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Development and Validation of Stability Indicating HPLC Method for Balsalazide in Bulk Drug Development

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Abstract: A rapid and reproducible RP-HPLC method was developed for the estimation of Balsalazide in bulk and subsequently validated for the parameters like accuracy, precision, linearity, range, robustness, ruggedness, % of recovery and limit of detection and limit of quantitation. Still now there were number of analytical methods were developed for the estimation and validation of Balsalazide alone and in ombined dosage form like UV-Visible spectroscopy, fluorimetry and RP-HPLC method but compared to those ethods the present study was a simple and selective LC method for the quantitative estimation of Balsalazide in bulk drugs. Good separation of Balsalazide disodium hydrate with good run times were obtained using a mobile phase A (phosphate buffer pH 7 and acetonitrile in the ratio of 950: 50 v/v) and mobile phase B (mixture of acetonitrile, methanol, tetrahedron furan and water in the ratio of 500: 300: 150: 50 v/v) at 1 ml/min flow rate. . The method was found to be precise as indicated by the repeatability analysis, showing %RSD less than 2. Results obtained are found to be reproducible.

1. Introduction

Balsalazide is chemically, 5-[(1E)-[4-[[(2-carboxyethyl) amino] carbonyl] phenyl] azo]-2-hydroxybenzoic acid. Balsalazide is chemically (E)-5-[[-4-[[(2-carboxyethyl) amino] carbonyl]phenyl]azo]-2-hydroxy benzoic acid. Balsalazide is an orally administered anti-inflammatory[1-3](gastrointestinal) drug. It is available in the form of disodium hydrate. It is used in the treatment of mild to moderate active ulcerative colitis. Balsalazide which has one molecule of 5-amino salicylic acid liked to a carrier via a diazo bond, is similarly split to release the active drug in the intestine. It is not official in any Pharmacopoeia. A thorough literature survey reveals that no HPLC method are reported however a few analytical methods have been reported for the estimation of balsalazide in bulk and pharmaceutical formulation including UV spectrophotometry[4-6].

2. Review of Literature

Several methods have been reported for the analysis of newer antifungal agents. Those include both classical and instrumental methods. The methods have been developed keeping in view of the requirements. Consequently, certain methods are also focused on the analysis of the drug from biological fluids. Analytical methods for quantification of the antifungal agents under study are discussed in this chapter. The following gives an account on some of the methods available in the literature. A RP-HPLC method was developed for the estimationof Balsalazide in bulk and Capsule dosage form and the method was proposed for the validation for the parameters like accuracy, precision, linearity, range, robustness, ruggedness, % of recovery and limit of detection and limit of quantitation[4]. Still now there were no. of analyticalMethods were developed for the estimation and validation of Balsalazide alone and in combined dosage form like UV-Visible spectroscopy, fluorimetry and RP-HPLC method but compared to those methods the present study was a simple and selective LC method for the quantification estimation of Balsalazide in capsule dosage form. Chromatogram Separation was achieved on a c18 column using Inertsil ODS 3V column, C18 (250 × 4.6 ID) mobile phase consisting of a mixture of KH2PO4:ACN:MEOH (50:30:20 vol./vol./v %)PH: 4.5 with detection of 304 nm. The retention time was found to be 2.487 min and linearity was observed in the range 90-210 µg/mL for Balsalazide. The method was found to be precise as indicated by the repeatability analysis, showing %RSD less than 2. A rapid and reproducible RP-HPLC chromatogram method was developed for the estimation of Balsalazide in its pure form as well as in pharmaceutical formulation [5]. Chromatogram was carried out on a C18 column using a mixture of water and acetonitrile as the mobile phase at flow rate of 0.8 mL/min and detection was done at 368 nm. The retention time of the drug was 3.685. The results obtained with the proposed methods are in good agreement with labeled amts. when marketed pharmaceutical preparations are analyzed. The recovery in the present method is in the range of 99.96-100.6. Results obtained are found to be reproducible.A simple, precise, rapid and accurate RP-HPLC method was developed and validated for the estimation of balsalazide in bulk and in capsule dosage forms[6]. Isocratic elution at a flow rate of 0.7 mL/min was employed on a

Phenomenax Luna C18 column (150 × 4.6mm; 5µ) at an ambient temp. The mobile phase consists of acetonitrile:methanol:triethylamine buffer (40:30:30% vol./vol.). The effluents were monitored at 254 nm and 20 µl of sample was injected. Nifedipine was used as an internal std. (IS). The retention times for balsalazide and IS were 3.42 and 5.07 min, resp. The method obeys Beer's law in the concentration. range of 10-50 µg/mL. The resp. linear regression equation being Y = 0.03727x + (-0.0084). The percentage assay of BSZ was 99.61% ± 0.106. The method was validated by determination its accuracy, precision and system suitability. The results of the study showed that the proposed RP-HPLC method is simple, rapid, precise and accurate, which is useful for the routine determination of BSZ in bulk drug and in its pharmaceutical dosage form.A HPLC method for determination of balsalazide disodium and its related substances was established. A Hypersil ODS Cr column was used with the mobile phase of 0.1 mol/L KHePOw soln. (pH 6.8)-methanol (65:35), at the detection wavelength of 254 nm and flow rate of 0.9 mL/min [7]. The calibration curve was linear in the concentration range of 0.06-0.6 mg/mL (r = 0.9999). A simple, precise, rapid, selective, and economic high-performance thin layer chromatogram (HPTLC) method was established for determination of balsalazide[9]. HPTLC method was developed using Chloroform: methanol (3.5:2, vol./vol.) as a Mobile Phase and Pre-coated silica gel G60-F254 aluminum sheet as a SP. Detection wavelength was 361 nm. In HPTLC linear range was 500-3000 ng/band, mean recoveries were found to be 99.99-100.04 % &Rf of a BAL was found to be 0.61. This HPTLC method is economic, sensitive, and less time consuming than other chromatogram procedures. It is a user-friendly and importance tool for analysis of tablet dosage forms.

A novel, sensitive, stability-indicating gradient RP-LC method has been developed for quantificationanalysis of balsalazide disodium and its related impurities both in the bulk drug and in pharmaceutical dosage forms. Efficient chromatogramseparation was achieved on a C18 stationary phase with a simple mobile-phase gradient prepd. from methanol and phosphate buffer [10]. The mobile-phase flow rate was 1.0 mL/min-1. Quantification was achieved by use of UV detection at 240 nm. Under these conditions resolution of balsalazide disodium from its three potential impurities was greater than 2.0. Regression analysis resulted in a correlation coefficient greater than 0.99 for balsalazide disodium and all three impurities. This method was capable of detecting the three impurities at 0.003% of the test concentration of 0.3 mg/mL-1, using an injection vol. of 10 µL. Inter-day and intra-day precision for all three impurities and for balsalazide disodium was within 2.0% RSD. Recovery of balsalazide disodium from the bulk drug (99.2-101.5%) and from pharmaceutical dosage forms (99.8-101.3%), and recovery of the three impurities (99.1-102.1%) was consistently good. The test soln. was found to be stable in 70:30 (vol./vol.) methanol-water for 48 h. When the drug was subjected to hydrolytic, oxidative, photolytic, and thermal stress, acidic and alkaline hydrolysis and oxidizing conditions led to substantial degradation. The RP-LC method was validated for linearity, accuracy, precision, and robustness. A novel, sensitive, stability-indicating gradient RP-LC method has been developed for quantificationanalysis of balsalazide disodium and its related impurities both in the bulk drug and in pharmaceutical dosage forms. Efficient chromatogramseparation was achieved on a C18 stationary phase with a simple mobile-phase gradient prepd. from methanol and phosphate buffer. The mobile-phase flow rate was 1.0 mL/min-1. Quantification was achieved by use of UV detection at 240 nm. Under these conditions resolution of balsalazide disodium from its three potential impurities was greater than 2.0. Regression analysis resulted in a correlation coefficient greater than 0.99 for balsalazide disodium and all three impurities. This method was capable of detecting the three impurities at 0.003% of the test concentration of 0.3 mg/mL-1, using an injection vol. of 10 µL. Inter-day and intra-day precision for all three impurities and for balsalazide disodium was within 2.0% RSD. Recovery of balsalazide disodium from the bulk drug (99.2-101.5%) and from pharmaceutical dosage forms (99.8-101.3%), and recovery of the three impurities (99.1-102.1%) was consistently good. The test solution was found to be stable in 70:30 (vol./vol.) methanol-water for 48 h. When the drug was subjected to hydrolytic, oxidative, photolytic, and thermal stress, acidic and alkaline hydrolysis and oxidizing conditions led to substantial degradation The RP-LC method was validated for linearity, accuracy, precision, and robustness.A HPLC method for determination of balsalazide disodium and its related substances was established. A Hypersil ODS Cr column was used with the mobile phase of 0.1 mol/L KHePOw soln. (pH 6.8)-methanol (65:35), at the detection wavelength of 254 nm and flow rate of 0.9 mL/min[11]. The calibration curve was linear in the concentration range of 0.06-0.6 mg/mL (r = 0.9999). Two simple and sensitive visible spectrophotometric methods (A and B) were developed for the quantificationestimation of balsalazide in bulk drug and dosage form. Method A is based on the

oxidation followed by complex formation reaction of balsalazide with 1,10-phenanthroline in presence of ferric chloride to form blood red colored chromogen with absorption maximum at 509 nm and Beer's law is obeyed in the concentration range of 6-18 μg/mL[12]. Method B is based on the oxidation followed by complex formation reaction of balsalazide with potassium ferricyanide in presence of ferric chloride to form a bluish green colored chromogen with absorption maximum at 790.0 nm and Beer's law is obeyed in the concentration range of 2-12 μg/mL. The developed methods were found to be precise and accurate. The results obtained are statistically validated and found to be reproducible.A simple, precise, rapid and accurate RP-HPLC method was developed and validated for the estimation ofBalsalazide in bulk and in capsule dosage forms. Isocratic elution at a flow rate of 0.7 ml/min was employed on aPhenomenax Luna C18 column (150*4.6mm; 5μ) at an ambient temperature. The mobile phase consists of Acetonitrile: Methanol: Triethylamine buffer (40:30:30% v/v). The effluents were monitored at 254nm and 20ml ofsample was injected. Nifedipine was used as an internal standard (IS). The retention times for Balsalazide and ISwere 3.42 and 5.07 min, respectively. The method obeys Beer's law in the concentration range of 10-50 mg/ml. Therespective linear regression equation being Y= 0.03727x + (-0.0084). The percentage assay of BSZ was 99.61% ±0.106. The method was validated by determining its accuracy, precision and system suitability[13]. The results of thestudy showed that the proposed RP-HPLC method is simple, rapid, precise and accurate, which is useful for theroutine determination of BSZ in bulk drug and in its pharmaceutical dosage form.

3. Aims and objectives

- To develop and validate RP-HPLC method for determination of Balsalazide in bulk and pharmaceutical formulation.
- To develop and validate RP-HPLC methods for estimation of Balsalazide in combination in synthetic mixture.
- Above mentioned method will be developed and validated statistically to ensure their accuracy, precision, repeatability, reproducibility and other analytical method validation parameters as mentioned in the various guidelines.

4. Experimental

4.1. Materials and reagents

All the chemicals, glassware and instruments are listed out below, which were used throughout the experimental work. Chemicals used were of analytical (AR) grade except specified and purchased from S.D fine chemical. Glassware used in the experimental work was calibrated in the laboratory and out of all only those were in calibration limit were selected for work1. The glassware was cleaned according to the pharmacopoeia procedure before they were used for the experiment.

4.2. Liquid chromatographs

4.2.1.RP-HPLC Method Development

Preparation of the mobile phase A

Prepare degassed mixture of phosphate buffer pH 7 and acetonitrile in the ratio of 950: 50 v/v

Preparation of the mobile phase B

Prepare degassed mixture of acetonitrile, methanol, tetrahedron furan and water in the ratio of 500: 300: 150: 50 v/v

Preparation of diluent:

Prepared degassed mixture of phosphate buffer and water in the ratio of 850:150 v/v

Preparation of Solutions:

Standard Preparation of Assay:

Accurately Weighed and transfer 75mg of balsalazide disodium hydrate and transferred to 100ml volumetric flask, add 70ml of diluent and sonicate to dissolve. Make up to 100 ml. filter through 0.45μ membrane filter.

Standard solution for related compounds:

Dilute 5 ml of standard solution for assay to 200ml with diluent. Further dilute 4 ml of this solution to 100 ml. filter through 0.45μ membrane filter.

Preparation of sample solution (10 μg/ml)

Accurately Weighed and transfer 75mg of Balsalazide disodium hydrate and transferred to 100ml volumetric flask, add 70ml of diluent and sonicate to dissolve. Make up to 100 ml. filter through 0.45μ membrane filter.

4.2.2.Chromatographic conditions:

Flow rate : 1 ml/min
Pump mode : gradient
Column :Hypersil BDSC8, 250 mm x 4.6 mm, 5μ.
Detector wave length : 254 nm
Column temperature : 40°C
Injection volume : 20 μL
Run time : 40 min
Diluent : Firstly dissolved in Methanol and made up with buffer

Method development was focused on the development and optimization of suitable sample preparation and chromatographic separation. Several tests were performed for optimizing the components of mobile phase in order to achieve good chromatographic peak shape and resolution[14-20]. Good separation of Balsalazide disodium hydrate with good run times were obtained using a mobile phase A (phosphate buffer pH 7 and acetonitrile in the ratio of 950: 50 v/v) and mobile phase B (mixture of acetonitrile, methanol, tetrahedron furan and water in the ratio of 500: 300: 150: 50 v/v) at 1 ml/min flow rate. The UV detector was programed at

254 nm for 40 minutes which was simple and fast resulting in sharp and symmetrical peaks.

5. Results and discussion for pregabalin:

5.1. Method optimization

The aim of this study was to develop a gradient RP-HPLC assay method for the analysis of pregabalin in formulation. Balsalazide, anantiepileptic drug similar to gabapentin produces its actions by binding to the alpha2-delta(α2δ) sub unit of the voltage-gated calcium channels. Balsalazide is an anticonvulsant drug used for neuropathic pain and asanad junct therapy for partial seizures with or with outs secondary generalization in adults. In case of RP-HPLC various columns are available, but as the main aim of the method is to resolve drug at retention time from excipients found in formulation, Hypersi**l** BDS C8, 250 mm x 4.6 mm, 5µ.particle size) was preferred over other columns.

Initial studies to optimise the mobile phase were involved with various mobile phases ratio containing acetonitrile and water in same proportion. In the study pregabalin (1 µg/ml) showed a retention time of 13.457 minute. In this case, the optimized mobile phase was constituted by different proportion of mobile phase A and mobile phase B. It was observed that satisfactory resolution of Balsalazide was obtained with flow rate of 1.0 ml/min at 254 nm (Figure.1.). The method parameter was optimized to analyse the pregabalin in oral suspension powder.

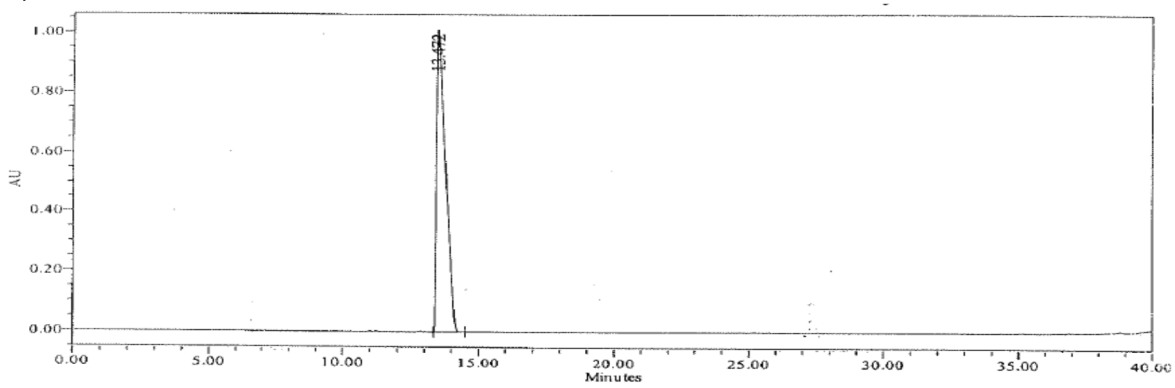

Figure.6.1. Balsalazide assay chromatogram at 254nm

5.2. Method validation:

A full method validation was performed according to guide-lines set by the ICH Guidelines. The validation of this procedure was performed in order to evaluate the method in terms of selectivity, sensitivity, range, linearity of response, accuracy, precision and intermediate precision.

5.3. Linearity

Linearity of the method was evaluated at five concentration levels by diluting the standard stock solution to give solutions in the concentration range from 80% to 120 %(Figure.1, Table.1). The results show that an excellent correlation existed between the peak area and concentration of analyte. The calibration curve was prepared by plotting the area under the response from the detector (AUC) line versus the concentration and analysed through linear regression. The response for the drug was linear ($r2$ = 0.9999) in the concentration. The linearity was observed in the expected concentration range, demonstrating its suitability for analysis.

% Concentration	Concentration (µg/mL)	Area	Statistical Analysis	
80	603.6	20259634	Slope	33385
90	678.1	22808767	Intercept	145788
100	756.3	25446554	% Y - Intercept	0.6
110	825.4	27671453	Residual Sum of Squares	43353
120	899.9	30177492	Correlation Coefficient	0.9999

Table.1. Linearity results of Balsalazide from 25% to 125% diluted samples with 25% interval

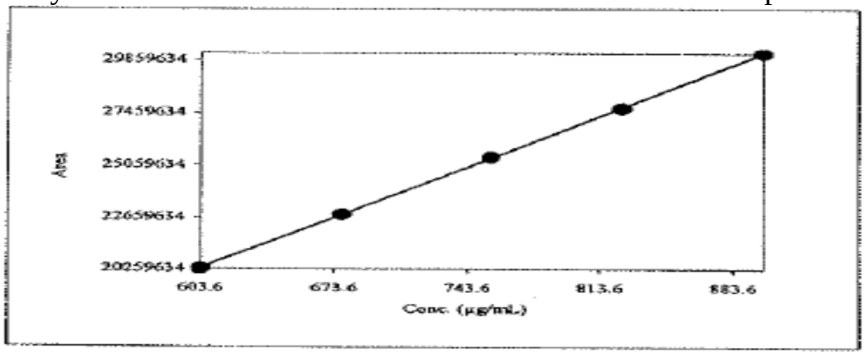

Figure. 1. Linearity plot of Balsalazide concentration Vs Response

5.4. Precision

The precision of the instrument was checked by repeatedly injecting (n = 5) solution of Balsalazide

a. System precision (% Repeatability)

(Table. 2 and Figure 2.). The results were reported in term of RSD i.e. 01%.

Injection ID	Assay	Injection ID	Assay
1	22810380	6	22761096
2	22830735	Mean	22794358
3	22801276	SD	27648
4	22800675	% RSD	0.1
5	22761984	95% Confidence Interval	± 29020

Table. 2. System precion results of Balsalazide

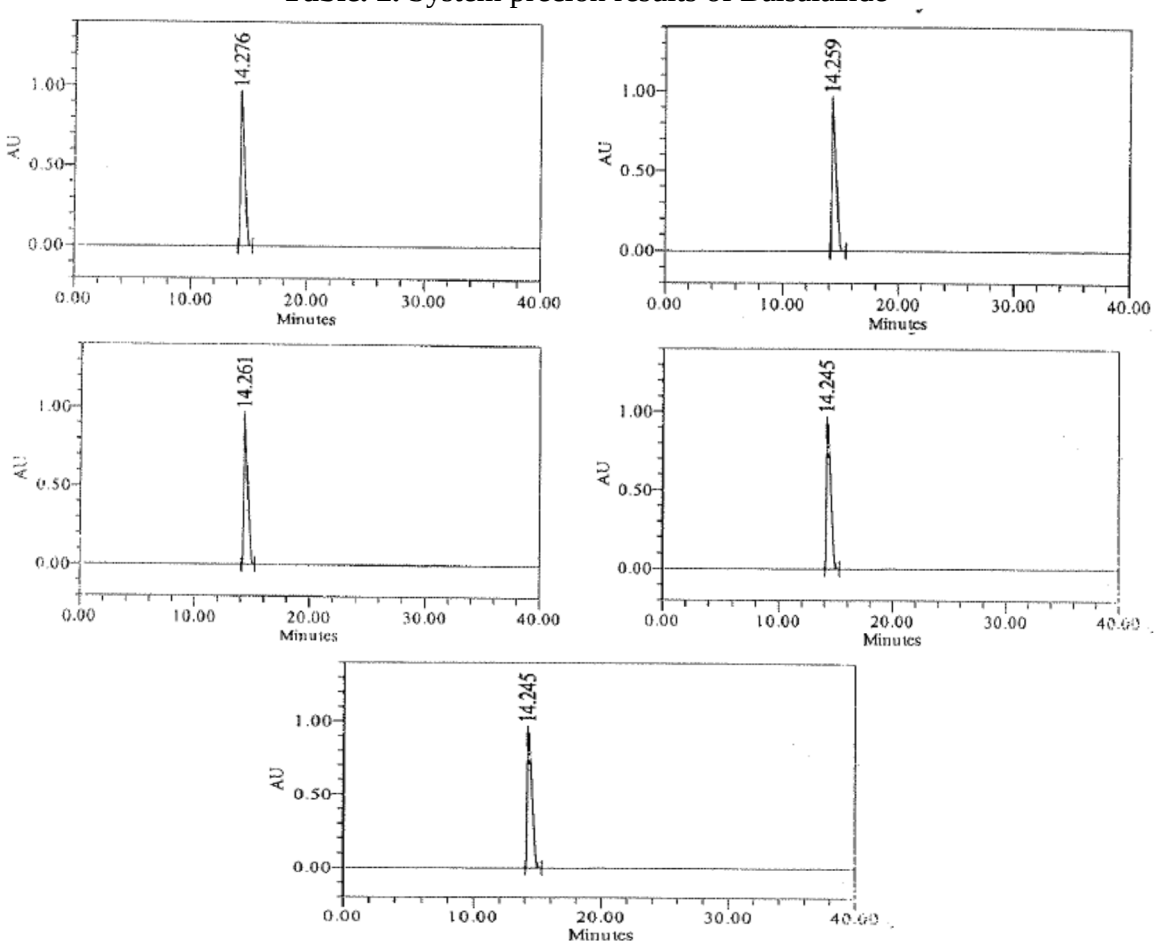

Figure.2. Repeatability chromatograms of Balsalazide

b. Intermediate precision:

Precision was evaluated in terms of intraday and interday precision. The intraday precision was investigated using three different concentrations of sample solutions prepared as discussed above, from stock solution. The intraday and interday precision of the proposed method was determined by analysing the corresponding concentration 3 times on the same day and on different days over a concentration of Balsalazide(Table.3). The results were reported in term of RSD i.e. 01%.

Assay			
Sample ID	**Assay (% w/w)**	**Statistical Analysis**	
1	99.4	Mean	99.3
2	99.3	SD	0.09
3	99.3	% RSD	0.1
4	99.4		
5	99.2	95% Confidence Interval	± 0.1
6	99.2		

Table. 3. System precion results of Balsalazide

5.5. Ruggedness: Six sample solutions were prepared individually using single batch of balsalazide disodium drug substance spiked with known related substances at specifications level as pet test method and injected each solution into HPLC as per methodology using different column, system and by another analyst on different day. 0.1% RSD was reported (Table.4.). The results are showing that the method is rugged for analyst to analyst, system to system, column to column and day to day variation.

RESULTS		
Sample ID	Assay (% w/w)	
	Set I	Set II
1	99.4	99.2
2	99.3	99.5
3	99.3	99.3
4	99.4	99.5
5	99.2	99.3
6	99.2	99.2
Mean	99.3	99.3
SD	0.09	0.14
%RSD	0.1	0.1
95% Confidence Interval	± 0.1	±0.1
Overall mean	99.3	
Overall SD	0.11	
Overall RSD (%)	0.1	
Overall 95% Confidence Interval	± 0.1	

Table. 4. Ruggedness results of the balsalazide disodium using different column

5.6. Accuracy: Sample solutions were prepared in triplicate at levels 80%, 100% and 120% of test concentrations for assay using balsalazide disodium drug substance: and by spiking with known substances to balsalazide disodium drug substance at levels 80%, 100%, and 120% of specification level as per the test method and injected each solution into HPLC as per methodology(Table.5.). Recovery %RSD of 80% 100% and 120% were reported 0.2, 0.3 and 0.9 respectively. 0.5 Overall % RSD was repowered for above spiked samples.

Concentration *I* Sample ID	**Amount added (mg)**	**Amount found (mg)**	**%Recovery**	**Statistical Analysis**	
80% Level Sample 1	54.87	54.98	100.2	Mean	100.2
80% Level Sample 2	54.69	54.70	100.0	SD	0.20
80% Level Sample 3	54.87	55.07	100.4	%RSD	0.2
100% Level Sample 1	68.29	67.99	99.6	Mean	99.9
100% Level Sample 2	68.57	68.57	100.0	SD	0.26
100% Level Sample 3	68.75	68.80	100.1	%RSD	0.3
120% Level Sample 1	81.71	80.68	98.7	Mean	99.6
120% Level Sample 2	82.81	83.10	100.4	SD	0.85
120% Level Sample 3	82.26	81.90	99.6	%RSD	0.9

Overall Statistical Analysis							
Mean	99.9	SD	0.53	% RSD	0.5	95% Confidence Interval	± 0.4

Table. 5. %Accuracy results of the balsalazide disodium

5.7. Stability of the solution: Balasalazide disodium test sample spiked with known related substances at specification level was prepared as test method and analyzed initially and at different time intervals by keeping the solutions at room temperature (25^0C) (Table.6.) and at refrigerator condition (6^0C)(Table. 7). % difference between the area obtained at initial and different time intervals were reported. Till 14^{th} hour % difference between the areas was less than 0.6% and the % difference between the areas of 14^{th} hour and 15th hour was 1.4%. % difference between the area obtained at initial and different time intervals were reported less than 0.7%.

5.8. Range: Range of analytical method can be obtained from the linearity and accuracy data. It can be concluded from the linearity and accuracy experiments that the range of analytical method for the assay is from 80% to 120% of test concentration in balsalazide disodium drug substance.

5.9. Robustness: According to the ICH (1994) guideline Q2A, 'robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage [21-24]. Method robustness was performed by applying small changes in the ratio of mobile phase,

injection volume, and column temperature and flow rate.

Time in Hours	Balsalazide Area	% Difference
Initial	22804353	-
1	22852287	0.2
2	22837249	0.1
3	22855983	0.2
4	22889269	0.4
5	22893599	0.4
6	22865061	0.3
7	22887806	0.4
8	22956512	0.7
9	23007087	0.9
10	22896983	0.4
11	22892938	0.4
12	23012289	0.9
13	22915875	0.5
14	22930859	0.6
15	23118210	l.4

Table.6. Stability of the solutions at room temperature (25^0C) with different time intervals.

Time in Hours	Balsalazide Area	% Difference
Initial	22372466	-
1	22413938	0.2
2	22319940	0.2
3	22323640	0.2
4	22399099	0.1
5	22436371	0.3
6	22404341	0.1
7	22341594	0.1
8	22467174	0.4
9	22437582	0.3
10	22431035	0.3
11	22473976	0.5
12	22506936	0.6
13	22446390	0.3
14	22402800	0.1
15	22433 136	0.3

Table.7. Stability of the solutions at room temperature (6^0C) with different time intervals.

Condition	Variation	System Suitability	
		USP Resolution	% RSD
STP	-	2.3	0.1
Flow Rate	-10%	2.7	0.1
	+ 10%	1.9	0.2
Wavelength	- 5 nm	2:3	0.1
	+ 5nm	2.2	0 .2
% Organic Mobile phase	2 % absolute	1.4	0.2
	+ 2 % absolute	2.2	0.1
Column oven Temperature	-5°C	2.8	0.0
	+5°C	1.8	0.1
pH	-0.2 units	1.5	0.2
	+0.2 units	2.1	0.2

Table. 8. Robustness results of the Balasalazide disodium

6. Conclusions:

A simple and rapid gradient RP-HPLC method was developed and validated for determining the process assay of Balasalazide disodium in bulk drugs and pharmaceuticals. Attempts were made to separate Balasalazide disodium from its process related impurities on different commercial C8 columns. The chromatographic conditions were optimized by studying the effects of temperature of the column and concentration and pH of ammonium acetate buffer. The developed method was found to be selective, sensitive, precise, linear, accurate and reproducible in determining the Balasalazide disodium and its potential impurities, which may be present at trace level in the finished products. The method could be of use for process development as well as quality assurance of Balasalazide disodium in bulk drugs as well as pharmaceutical formulations. The method is specific as there is no interference of blank at the retention time of Balsalazide disodium dihydrate. It is very fast, with good reproducibility and good response. Validation of this method was accomplished, getting results meeting all requirements. It allows reliable analysis of Balsalazide disodium dihydrate in bulk and its pharmaceutical dosage forms.

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