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CHARACTERIZATION AND VALIDATION OF IMPURITIES RELATED TO PHARMACEUTICAL BULK DRUG (API) BY USING SOME ANALYTICAL TECHNIQUES

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ABSTRACT: Active Pharmaceutical Ingredient (API) of pharmaceutical bulk drug, four impurities were identified and were detected by a newly developed Reverse phase high performance liquid chromatographic (HPLC) method. The R.S.D.'s for SM-I, SM-II, SM- III and SM were found to be 1.48%, 1.71%, 1.67%, 6.37% respectively. These values are within the acceptance criteria of 10%.The limit of quantification values for SM-I, SM-II and SM-III were found to be 0.006 %, 0.006 %, and 0.006 % w.r.t. analyte concentration (500 µg /cm^3), respectively. For determining method accuracy, known as unclean LOQ, the specified limit of 80%, 100% and 120% of the SM bulk sample (test preparation) was pointed. For confirming method precision six different test preparations of samples of SM were analyzed. Identified impurities were characterized by LC/MS/MS method. Identified impurities were unknown. Structural determination of such impurities was carried out by LC/MS/MS using electro spray ionization source and an ion trap mass analyzer. Structural identification by using nuclear magnetic resonance (NMR) and infrared (IR) spectroscopy. The method was validated according to ICH guidelines with respect to Specificity, Precision, Accuracy, Linearity and Robustness.

INTRODUCTION: Sulfa methoxypyrazine (SM) such as N1 - (3-Methoxypyrazin – 2 - yl) Sulphanilamide is a long-acting sulfonamide which has been used orally for the treatment of respiratory and urinary tract infections [1]. It gives the combination with pyrimethamine [2, 3] in the treatment of malaria. It has also been given in the ratio 4 parts of Sulfa methoxypyrazine to 5 parts of trimethoprim as a combination with uses similar to those of co-trimoxazole [4].

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A few bio-analytical techniques were reported in the literature for the quantitative determination of Sulfa methoxypyrazine (SM) concentration in biological fluids using liquid chromatography and mass spectroscopic method [5, 6, 7]. However, so far there is no published report, describing the complete characterization of related impurities in Sulfa methoxypyrazine as active pharmaceutical ingredient (API). Some part of the paper is reported by using LC/MS/MS and isolation/synthesis of related substances in Sulfa methoxypyrazine Active Pharmaceutical Ingredient [8].

Impurity profile of a drug substance is critical for its security valuation and manufacturing process. It is required to identify and characterize the impurities in the pharmaceutical bulk drugs, if

impurities are present above then the acceptable limits of 0.1 % [9]. The present study deals with the identification and structural explanation of the process related impurities, which were found in the pharmaceutical bulk drugs Sulfa methoxypyrazine. Though, different methods of synthesis of Sulfa methoxypyrazine are reported, the selected route was safe, feasible & economical [10]. However, the literature survey does not give any details regarding these impurities. Impurity profiling of drugs is the most important issue in the modern pharmaceutical analysis [11, 12] for developing process technology to manufacture high purity drug substance. During process development studies, four impurities were detected in both crude and pure samples of SM using a newly developed gradient reversed phase HPLC method. This paper also deals with the analytical method validation of a new HPLC method for quantitative determination of these impurities.

MATERIALS AND METHODS:

Materials: Samples of API were obtained from pharmaceutical laboratories, Chemical Research Division, Mumbai, India. HPLC grade CH_3CN and perchloric acid (70%) were purchased from Merck India Limited. Chloroform(d) and DMS (d) (for NMR) were purchased from Aldrich Company.

Methods: High Performance Liquid Chromatography: The Samples were analyzed on Alliance 2690 HPLC (Waters, Milford, MA, USA) system equipped with 2487 UV\detector. A Unisphere C18 column (150 mm x 4.6 mm i.d. 5 µm) was used for chromatographic separation. The mobile phase consisting of A: 1 cm^3 perchloric acid (70%) in 1000 cm^3 of water and B: acetonitrile, with timed gradient programmer *T*min /A: B: *T*0/85:15; *T*10/85:15; *T*30/50:50; *T*40/85:15; *T*45/85:15 with flow rate of 0.8 ml per minute were used. The column oven temperature was maintained at 30 °C. The injection volume was 20µL and the detector wavelength was fixed at 270 nm.

Liquid Chromatography-Tandem Mass Spectrometry (LC/MS/MS): The MS and MS/MS studies were performed on LCQ Advantage (Thermo Electron, San Jose, CA) ion trap mass spectrometer. The source voltage was maintained at 3.0 kV and capillary temperature at 250 °C. Nitrogen was used as both sheath and auxiliary gas. The mass to charge ratio was scanned across the various range. MS/MS studies were carried out by keeping normalized collision energy at 25-30% and an isolation width of 6 amu. The HPLC consisted of an Agilent-1100 series quaternary gradient pump with a degasser, an auto sampler and column oven. A C18 column (ProntoSIL Kromabond column 150 mm x 4.6 mm i.d. 5 µm) was used for separation. The mobile phase consisting of A: 1 cm^3 Trifluoracetic acid in 1000 cm^3 water and B: acetonitrile, with timed gradient programme *T*min/A: B: *T*0/85:15; *T*10/85:15; *T*30/50:50; *T*40/85:15; *T*45/85:15 with flow rate of 0.8 ml per minute were used.

NMR Spectroscopy*:* ^1H and ^{13}C NMR spectra of the synthesized/isolated impurities were recorded on Bruker 400MHz instrument. The ^1H and ^{13}C chemical shift values were reported on the δ scale (ppm) relative to $CDCl_3$ (7.26 ppm).

Preparative Liquid Chromatography: Impurities were isolated from the bulk sample using Waters Auto purification system consisting of 2525 binary gradient pump, a 2487UV detector and 2767 sample manager (Waters, Milford MA, USA). A Peerless Basic C18 column (150mm×21.2mm i.d., particle size 5µm) was used for the separation. The mobile phase was consisted of a mixture of water and acetonitrile in the ratio of 85:15 and was pumped at flow rate 25 cm^3 /min. The detection was monitored at 270 nm.

Preparation of Solutions for Validation of HPLC Method: The test preparation solution of 500 µg/cm^3 of SP bulk drug sample were prepared by using the diluents (mixture of 0.1% perchloric acid (75%) in water and acetonitrile, ratio is 85:15. A stock solution of mixture of impurities were prepared by dissolving 0.5mg/cm^3 each of SM-I, SM-II, SM-III and SM. From this stock solution, a standard solution containing 0.5µg/cm^3 each of SM-I, SM-II, SM-III and SM were prepared. This standard solution was also used for checking system suitability parameters.

RESULTS AND DISCUSSIONS:

Detection of Impurities by HPLC: Using HPLC analysis method are described and having the presence of four impurities at RRTs 0.20, 0.25,

0.54 and 1.34 with respect to principle peak. The target impurities under study are marked as Sulphanilamide used as a starting material, SM-I, SM-II, and SM-III, respectively. The typical chromatogram of crude SM sample highlighting the retention time of impurities.

Identification of Impurities by LC/MS/MS: The previous work of characterization is ideological to produce the mass data for the parent drug molecule so that, it may be easily compared and achieve, the process related impurities may be formed during the synthetic reaction. The spectra of SM exhibits a protonated molecular ion peak $[M+H]^+$ 281 (**Fig. 1**) (molecular mass of SM is 280) in electro spray ionization in positive mode, the most plausible position of protonation was at NH_2 and NH. The MS/MS spectrum taken for the protonated SM molecule showed prominent peak at 156 (**Fig. 2**) which is due to NH-SO_2 bond giving rise to $C_6H_6NO_2S^+$ probable fragmentation are shown in (**Fig. 3**).

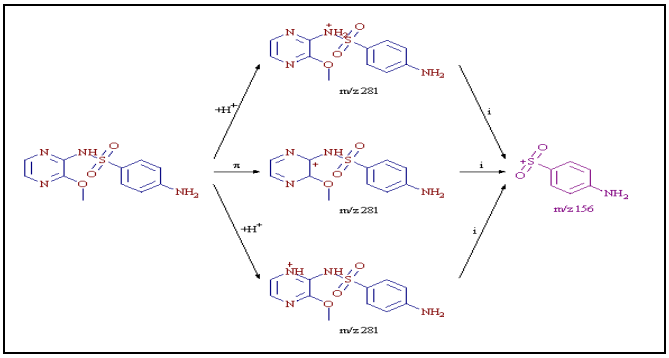


FIG. 1: PLAUSIBLE SCHEME FOR FRAGMENTATIONS OF SM

SM-I showed a protonated molecular ion peak $[M+H]^+$ 267 having molecular mass of 266, which under goes fragmentation to form $C_6H_6NO_2S^+$ for 156 by loss of $C_4H_5N_3O$ ion.

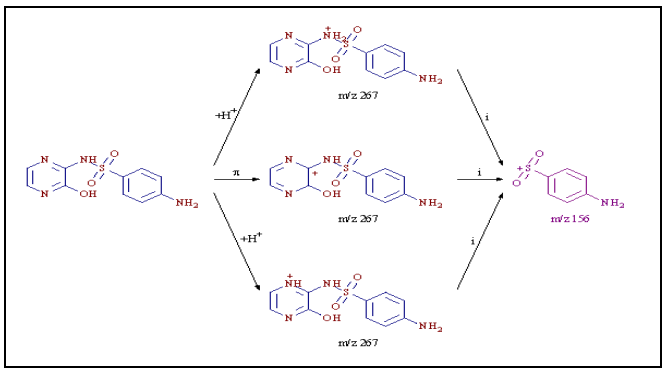


FIG. 2: PLAUSIBLE SCHEME FOR FRAGMENTATIONS OF SM-I

SM-II similarly showed $[M+H]^+$ of 251 for molecular mass of 250 and a loss of $C_4H_5N_3^{\bullet+}$ giving daughter ion of mass 156.

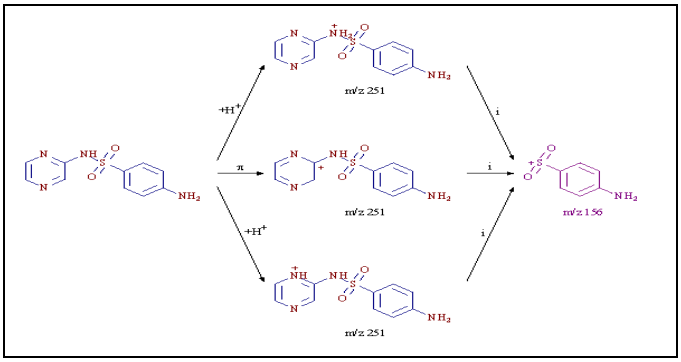


FIG. 3: PLAUSIBLE SCHEME FOR FRAGMENTATIONS OF SM-II

SM-III, which is an isomer, showed similar fragmentation that of SM. From all the mass fragmentation as discussed above, showed similar daughter ions of 156 for $C_6H_6NO_2S^+$ which revealed that these impurities are structurally similar.

SM-I: showed a protonated molecular ion peak $[M+H]^+$ 267 having molecular mass of 266, which under goes fragmentation to form $C_6H_6NO_2S^+$ for 156 by loss of $C_4H_5N_3O$ ion.

SM-II: similarly showed [M+H]+ of 251 for molecular mass of 250 and a loss of $C_4H_5N_3$ $\bullet^+$ giving daughter ion of mass 156.

SM-III: which is an isomer, showed similar fragmentation that of SM (**Fig. 4**). Since all the mass fragmentation as discussed above, showed similar daughter ions of 156 for $C_6H_6NO_2S^+$ indicated that such impurities are structurally similar. SM-III and SM were having same molecular mass and may be Regio isomer of each other; later it was fixed to confirm the structure by NMR. Hence NMR of all the impurities and the product was carried out for comparison and further confirmation of structure.

Since SM-III and SM were having same molecular mass and may be diasteriomer, hence it was mandatory to confirm the structure by NMR. Hence NMR of all the impurities and the product was carried out for comparison and further confirmation of structure.

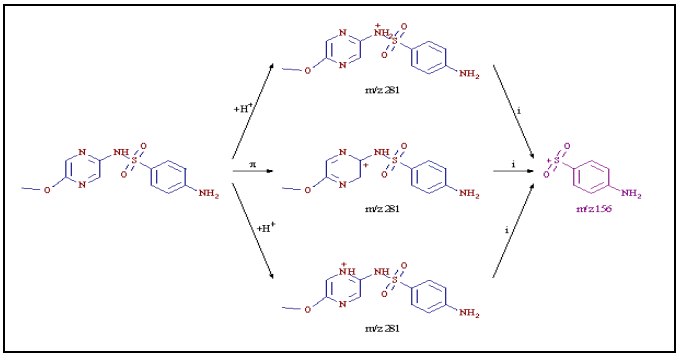


FIG. 4: PLAUSIBLE SCHEME FOR FRAGMENTATIONS OF SM-III

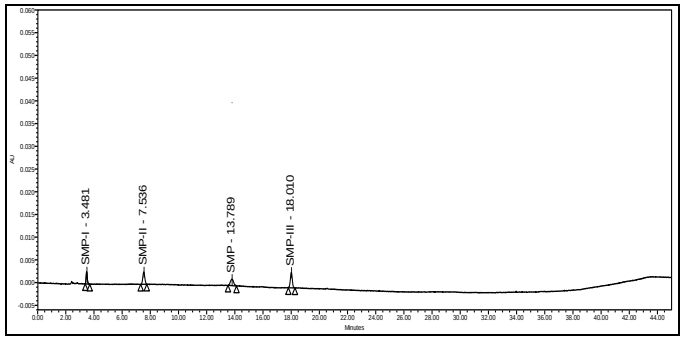


FIG. 5: CHROMATOGRAM OF SYSTEM SUITABILITY SOLUTION

Analytical Method Validation by HPLC: The validation study allowed the assessment of the process for its appropriateness for routine analysis. The new advanced system for SM and its related impurities was validated according to ICH guidelines [9]. The validation study was accepted for the analysis of SM-I, SM-II and SM-III. The system appropriateness parameters obtained for related substance process are given (**Fig. 5**). Forced degradation studies were also performed (Acid, Base) for SM bulk drug sample to demonstrate the stability indicating power of the newly developed HPLC method.

Specificity: Specificity is the capacity of analytical process to found the amount of analyte response in the existence of its potential impurities and degradants. To specificity the HPLC technique for the determination of injecting individual impurity samples, wherein no interference was observed for any other components. The chromatograms were checked for the presence of any extra peak. Peak purity of these samples under stressed conditions was verified using a PDA detector. The purity of the principle and other chromatographic peaks was found to be acceptable. This study confirmed the stability indicating power of the HPLC method.

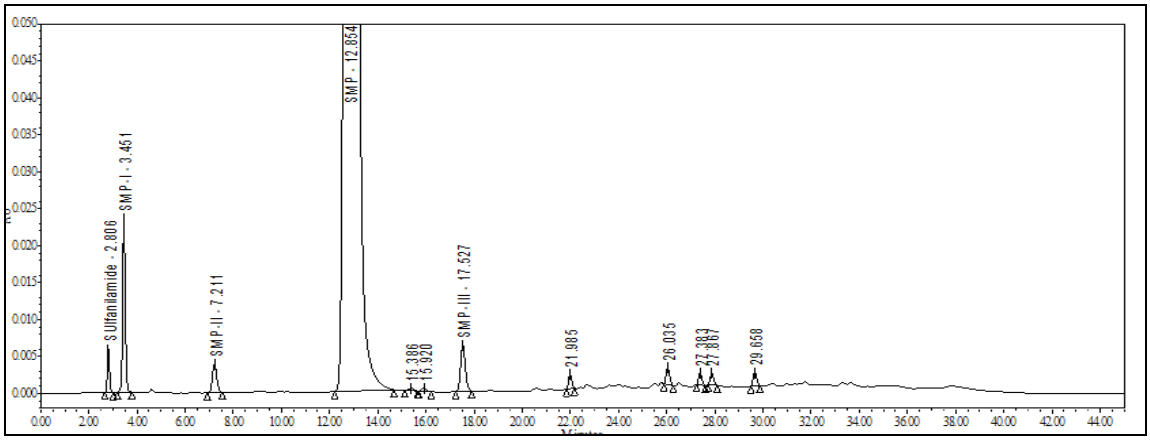


FIG. 6: A TYPICAL CHROMATOGRAM OF SM SAMPLE

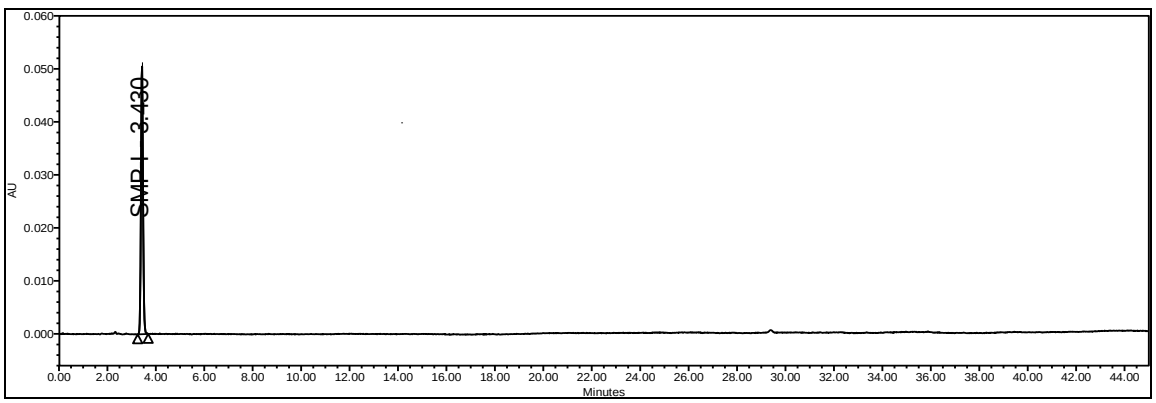


FIG. 7(a): CHROMATOGRAM OF SM-I IN IDENTIFICATION

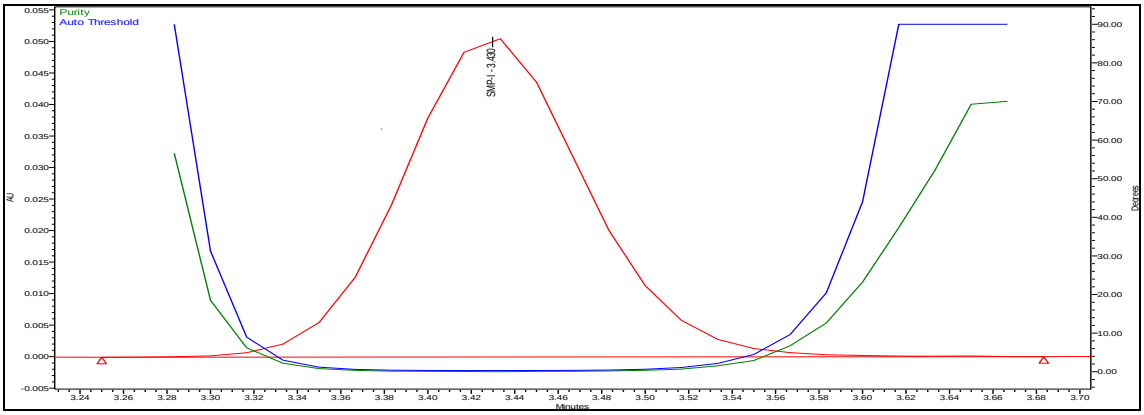


FIG. 7 (b): PDA PEAK PURITY SPECTRUM OF SM-I

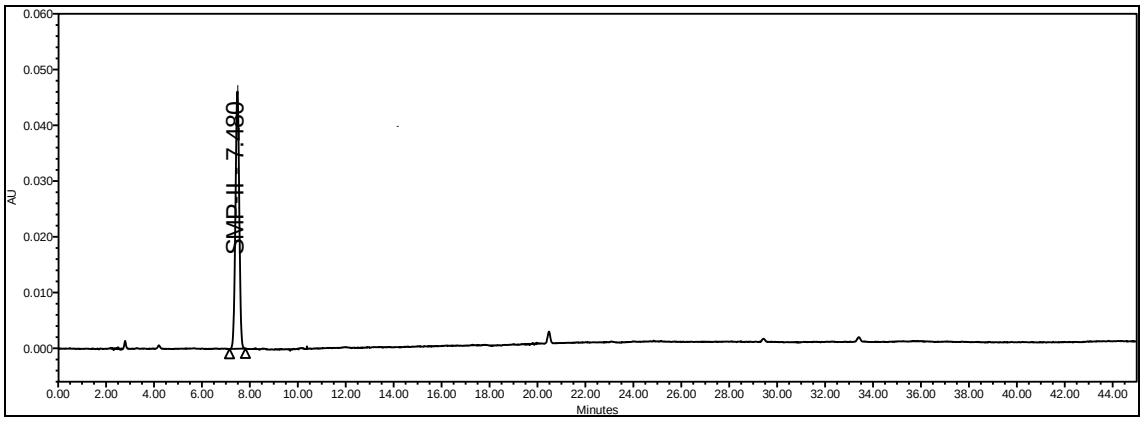


FIG. 8 (a): CHROMATOGRAM OF SM-II IN IDENTIFICATION

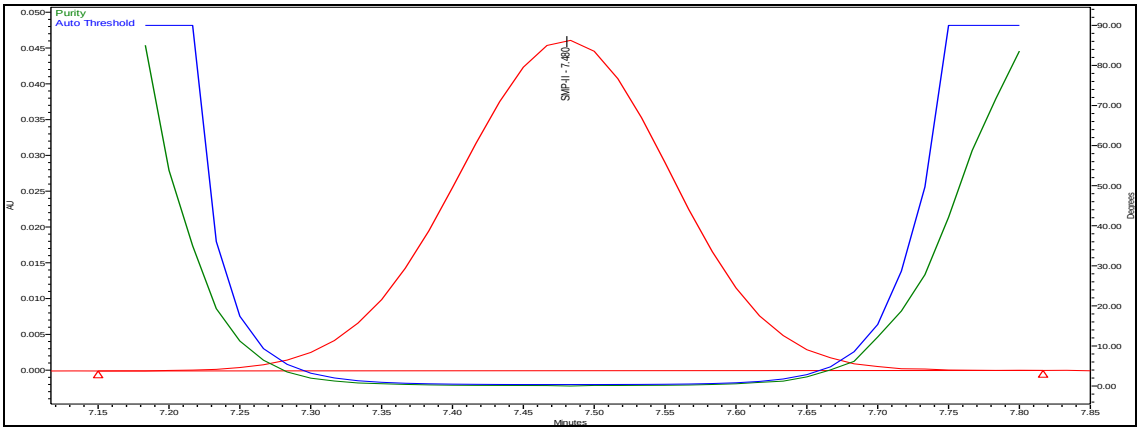


FIG. 8 (b): PEAK PURITY PDA SPECTRUM OF SM-II

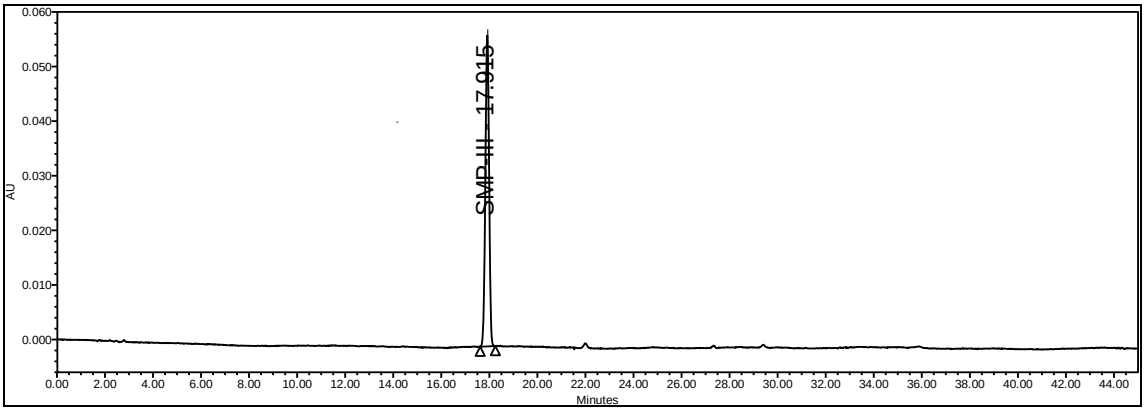


FIG. 9 (a): CHROMATOGRAM OF SM-III IN IDENTIFICATION

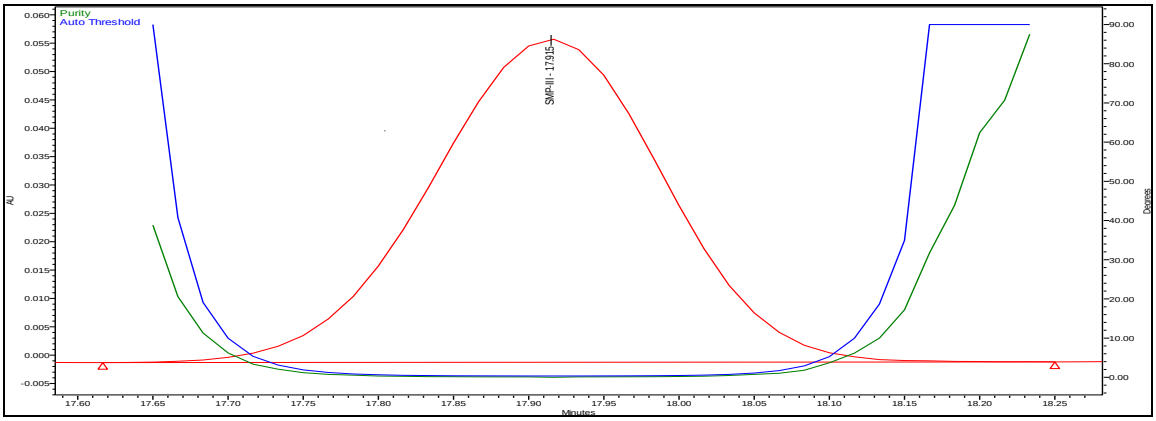
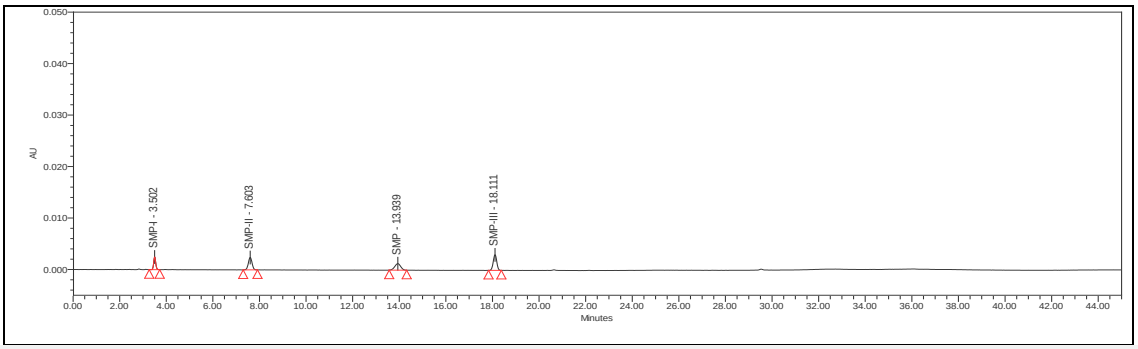


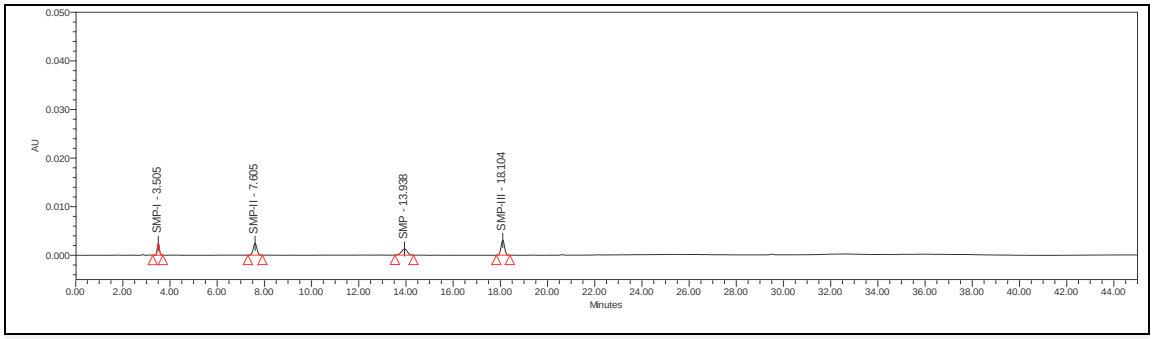
FIG. 9 (b): PEAK PURITY PDA SPECTRUM OF SM-III

Precision: The ability of the method to precisely quantify impurities was determined by calculating the relative standard deviation (RSD) for response (peak area) of each impurity in the standard solution (a mixture of impurities) a copy of the injections. The R.S.D.'s for SM-I, SM-II, SM and SM-III were found to be 1.48%, 1.71%, 6.37% and 1.67%, respectively. These values are within the acceptance criteria of 10%. For confirming method precision six different test preparations of samples of SM were analyzed. The determined R.S.D. of these results was found to be under acceptable limit.



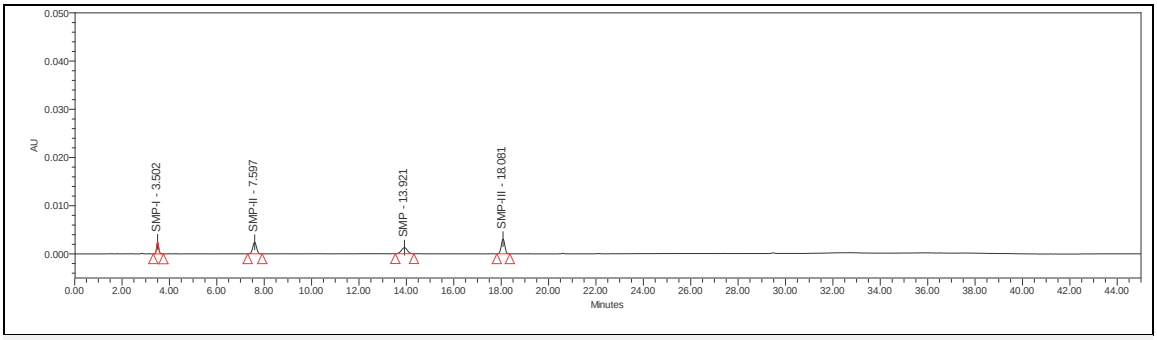
Name	Retention Time	Area	% Area	Height
SM-I	3.502	15391	15.99	2474
SM-II	7.603	26782	27.82	2414
SM	13.939	22083	22.94	1256
SM-III	18.111	32027	33.26	3043

FIG. 10(a): CHROMATOGRAM OF MIX STANDARD SOLUTION (INJECTION-01) IN PRECISION



Name	Retention Time	Area	% Area	Height
SM-I	3.505	15340	15.89	2496
SM-II	7.605	26965	27.93	2434
SM	13.938	22220	23.02	1265
SM-III	18.104	32019	33.17	3066

FIG. 10(b): CHROMATOGRAM OF MIX STANDARD SOLUTION (INJECTION-02) IN PRECISION



Name	Retention Time	Area	% Area	Height
SM-I	3.502	15422	15.99	2494
SM-II	7.597	26795	27.78	2423
SM	13.921	22164	22.98	1260
SM-III	18.081	32056	33.24	3057

FIG. 10 (C): CHROMATOGRAM OF MIX STANDARD SOLUTION (INJECTION-03) IN PRECISION

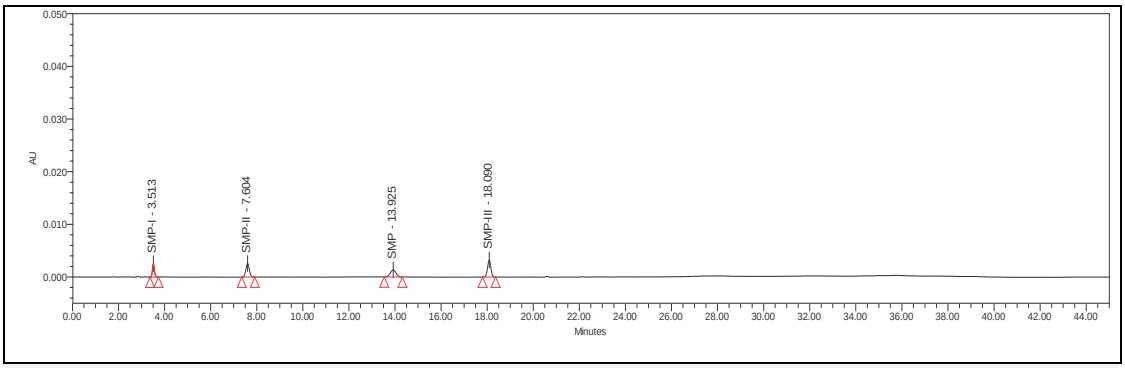
TABLE 1: EVALUATION DATA OF PRECISION STUDY

No. of injection	SM-I	SM-II	SM	SM-III
1	15391	26782	22083	32027
2	15340	26965	22220	32019
3	15422	26795	22164	32056
4	15371	26767	22466	32124
5	15477	26920	22080	32148
6	15351	26613	22066	32095
Mean	15392	26807	22179.83	32078.17
SD	50.8960	124.6900	152.3528	52.6704
%RSD	0.33%	0.47%	0.69%	0.16%

TABLE 2: SYSTEM SUITABILITY REPORT IN PRECISION

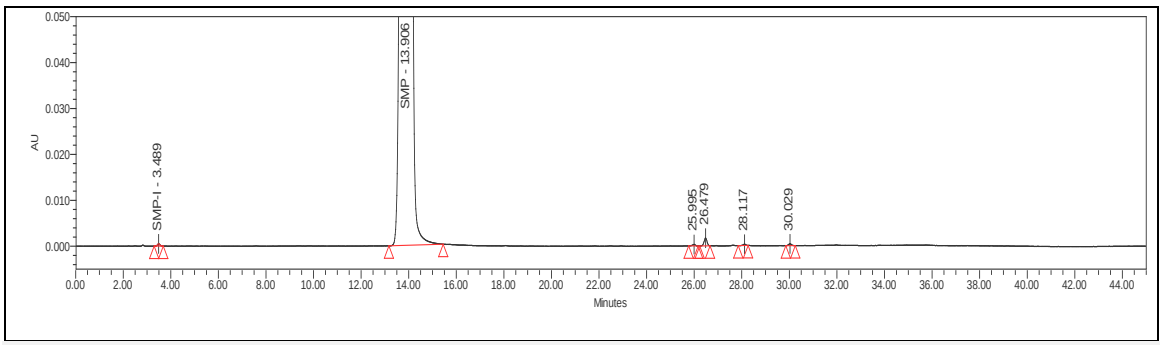
Component	Tailing factor	Theoretical plates	% RSD
SM-I	1.10	8547	1.48
SM-II	1.02	13763	1.71
SM	0.98	17644	1.67
SM-III	1.06	86953	6.37

Accuracy: For determining method accuracy, known as unclean LOQ, the specified limit of 80%, 100% and 120% of the SM bulk sample (test preparation) was spiked into. The unclean recovery was calculated individually.



Name	Retention Time	Area	% Area	Height
SM-I	3.513	15523	15.75	2561
SM-II	7.604	27284	27.68	2565
SM	13.925	22822	23.16	1351
SM-III	18.090	32928	33.41	3229

FIG. 11: CHROMATOGRAM OF MIX STANDARD SOLUTION OF ACCURACY



Name	Retention Time	Area	% Area	Height
SM-I	3.489	3836	0.01	514
SM-II	7.400			
SM	13.906	28709615	99.91	1631760
SM-III	18.000			
	25.995	2715	0.01	314
	26.479	14744	0.05	1694
	28.117	2152	0.01	284
	30.029	3362	0.01	394

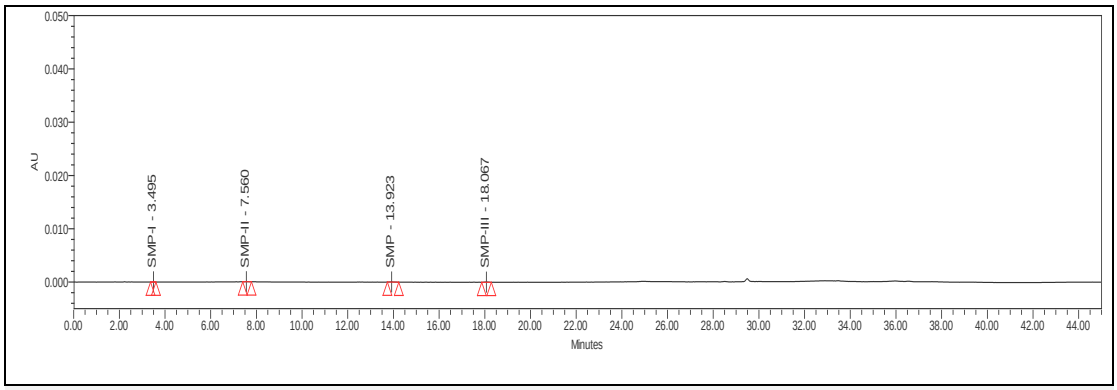
FIG. 12: CHROMATOGRAM OF PARENT SAMPLE IN ACCURACY

TABLE 3: ACCURACY OF IMPURITIES

	Amount added (µg/cm^3)	Amount recovered (µg/ cm^3)	Recovery (%)	Mean
At LOQ level				
SM-I	0.0315	0.0279	88.67	104.22
	0.0315	0.0339	107.67	
	0.0315	0.0366	116.33	
SM-II	0.0309	0.0342	113.67	112.45
	0.0309	0.0337	109.00	
	0.0309	0.0354	114.67	
SM-III	0.0303	0.0333	110.00	107.78
	0.0303	0.0316	104.33	
	0.0303	0.0330	109.00	
At 80% level				
SM-I	0.4200	0.4012	95.53	98.48
	0.4200	0.4158	99.00	
	0.4200	0.4238	100.90	
SM-II	0.4120	0.4098	99.48	99.38
	0.4120	0.4051	98.33	
	0.4120	0.4133	100.33	
SM-III	0.4040	0.4073	100.83	100.86
	0.4040	0.4080	100.98	
	0.4040	0.4071	100.78	
At 100% level				
SM-I	0.5250	0.5144	97.98	101.17
	0.5250	0.5396	102.78	
	0.5250	0.5394	102.74	
SM-II	0.5150	0.5081	98.66	98.28
	0.5150	0.5061	98.26	
	0.5150	0.5043	97.92	
SM-III	0.5050	0.5075	100.48	99.87
	0.5050	0.5019	99.40	
	0.5050	0.5036	99.72	
At 120% level				
SM-I	0.6300	0.5844	92.76	96.78
	0.6300	0.6191	98.27	
	0.6300	0.6256	99.30	
SM-II	0.6180	0.6018	97.38	97.56
	0.6180	0.6011	97.27	

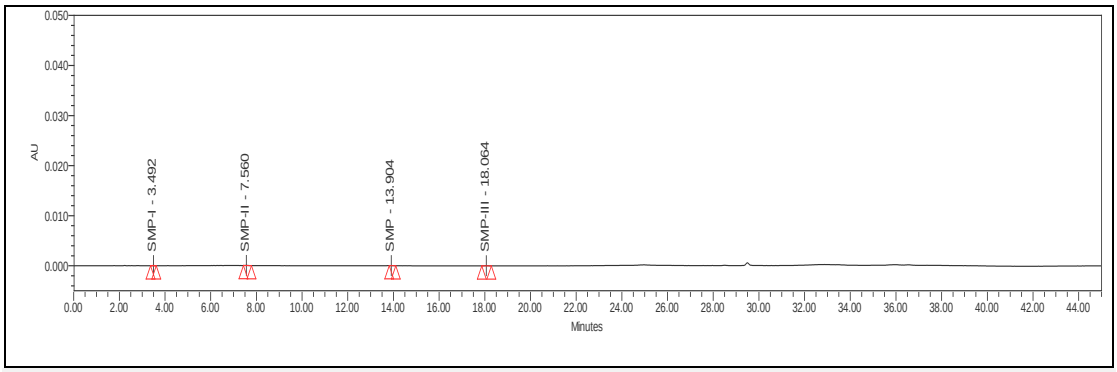
	0.6180	0.6060	98.05	
SM-III	0.6060	0.5996	98.95	98.61
	0.6060	0.5961	98.37	
	0.6060	0.5969	98.50	

Limit of Detection (DL) and Limit of Quantification (QL): Detection limit and quantitation limit for all impurities was estimated by signal to noise (S/N) method. The limit of detection values for SM-I, SM -II and SM -III were 0.002 %, 0.002 % and 0.002 % w.r.t. analyte concentration (500 µg /cm^3) respectively. The limit of quantification values for SM-I, SM-II and SM-III were found to be 0.006 %, 0.006 %, and 0.006 % w.r.t. analyte concentration (500 µg /cm^3), respectively.



Name	Retention Time	Area	% Area	Height
SM-I	3.495	277	15.22	49
SM-II	7.560	460	25.22	42
SM	13.923	523	28.69	41
SM-III	18.067	563	30.87	46

FIG. 13: CHROMATOGRAM OF LOQ STANDARD SOLUTION



Name	Retention Time	Area	% Area	Height
SM-I	3.492	317	21.74	54
SM-II	7.560	363	24.87	34
SM	13.904	199	13.60	22
SM-III	18.064	581	39.79	52

FIG. 14: CHROMATOGRAM OF LOD STANDARD SOLUTION

Linearity: On drawing a plot at six concentration levels in triplicate, covering a range of LOQ to 150%, linear calibration plots were achieved. For SM-I, the corresponding regression equation was $y = 28876x+348.97$, with correlation coefficient (r) is 0.9996. For SM-II, corresponding regression equation was $y = 52747x-0.2397$, with correlation coefficient (r) is 0.9990. For SM-III, corresponding regression equation was $y = 63972x+434.54$, with the correlation coefficient (r) is 0.9983. The results showed a good correlation between the peak area and concentration of impurities.

TABLE 4: LINEARITY TABLE FOR SM-I IMPURITY

Level	Conc.(ppm)	Mean Area	Req area
LOQ%	0.0315	1064.33	1259
50%	0.2500	7716.33	7568
80%	0.4200	12739.00	12477
100%	0.5250	15579.67	15509
120%	0.6300	18237.33	18541
150%	0.7875	23105.33	23089

Correlation coefficient =0.9996
Slope =28876.00
Intercept =348.97

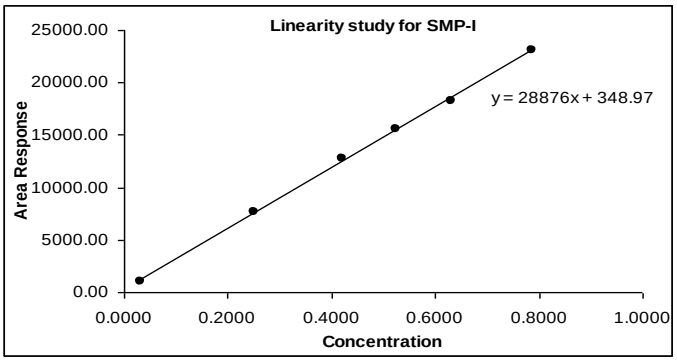
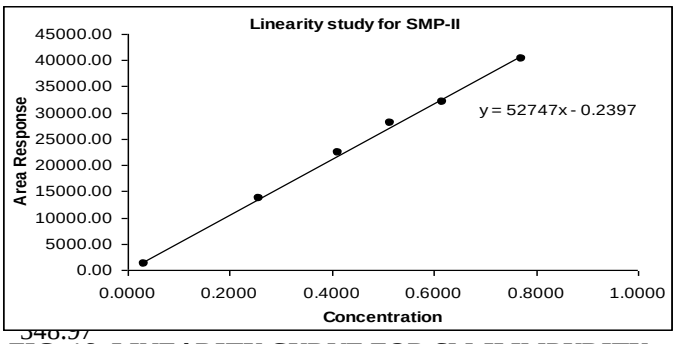


FIG. 15: LINEARITY CURVE FOR SM-I IMPURITY

TABLE 5: LINEARITY TABLE FOR SM-II IMPURITY

Level	Conc.(ppm)	Mean Area	Regg area
LOQ%	0.0309	1064.33	1630
50%	0.2575	13742.67	13582
80%	0.4120	22331.00	21731
100%	0.5150	27975.67	27164
120%	0.6180	32023.67	32597
150%	0.7725	40313.67	40747

Correlation coefficient =0.9990
Slope =52746.63
Intercept =-0.24



348.97

FIG. 16: LINEARITY CURVE FOR SM-II IMPURITY

TABLE 6: LINEARITY TABLE FOR SM-III IMPURITY

Level	Conc.(ppm)	Mean Area	Freq area
LOQ%	0.0303	1791.33	2373
50%	0.2525	16582.33	16587
80%	0.4040	26540.33	26279
100%	0.5050	34512.67	32740
120%	0.6060	38447.33	39202
150%	0.7575	48201.00	48893

Correlation coefficient = 0.9983
Slope =63972.03
Intercept =434.54

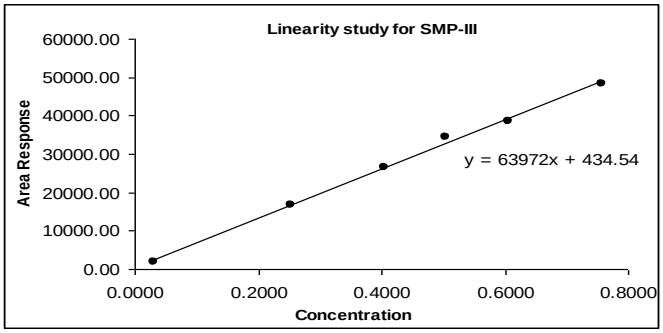


FIG. 17: LINEARITY CURVE FOR SM-III IMPURITY

Robustness: In all the deliberately varied chromatographic conditions (column temperature, flow rate and column make), the chromatogram for system suitability solution for related substance showed no significant change in system suitability parameters.

TABLE 8: CHANGE IN COLUMN TEMPERATURE [± 3 °C]

Name of component	Change in column temperature [± 3°C]											
	27°C				30°C				33°C			
	RT (min)	Area	Tailing factor	Theoretical plates	RT (min)	Area	Tailing factor	Theoretical plates	RT (min)	Area	Tailing factor	Theoretical plates
SM-I	3.61	15883	1.11	8234	3.48	16090	1.1	8547	3.41	15713	1.11	8721
SM-II	7.98	27718	1.03	13475	7.47	28343	1.02	13763	7.17	27990	1.03	13560
SM	14.42	24269	0.98	22606	13.75	23764	0.98	17644	13.35	23696	0.99	15303
SM-III	18.53	33571	0.99	93277	17.88	31596	1.06	86953	17.46	32934	1.00	73604

* Data taken from Precision study

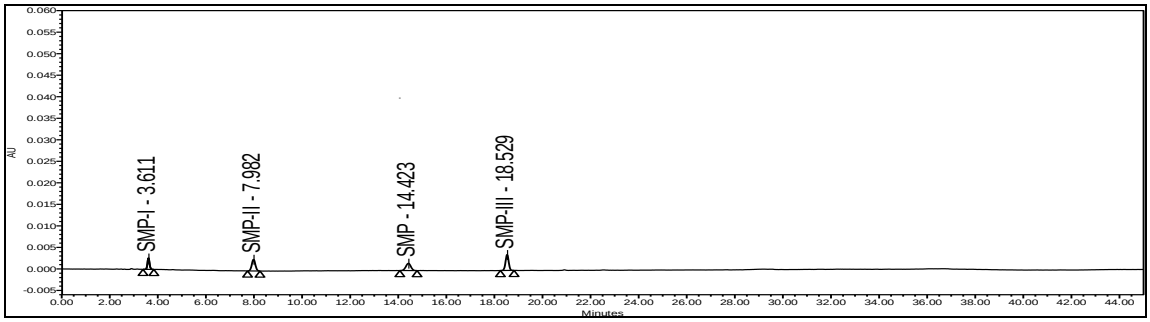


FIG. 18: CHROMATOGRAM OF SYSTEM SUITABILITY AT COLUMN TEMPERATURE 27 °C

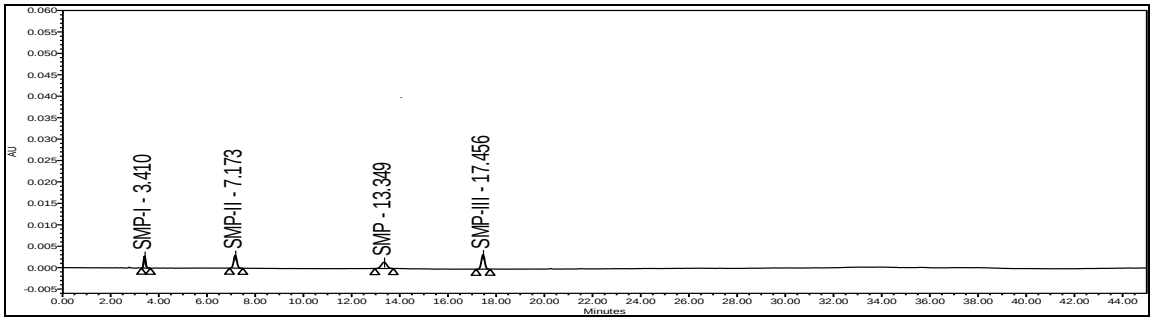


FIG. 19: CHROMATOGRAM OF SYSTEM SUITABILITY AT COLUMN TEMPERATURE 33 °C

TABLE 9: CHANGE IN FLOW RATE [± 0.2 cm^3/min]

Name of component	Change in flow rate [± 0.2 cm^3/min]											
	0.6 ml/min				0.8 ml/min				1.0 ml/min			
	RT (min)	Area	Tailing factor	Theoretical plates	RT (min)	Area	Tailing factor	Theoretical plates	RT (min)	Area	Tailing factor	Theoretical plates
SM-I	4.60	21364	1.13	9412	3.48	16090	1.1	8547	2.81	7423	1.07	12569
SM-II	9.88	37923	1.02	14450	7.47	28343	1.02	13763	6.02	22353	1.01	12350
SM	17.20	32726	0.99	42320	13.75	23764	0.98	17644	11.11	18707	1.01	14002
SM-III	20.55	46265	1.02	110834	17.88	31596	1.06	86953	15.88	26145	0.98	55785

* Data taken from Precision study

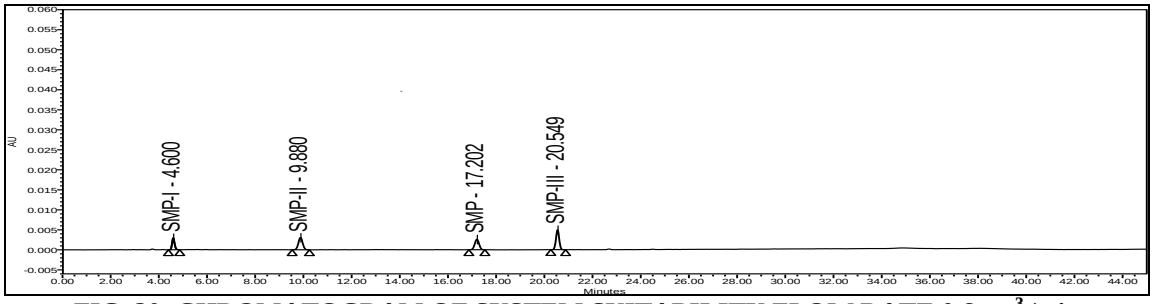


FIG. 20: CHROMATOGRAM OF SYSTEM SUITABILITY FLOW RATE 0.6 cm^3 /min

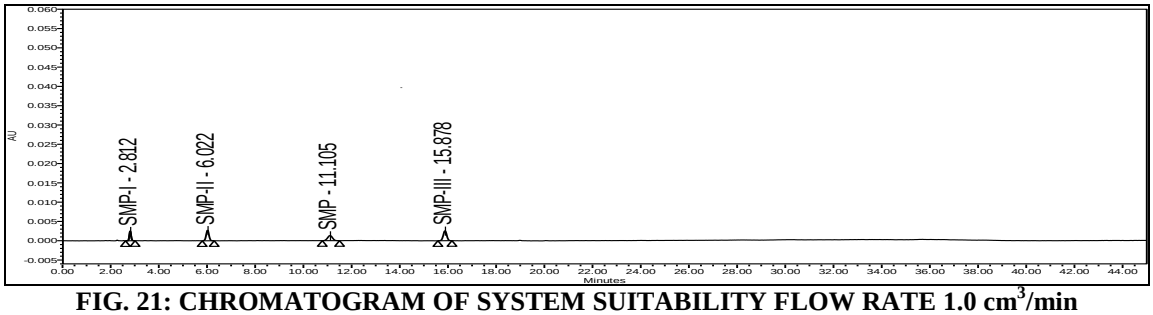


FIG. 21: CHROMATOGRAM OF SYSTEM SUITABILITY FLOW RATE 1.0 cm^3/min

TABLE 10: CHANGE IN COLUMN MAKES

Name of component	Change in column make															
	Pronto SIL Kromabond (150X4.6X5µm)				*Unishere (150X4.6X5µm)				Pronto SIL Kromaplus (150X4.6X5µm)				Kromasil (150X4.6X5µm)			
	RT (min)	Area	Tailing factor	Theoretical plates	RT (min)	Area	Tailing factor	Theoretical plates	RT (min)	Area	Tailing factor	Theoretical plates	RT (min)	Area	Tailing factor	Theoretical plates
SM-I	3.42	15828	1.16	5022	3.48	16090	1.1	8547	3.42	15799	1.03	6442	3.56	16103	1.46	4768
SM-II	7.09	28044	1.14	6675	7.47	28343	1.02	13763	7.25	28500	0.92	8176	7.46	28943	1.55	6742
SM	12.72	22736	1.13	7206	13.75	23764	0.98	17644	13.25	24171	0.92	8316	13.51	21531	1.34	7909
SM-III	17.38	33488	1.14	32742	17.88	31596	1.06	86953	17.6	33697	0.92	42254	17.84	34362	1.5	35924

* Data taken from Precision study

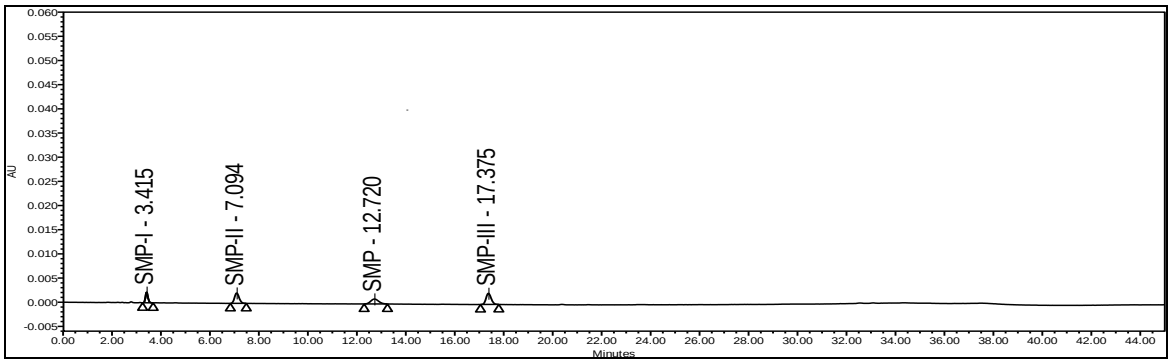


FIG. 23: CHROMATOGRAM OF SYSTEM SUITABILITY USING PRONTO SIL KROMABOND COLUMN (150*4.6*5um)

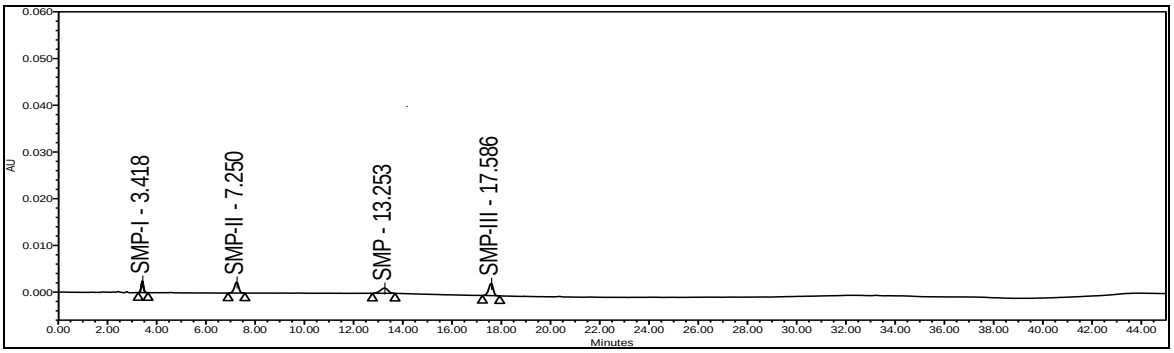


FIG. 24: CHROMATOGRAM OF SYSTEM SUITABILITY USING PRONTO SIL KROMAPLUS COLUMN (150*4.6*5um)

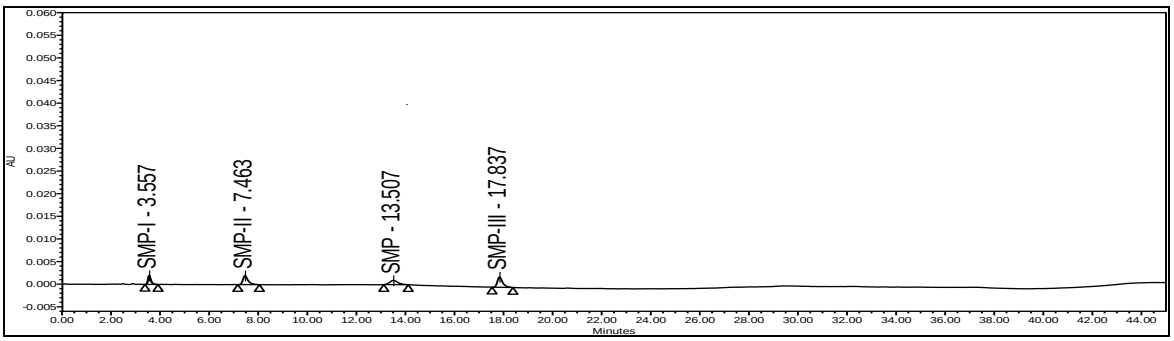
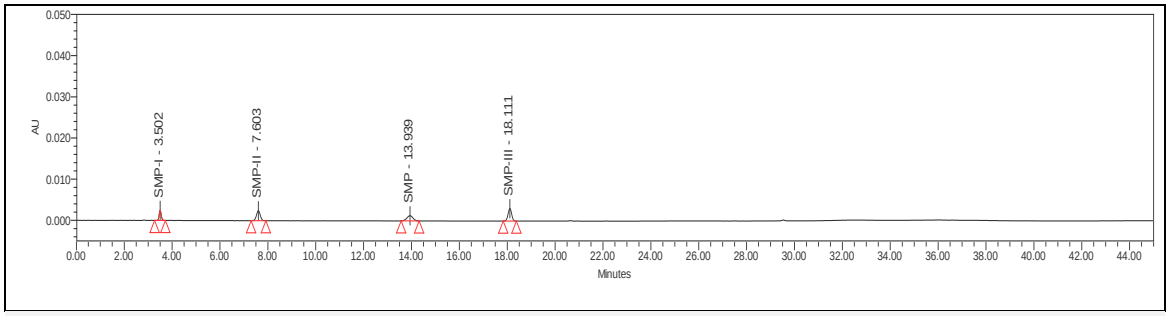


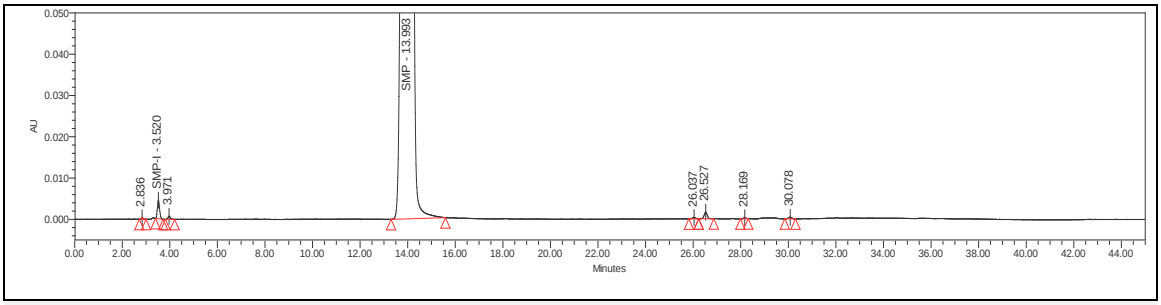
FIG. 25: CHROMATOGRAM OF SYSTEM SUITABILITY USING KROMASIL C18 COLUMN (150*4.6*5um)

Solution Stability: The solution stability of SM sample and its related impurities was carried out by leaving both solutions in tightly capped HPLC vials at 25 °C for 16 hours in an auto sampler. No significant changes were observed in the area of impurities in standard solution after 16 hours.



Name	Retention Time	Area	% Area	Height
SM-I	3.502	15391	15.99	2474
SM-II	7.603	26782	27.82	2414
SM	13.939	22083	22.94	1256
SM-III	18.111	32027	33.26	3043

FIG. 26: CHROMATOGRAM OF MIX STANDARD FRESHLY PREPARED IN SOLUTION STABILITY [0 HRS]



Name	Retention time	Area	% Area	Height
	2.836	2028	0.01	349
SM-I	3.520	29907	0.10	4502
	3.971	5546	0.02	755
SM-II	7.400			
SM	13.993	28550955	99.79	1602137
SM-III	18.000			
	26.037	3084	0.01	340
	26.527	14625	0.05	1588
	28.169	1898	0.01	253
	30.078	3422	0.01	381

FIG. 27: CHROMATOGRAM OF TEST SOLUTION IN SOLUTION STABILITY [AFTER 16 HRS]

Isolation and structural elucidation of SM-I : During the synthesis of SM *i.e.* from SCP to SM due to the basics condition of the reaction mass there is hydrolysis of methoxy group taking place which give rise to SM-I impurity which is then isolated by preparative HPLC (described in Section 2.3.6). 95% of chromatographic purity found.^{1}H and ^{13}C NMR spectral data (refer **Table 11**) the proposed structure was confirmed. The MS / MS spectrum of the isolated Faux direct infusion mode using a combination of MS / MS spectrum was the same as the match SM-I (Refer **Fig. 28** and **29**).

Synthesis and structural elucidation of SM-II: Since SM-II cannot be isolated from the reaction mixture SM synthesis, it was synthesized independently. Due to the presence of 2-chloropyrazine as an impurity in 2,3-dichloropyrazine used as raw material in synthetic route of SM there is formation of SM-II which remains unreacted and get carry forward to SM final. This impurity was prepared synthetically by using the same synthetic route as that of SM but instead of 2,3-dichloropyrazine the starting material used was 2-chloropyrazine (**Fig. 30**). The chromatographic purity was found to be 97%. ^{1}H and ^{13}C NMR spectral data (refer to **Table 11**) confirmed the proposed structure. Direct infusion of the compound synthesized from unclean condition using MS / MS spectrum was a match to the MS/MS spectrum of SM-II.

Synthesis and structural elucidation of SM-III: 2,6-dichloropyrazine which is isomer presence in 2,3-dichloropyrazine as an impurity under goes similar reaction of SM formed SM-III. SM-III is

synthesized by using 2,6-dichloropyrazine instead of 2,3-dichloropyrazine in synthetic process of SM (**Fig. 31**). The chromatographic purity was found to be 96%. ^{1}H and ^{13}C NMR spectral data (refer to **Table 11**) confirmed the proposed structure. Direct infusion of the compound synthesized from unclean condition using MS / MS spectrum was a match to the MS/MS spectrum of SM-III.

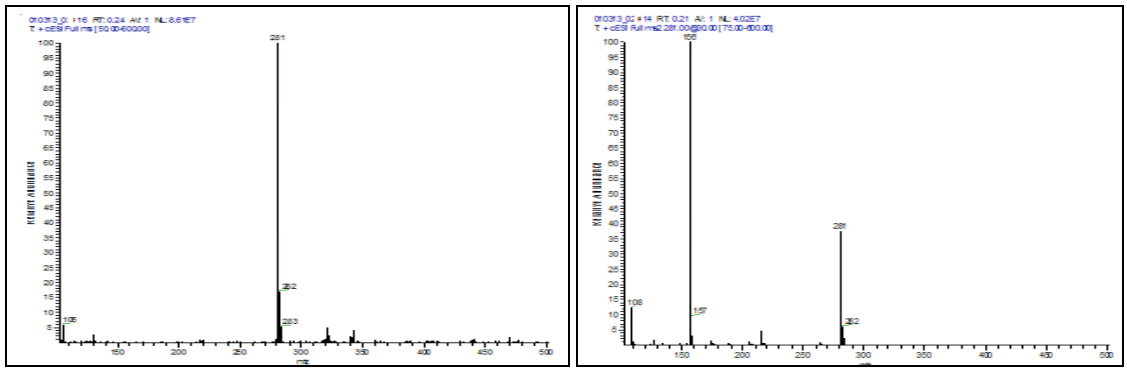


FIG. 28: MASS SPECTRUM OF SM P, (b) ms/ms SPECTRUM OF SM

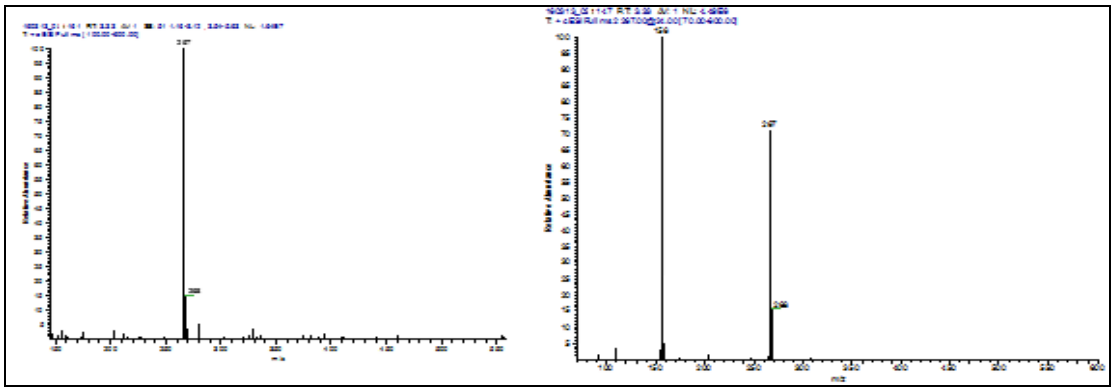


FIG. 29: (a) MASS SPECTRUM OF SM – I, (b) ms/ms SPECTRUM OF SM -I

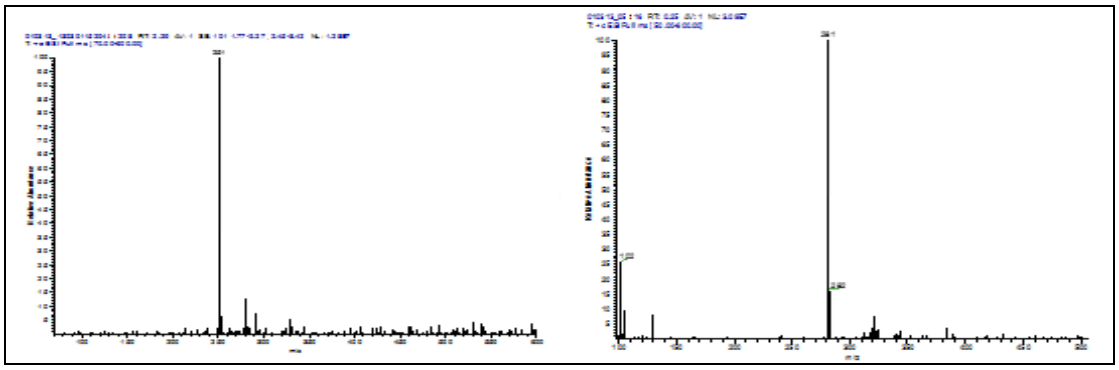


FIG. 30: (a) MASS SPECTRUM OF SM-II, (b) ms/ms SPECTRUM OF SM-II

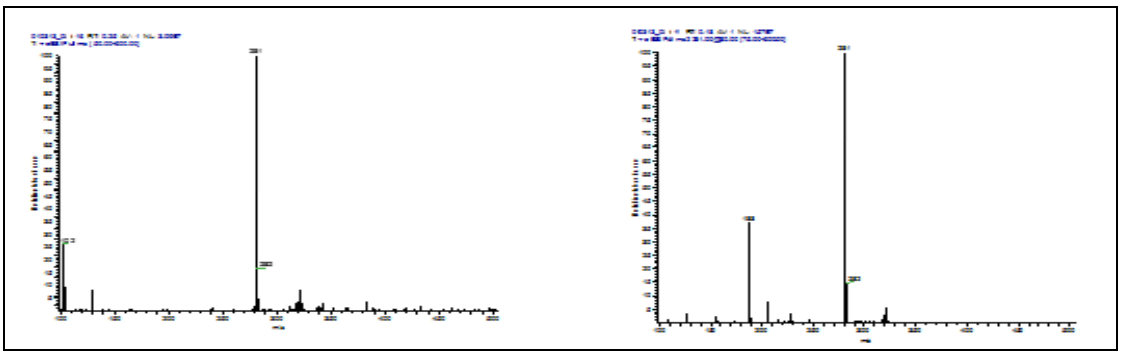
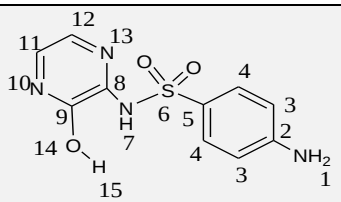
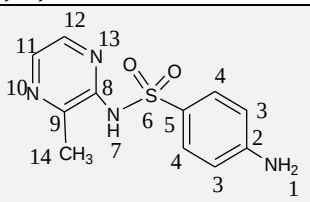


FIG. 31: (a) MASS SPECTRUM OF SM-III, (b) ms/ms SPECTRUM OF SM-III

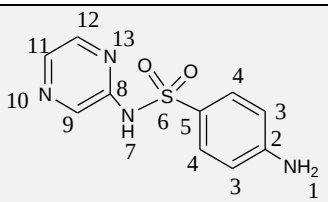
TABLE 11: NMR DATA OF SM-I, II, III AND SM



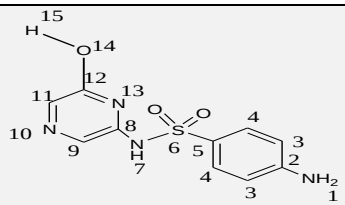
SM



SM-I



SM-II



SM-III

Position	Integration	δ ppm	Multiplicity J(H)z	13C &ppm	Integration	δ ppm	Multiplicity J(H)z	13C& ppm	Integration	δ ppm	Multiplicity J(H)z	13C& PPM	Integration	δ ppm	Multiplicity J(H)z	13C & ppm
1	2Ha	6.01	Brs	-	2Ha	4.39	Brs		2Ha	6.08	Brs		2Ha	6.08	Brs	
2	-	-	-	153.3	-	-		151.6				153.8				151.6
3	2Ha	6.57	d(8.8)	112.6	2Ha	6.61	d(8.8)	116.6	2Ha	6.58	d(8.8)	153.9	2Ha	6.56	d(8.8)	116.6
4	2H	7.67	d(8.8)	130.2	2Ha	7.81	d(8.8)	128.1	2Ha	7.58	d(8.8)	129.8	2Ha	7.55	d(8.8)	128.1
5	-	-	-	125.6	-	-		129.1				124.5				129.7
6	-	-	-	-	-	-										
7	1Hb	10.34	Brs	-	1Hb	11.7	Brs		1Hb	11.0	Brs		1Hb	11.11	Brs	
8	-	-	-	149.9	-	-		152.7				7.8				154
9	1Ha	-	d(1.2)	138.8	1Ha	-		145.7	1Ha	7.8		7.8	1Ha	8.32	d(1.2)	123
10	-	-	-	-	-	-				7.8						
11	1Ha	7.71	S	133.9	1Ha	6.05	d(4.3)	125.5	1Ha				1Ha	8.18	s	132.4
12	1Ha	7.71	S	133.6	1Ha	6.9	d(4.3)	125.5	1Ha				1Ha	8.18	s	161.1
13	-	--	-	--	-	-										
14	-	-	-	-	1Hb	8.44	Brs									
15	2Hb	3.89	S	54.1	-	-			3H	3.73	S	3.73				55.9

s-singlet, d-doublet, brs-broad singlet. [a] Refer the structural formula in Figure.[b]^{1}H-^{1}H coupling constants

CONCLUSION: The present study details the identification and determination of structure of four process related impurities are found in the product (SM). Reverse phase HPLC using a gradient of a newly developed method, the impurities were detected in samples of crude and refined SM during process development studies. An exhaustive study was carried out LC / MS / MS for the identification of impurities using. The spectroscopy using different technologies and their synthesis was followed by characterization. For a quantitative estimate of the contaminants in the paper elaborates on a new HPLC method validation. The specificity of the HPLC method was confirmed by injecting samples of individual impurity and it was observed that there was no interference for any of the components.

The ability of the method to precisely quantify impurities was determined by calculating the relative standard deviation (RSD) for response (peak area) of each impurity in the standard solution (a mixture of impurities) a copy of the injections. For determining method accuracy, known as unclean LOQ, the specified limit of 80%, 100% and 120% of the SM bulk sample (test preparation) was spiked into. Signal to noise (S/N) method was used for Detection limit (DL) and quantitation limit (QL) for all impurities. On drawing a plot at six concentration levels in triplicate, covering a range of LOQ to 150 %, linear calibration plots were achieved. The stability of solution of SM sample and its related impurities was determined by placing both solutions in tightly capped HPLC vials at 25 °C for 16 hrs in an auto sampler. There are no significant changes were noted in the area of impurities in standard solution after 16 hours. But in sample solution area of SM-I have been increased and one unknown impurity at RRT 0.28 is generated after 16 hours. From stability data generated it is concluded that standard solution is stable for 16 hours and sample solution should be prepared freshly.

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CONFLICTS OF INTEREST: Nil

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