluent and mixed (Prepared 6 individual sets of sample preparation with spiked samples).

Table 6 Comparative data of method precision and intermediate precision in spiked test.

Spiked Test sets	% of impurities (in µg/mL)							
	4-amino benzene sulphonamide		Single Max		Total Unknown		Total Impurity	
	MP	IP	MP	IP	MP	IP	MP	IP
1	0.60	0.61	0.12	0.14	0.20	0.22	0.80	0.83
2	0.60	0.61	0.13	0.14	0.22	0.22	0.82	0.83
3	0.61	0.61	0.13	0.14	0.21	0.22	0.82	0.83
4	0.61	0.61	0.13	0.14	0.21	0.22	0.82	0.83
5	0.61	0.61	0.15	0.14	0.25	0.22	0.86	0.83
6	0.61	0.61	0.15	0.14	0.23	0.22	0.84	0.83
Mean	**0.61**	**0.61**	**0.14**	**0.14**	**0.22**	**0.22**	**0.83**	**0.83**
SD	**0.00516**	0.000	**0.01225**	0.000	**0.01789**	0.000	**0.02066**	0.000
% RSD	**0.85**	**0.000**	**8.75**	**0.000**	**8.13**	**0.000**	**2.49**	**0.000**
Mean Difference between MP and IP	**0.00**		**0.00**		**0.00**		**0.00**	

*MP: Method Precision, IP: Intermediate Precision, RSD: Relative Standard Deviation, SD: Standard Deviation.

Robustness

Robustness is performed by small but deliberate changes in method parameters, by differencing column oven temperature, flow rate, gradient programme change. The single maximum unknown impurity should not be more than 0.1 % and total impurities should not be more than 10.0% showed in **Table 7**.

Table 7 Comparative data of robustness in spiked test.

Parameters	Change	4-amino benzene sulphonamide		Single Max. Unknown Impurity		Total unknown impurities		Total impurity	
		% Impurity	Diff.	% Impurity	Diff.	% Impurity	Diff.	% Impurity	Diff.
As such Preparation	No Change	0.63	NA	0.12	NA	0.22	4.55	0.85	NA
Flow rate (mL/min)	0.9	0.64	0.01	0.12	0.00	0.21	0.00	0.85	0.00
	1.1	0.66	0.03	0.13	0.01	0.22	4.55	0.88	3.53
Column Oven Temp.	22°C	0.63	0.00	0.12	0.00	0.21	0.00	0.84	1.18
	28°C	0.63	0.00	0.12	0.00	0.22	0.00	0.85	0.00
Gradient Composition Mobile phase B	−20%	0.64	0.01	0.12	0.00	0.22	0.00	0.86	1.18
	+20%	0.63	0.00	0.12	0.00	0.22	0.00	0.85	0.00

As such parameters, a) flow rate, b) column oven temperature, c) gradient composition and sample preparation see specificity study and **Table 1**.

Conclusions

The chemical contaminant known as 4-amino benzene sulphonamide is carcinogenic, extremely hazardous, and has the potential to cause cancer. A reliable high performance liquid chromatographic method for figuring out how much organic impurity (4-amino benzene sulphonamide) is in sulphonamide hydrochloride was made and tested. This leads one to the conclusion that the procedure will prove useful for both speedy screening and routine analysis. For the routine analysis of bulk drugs containing sulphonamide hydrochloride as active components, this method is rapid, faster, more cost effective, and requires less time than other methods which is been existed.The method was used to determine the concentration of the impurity. YMC-Triart C8 (250×4.6 mm^2) 5 column was utilized during the process of developing the RP-HPLC procedure. The flow rate of the mobile phase was set at 1.0 mL/min, and the run time was set at 40 min. The injection volume was set at5µg/L in relation to the gradient programme, the temperature of the column oven at 25 °C, and the detection wavelength at 265 nm, accordingly. In light of the results of analytical validation, an analyte is highly resolved and selective. In the range of LOQ to 200 % level with respect to limit concentration of sulphonamide hydrochloride, the method is accurate and exact as well as linear, and the observed correlation coefficient is 0.999.

Acknowledgements

Authors express their sincere gratitude to SSS's Divine College of Pharmacy, Satana, India, Nashik for continuous motivation, support, and guidance for research activity and for providing all required facilities to accomplish the entitled work.

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