The determination of impurities in Sapropterin Di-HCl powder for oral solution by Chromatography: A Robust, Stabilized Approach revealing Forced Degradation Studies

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Abstract

This research study presents a comprehensive and validated chromatographic method for the precise quantification of Sapropterin Di hydrochloride (SAPR) and its associated impurities using RP-HPLC technique (Liquid chromatography with Reverse phase). The method employs specific chromatographic conditions including a 265 nm detector, Phenomenex Luna SCX column and 35°C column temperature, along with mobile phases A and B created using pH 2.5 buffer solutions and acetonitrile. The validation process covers parameters of system suitability, specificity, limit of detection (LOD) and limit of quantification (LOQ) for impurities and SAPR as well as forced degradation studies to confirm stability indication. Parameters such as precision, accuracy, linearity, range, ruggedness, solution stability and robustness were thoroughly validated.

This study also discusses the suitability of 0.45µm PVDF and nylon filters for sample preparation and emphasizes the role of the validated method in enhancing pharmaceutical quality control for batch analysis release and stability studies of SAPR powder for oral suspension. The precise quantification of SAPR and its associated impurities are crucial in pharmaceutical analysis. This validated method ensures consistent and accurate analysis.

Keywords: SAPR, oral suspension, forced degradation studies, Impurities, mobile phases.

Introduction

SAPR plays a crucial role as an enzyme cofactor and is obtainable in an oral form, synthesized as the hydrochloride salt of tetrahydrobiopterin (BH4), a compound naturally occurring in the body[2,4,10,14]. This synthetic variant, referred to as SAPR, is a pivotal element in the synthesis of nitric oxide, underscoring its significance in biological processes. Possessing a molecular weight of 277.09 g/mol, SAPR represents a substantial constituent in pharmaceutical applications. The molecular attributes of SAPR encompass a weight of 277.09 g/mol and as a raw material, it requires storage within the temperature range of 2 to 8°C. These specific storage conditions underscore the compound's susceptibility to environmental factors, emphasizing the necessity of preserving its stability for effective utilization.

SAPR manifests distinctive electrochemical properties, demonstrating oxidation potential at +0.27 V and reduction at -0.16 V. These features are pivotal in comprehending the compound's conduct in diverse reactions and environments, contributing to its function as an enzyme cofactor. The researchers examined impurity separation in SAPR within a pharmaceutical product presented in oral suspension form. This signifies a pragmatic application of SAPR in the pharmaceutical industry and accentuates the importance of ensuring the purity of the compound for therapeutic purposes.

This research provides analytical method for quantification of impurities in SAPR powder oral suspension[1,5,7]. Prasad et al[15] developed a method to estimate SAPR by using UV Spectroscopy and method was developed based on oxidation NBS.

Scudellaro et al[12] developed a method to estimate the impurities available in SAPR containing drugs in stability studies by using Column Partisil® column, 250 X 4.6 mm 10 SCX-250A i.d, five µm, sodium di hydrogen phosphate buffer with pH 3.0 with 1.20 mL/min flow and the 20 minutes of chromatographic run, 20 µL injection volume at wavelength of 265 nm[12]. Different authors[3,6,8,9,11,13] described various drugs and their studies. We developed HPLC method to separate these impurities from SAPR in SAPR powder for oral solution. The impurities identified are 1(a), 1(b), 1(c) and 1(d) for SAPR, IMPT-1, IMPT-2 and IMPT-3 as represented in the figure 1.

Material and Methods

Standard, Impurities, Chemicals and Reagents: For this analysis; SAPR (99.97% purity), IMPT-1 (93.77% purity), IMPT-2 (97.78% purity) and IMPT-3 (98.0% Purity) were obtained from Dr Reddy's Laboratory, Hyderabad, India. Analytical/HPLC-grade orthophosphoric acid, Sodium dihydrogen orthophosphate, acetonitrile, hydrochloric acid and ascorbic acid were procured from different manufactures

Instrumentation and Software: Liquid chromatographs are equipped with UV or PDA detectors in the wavelength range of 200-400 nm. The HPLC system consists of several components including gradient pumps, auto samplers (Sample Manager SM.), vapour columns, UV and PDA detectors. Empower-3 software was used to collect data. The HPLC column used was Phenomenex Luna SCX, 250 x 4.6mm, 5µm. A Sartorius balance, a pH meter from Lab India and a Metler Toledo balance were also used. Other equipments include ultrasonic generators from Hwashin, centrifuges from Lab Companion, Hemel, photo stability chambers and thermal ovens from Newtronic.

Optimization of Chromatographic Conditions: Chromatographic conditions were performed according to Phenomenex Luna SCX, 250 × 4.6 mm, 5 µm column, 35°C column oven temperature, 10°C sample temperature, gradient program at a rate of flow 0.70 mL/min, run time in a time interval of 65 min. To get separation gradient flow programme with mobile phase A pH 2.5 Buffer solution dissolve 6.0 g of sodium dihydrogen phosphate (NaH_2PO_4) in 1000mL with water. Adjust the pH to 2.5 by dilute ortho phosphoric acid (10% v/v) along with acetonitrile in the ration of 95:5 respectively. Mobile phase B pH 2.5 Buffer solution - dissolve 12.0 g of Sodium dihydrogen phosphate (NaH_2PO_4) in 1000mL with water. Adjust the pH to 2.5 by dilute ortho phosphoric acid (10% v/v) as well as acetonitrile in the ration of 80:20. Gradient programme represented in the table 1. HPLC System recorded the chromatograms by using injection volume of 20µL, at 265 nm wave length.

Preparation of System suitability and standard solution: Diluent is freshly prepared as 0.2% ascorbic acid solution which is used for analytical solution preparation and blank injection. System suitability solution contains SAPR 1.00 mg/ml and impurity 0.05 mg/ml. Dilute standard solution, prepare SAPR 0.003 mg /ml. Inject 10µL of blank, system suitability solution. Record the chromatogram and measure the peak responses at 265nm as in fig. 2.

Preparation of Sample Solutions (CDB- 250 µg/mL, CDH - 500 µg/mL): For this estimation, 10 sachets were taken. Tap each sachet in such a way to bring powder at the bottom of sachet. Cut the sachet at the top corner and transfer the content to a poly bag and mix the content thoroughly. 1.00mg/mL SAPR di-hydro-chloride powder for oral solution was prepared by using diluent with sonicating the volumetric flask for 10 minutes with intermediate shaking. Ensure that all the content is completely dissolved. A typical sample chromatogram is presented in figure 3.

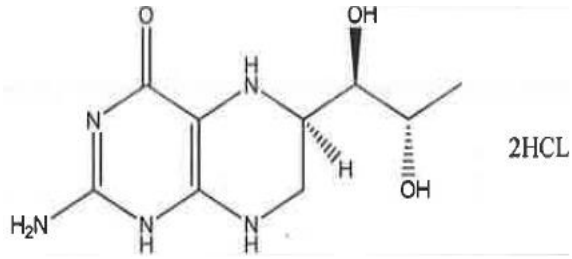

Fig. 1(a): SAPR Structure

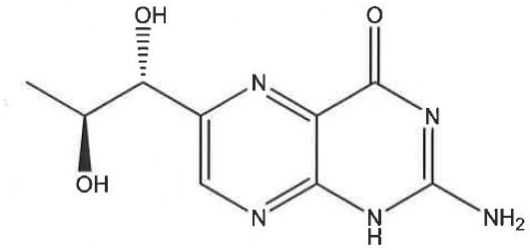

Fig. 1(b): IMPT-1

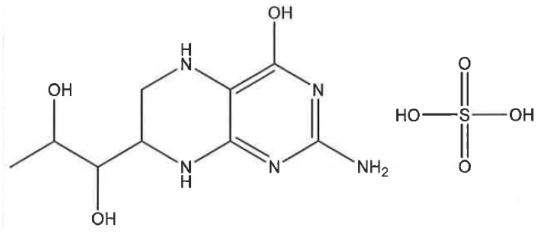

Fig. 1(c): IMPT-2

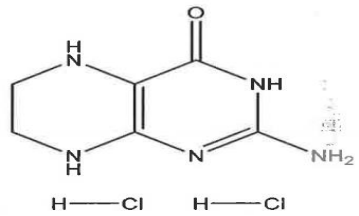

Fig. 1(d): IMPT-3

Fig. 1(a): SAPR Structure; Fig. 1(b): IMPT-1 structure; Fig. 1(c): IMPT-2; structure; Fig. 1(d): IMPT-3 structure

Table 1
Gradient Program

Time (in min)	% of Mobile Phase A	% of Mobile Phase B (%)
0.01	100	0
5	100	0
35	55	45
40	50	50
50	0	100
55	0	100
60	100	0
65	100	0

Results and Discussions

Method Validation: International Conference on Harmonization (ICH) guidelines are referred while performing analytical method validation.

Specificity: Study was performed by injecting placebo, system suitability solution, known impurities of IMPT-1, IMPT-2 and IMPT-3spiked on sample solution (Sample along with impurity) into chromatographic system. There was no interference due to placebo and blank at retention time of SAPR, IMPT-1, IMPT-2 and IMPT-3 as tabulated in table 3. A typical chromatogram of spiked sample is given in figure 4.

Stressed Condition Studies: Various forced degradation studies using specificity by stressed condition were performed by preparing the sample solution, placebo, acid stressed sample as concentrated HCl for about 6 hours 35 minutes at 75°C on water bath; alkali stressed sample as 1N NaOH for about 1 hour 06 minutes at room temperature, hydrogen peroxide stressed 30% H_2O_2 for about 1 hour at room temperature; water for about 7 hours 10 minutes at 60°C on water bath; sample exposing to UV and visible light, sample exposing to heat for about 24 hours 33 minute at 105°C temperature. Overall degradation products well separated from SAPR and related impurities. Under the different stress conditions, product has degraded and mass balance was obtained. Finally, it was concluded that the SAPR undergoes degradation. SAPR peak purity was identified by Empower software and it was observed that purity angle < purity threshold. Stress study results are tabulated in table 4 and 5.

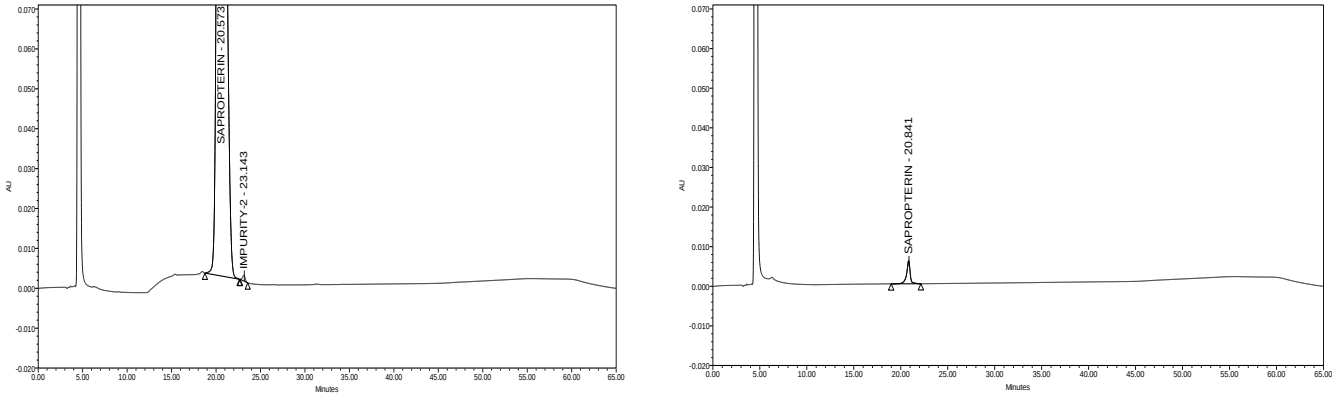

Figure 2: Typical chromatogram of Peak identification and Diluted Standard

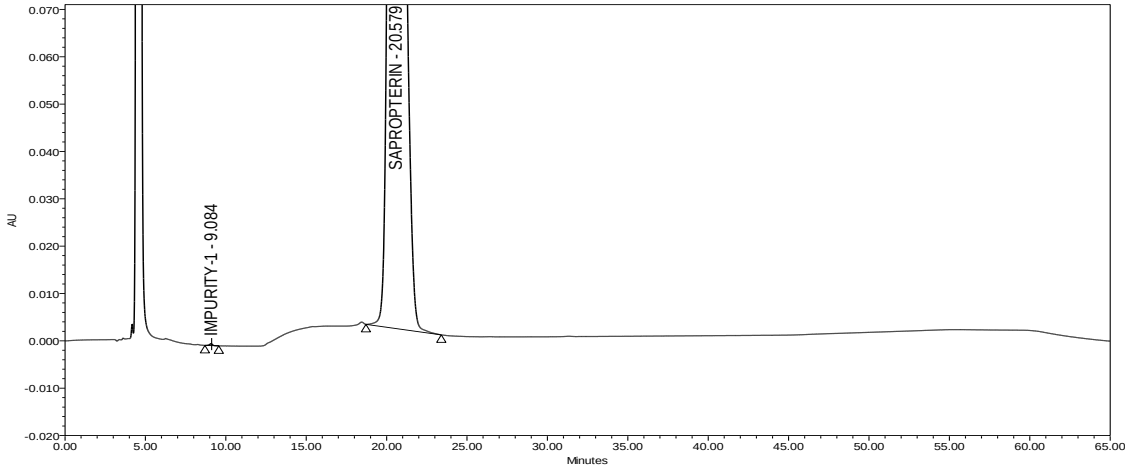

Figure 3: Typical chromatogram of SAPR Powder for orals solution sample solution

Table 2
Table of Results of System Suitability

System suitability parameters	Observed value
Resolution between SAPR and IMPT-2 peaks	4.2
Tailing factor for SAPR peak from Standard solution chromatogram	0.8
SAPR peak theoretical plates in standard solution chromatogram	19028
%RSD of SAPR peak replicate of standards	0.8
%RSD of IMPT-2 from System suitability	1.0

LOD and LOQ: Prepare a total of six test preparations at LOQ strength over placebo after that inject into the system of HPLC. % RSD of SAPR and their known foreign substances from six replicate preparations were found within the limit. Accuracy of known impurities and SAPRat about LOQ was conducted. Triplicate solutions were prepared at LOQ level by spiking IMPT-1, IMPT-2 and IMPT-3 on test (API+ Placebo) and injected into the HPLC. LOQ accuracy was calculated for SAPR. % recovery of known impurities and of SAPR was found well in the acceptance limit. The results are represented in the table 6.

Linearity: A series of solutions of IMPT-1, IMPT-2, IMPT-3 and SAPR peaks (for unknown foreign substances) with strength ranging from about limit of quantification to 150 % level were prepared. The specification were prepared after implanting into HPLC system. The correlation coefficient and % bias to 100% level, for known foreign substances and main peak were found within the limit. The linearity results are represented in the table 7.

Table 3
Retention time of Active and Impurities

Names	Retention time
SAPR	20.58
IMPT-1	9.09
IMPT-2	23.14
IMPT-3	28.84

Table 4
Stress study result for SAPR

S.N.	Conditions	% Net Degradation	% Mass Balance	PAG	PTH	Flag
1	As Such	NA	NA	0.388	1.027	No
2	Acid-Concentrated HCl for about 6 hours 35 minutes at 75°C on water bath.	1.4360	100.7	0.462	1.034	No
3	Base-1N NaOH for about 1 hour 06 minutes at room temperature.	3.8349	99.0	0.435	1.026	No
4	Peroxide-30% H_2O_2 for about 1 hour at room temperature	0.3956	100.0	0.556	1.053	No
5	Water for about 7 hours 10 minutes at 60°C on water bath.	2.4358	101.4	0.442	1.040	No
6	Thermal-Dry heating for about 24 hours 33 minute at 105°C temperature.	0.3033	100.7	0.427	1.035	No
7	Photo stability-Exposed to UV light 200 Watts hr/square meter and Visible light for 1.2 million lux hours.	0.0096	100.8	0.346	1.031	No
8	Humidity 90% RH and 25°C for 3 days	2.1285	100.1	0.238	1.040	No

Table 5
% Known from Stress study

S.N.	Conditions	IMPT-1	IMPT-2	IMPT-3
1	As Such	0.0126	0.0321	ND
2	Acid-Concentrated HCl for about 6 hours 35 minutes at 75°C on water bath.	0.1071	ND	ND
3	Base-1N NaOH for about 1 hour 06 minutes at room temperature.	0.0701	ND	0.0659
4	Peroxide-30% H_2O_2 for about 1 hour at room temperature	0.0887	ND	0.0282
5	Water for about 7 hours 10 minutes at 60°C on water bath.	1.3008	ND	ND
6	Thermal-Dry heating for about 24 hours 33 minute at 105°C temperature.	0.2367	ND	ND
7	Photo stability-Exposed to UV light 200 Watts hr/square meter and Visible light for 1.2 million lux hours.	0.0597	ND	ND
8	Humidity 90% RH and 25°C for 3 days	1.1550	0.0398	ND

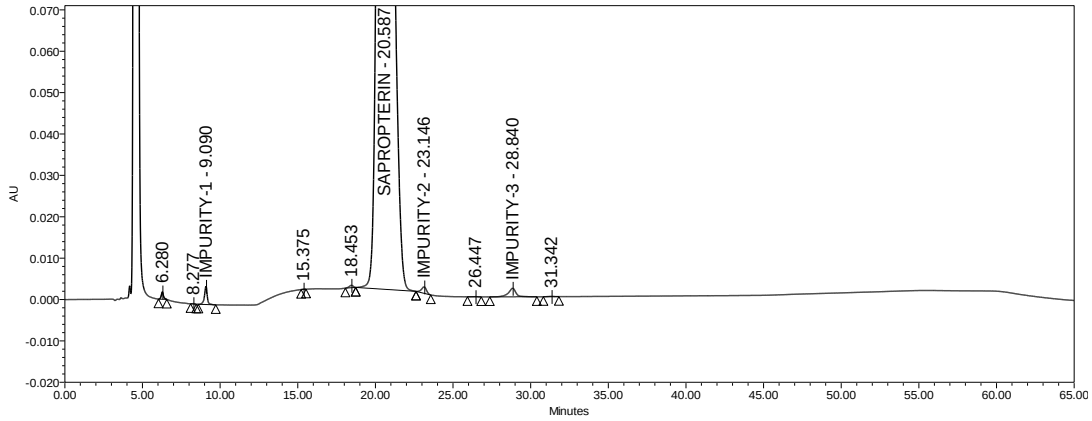

Figure 4: Typical chromatogram of SAPR POS Spiked sample solution

Table 6
Results of LOD and LOQ

Impurity name	LOQ (%)	LOD (%)	LOQ	
			Precision(%RSD)	Accuracy (% Recovery)
SAPR	0.028	0.006	2.3	103.7
IMPT-1	0.036	0.009	0.4	108.0
IMPT-2	0.032	0.009	1.9	93.4
IMPT-3	0.034	0.012	0.3	104.5

Table 7
Results of Linearity

Name of the impurity	Correlation coefficient	Slope	Intercept	Bias at 100%
IMPT-1	0.999969	50796.319290	681.103631	1.31
IMPT-2	0.999939	53666.249664	353.799246	0.65
IMPT-3	0.999922	67994.329332	704.357598	1.05
SAPR	0.999706	49316.700112	5483.290664	3.81

Precision (Repeatability): Determine the precision by spiking of known impurities to get the specification level on test preparation and SAPR (for unknown foreign substances) on Placebo. Calculated % foreign substances and % RSD results were found within the limit for known impurities and SAPR.

The precision of test method was determined by spiking of known impurities at specification level on test preparation. Precision of SAPR (for unknown impurities) was performed on placebo. Calculated % impurities and % RSD, results were found within the limit for known impurities and SAPR (for unknown foreign substances).

Accuracy: This parameter is evaluated on test solution by spiking foreign substances at levels ranging LOQ to 150% of foreign substances level of specification in test solution. Additionally, SAPR was spiked into the placebo at level of specification. % recovery values were within acceptable limits of 85.0% to 115.0%. Results are tabulated in table 8.

Stability of standard and sample solution at Room Temperature: Standard stability for SAPR was established on room temperature for a period of 24 hours. Two test solution spiked by known impurities at stability level of specification are constructed as per test method and kept on room temperature and injected at after 20 hours and 24 hours of study. It was established that used solutions of sample are highly stable on room temperature in a time interval of 20 hours.

Stability of standard and sample solution at 2°C-8°C: Standard stability was established at 2°C-8°C for a period of 5 days. Two test solution spiked with acknowledged impurities at stability level of specification were constructed as per test method and kept at 2°C-8°C and injected initial, after day 1, day 2 and day 5. By study, it is entrenched that these standard as well as solutions of sample were stable at a temperature of 2°C-8°C in a time interval of 5 days.

Bench top stability of Mobile phase: A 5-day study was conducted to determine stability of benchtop mobile stage. After 1, 2 and 5 days, the same set was used to add blank solutions, standard solutions, peak concentrations and innovative solution to the HPLC system by increasing the known impurities at the specification level. It was seen that mobile time remained constant on the bench for 5 days.

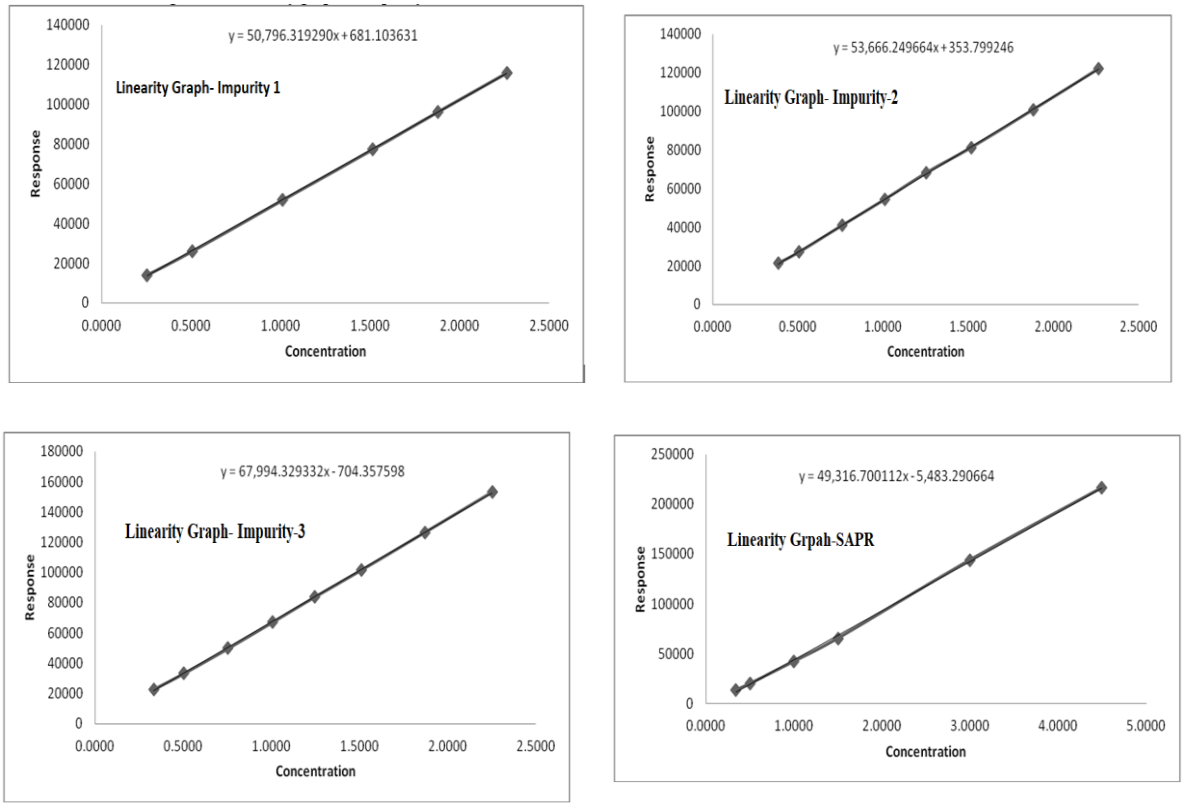

Figure 4: Linearity Graph SAPR, IMPT-1, IMPT-2 and IMPT-2

Table 8
Results of Accuracy and Precision

Parameter	IMPT-1	IMPT-2	IMPT-3	SAPR
LOQAccuracy	108.0,	93.4,	104.5	103.7
50% Accuracy	93.1	96.0	101.5	90.2
100% Accuracy	96.5	104.4	103.2	91.8
150% Accuracy	90.4	107.8	105.5	93.2
Repeatability (%RSD)	0.4	0.5	0.4	0.8
Intermediate precision (%RSD)	0.4	0.7	1.6	2.1
Cumulative (%RSD)	6.1	0.9	2.6	2.5

Robustness: Within the scope of the robustness study, it is determined that acceptable change in rate of flow ranged as 0.5 mL/min to 1.8 mL/min. Allowable variation in column oven temperature is in a temperature range as 30°C to 50°C. Permissible change in pH buffer (pH buffer in mobile phase A) is from pH 2.3 to pH 2.5.

Filter validation: Filter validation was performed by preparing a test sample on two different filters (e.g. 0.45 µm PVDF and 0.45 µm Nylon), adding known impurities of impurity content and injecting it into the HPLC system according to the test method. Centrifuge a portion of the above solution and filter a portion of each test solution through two filters and discard the first 3 mL of the sample solution. The discrepancy between the percentage of impurity and total foreign substances and between centrifuged and PVDF filter samples is within limits. The difference between the percentage of foreign matter and total impurities of centrifuged as well as nylon filter samples is also within limits. Therefore, it was concluded that PVDF and nylon filters were suitable to filtration.

Conclusion

Validation of the HPLC method IMPT-1, IMPT-2 and IMPT-3 for SAPR powder for oral solution was carried out according to ICH rules and regulations and this analysis was determined to be particularly suitable for effects from placebo and degradation products, linearity, method precision, robustness (medium level precision), accuracy, LOD and LOQ design, LOQ precision, LOQ accuracy, range (linearity, precision and accuracy), stability, robustness. The construction method was designed for use in routine inspections for quality control.

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