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Development and validation of UHPLC method for simultaneous estimation of sulbutamol sulphate and Beclomethasone Dipropionate

Venkata naveen.T[1], B. Gopinath[2] and V.Vijay Bhaskar[3]

[1, 2] Dept. of Pharmaceutical Analysis, National College of Pharmacy, Shimoga.,

[3] Scientist, G7 Synergone Pvt.Ltd, Bangalore

PHARMACEUTICAL SCIENCES RESEARCH ARTICLE

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ABSTRACT

The purpose of research is to Develop and validate the sulbutamol sulphate and Beclomethasone Dipropionate of UHPLC method for simultaneous estimation of. From the results it was concluded that UHPLC is very good method interms of accuracy, precision (repeatability, intermediate precision), specificity, LOD, and analysis of marketed formulation. This method is robust to the little variations in the, amount of methanol in the mobile phase and column temperature.

KEYWORDS: *simultaneous estimation, UHPLC method, sulbutamol sulphate and Beclomethasone Dipropionate.*

INTRODUCTION

Rationale for Selection of Drugs

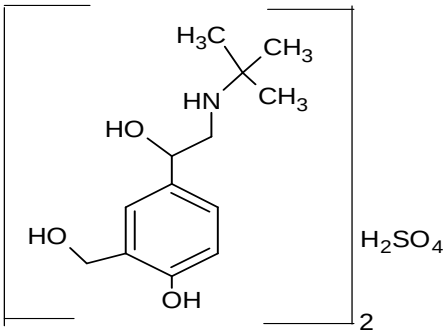
Bronchial asthma: Bronchial asthma is characterized by hyper responsiveness of trachea bronchial smooth muscle to a variety of stimuli, resulting in narrowing of air tubes, often accompanied by increased secretion, mucosal edema & mucus plugging.[7]

Salbutamol Sulphate: It is a stimulant of β_2 adrenergic receptors and it causes bronchodilation. It is used as an anti asthmatic drug. It relieves and prevents bronchospasm in patients with reversible obstructive airway disease, Prevents exercise induced bronchospasm.[7]

Beclomethasone Dipropionate: It is a steroidal drug used in asthma, it is used to control bronchial asthma in patients requiring chronic treatment, prophylaxis & treatment of allergic & vasomotor rhinitis. This drug restores responsiveness to sympathomimetic drugs like Salbutamol Sulphate once resistance to them has been developed.[7]

There may be many drugs in individual form, and in combination forms available in market, but Salbutamol Sulphate in combination with Beclomethasone Dipropionate is a good combination in market used for management of bronchial asthma.

Salbutamol Sulphate[8, 9, 10]



Mol. Formula :$(C_{13}H_{21}NO_3)_2,H_2SO_4$; Mol. Weight: 576.7; Syn: Albuterol sulphate:C.A.S. No: 51022-70-9; Merck index: 215.

Salbutamol Sulphate is (RS)-1-(4-hydroxy-3-hydroxymethylphenyl)-2-(tert-butylamino) ethanol sulphate.

Physical and chemical properties:

Physical state and appearance: Solid, white or almost white crystalline powder.

Solubility: Freely soluble in water, slightly soluble in ethanol (95 %), ether and very slightly soluble in dichloromethane.

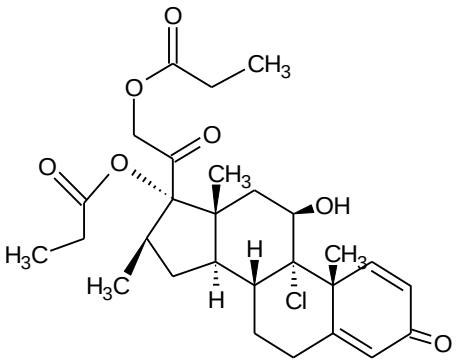
Stability: The product is stable.

Corrosivity: Non-corrosive in presence of glass.

Polymerization: Will not occur.

Storage: Store in tightly closed container protected from light.

Beclomethasone Dipropionate[8, 10, 11]



Mol.Formula: $C_{28}H_{37}ClO_7$;Mol. Weight: 521.1; Syn: Beclometasone Dipropionate; C.A.S. No: 5534-09-8 100; Merck index: 1020.

Beclomethasone Dipropionate is 9α-chloro-11β-hydroxy-16β-methyl-3, 20-dioxopregna-1, 4-iene-17, 21-diyldipropionate.

Physical and chemical properties:

Physical state and appearance**:** Solid, a white to creamy-white, crystalline powder.

Solubility: Freely soluble in acetone, chloroform, sparingly soluble in ethanol (95 %) and practically insoluble in water.

Stability: The product is stable.

Corrosion: Not reported to be corrosive.

Polymerization: Will not occur.

Storage: Store in tightly closed container protected from light.

ULTRA HIGH PERFORMANCE LIQUID CHROMATOGRAPHY.[13]

Definition: This new category of analytical separation science retains the practicality and principles of HPLC While increasing the overall interlaced attributes of speed, sensitivity, and resolution.UHPLC Systems take advantage of technological strides made in particle chemistry performance, system optimization, detector design, data processing and control. When taken together, these achievements have created a step-function improvement in chromatographic performance.

OBJECTIVE

A. To develop reverse phase ultra high performance liquid chromatographic method for simultaneous estimation of Beclomethasone Dipropionate and Salbutamol Sulphate in Bulk drug and pharmaceutical formulation.
B. Validation of the method according to ICH guide lines.

REVIEW OF LITERATURE

METHODS REPORTED ON SALBUTAMOL SULPHATE

1. **Selective extraction of Salbutamol from human plasma with the use of phenylboronic acid.**[19]

It involves the retention of a phenylboronate–Salbutamol complex on an end-capped C_{18} solid-phase sorbent to determine the level of Salbutamol in human plasma samples. Propranolol, a β-blocker, was chosen as the internal standard for this assay. In this solid-phase clean-up method, 50 mM sodium carbonate buffer, pH 9.60, was used for conditioning the column as well as washing the endogenous interference.

2. **Determination of β-agonists in liver and retina by liquid chromatography-tandem mass spectrometry.**[20]

This procedure uses enzymatic digestion, liquid–liquid extraction, and cleanup on oasis HLB solid-phase extraction cartridges, followed by determination of the residues by LC-tandem quadrupole mass spectrometer using atmospheric pressure chemical ionization in the positive ion mode.

3. **HPLC versus SFC for the determination of Salbutamol Sulphate and its impurities in pharmaceuticals.**[21]

It uses reverse phase high performance liquid chromatography (RP-HPLC) with diode array detection (DAD). The best separation was achieved using a gradient of 0.1 M ammonium acetate pH 3.0 and acetonitrile.

4. **Determination of clenbuterol, Salbutamol, and cimaterol in bovine retina by electrospray ionization-liquid chromatography-tandem mass spectrometry.**[22]

The tissue was homogenized in alkaline buffer and spiked to give 10, 15, and 20 ng of each of the 3 analytes together with the internal standards d_6-salbutamol and d_6-clenbuterol. The mixture was incubated with protease enzyme to release any protein-bound analytes and then made alkaline before extraction with isobutanol. The extract was

dissolved in water and transferred to a clenbuterol immunoaffinity column. After washing, the analytes were eluted and analyzed by ESI/LC/MS/MS using a C_{18} column with acetic acid-methanol as mobile phase.

5. **Determination of Salbutamol in human plasma and urine by high-performance liquid chromatography with a coulometric electrode array system.[23]**

It uses using high-performance liquid chromatography (HPLC) with a coulometric electrode array system. The mobile phase component A is 30 mM sodium dihydroxy phosphate-30 mM triethylamine and is adjusted to pH 6.0 with 20 % phosphate acid. The mobile phase component B is methanol. The optimized mobile phase composition was A and B in the proportion of 90:10 (v/v). Paracetamol is selected as the external standard.

6. **Determination of Salbutamol and detection of other beta-agonists in human postmortem whole blood and urine by GC-MS-SIM.[24]**

A sensitive and quantitative method for the determination of Salbutamol and the detection of terbutaline, clenbuterol, fenoterol, and isoprenaline in postmortem human whole blood and urine. It describes solid-phase extraction, formation of trimethylsilyl derivatives, and analysis by gas chromatography-mass spectrometry-selective ion monitoring.

7. **Determination of Salbutamol in human plasma and urine by high-performance thin-layer chromatography.[25]**

Salbutamol is extracted using solid-phase techniques and converted to an indoaniline dye by reaction with dimethyl-p-phenylenediamine. The indoaniline dye is separated using HPTLC and quantified by absorption microdensitometry at 650 nm.

8. **Analysis of Salbutamol and related impurities by derivative spectrometry.[26]**

Ultraviolet derivative spectrometry has been proposed for the analysis of Salbutamol and related impurities. The assay of Salbutamol aldehyde, 5-formyl-saligenin, and Salbutamol ketone was performed in sodium hydroxide 0.1 mol/l solutions, using first and second derivative spectra.

9. **Derivatization procedures for the detection of beta (2)-agonists by GC/MS analysis.[27]**

The study was performed on the beta (2)-agonists bambuterol, clenbuterol, fenoterol, formoterol, Salbutamol, salmeterol and terbutaline. Different derivatizating agents were employed, aiming to obtain derivatives with high selectivity to be used in the gas chromatographic/mass spectrometric analysis of beta (2)-agonists in biological samples.

10. **Determination of Salbutamol in syrups by capillary electrophoresis with contactless conductivity detection.[28]**

This paper describes the separation and quantification of Salbutamol in pharmaceutical products (Salbutamol syrups) by capillary electrophoresis (CE) with contactless conductivity detection (C^4D).

METHODS REPORTED ON BECLOMETHASONE DIPROPIONATE

1. **Simultaneous determination of Beclomethasone, Beclomethasone monopropionate and Beclomethasone Dipropionate in biological fluids using a particle beam interface for combining liquid chromatography with negative-ion chemical ionization mass spectrometry.[29]**

A new simple and sensitive assay has been developed for the simultaneous quantitative measurement of Beclomethasone Dipropionate and its hydrolysis products in human plasma and urine. Beclomethasone 17, 21-dipropionate, Beclomethasone 17-monopropionate, Beclomethasone and the internal standard, dexamethasone 21-acetate, were measured by combined liquid chromatography and negative-ion chemical ionization mass spectrometry with methane as the reagent gas.

2. **The measurement of Beclomethasone Dipropionate entrapment in liposomes: a comparison of a microscope and an HPLC method.[30]**

The purpose of this study was to examine the methodologies that may be used to estimate the maximum incorporation (< 5 mole % drug) of Beclomethasone Dipropionate (BDP) in dipalmitoylphosphatidylcholine (DPPC) multilamellar liposomes.

3. **Screening for anabolic steroids in doping analysis by liquid chromatography/electrospray ion trap mass spectrometry.**[31]

A fast and selective LC/MS/MS method for the screening of four anabolic steroids in human urine has been developed and validated. Liquid-liquid extraction with diethyl ether was applied after enzymatic hydrolysis. Analyses were performed on an ion trap mass spectrometer equipped with electrospray ionisation.

4. **HPLC determination of Beclomethasone Dipropionate and its degradation products in bulk drug and pharmaceutical formulations.**[32]

An HPLC method for the simultaneous determination of Beclomethasone Dipropionate and its principal degradation products has been developed. The only sample treatment necessary for the analysis is its dilution with methanol.

5. **Detection of corticosteroids in injection sites and cocktails by MS^n.**[33]

For the analysis of injection sites and of suspect cocktails (found at the farm), a multiple mass spectrometric (MS^n) method was developed. The method is based on rapid extraction of the matrix with methanol and direct infusion of the extract into the interface of the mass spectrometer.

6. **LC/MS-MS method for the determination of Beclomethasone Dipropionate and Beclomethasone-17-monoproprionate in human plasma.**[34]

1ml of a mixture of EDTA human plasma and internal standard (budesonide) was extracted with tert-butylmethylether. After evaporation and reconstitution in injection solvent, the organic phase was injected onto a synergi fusion-RP column with 4 µm particle size, 100 x 2.00 mm I.D. (Phenomenex). Elution was by a gradient of 2 mM ammonium acetate in a mixture of acetonitrile/high purity water containing 0.2 % formic acid at a flow rate of 0.3 ml/min.

7. **On-line high-performance liquid chromatography method for analyte quantitation from pressurized metered dose inhalers.**[35]

For the MDI systems in this study, an acetonitrile-water (90:10, v/v) mobile phase at a flow rate of 0.9 ml/min was found to give acceptable chromatography for BDP on a Apollo C_{18} 5 µm, 150 mm x 4.6 mm column (alltech associates, deerfield, IL, USA). Ultraviolet detection was done at 240 nm and the retention time of BDP was 2.7 min. The on-line HPLC method was characterized to be accurate, precise, sensitive, and specific.

8. **Quantitative, highly sensitive liquid chromatography–tandem mass spectrometry method for detection of synthetic corticosteroids.**[36]

Stable isotopes of cortisol-9, 11, 12, 12-d_4 and triamcinolone-d_1 acetonide-d_6 were added as internal standards to calibrators, controls, and unknown samples. After acetonitrile precipitation, these samples were extracted with methylene chloride, and the extracts were washed and dried. Reconstituted extract (15 µl) was injected on a reversed-phase column and analyzed by LC-MS/MS in positive-ion mode.

METHOD : REVERSE PHASE UHPLC METHOD.

INSTRUMENT USED[14]**:**

- Make : Agilent
- Model : RRLC 1200 series
- Pump : Binary pump with gradient system
- Degasser : Vacuum degasser
- Injector : Auto sampler with temp control
- Detectors : Diode array detector, UV, multiple wavelength detector
- Software : Chemstation.
- Columns : Broad range of more than 140 RRHT columns of 1.8 µm for wide applicability and thermostated column compartment
- Flow range : 0.05 - 5.0 ml/min
- 2000 samples/day on high throughput configuration, with alternating column regeneration (ACR) using system setup with two pumps, two columns and a 2-Position/10-Port valve for alternating column switching.

CHEMICALS USED:

- Millipore water, (ammonium acetate, dil acetic acid, methanol)-HPLC grade

EXPERIMENTAL

A. Selection of chromatographic conditions for analysis.

1. Selection of column:

As the method is reverse phase, two columns (C_8 and C_{18}) are initially chosen. Finally Agilent zorbax. Eclipse XDB –C_8 {3(i.d)*50mm (length)}, 1.8 µm particle size is chosen.

2. Selection of mobile phase:

The mixed standard solutions of SS and BDP were injected into the UHPLC system and run in different solvent systems. They are:

- Water and methanol
- Methanol and ammonium acetate buffer without adjusting pH
- Methanol and 0.1% v/v of formic acid in water
- Methanol and ammonium acetate buffer with pH adjustment to 4.5.

It was found that methanol and ammonium acetate buffer with pH adjustment to 4.5, gives satisfactory results as compared to other mobile phases. This mobile phase system was tried in different proportions. Finally the optimal composition of the mobile phase was determined to be 80:20 of methanol and ammonium acetate buffer with pH adjusted to 4.5.

3. Selection of flow rate:

Various flow rates are tried. They are:

- 0.6 ml
- 0.5 ml
- 0.4 ml
- 0.3 ml
- 0.2 ml

Finally 0.4 ml is considered to be the best flow rate.

4. **Selection of analytical wavelength:**

10 µg/ml solutions of Salbutamol sulphate and Beclomethasone Dipropionate were prepared individually with mobile phase. Solutions were scanned using double beam UV visible spectrophotometer (U-2900 of Hitachi) in the "Spectrum mode" between the range of 400 nm to 200 nm by overlaying. From the overlay spectra, 230.0 nm was selected as analytical wavelength.

5. Run time:

By checking various chromatograms, the run time is considered as 3.5 mins.

6. Chromatographic conditions selected after trails:

Method	:	Isocratic reverse phase
Mobile phase	:	Methanol + ammonium acetate buffer of pH 4.5 (80 %: 20 %) v/v
Run time	:	3.5 mins
Flow rate	:	0.4 ml/min
Temp of column	:	40^0C
Detection	:	230 nm
Pressure	:	600 bar
Sample injection size	:	5 µl
Column	:	C_8

B. Preparation of mobile phase:

Take 1000 ml volumetric flask, add 200 ml of ammonium acetate buffer of pH 4.5. To this add 500 ml of methanol initially and shake well and finally make up the volume to 1000 ml with methanol. Filtered before use.

❖ **Preparation of ammonium acetate buffer of pH 4.5:**

It involves 2 steps.

- First step: Preparation of 10 mM ammonium acetate solution

Weigh 385 mg of ammonium acetate and dissolve in 500 ml of water.

- Second step: Adjust the pH of the solution to 4.5 by using dil acetic acid (Adding drop by drop and checking with pH meter)

C. Preparation of standard stock solutions:

<u>All the standard stock solutions are mixed in origin i.e. combination of Beclomethasone Dipropionate and Salbutamol sulphate.</u>

❖ Stock A:

Dissolve 10 mg and 20 mg of Beclomethasone Dipropionate and Salbutamol Sulphate respectively in 10 ml of methanol.

This contains 1000 µg/ml of Beclomethasone Dipropionate 2000 µg /ml of Salbutamol Sulphate.

❖ Stock B:

Dilute 1ml of stock A to 10 ml with mobile phase.This contains 100 µg /ml of Beclomethasone Dipropionate200 µg /ml of Salbutamol Sulphate

❖ Stock C:

Dilute 1ml of stock B to 10 ml with mobile phase.This contains 10 µg /ml of Beclomethasone Dipropionate 20 µg /ml of Salbutamol Sulphate

❖ Stock D:

Dilute 2 ml of stock B to 10 ml with mobile phase.This contains 20 µg /ml of Beclomethasone Dipropionate. 40 µg/ml of Salbutamol Sulphate

❖ Stock E:

Dilute 4 ml of stock B to 10 ml with mobile phase.This contains 40 µg/ml of Beclomethasone Dipropionate and 80 µg /ml of Salbutamol Sulphate respectively.

D. Selection of analytical concentrations:

Appropriate aliquots were pipetted out from the standard stock solutions into a series of 10 ml volumetric flasks. The volume was made up to the mark with mobile phase to get a set of solutions having the concentrations ranging from 0.59 to 37.76 µg/ml of Beclomethasone Dipropionate and 4.72 to 75.52 µg/ml of Salbutamol Sulphate.

Table: 4.4. Concentrations of BDP and SS in the mixed standard solutions.

Sl.No	Conc. of BDP (µg/ml)	Conc. of SS (µg/ml)
1	0.59	1.18
2	1.18	2.36
3	2.36	4.72
4	4.72	9.44
5	9.44	18.88
6	18.88	37.76
7	37.76	75.52

Six replicates of each concentration were prepared and from these six solutions, 5 µl of volume is injected into the UHPLC system and their chromatograms were recorded under the chromatographic conditions. Peak areas were recorded for all the peaks and a standard calibration curve of peak area against concentration was plotted.

E. Analysis of formulation:

Dissolve 20 capsules in 50 ml of methanol in 50 ml volumetric flask. This gives 160 µg/ml of Salbutamol Sulphate and 80 µg/ml of Beclomethasone Dipropionate. Take 10 ml from this solution and filter by using syringe filter of 0.4 micron. From the filtered portion take 3.625 ml and dilute to 10 ml with mobile phase. This gives 58 µg/ml of Salbutamol Sulphate and 29 µg/ml of Beclomethasone Dipropionate respectively.

METHOD VALIDATION.[17]

A. ACCURACY.

Application of the analytical procedure to synthetic mixtures of the drug product components to which 80 %, 100 %, 120 % of the drug substance to be analyzed have been added.

❖ **80 % level of recovery:**

Dissolve 20 capsules in 25 ml of methanol in 50 ml volumetric flask, add 6.4 mg and 3.2 mg of SS, and BDP respectively. Shake for 20 mins and make up the volume with methanol.

Take 10 ml from this solution and filter by using syringe filter of 0.4 micron. From the filtered portion take 1.1 ml and dilute to 10 ml with mobile phase. This gives 31.968 µg/ml of Salbutamol Sulphate and 15.984 µg/ml of Beclomethasone Dipropionate respectively. Three replicates were prepared and analyzed.

❖ **100 % level of recovery:**

Dissolve 20 capsules in 25 ml of methanol in 50 ml volumetric flask, add 8 mg and 4 mg of SS, and BDP respectively. Shake for 20 mins and Make up the volume with methanol.

Take 10 ml from this solution and filter by using syringe filter of 0.4 micron. From the filtered portion take 2.06 ml and dilute to 10 ml with mobile phase. This gives 65.92 µg/ml of Salbutamol Sulphate and 32.96 µg/ml of Beclomethasone Dipropionate respectively. Three replicates were prepared and analyzed.

❖ **120 % level of recovery:**

Dissolve 20 capsules in 25 ml of methanol in 50 ml volumetric flask, and add 9.6 mg and 4.8 mg of SS, and BDP respectively. Shake for 20 mins and make up the volume with methanol.

Take 10 ml from this solution and filter by using syringe filter of 0.4 micron. From the filtered

portion take 1 ml and dilute to 10 ml with mobile phase. This gives 35.2 µg/ml of Salbutamol Sulphate and 17.6 µg/ml of Beclomethasone Dipropionate respectively. Three replicates were prepared and analyzed.

All the prepared solutions were analyzed in the selected chromatographic conditions.

B. PRECISION:

Precision is carried at 2 levels: repeatability, and intermediate precision.

❖ **Repeatability:**

Six determinations at 100 % of test concentration i.e. 75.52 µg/ml and 37.76 µg/ml of Salbutamol Sulphate and Beclomethasone Dipropionate respectively. This is carried on six mixed standard solutions. The procedure for the preparation of mixed standard solutions of Salbutamol Sulphate and Beclomethasone Dipropionate remains same as explained in selection of analytical concentrations. The solutions are analyzed by using the selected chromatographic conditions and the results are validated statistically.

❖ **Intermediate precision:**

Carried on 3 days by 3 different analysts using a set of six sample mixtures containing 37 µg/ml and 74 µg/ml of Beclomethasone Dipropionate and Salbutamol Sulphate respectively. Solutions were prepared and analyzed at same time on different days. The variations of the results on different days was analyzed and statistically validated.

C. SPECIFICITY:

It involved demonstration of the discrimination of the analyte in the presence of excipients. This is done by spiking pure substances with appropriate levels of excipients and demonstrating that the assay result is unaffected by the presence of that material.

It involves 2 steps

❖ First step:

Preparation of a set of six mixed standard solutions containing 37.76 µg/ml and 75.52 µg/ml of Beclomethasone Dipropionate and Salbutamol Sulphate respectively and analyzed on UHPLC.

❖ Second step:

Addition of 5 mg of excipients to each of the 10 ml of above solutions containing 37.76 µg/ml and 75.52 µg/ml of Beclomethasone Dipropionate and Salbutamol Sulphate respectively, shake for 20 mins, filter using syringe filter, filterate is used for analyzing.

D. RANGE:

Range is tested from 50 % to 120 % of test concentration. 3 replicates were prepared for each concentration.

❖ 50 % test concentration:

It includes the solution which contains 18.88 µg/ml and 37.76 µg/ml of Beclomethasone Dipropionate and Salbutamol Sulphate respectively. The method of preparation is same as given in selection of analytical concentrations.

❖ 100 % test concentration:

It includes the solution which contains 37.76 µg/ml and 75.52 µg/ml of Salbutamol Sulphate and Beclomethasone Dipropionate respectively. The method of preparation is same as given in selection of analytical concentrations.

❖ 105 % test concentration**:**

It includes the solution which contains 79.296 µg/ml and 39.648 µg/ml of Salbutamol Sulphate and Beclomethasone Dipropionate respectively.

➢ Preparation:

Dissolve 7.9296 mg of Salbutamol Sulphate and 3.9648 mg of Beclomethasone Dipropionate in 10 ml of methanol. This contains 792.96 µg/ml and 396.48 µg/ml of Salbutamol Sulphate and Beclomethasone Dipropionate respectively. Dilute 1 ml of this solution to 10 ml with mobile phase.

❖ 120 % test concentration:

It includes the solution which contains 90.624 µg/ml and 45.312 µg/ml of Salbutamol Sulphate and Beclomethasone Dipropionate respectively.

➢ Preparation:

Dissolve 9.0624 mg of Salbutamol sulphate and 4.5312 mg of Beclomethasone Dipropionate in 10 ml of methanol. This contains 9062.4 µg/ml and 4531.2 µg/ml of Salbutamol Sulphate and Beclomethasone Dipropionate respectively. Dilute 1 ml of this solution to 10 ml with mobile phase, this gives 90.624 µg/ml of Salbutamol Sulphate and 45.312 µg/ml of Beclomethasone Dipropionate respectively.

E. LIMIT OF DETECTION:

The procedure for the determination of limit of detection is based on the signal to noise ratio

F. LIMIT OF QUANTITATION:

The procedure for the determination of limit of quantitation is based on the signal to noise ratio

ROBUSTNESS:

The solutions containing 37 µg/ml of Beclomethasone Dipropionate and 74 µg/ml of Salbutamol Sulphate was injected three times under different parameters like deliberate variations in percentage of methanol in the mobile phase and column temperature**.**

❖ **Temperature variations:**

Three replicates were taken and each solution is analyzed at different temperatures i.e. 38^{O}C, 40^{O}C and 42^{O}C. After analyzing in the UHPLC, the results are compared, interms of retention time, tailing factor and % amount of Beclomethasone Dipropionate and Salbutamol Sulphate obtained.

❖ **Variations in the percentage of methanol in the mobile phase:**

Three replicates were taken and each solution is analyzed at different concentrations of methanol in the mobile phase i.e. 79 ml, 80 ml and 81 ml. After analyzing in the UHPLC, the results are compared, interms of retention time, tailing factor and % amount of Beclomethasone Dipropionate and Salbutamol Sulphate obtained.

METHOD : REVERSE PHASE UHPLC METHOD.

UHPLC method is carried out on Agilent RRLC-1200 series with Chemstation software at G7 Synergone Pvt.Ltd, Bangalore

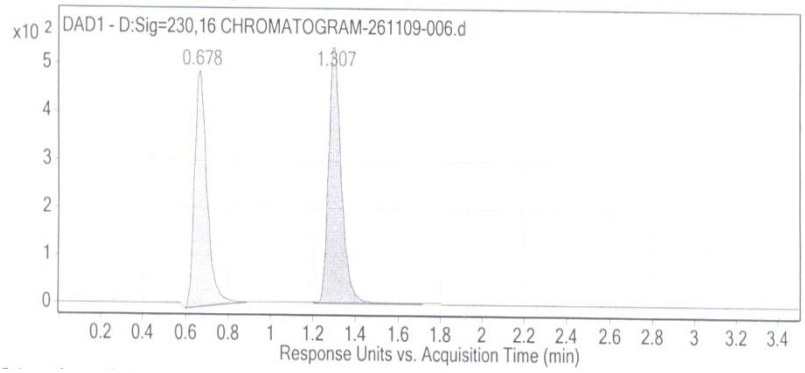


Fig: 5.16. Chromatogram of mixture of BDP and SS.

Table: 5.54. Result of calibration curve for SS at 230 nm by RP–UHPLC Method.

Concentration (µg/ml)	Peak area
4.72	290.88
9.44	331.21
18.88	405.8
37.76	570.5
75.52	898.93

Fig: 5.17. Calibration curve of SS at 230 nm by RP–UHPLC Method.

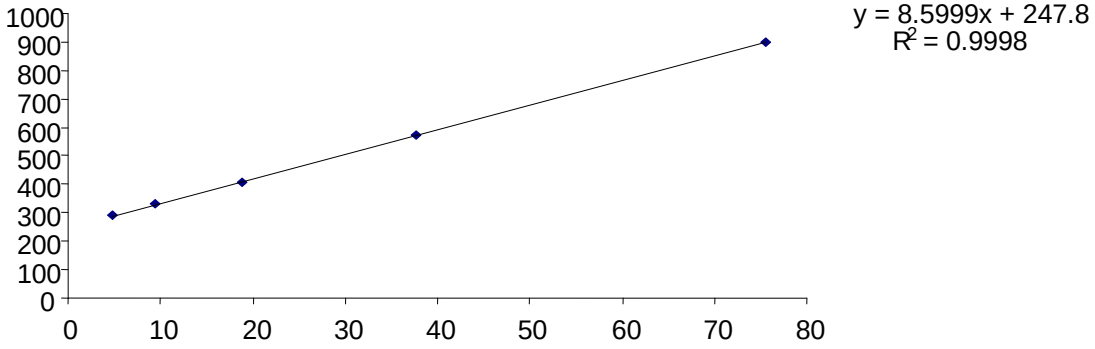


Table: 5.55. Result of calibration curve for BDP at 230 nm by RP–UHPLC Method.

Concentration (µg/ml)	Peak Area
0.59	5.97
1.18	11.49
2.36	23.41
4.72	48.53
9.44	96.55
18.88	196.56
37.76	395.75

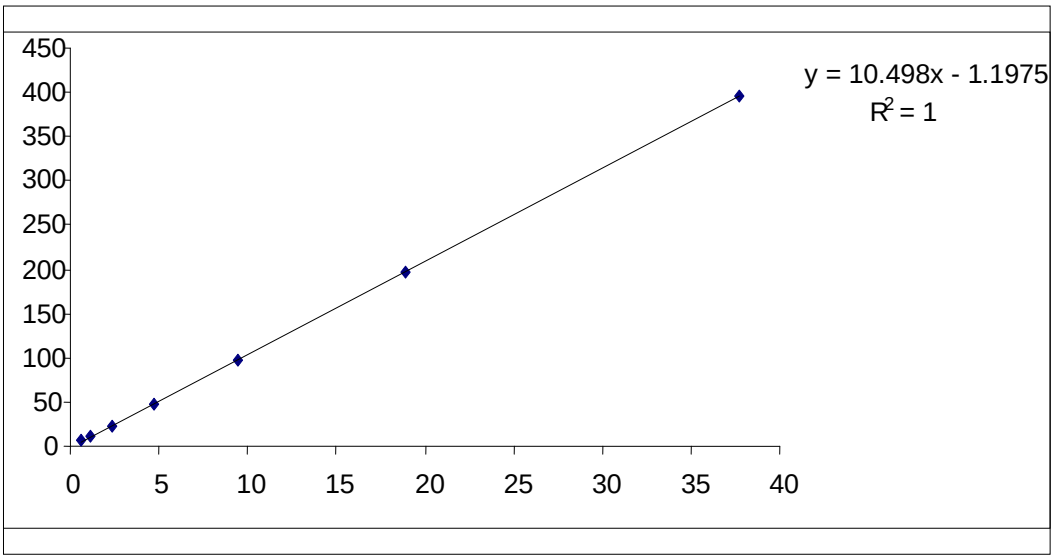


Fig: 5.18. Calibration curve of BDP at 230 nm by RP–UHPLC Method.

Table: 5.56. Data of SS and BDP at 230 nm by RP–UHPLC method.

Parameter	BDP	SS
Linearity (µg/ml)	0.59-37.76	4.72-75.52
Slope	10.498	8.5999
Intercept	-1.1975	247.8
Limit of detection (µg/ml)	0.0601	0.162
Limit of quantification (µg/ml)	0.1821	0.493

Table: 5.57. Assay results of formulation.

Sl. No.	Amount present (µg/cap)		Amount obtained (µg/cap)		Label Claim (%)	
	BDP	SS	BDP	SS	BDP	SS
1	200	400	198.184	404.856	99.092	101.214
2	200	400	199.086	403.696	99.543	100.924
3	200	400	198.594	405.012	99.297	101.253
4	200	400	199.702	405.064	99.851	101.266
5	200	400	198.152	404.968	99.076	101.242
6	200	400	199.85	404.668	99.925	101.167

- **The concentration of BDP and SS is 99.464 µg/ml and 101.178 µg/ml.**

Table: 5.58. Determination of accuracy of BDP and SS.

Level of recovery	Amount present (µg/20cap)		Amount of standard drug added (µg/ml)		Total Amount recovered (µg)		% Recovery	
	BDP	SS	BDP	SS	BDP	SS	BDP	SS
80%	4000	8000	3200	6400	7134.62	14579.136	99.092	101.244
	4000	8000	3200	6400	7137.14	14620.032	99.127	101.528
	4000	8000	3200	6400	7151.04	14557.536	99.32	101.094
100%	4000	8000	4000	8000	7971.04	16171.84	99.638	101.074
	4000	8000	4000	8000	7945.52	16207.68	99.319	101.298
	4000	8000	4000	8000	7958.08	16179.84	99.476	101.124
120%	4000	8000	4800	9600	8687.36	17829.856	99.721	101.306
	4000	8000	4800	9600	8665.272	17814.016	99.469	101.216
	4000	8000	4800	9600	8642.656	17874.736	99.212	101.561

Table: 5.59.The % recovery of BDP and SS.

Components	80%	100%	120%
BDP	99.179	99.477	99.467
SS	101.289	101.165	101.361

Table: 5.60. Repeatability data for BDP.

Sl.No	Amount of BDP prese (µg/ml)	Amount of B obtained (µg/ml)	Label claim (%)
1	37.76	37.792	100.086
2	37.76	37.661	99.738
3	37.76	37.422	99.105
4	37.76	37.536	99.407
5	37.76	37.398	99.042
6	37.76	37.567	99.491

Table: 5.61. Repeatability Data for SS.

Sl.No	Amount of SS prese (µg/ml)	Amount of SS obtain (µg/ml)	Label claim (%)
1	75.52	74.853	99.117
2	75.52	75.045	99.371
3	75.52	74.745	98.973
4	75.52	74.560	98.729
5	75.52	74.968	99.269
6	75.52	75.043	99.369

Table: 5.62. Statistical Validation Data for repeatability.

Components	Mean*	Standard deviation*	Co-efficient variation*
BDP	99.478	0.392	0.395
SS	99.138	0.252	0.255

*n = 6

Table: 5.63. Determination of intermediate precision of SS and BDP.

Sl. No.	Amount present (µg/ml)		Amount obtained (µg/ml)		Label Claim (%)	
	BDP	SS	BDP	SS	BDP	SS
DAY – I, ANALYST-I						
1	37	74	37.762	73.399	102.059	99.187
2	37	74	37.881	73.682	102.38	99.570
3	37	74	37.814	73.459	102.21	99.269
4	37	74	38.021	73.417	102.76	99.213
5	37	74	37.899	73.460	102.43	99.271
6	37	74	37.862	73.43	102.33	99.233
DAY – II, ANALYST-II						
1	37	74	38.085	74.071	102.93	100.095
2	37	74	37.680	73.420	101.837	99.216
3	37	74	37.712	73.611	101.926	99.475
4	37	74	37.773	73.719	102.09	99.621
5	37	74	37.891	73.758	102.41	99.674
6	37	74	37.802	74.055	102.17	100.075
DAY – III, ANALYST-III						
1	37	74	37.613	74.167	101.656	100.225
2	37	74	37.519	74.324	101.402	100.437
3	37	74	37.562	74.277	101.528	100.375
4	37	74	37.546	74.315	101.476	100.426
5	37	74	37.531	73.943	101.437	99.923
6	37	74	37.555	74.156	101.500	100.211

Table: 5.64. Statistical validation data for intermediate precision.

Components	Mean*	Standard deviation*	Co-efficient of variatio
BDP	102.029	0.465	0.456
SS	99.749	0.470	0.471

*n = 18

Table: 5.65. Data of BDP and SS before spiking with excipients.

Sl.No.	Amount present (µg/ml)		Amount obtained (µg/ml)		Label Claim (%)	
	BDP	SS	BDP	SS	BDP	SS
1	37.76	75.52	38.198	75.330	101.161	99.749
2	37.76	75.52	38.103	75.445	100.91	99.901
3	37.76	75.52	37.863	75.344	100.275	99.767
4	37.76	75.52	37.827	75.436	100.18	99.889
5	37.76	75.52	37.840	75.379	100.213	99.814
6	37.76	75.52	37.957	75.350	100.523	99.776

Table: 5.66. Data of BDP and SS after spiking with excipients.

Sl.No.	Amount present (µg/ml)		Amount obtained (µg/ml)		Label Claim (%)	
	BDP	SS	BDP	SS	BDP	SS
1	37.76	75.52	38.166	75.314	101.077	99.728
2	37.76	75.52	38.130	75.383	100.98	99.819
3	37.76	75.52	38.218	75.325	101.213	99.742
4	37.76	75.52	37.808	75.466	100.128	99.929
5	37.76	75.52	37.865	75.427	100.279	99.877
6	37.76	75.52	37.971	75.326	100.561	99.744

Table: 5.67. Effect of excipients on the method.

Components	% of conc. before spike	% of conc. after spike	Difference in the % of conc.
BDP	100.543	100.706	0.163
SS	99.816	99.806	0.01

Table: 5.68. The range of BDP.

Sl.No	% level of conc.	Amount prese (µg/ml)	Amount obtain (µg/ml)	Label claim (%)
1	50	18.88	18.929	100.263
2	100	37.76	37.728	99.916
3	105	39.648	36.640	92.414
4	120	45.312	39.527	87.235

Table: 5.69. The range of SS.

Sl.No	% level of conc.	Amount prese (µg/ml)	Amount obtain (µg/ml)	Label claim (%)
1	50	37.76	37.409	99.072
2	100	75.52	75.831	100.412
3	105	79.296	100.801	127.12
4	120	90.624	75.816	83.66

- **The range is only upto 100 % of test concentration for BDP (37.76 µg/ml) and SS (75.52 µg/ml).**

Table: 5.70. Robustness results for variations in amount of methanol in the mobile phase (v/v).

Method parameter	Level	Retention time		Tailing factor		Amount obtained (%)	
% of methanol		SS	BDP	BDP	SS	BDP	SS
79	-1	0.643	1.259	1.1	1.1	101.26	100.163
80	0	0.648	1.260	1.1	1.1	101.64	100.321
81	+1	0.642	1.264	1.2	1.2	101.58	100.217

Table: 5.71. Robustness Results for variations in column temperature.

Method parameter	Level	Retention time		Tailing factor		Amount obtained (%)	
Columns temperature		SS	BDP	BDP	SS	BDP	SS
38ºC	-2	0.645	1.267	1.1	1.1	100.427	99.537
40ºC	0	0.644	1.268	1.1	1.1	100.324	99.647
42ºC	+2	0.643	1.265`	1.1	1.1	100.273	99.421

Table: 5.72. Statistical validation of robustness results For variations in method parameters.

Method parameters	Mean*		Standard deviation*		Co-efficient of variation*	
	BDP	SS	BDP	SS	BDP	SS
% of methanol(V/V)	1.263	0.644	0.003	0.003	0.285	0.498
Column temp(ºC)	1.266	0.645	0.001	0.001	0.12	0.155

Table: 5.73. System suitability parameters of SS and BDP.

Parameters	BDP	SS
Retention time (mins)	1.301±0.01921	0.676±0.00979
Tailing factor	1.1	1.1
Asymmetric factor	0.87	0.92
Resolution factor	6.2	
Separation factor	6.391	
Theoretical plate/M	18200	6240

CONCLUSION

UHPLC:

From the results it was concluded that UHPLC is very good method interms of accuracy, precision (repeatability, intermediate precision), specificity, LOD, and analysis of marketed formulation. This method is robust to the little variations in the, amount of methanol in the mobile phase and column temperature. Even the system suitability parameters like tailing factor, assymetric factor, resolution, separation factor and theoretical plates are very good. Samples are analyzed simultaneously without any separation within a very shorter duration of time, so the method can be directly applied in daily laboratory routine.

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***Corresponding Author:**
Venkata naveen.T*
Dept. of Pharmaceutical Analysis,
National College of Pharmacy,
Shimoga