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Characterization of degradation products of the Balsalazide by Mass spectrometry: Optimization of stability-indicating HPLC method for separation and quantification of process related impurities of Balsalazide

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Abstract: The study aimed to investigate a novel approach by utilizing liquid chromatography (LC) and liquid chromatography-mass spectrometry (LC–MS) to separate, identify and characterize very nominal quantities of degradation products (DPs) of balsalazide along with its process related impurities without isolation from their reaction mixtures. The impurities along with balsalazide were resolved on spherisorb ODS2 (250×4.6 mm, 5.0 µm) column at room temperature using 0.2 M sodium acetate solution at pH 4.5 and methanol in the ratio of 55:45 (v/v) as mobile phase pumped isocratically at 1.0 mL/min as mobile phase and UV detection at 255 nm. The method shows sensitive detection limit of 0.003 µg/mL, 0.015 µg/mL and 0.009 µg/mL respectively for impurity 1, 2 and 3 with calibration curve liner in the range of 50-300 µg/mL for balsalazide and 0.05-0.30 for its impurities. The balsalazide pure compound was subjected to stress studies and a total of four degradation products (DPs) were formed during the stress study and all the DPs were characterized with the help of their fragmentation pattern and the masses obtained upon LC–MS/MS. The DPs were identified as *3-({4-[(E)-(4-hydroxyphenyl) diazenyl]benzoyl}amino)propanoic acid* (DP 1), *4-[(E)-(4-hydroxyphenyl)diazenyl] benzamide* (DP 2), *5-[(E)-(4-carbamoylphenyl)diazenyl]-2-hydroxybenzoic acid* (DP 3) and *3-({4-[(E)-phenyldiazenyl]benzoyl}amino)propanoic acid* (DP 4). Based on findings, it was concluded that, the proposed method was successfully applicable for routine analysis of balsalazide and its process related impurities in pure drug and formulations and also applicable for identification of known and unknown impurities of balsalazide.

Key words: balsalazide, impurities, HPLC method, stress degradation studies, LCMS characterization, degradation products

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1. Introduction

The efficacy of a pharmaceutical product (drug) strictly associated with its microbial, chemical and physical stability. The presence of impurities and the possible formation of degradants / degradation products (DPs) in a drug product can affect its distribution, absorption, metabolism and excretion and have important repercussions on its safety profile.[1] In order to reduce the risk of failure due to stability and ascertain potentially toxic DPs, the pharmaceutical companies routinely performing forced/stress degradations in the early stage of drug development process.[2] The investigation of drug degradation behavior toward various stressed conditions and structural characterization of DPs was considered as an integral part in the process of development of a pharmaceutical product.[3] Furthermore, it was pivotal in the drug manufacturing process, determination of its shell life, formulation as well as packaging. Hence, stress degradation studies aim to accelerate the DPs formation by drug exposer to various physic-chemical stress conditions to evaluate its stability and degradation pathways.[4]

The regulatory agencies like FDA (Food & Drug Administration), WHO (World Health Organization), and ICH (International Council for Harmonisation) etc., recommend stress exposure of drug to acid, base, dry heat, oxidation (peroxide), and UV light conditions among others along with their protocols including pH, temperature and stress exposer time.[5,6] The High-performance liquid chromatography (HPLC) or ultra HPLC coupled with high resolution mass spectrometry (HRMS) and/or with suitable detectors represent most commonly employed analytical techniques for both structural and quantitative evaluation of DPs.[7]

Balsalazide (*Fig.* 1) belongs to aminosalicylate class, anti-inflammatory drug prescribed to treat inflammatory bowel disease and ulcerative colitis.[8] Balsalazide is a prodrug of mesalamine which has an inert carrier molecule instead of the sulfapyridine moiety of sulfasalazine. It is designed to deliver 5-aminosalicylic acid to the colonic mucosa without the sulfapyridine-associated side-effects encountered with sulfasalazine.[9] The common side effects possible during the use of balsalazide includes hives, difficulty breathing, swelling of face, burning when urinate, fever, stomach pain, cramps, bloody diarrhea, swelling, weight gain and

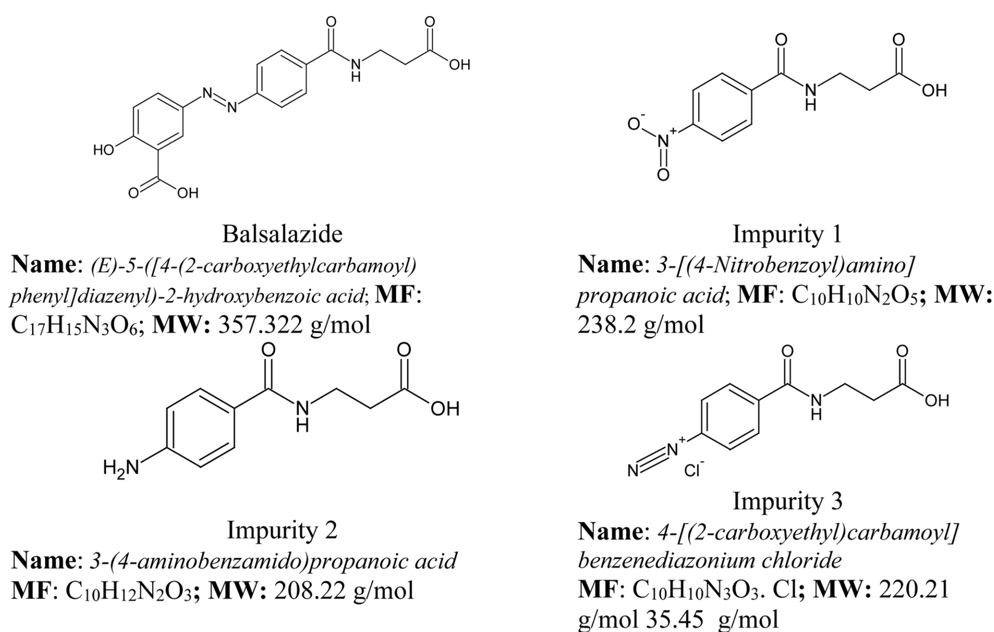

Fig. 1. Details of balsalazide and its process related impurities

upper stomach pain. It was commonly administered as disodium salt.

The literature survey was conducted for identification of analytical methods reported for quantification of balsalazide. In literature, few analytical HPLC methods[10-13] reported for quantification of balsalazide in formulations. One HPTLC-Densitometry method[14] published for quantification of balsalazide in pharmaceutical formulations. Few UV visible spectrophotometer methods reported for estimation of balsalazide in formulations.[15-18] The literature review confirms that there is no method reported for quantification of process related impurities and characterization of stress degradation products. Hence this study aimed to develop a simple stability indicating HPLC method for resolution and quantification of process related impurities of balsalazide. Further, the study also intended to identify the stress degradation products formed during the stress study of balsalazide using LCMS/MS analysis. The details of process related impurities in the study were presented in *Fig.* 1.

The origin of impurities in the final product of balsalazide was assessed by observing the route of synthesis and the synthesis mechanism[19] of balsalazide disodium salt was presented in *Fig.* 2. The intermediate products formed in the process of synthesis of balsalazide were remains in the final product as impurities. The intermediate products **3**, **4** and **5** as shows in *Fig*. 2 were remains in the final product as impurity 1, 2 and 3 respectively. There is a significant need to evaluate the quantity of these impurities in the final product and hence this study aimed to resolve and quantity the process related impurities in balsalazide.

2. Experimental

2.1. Chemicals and reagents

The analytical standard compound balsalazide with purity of 98.55 %, its impurity 1, 2 and 3 were procured from Intas Pharmaceuticals Limited, Secunderabad, Telangana. The capsule formulation containing 750 mg of balsalazide with brand Balacol® was purchased from local market. The HPLC grade methanol, acetonitrile, Milli-Q® water were purchased from Merck chemicals, Mumbai. The analytical reagent grade chemicals such as acetic acid, sodium acetate, hydrochloric acid (HCl), sodium hydroxide (NaOH), and hydrogen peroxide were purchased from Fisher scientific, Mumbai.

2.2. Instrumentation

The HPLC analysis was performed on Agilent 1100 (USA) instrument that coupled with quaternary pump (G1311 A) for solvent delivery. The analytes were injected through temperature adjustable auto sampler (G 1329A) having injection capacity of 0.1-1500 μL. The column eluents were detected using programmable ultraviolet (UV) detector (G 1314 A) and the chromatographic integrations were carried using Agilent chem-station software. The LCMS analysis was performed on Waters LCMS (Japan) equipped with triple quadruple mass detector and

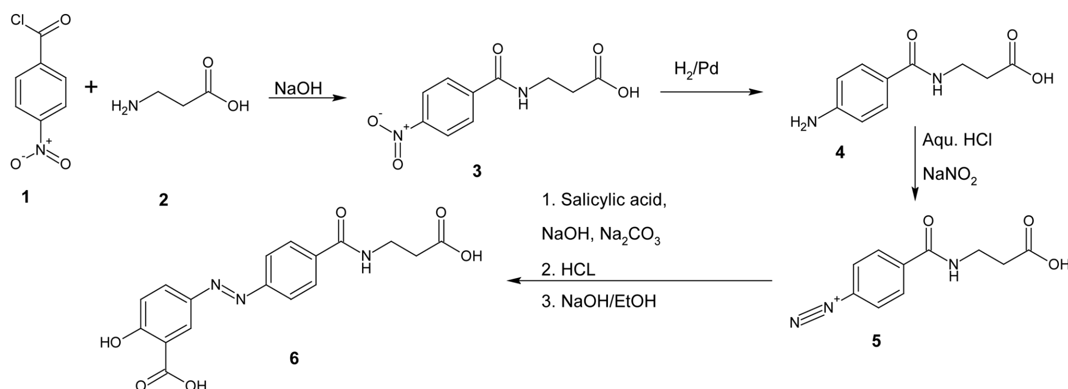

Fig. 2. Route of synthesis of balsalazide disodium salt.

MassLynx software.

2.3. Standard solution preparation

The standard balsalazide and its impurities at 1 mg/mL (1000 μg/mL) were prepared separately by accurately weighing 25 mg of analyte in 25 mL of volumetric flask containing 15 mL of methanol. The analytes were dissolved in solvent using an ultrasonic bath sonicator. Then the analytes were filtered through 0.2 μ membrane filter and the final volume was made up to the mark using same solvent to obtain 1000 μg/mL concentration of balsalazide and its impurities separately. During the analysis, selected volume of required concentration of individual analyte was mixed separately.

2.3.1 Test solution preparation

The Balacol® capsules with 750 mg strength were used for the preparation of formulation solution. The tablets were fine powdered using clean, dry mortar - pestle and the tablet powder equivalent to 25 mg of balsalazide was weighed accurately. The weighed tablet powder was taken in a 25 mL volumetric flask containing 15 mL methanol. Then the drug was dissolved completely in solvent and the final volume made up to the mark. Then, it was filtered and was diluted to standard concentration and the dilute solution was used for evaluation of formulation assay.

2.4. Method development

The suitable detector wavelength for maximum detection of balsalazide along with its impurities was identified using UV-visible spectrophotometer. The standard solution at 10 μg/mL concentration of balsalazide and its impurities was scanned using spectrophotometer separately and the overly absorption spectra confirm the suitable wavelength. Various configurations of stationary phases with different manufactures were varied for the best resolution of balsalazide and its impurities. The mobile phase was optimized by change in different solvent compositions with different pH ranges and different flow rates were varied. The conditions that resolve the analytes was further studied for validation.

2.5. Method validation

The method developed in the study was validated for its acceptable performance to ensure suitability of indent purpose. The parameters such as range, linearity, accuracy, precision, specificity, detection limit, quantification limit, ruggedness and robustness experiments were executed as per reported literature [20-23] and ICH guidelines [24].

The limit of quantification (LOQ) and limit of detection (LOD) for impurities were confirmed by measuring response at signal to noise ratio of 10:1 and 3:1, respectively, by injecting progressively known concentrations of impurity solutions in the optimized HPLC method. The precision experiment was also performed at LOQ level by injecting six independent preparations of impurities and the %RSD (relative standard deviation) of the peak areas observed for each impurity was determined.

Linearity was evaluated by analysing the mixed calibration standard solutions at six concentration levels. The linearity solutions were prepared from LOQ level to 150 % of specification limit. Linear regression equations were plotted with the least squares linear regression method. The method precision was investigated by analysing six individual preparations in the same day for intraday precision, three different days by same analyst for interday precision and six times by six different analysts in the same day in ruggedness. The method robustness was verified by introducing minor variations in the developed method parameters, including the mobile phase mixture, pH, and detector wavelength and column temperature. The selectivity, resolution between each nearby peak and the % change in the peak area response were evaluated in each of the altered condition. The method accuracy was evaluated by spiking pre-analysed samples with four different concentration levels in triplicate, i.e., at LOQ, 50 %, 100 % and 150 % levels of the specification limit. The % recovery in each analysis and the % RSD of recovery in each spiked level was summarized for evaluating the method accuracy.

The specificity and stability indicating power of the developed method was evaluated by performing stress degradation study. Various stress studies like

acid, base, peroxide, thermal and ultraviolet stress studies were performed for standard balsalazide. The placebo, balsalazide pure drug and tablet powder were exposed 5 mL of hydrochloric acid (1 N), 5 mL of sodium hydroxide (1 N) and 5 mL of hydrogen peroxide (3.0 %) separately for acid, base and peroxide degradation study respectively for 12 hours. Then, the stress exposed samples were neutralized with suitable diluent. The samples were kept in a photolytic chamber irradiated with UV light at 1.2 million Lux/h and 103,959 Wh/m^2 for UV light degradation study and samples were kept in an air oven at 80 °C for 24 hours for thermal degradation study. The all the stress exposed samples were diluted to target concentration and then the diluted samples were analysed in the developed method. The resultant chromatograms and chromatographic response was utilized for evaluating the stability indicating nature of the proposed method.

The stress degradation compounds (DPs) were characterized using mass spectral analysis. The mass spectral analysis was performed by allowing 40 % of the column eluents in to the column using a splitter and the samples were recorded using mass detector that was operated in ESI (Electrospray ionization) positive ion mode. The mass detector was operated with adequate conditions such as capillary voltage (3500 V), fragmentor voltage (60 V) and skimmer voltage (60 V). Nitrogen gas was utilized for drying (300 ºC, 8 L/h) and nebulization (50 Psi). The spectra throughout the analysis were recorded under similar experimental conditions and 20-30 average scans were conducted.

The proposed method was applied for identification and quantification of process related impurities of balsalazide in formulations. The Balacol® formulation solution spiked with known concentration of impurities and the un-spiked solution was analysed in the proposed method. The % assay of impurities was calculated by comparing peak area response of each impurity in its corresponding calibration curve.

3. Results and Discussions

As the literature doesn't show any analytical method for quantification of process related impurities of balsalazide, this study aimed to develop a simple HPLC method for identification and quantification of process related impurities 1, 2 and 3 of balsalazide.

Different configurations of columns including amino, octadecylsilane (ODS), phenyl–hexyl and cyano columns were studied for best resolution of balsalazide and its impurities in the study. The mobile phase composition with suitable pH range was optimized by varying different composition of mobile phase solvents including buffer strength and composition. The acetate and phosphate buffers with pH range of 3-6 were studied for best resolution of balsalazide and its impurities. The hydrophilic ionisable functional groups in the analytes such as –COOH, –C=O and –NH etc., were effectively resolved with mobile phase containing pH buffers and hence various buffers with different pH range was studied for effective resolution. In the use of phosphate buffer in the mobile phase produces asymmetric peaks with poor resolution was noticed. The presence of acetonitrile in the mobile phase doesn't resolve the analytes. Hence these were not studied in the optimization process. The mobile phase with low pH value shows negative influence on the column life and hence mobile phase was selected such that the pH doesn't influence the column life. Successful separation of balsalazide and its impurities was achieved using spherisorb ODS2 (250 × 4.6 mm, 5.0 μm) column using 0.2 M sodium acetate solution at pH 4.5 and methanol in the ratio of 55:45 (v/v) as mobile phase pumped isocratically at 1.0 mL/min as mobile phase. The temperature of the column oven was maintained at 35 ºC and the sample injection volume of 20 μL was kept constant throughout the analysis. Detection wavelength was selected as 255 nm based on the observations that the detector response was optimum, when compared to the determinations made at other wavelengths for all analytes.

In this condition, the shape of the peaks corresponds to balsalazide and its impurities were noticed to be symmetric and the resolution between the nearby peaks was observed to be more than 2. The compounds in the standard solution were identified by injecting

the individual standard solution and comparing the individual retention time with standard. The retention time of the analytes was noticed to be 8.07 min for balsalazide, 4.88 for impurity 1, 6.21 min for impurity 2 and 3.16 min for impurity 3. The column efficiency for balsalazide and its all impurities was noticed to be lesser than the limit in case of tail factor and greater than acceptable limit in case of theoretical plates and resolution and results were illustrated in *Table* 1. Placebo and the standard balsalazide solution spiked with impurities chromatogram were presented in *Fig.* 3 and based on chromatograms it was confirmed that the method was specific for the separation and detection of process related impurities of balsalazide.

In each method validation run, the system suitability of peaks corresponds to balsalazide and its impurities

Table 1. Summary of the method validation results observed in the developed method

Parameter	Results			
	Balsalazide	Impurity 1	Impurity 2	Impurity 3
System suitability$				
t_R (min)	8.07	4.88	6.21	3.16
RRT	--	0.60	1.27	0.51
RRF	--	0.08	0.05	0.06
R_S	9.24	5.27	4.96	--
A_S	1.01	1.02	0.93	1.06
N	11258	7810	9637	5274
Linearity				
Range (µg/mL)	50-300	0.05-0.30	0.05-0.30	0.05-0.30
Slope	5004.8	399146	227631	263698
Intercept	4924	2193.2	1599.7	2114.8
r^2	0.9991	0.9999	0.9996	0.9998
Precision$$				
Intraday	0.12	0.20	0.49	0.28
Interday (day 1)	0.72	0.39	0.64	0.52
Interday (day 2)	0.40	0.29	0.59	0.37
LOQ level	--	0.28	0.19	0.30
Accuracy at 50 % level$				
Prepared (µg/mL)	50	0.05	0.05	0.05
Recovered (µg/mL)	49.447	0.049	0.050	0.050
% Recovery	98.89	98.99	99.21	99.29
% RSD	0.75	0.59	0.71	0.51
Accuracy at 100 % level$				
Prepared (µg/mL)	100	0.10	0.10	0.10
Recovered (µg/mL)	99.14	0.099	0.100	0.100
% Recovery	99.14	98.71	100.19	100.00
% RSD	0.89	0.38	1.24	0.93
Accuracy at 150 % level$				
Prepared (µg/mL)	150	0.15	0.15	0.15
Recovered (µg/mL)	148.29	0.149	0.151	0.150
% Recovery	98.86	99.30	100.53	100.02
% RSD	0.40	0.48	0.59	1.43
Sensitivity				
LOD (µg/mL)	--	0.003	0.015	0.009
LOQ (µg/mL)	--	0.01	0.05	0.03

t_R (min) = retention time; RRT = relative retention time; RRF = relative response factor; R_S = resolution; A_S = tail factor; N = No. of theoretical plates; r^2 = slope; $average of three determinations; $$average of six determinations

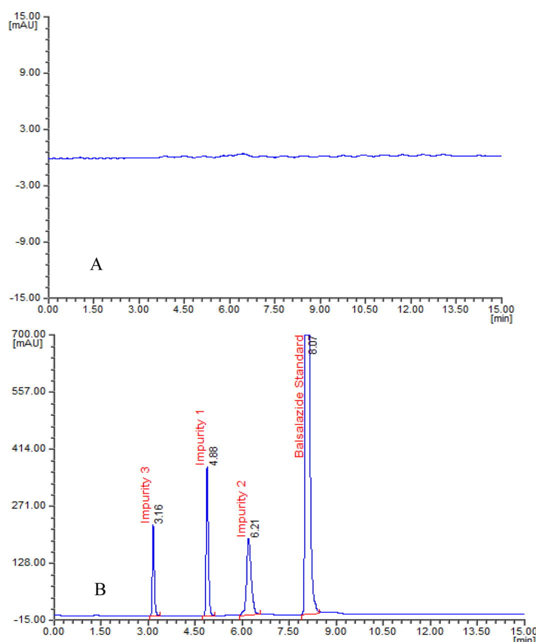

Fig. 3. Chromatograms observed in specificity study. (A) chromatogram observed for analysing the placebo solution in the developed method that doesn't show any chromatographic detections; (B) standard chromatogram observed for analysing balsalazide pure drug solution spiked with 0.1 % impurities that clearly show well resolved, retained symmentic peaks corresponds to analytes in the study)

were verified. The acceptance criteria in each validation run was considered as theoretical plates (N) of > 2000, resolution (R_S) between adjacent peaks was > 2 and tailing factor (A_S) of ≤ 2.0 as per the guidelines. The N, R_S, and A_S of peaks observed for balsalazide and its impurities were within the acceptable limits (*Table* 1), so the optimized method is suitable for analysis of these compounds.

The sensitivity of the method for the detection of impurities was identified by determining the LOD and LOQ of impurities in the developed method which was performed by adoption s/n ratio method. Based in the results observed, the LOD was confirmed as 0.003 µg/mL, 0.015 µg/mL and 0.009 µg/mL respectively for impurity 1, 2 and 3 proved that the method was very sensitive and can detect the analytes at very low concentrations. The sensitive results were presented in *Table* 1.

The calibration curve dilutions of the impurities were prepared based on the sensitive results of the impurities and the quantification limit was considered minimum concentration in calibration range. The concentration of balsalazide was considered such that the solution contains 0.1 % each impurity. Two calibration curve ranges with six points each were obtained separately for balsalazide and its impurities by analysing the calibration standards of balsalazide prepared according to the procedure described above and plotting the analyte peak area (A) against analyte strength. The equations of the calibration curves were determined using linear least squares regression analysis and results were presented in *Table* 1. The calibration curve was linear in the concentration level of 50-300 µg/mL and 0.05-0.30 respectively for balsalazide and its studied impurities. These results show good linearity, indicating that this method could be applied to quantify impurities at very low concentrations.

The method precision and reproducibility was performed as intra, interday precision and ruggedness experiments and the results were calculated on control samples to verify its applicability to the assay of balsalazide and its impurities. The % RSD of balsalazide and its impurities was calculated to be less with in the acceptable limit of less than 2 (*Table* 1) proves that the method was precise and rugged for the analysis of balsalazide and its impurities. The accuracy of the method was based on the recovery (R %) of known amounts of analyte in placebo by and was calculated by using the formula:

$$R\% = C_{found} \times 100 / C_{taken}$$

The accuracy of the method was investigated by performing three consecutive replicate injections of three control samples with concentration of 50 µg/mL, 100 µg/mL and 150 µg/mL for balsalazide spiked with 0.1 % of studied impurities. The acceptable % recovery of 98-102 was observed for balsalazide and impurities studied and the % RSD in each spiked level was found to be less than 2 which is the acceptable limit determining the accuracy of the method. The summary results observed in system suitability, linearity, precision, accuracy and sensitivity study in

the proposed method were presented in *Table* 1.

The method robustness evaluated by making deliberate small modifications in the proposed method conditions and the effectiveness of the method for the resolution and quantification of balsalazide and its impurities was assessed. In this study the mobile phase composition was changed as 50:50 (v/v) and 60:40 (v/v) of sodium acetate and methanol respectively in MP 1 and MP 2. The pH of the mobile phase was altered as 4.6 in pH 1, 4.4 in pH 2, detector wavelength altered as 250 nm in WL 1 and 260 nm in WL 2 whereas the column temperature was altered as 30 ^oC in CT 1 and 40 ^oC in CT 2 respectively. In all the altered conditions, the % change in the peak area response and the system suitability of balsalazide and its impurities were summarised in *Table* 2. In all the altered conditions, no prominent changes were observed proved that the method was robust.

The forced degradation studies were performed to identify the probable degradation products that can helpful for establishing the degradation pathway and subsequently the molecules intrinsic stability. The stability indicating ability of an analytical method considered as powerful tool for establishing the products shelf life. Hence, the stability indicating ability of the proposed method was assessed and the degradation products (DPs) formed in the study were characterized using LCMS/MS analysis. The % degradation was calculated to be 6.31 % in acidic, 8.18 % in base, 4.93 % in peroxide, 2.31 % in thermal and 3.08 % in UV light degradation study. Based on the degradation results, it was observed that there is no significance degradation was noticed in UV light and thermal degradation study without any degradation products detected in the chromatogram. A very high % degradation of 8.18 % was noticed in base degradation study and the chromatogram resolves DP 1, 2 and 4 at t_R of 3.58 min, 5.53 min and 8.69 min

Table 2. Robustness study results in the proposed method

S No	Changed condition	Parameter	Results observed			
			Balsalazide	Impurity 1	Impurity 2	Impurity 3
1	MP 1	% change	0.62	0.30	0.84	0.25
		t_R	8.05	4.85	6.19	3.15
		N	10310	8263	8826	5580
2	MP 2	% change	0.99	0.20	0.14	0.10
		t_R	8.02	4.83	6.18	3.18
		N	10543	8443	9025	5701
3	pH 1	% change	0.30	0.66	0.94	0.29
		t_R	8.09	4.89	6.23	3.11
		N	10974	8060	9394	5443
4	pH 2	% change	0.32	0.34	0.69	0.24
		t_R	8.06	4.84	6.21	3.13
		N	11618	7543	9307	5443
5	WL 1	% change	0.28	0.64	0.13	-0.71
		t_R	8.04	4.85	6.25	3.15
		N	11539	7517	9276	5406
6	WL 2	% change	0.07	0.62	0.72	0.30
		t_R	8.08	4.86	6.22	3.16
		N	12361	7507	9263	5791
7	CT 1	% change	0.77	0.29	0.96	0.45
		t_R	8.06	4.87	6.21	3.11
		N	10974	8060	9394	5443
8	CT 2	% change	0.92	0.07	0.74	0.20
		t_R	8.07	8.89	6.20	3.16
		N	11539	7517	9276	5406

respectively. The chromatogram observed in acid degradation study resolve two DPs at the retention time of 5.54 min (DP 2) and 8.70 min (DP 2) along with impurity 2. Peaks correspond to DP 1 and DP 3 was retained at 3.58 min and 7.51 min respectively in the chromatogram of peroxide degradation and the chromatogram doesn't show any peak corresponds to known impurities.

Peak purity test results obtained from the PDA detector confirmed that the balsalazide peak was pure and homogeneous in all the analysed stress samples. The mass balance of stressed samples was found in the range of 96.03-98.76 %. Peak purity test results obtained confirmed that the balsalazide peak was homogeneous and pure in all the analysed stress samples. Insignificant change in assay of balsalazide in the presence of impurities and peak purity results of stress samples confirm the specificity and stability-indicating ability of the developed method. *Table* 3 presents the results and *Fig.* 4 shows the representative chromatograms observed in forced degradation study.

Table 3. Results achieved in forced degradation study of balsalazide

Stress condition	% degradation[$] of balsalazide	% assay[$] of balsalazide	% Mass balance[$] (assay + total impurities	Remark
Acid	6.31	93.69	98.25	Two DPs (DP 2 and 4) were identified
Base	8.18	91.82	98.76	Three DPs (DP 1, 2 and 4) were identified
Peroxide	4.93	95.07	98.53	Two DPs (DP 1 and 3) were identified
Thermal	2.31	97.69	96.25	No degradation product identified
UV light	3.08	96.92	96.03	No degradation product identified

[$]average of three replicate experiments

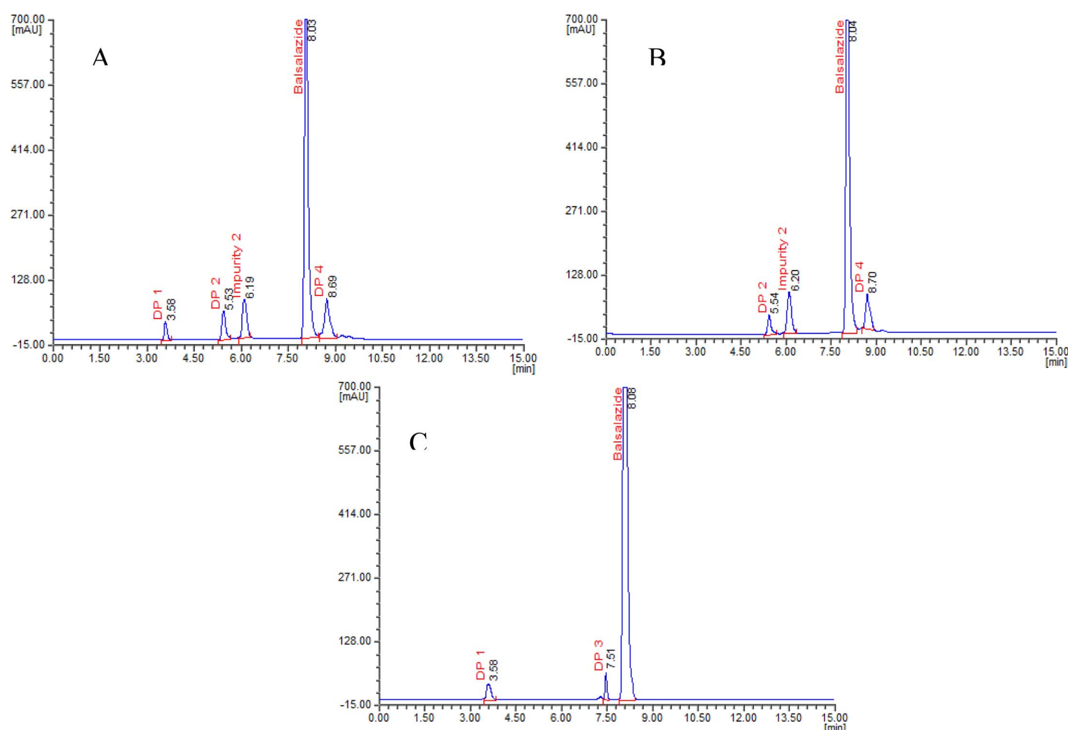

Fig. 4. Chromatograms observed in stress degradation study of balsalazide. (A) Base degradation chromatogram clearly showing well resolved peaks for DP 1, DP 2 and DP 4, (B) Acid degradation chromatogram showing peaks correspond to DP 2 and DP 4, (C) Peroxide degradation chromatogram showing peaks correspond to DP 1 and DP 3

3.1. LCMS/MS Characterization of DPs

The DPs generation in forced degradation of balsalazide were characterised using LCMS/MS analysis. Based on the retention time of DPs observed in the forced degradation chromatogram, it was confirmed that 4 DPs were formed and were designated as DP 1 to 4. All the DPs along with standard balsalazide exhibited abundant protonated molecular ions in positive ionization mode ($[M+H]^+$). The collision induced dissociation spectra of the molecular ions of balsalazide and its DPs were recorded to obtain its structural information. The mass spectra of the DPs observed in the study were shown in *Fig*. 5.

The ESI/MS spectrum of DP 1 identified at t_R of 3.5 min show abundant parent ion at m/z of 314 (m+1) which was formed by losing carboxylic acid group attached to aromatic ring of balsalazide. The fragmentation spectra shows abundant fragment ion at

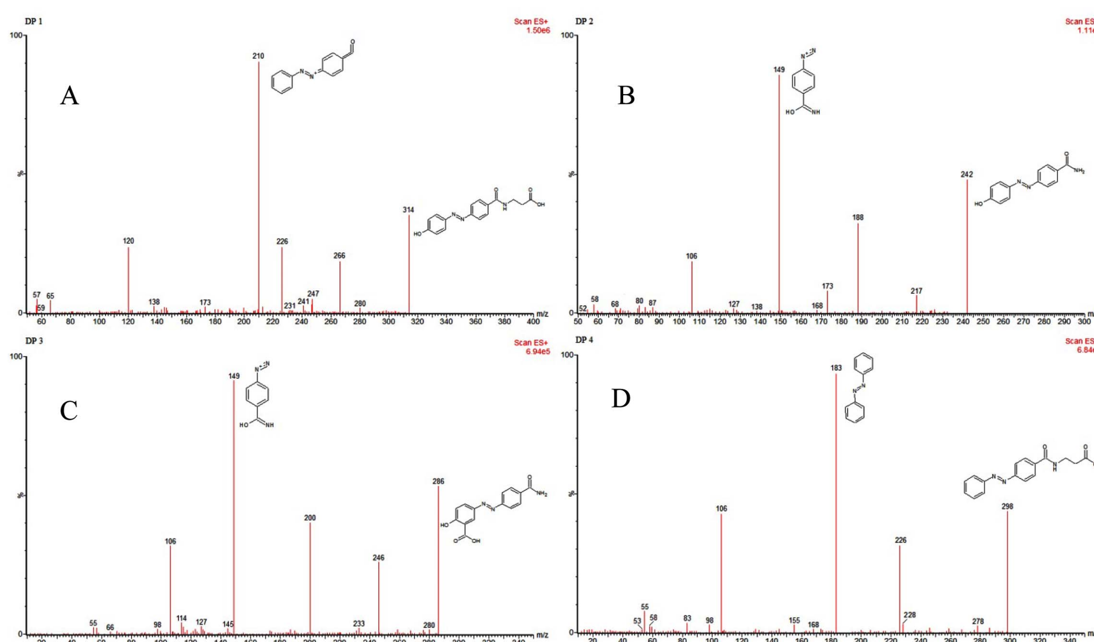

Fig. 5. Mass spectra of DPs formed during forced degradation study. (Mass fragmentation spectra identified at t_R of 3.5 min (A), 5.5 min (B), 7.5 min (C) and 8.6 min (D) for DP 1, DP 2, DP 3 and DP 4 respectively)

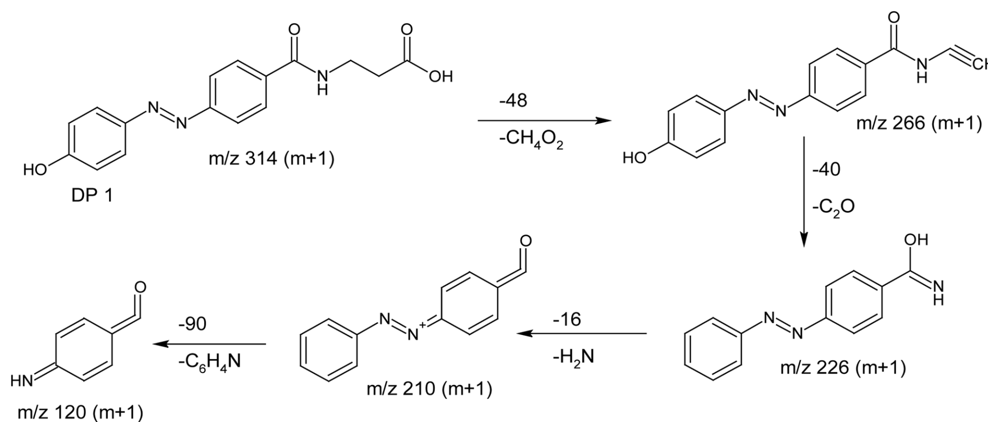

Fig. 6. Mass fragmentation pattern of DP 1.

m/z of 210 (m+1) with molecular formula $C_{13}H_9N_2O$. Based on the fragmentation pattern, the molecular structure of DP 1 was proposed with chemical name of *3-({4-[(E)-(4-hydroxyphenyl) diazenyl]benzoyl}amino) propanoic acid* and its fragmentation mechanism was presented in *Fig.* 6.

In DP 2 was identified at t_R of 5.5 min in both acid and base degradation chromatograms and it was characterized using LCMS/MS study. The fragmentation spectra of DP 2 as shown in *Fig.* 5B show fragment corresponds to parent ion at m/z of 242 (m+1) confirms the molecular weight of DP 2 as 341. The DP 2 was formed due to the breakage of C-N bond of β-Alanine and losing $–C_3H_4O_2$ of DP 1. The fragmentation spectra shows abundant fragment ion at m/z of 149 (m+1) with $C_7H_6N_3O$. Based on the mass spectral results, the DP 2 was confirmed as *4-[(E)-(4-hydroxyphenyl)diazenyl]benzamide*. The peak purity CID studies of DP 2 confirm it as one of the degradation product of DP 1 observed in the study. The elemental compositions of molecular ion of DP 2 and all its fragmentation ions have been confirmed by accurate mass measurements. The proposed fragmentation pattern of DP 2 was presented in *Fig*. 7.

The peroxide degradation chromatogram shows DP 3 at t_R of 7.5 min and its mass spectra (*Fig.* 5C). The mass spectra clearly show parent ion at m/z of 286 (m+1) with possible molecular formula of $C_{14}H_{11}N_3O_4$. The DP 3 was formed by losing $C_3H_4O_2$ from balsalazide due to breakage of aromatic N-C bond of β-Alanine. The mass spectra also show abundant fragment ion at m/z of 149 (m+1) suggest the molecular structure of DP 3. Based on the results, the DP 3 was identified as *5-[(E)-(4-carbamoylphenyl)diazenyl]-2-hydroxybenzoic acid*. The fragmentation pattern of DP 3 of balsalazide proposed based on the mass fragmentation was presented in *Fig.* 8.

The acid and base degradation chromatograms

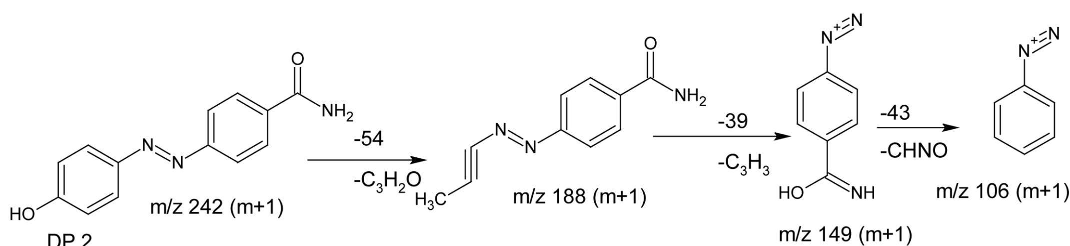

Fig. 7. Mass fragmentation pattern of DP 2.

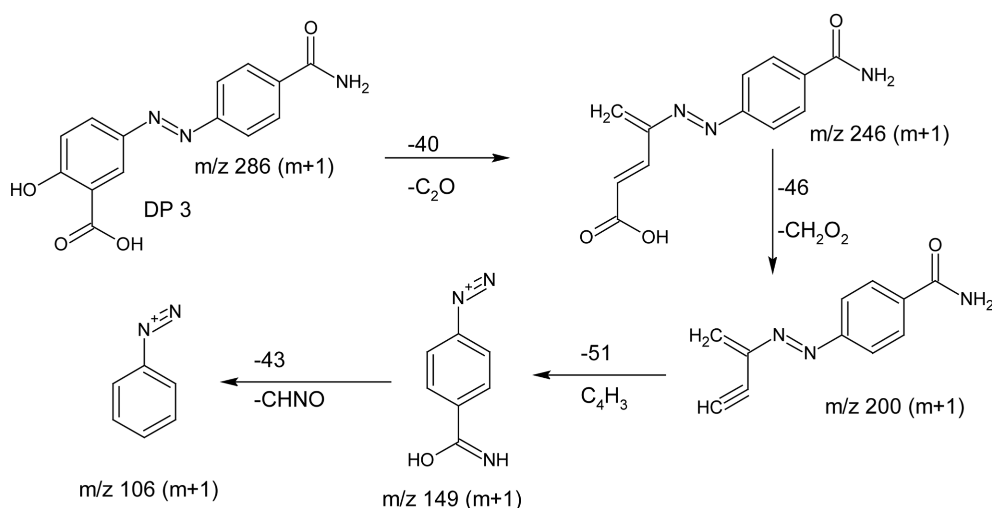

Fig. 8. Mass fragmentation pattern of DP 3.

clearly resolve DP 4 at t_R of 8.7 min and the product was generated with molecular formula of $C_{16}H_{15}N_3O_3$ by losing CH_2O_3 of balsalazide and the compound was confirmed as *3-({4-[(E)-phenyldiazenyl]benzoyl} amino)propanoic acid*. The peak purity CID studies of DP 4 confirm it as one of the degradation product of DP 1 observed in the study. The mass spectrum (*Fig.* 5D) shows abundant fragment ion at m/z of 183 (m+1) which was generated due to lose of $C_5H_4NO_3$ from DP 4. The elemental compositions of molecular ion of DP 4 and all its fragmentation ions have been confirmed by accurate mass measurements. The proposed fragmentation pattern of DP 4 identified in the study was presented in *Fig*. 9.

Based on the results achieved, it was confirmed that four different DPs were identified in forced degradation study of balsalazide and these compounds were characterized using LCMS/MS analysis. The molecular structures of DPs formed in the study were presented in *Fig.* 10.

The method was adopted for detection and quantification of process related impurities in formulations and in this study, the Balacol® brand formulation of balsalazide was selected. The prepared impurities spiked

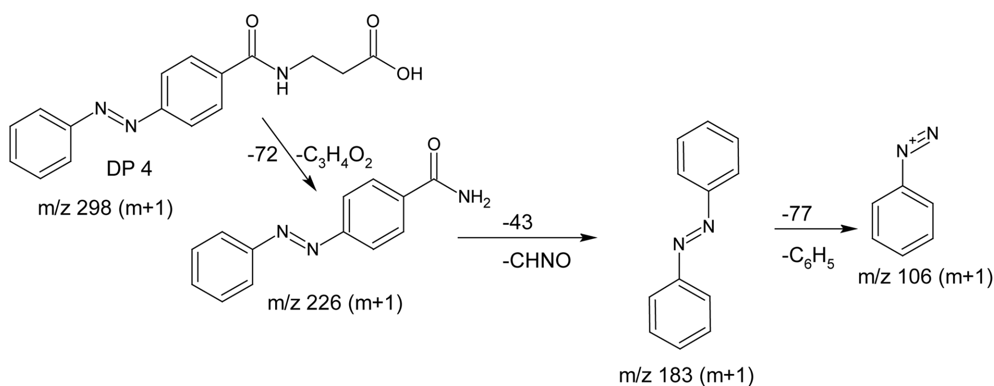

Fig. 9. Mass fragmentation pattern of DP 4.

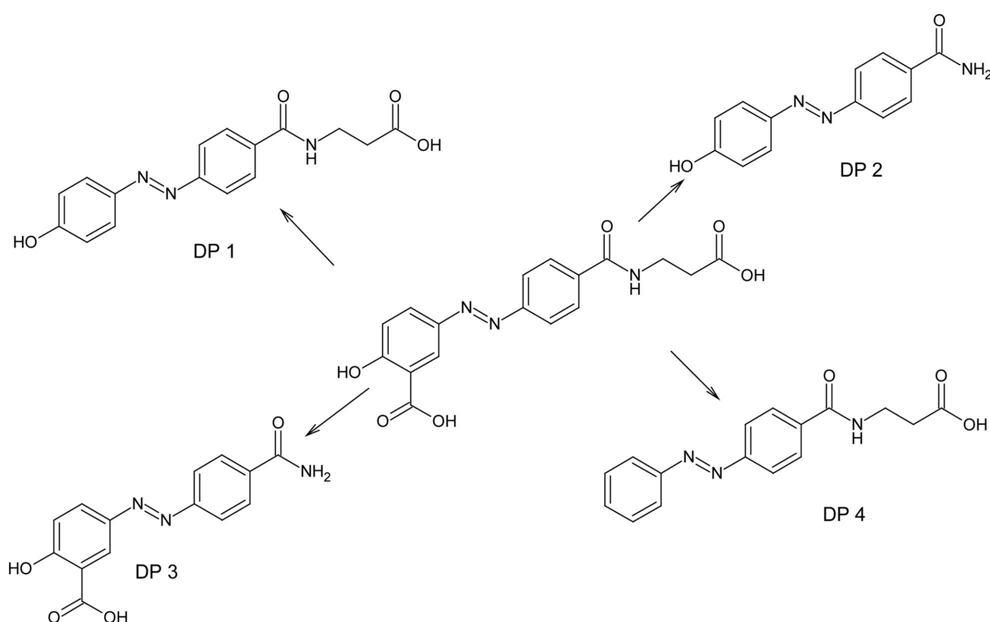

Fig. 10. DPs of balsalazide characterized in the study.

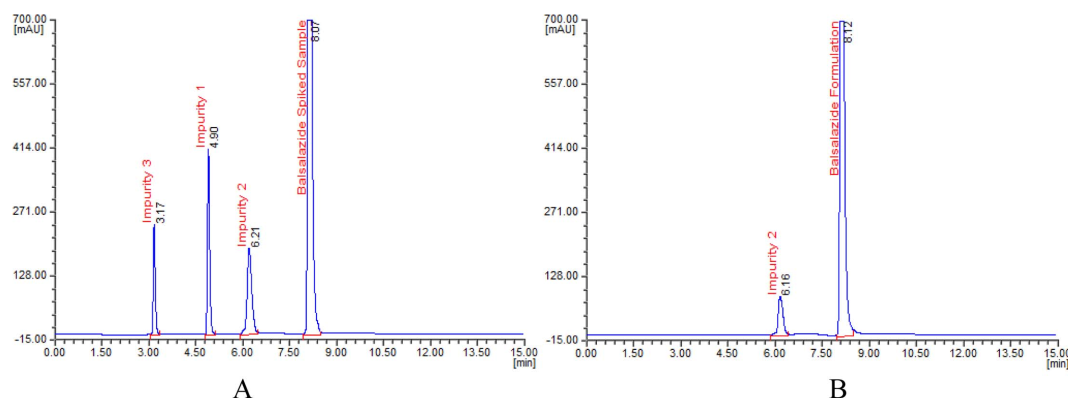

Fig. 11. Chromatogram observed during the analysis of Balacol® formulation solution in the proposed method. (A) Balacol® formulation solution spiked with 0.1 % of each impurity in the study; (B) Balacol® formulation solution spiked with no impurities

and un-spiked formulation solution of balsalazide was analysed in the proposed method. The impurities spiked formulation solution clearly resolves the impurities along with standard balsalazide. The chromatogram observed for un-spiked formulation solution show peak corresponds to impurity 2 only and other impurities were not detected. This proved that the impurity 1, 3 and 4 were not present in the formulation or they are less than the detection limit of the method. The % assay of the impurity 2 detected was calculated to be 0.05 % which was considered to be negligible. There is no detection of formulation excepients as well as other un-known impurities in both spiked and un-spiked formulation analysis and hence this method was confirmed to be suitable for the routine analysis of balsalazide and its process related impurities. *Fig*. 11 shows the spiked and un-spiked formulation chromatograms identified in the developed method.

4. Conclusions

In this paper, we present and validate a stability-indicating isocratic HPLC method for the determination of process related impurities of balsalazide. The drug was submitted to different degradation conditions (acidic, basic, peroxide, thermal and UV light stress). A number of DPs, depending on the stress, appear in the chromatograms. The drug was found to be strongly degraded under base, acid conditions showing high degradation and less degraded under thermal stress with the appearance of no degradation compounds. The structures of the DPs are established by LCMS/MS and this will helpful for assessing the degradation pathway and stability of balsalazide. The isocratic selected HPLC method is validated and is specific, linear, precise and accurate that can helpful for identification and quantification of process related impurities and degradation compounds of balsalazide.

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