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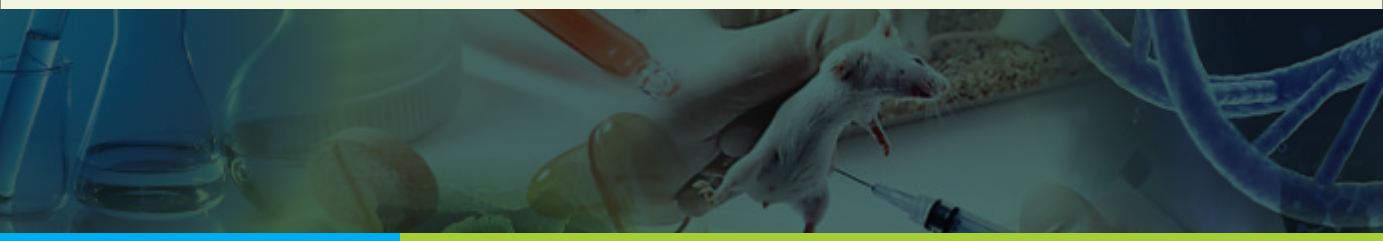

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Content

EDITORIAL MESSAGE

It gives us great pleasure to introduce this special issue of Nirma University Journal of Pharmaceutical Sciences (NUJPS). Welcome to the first issue of Nirma University Journal of Pharmaceutical Sciences in 2023. We would like to take this opportunity to express our gratitude to the readers, authors, expert reviewers, and respected editorial board for their contribution to Journal.

I hope with full commitment and determination, Nirma University Journal of Pharmaceutical Sciences (NUJPS) will serve its cause and goal in the field of Pharmaceutical Sciences. With the support of our colleagues, students, alumni, fellow researchers, scientists and well-wishers, we will scale up the status of Nirma University Journal of Pharmaceutical Sciences.

We plan for more supplements on important scientific topics and academic activities in India and abroad.

Members of the editorial team are determined to make the NUJPS a really significant journal.

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RESEARCH ARTICLE

DEVELOPMENT AND VALIDATION OF STABILITY INDICATING RP-HPLC METHOD FOR ESTIMATION OF VERICIGUAT IN MARKETED FORMULATION

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ABSTRACT

For the estimation of Vericiguat in the API and Marketed formulation, Following ICH criteria, an accurate, precise, specific, and robust stability indicating RP-HPLC technique was designed and validated. The development of method utilized by Zorbax eclipse plus C18 (250 mm × 4.6mm × 5µm) column with mobile phase of 10mM Potassium dihydrogen phosphate: Methanol (60:40 v/v) in isocratic mode. The flow rate kept at 1.0 mL/min at 256 nm wavelength. Vericiguat has retention time 6.9 min. Developed method to be validated according to ICH guideline in term of linearity, Accuracy, Precision, Robustness, and Forced degradation studies was performed. Linearity was achieved with a correlation coefficient was 0.9995 between the concentration 50 µg/mL to 150 µg/mL. Percentage recovery was discovered within the limits of 98% to 102%. %RSD was found to be less than 2% which is the specified in range. Studies of forced degradation reveal that Acid and Alkali have the highest rates of degradation. Impurity peak eluted at 3.1 and 3.0 mins in Acid and Alkali degradation respectively. Vericiguat did not degrade in Thermal and Photolytic degradation.

Key Words: RP-HPLC, Vericiguat, Validation, Eclipse plus C18 column, ICH guideline

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INTRODUCTION

Heart failure indicates that the heart is unable to pump enough blood for the body's metabolism, with the main symptoms being fatigue, fluid retention, and dyspnea. Systolic heart failure is defined by the heart's inability to adequately evacuate enough blood during the contraction phase.[1] Reduced ejection fraction with less than 40 percent of the blood move from the heart with each beat. Because of decreased blood flow, your entire body may get weary and cause issues. Heart failure (HF) has arisen as a global pandemic with 26 million individuals affected and the costing of global health system an estimated US$31 billion worldwide.[2] According to data from Register General of India (RGI) and Indian Council of Medical Research (ICMR) heart failure become the leading cause of death among the Indians. The nitric oxide (NO)-sGC-cyclic guanosine monophosphate (cGMP) has a essential effect on regulation of cardiovascular system. Vericiguat directly activates sGC, independently of and in synergy with NO, to create more cGMP, resulting in smooth muscle relaxation and vasodilation, which may improve cardiac function. [3,4].

In order to reduce the risk of cardiovascular death, heart failure (HF) hospitalization after an HF hospitalization, or the demand for outpatient IV diuretics, vericiguat is advised in patients with symptomatic chronic HF and an ejection fraction less than 45%. [5]. Vericiguat was approved by USFDA in January 2021 and CDSCO approval in 25th February 2022. It is available as Verquvo® by Bayer Zydus Healthcare pharmaceuticals. The dose of Vericiguat is starting from 2.5 mg once daily. The chemical name of Vericiguat is Methyl (4,6-diamino-2 (5-fluoro-1- (2-fluorobenzyl) -1H-pyrazolo [3,4-b] pyridine-3-yl) pyrimidin-5-yl) carbamate. vericiguat is white powder with a molecular weight of 426.39g/mole and a molecular formula of C19H16F2N2 O2. Vericiguat is slightly soluble in water and acetone acetone, very slightly soluble in acetone, methanol, ethanol, ethyl acetate freely soluble in dimethyl sulphoxide (DMSO). Survey of literature search there are only one RP-HPLC method for the quantification of vericiguat in marketed formulation at public domain [6]. The process of developing new drugs includes essential stress testing. Guidelines from the International Conference on Harmonization (ICH) Q3A(R2) and Q3B(R2) emphasize the need for studies to be done on drugs to determine their inherent stability characteristics [7].

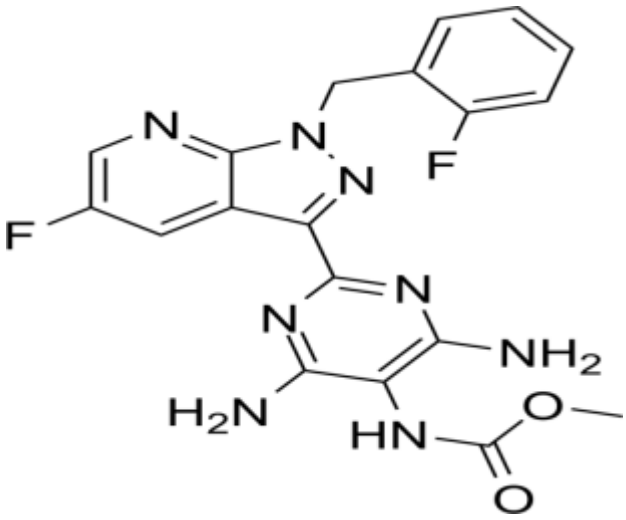

Figure: 1 chemical Structure of Vericiguat MATERIALS AND METHODS

Reagents and chemicals

Vericiguat tablets (marketed formulation) was purchased from the local market. HPLC grade chemicals are used for the development of method. HPLC grade Methanol and Hydrochloric acid were procured from Rankem lab. Potassium dihydrogen phosphate procured from Avantor Pvt.ltd. Other chemicals such as Sodium hydroxide, Hydrogen peroxide was procured from central drug house(p) ltd.

Instrumentation

The estimation of Vericiguat carried out by using Agilent 1260 infinity II HPLC system with photodiode array detector using Open lab CDS software as an integrator. pH meter (lab line), Analytical Balance (Mettler Toledo), Sonicator and centrifuge (Remi). The column used for separation of vericiguat was Zorbax Eclipse plus C18 (250 mm x 4.6 mm x 5 µm).

Preparation of Mobile phase

Mobile phase consists of 10mM Potassium dihydrogen phosphate buffer and Methanol in ratio of (60:40 v/v). The buffer solution was sonicated for 10 min and then filtered through 0.45 µm membrane filter and degassed before use.

Preparation of Diluent

The diluent made up of 10mM Potassium dihydrogen phosphate: Methanol (60:40 v/v).

Preparation of standard stock solution

Standard stock solution of vericiguat was prepared by dissolving accurately weighted 50 mg of vericiguat was taken into 50 mL volumetric flask and added 30 mL of diluent 10mM Potassium Dihydrogen Phosphate: Methanol (60:40 v/v). Sonicate

for 5 mins to properly dissolve. Volume was made up to the mark with the same diluent to get 1000 µg/mL standard stock solution.

Preparation of Standard solution (WSS)

From standard stock solution 1mL of solution was placed in a 10 mL volumetric flask and then diluted with 10 mL of diluent 10mM Potassium dihydrogen phosphate: methanol (60:40 v/v). The resulting solution has a 100 µg/mL concentration.

Sample Preparation for Formulation Analysis

10 tablets of Vericiguat were weighted individually and an average weight of 10 tablets was calculated. Taken equivalent weight 50 mg transferred to a 50 ml volumetric flask and dissolved in 30 ml of diluent. The mixture was vortex and sonicated for 30 min with intermittent shaking. Cooled the flask at room temperature then make up volume with diluent 10mM Potassium dihydrogen phosphate: methanol (60:40 v/v). Centrifuge at 5000 rpm for 10 mins to get clear supernant. Solution was then filtered through a 0.45 µm nylon syringe filter. Filtered solution was collected and 1mL of filtered solution was diluted up to 10 mL to achieve 100 µg/mL concentration.

Method development and Optimized Chromatographic condition

A RP-HPLC method was developed with the purpose of achieving a sufficient resolution of vericiguat and its degradation product. In this study zorbax eclipse plus C18 (250 mm x 4.6 mm x 5 µm) chosen for the achievement of good separation and resolution between drug and impurities. Then various mobile phase compositions and ratios were tried for better separation. Preliminary trials taken at isocratic mode using different ratio of water and methanol,0.1% formic acid in water and methanol,10mM potassium dihydrogen phosphate and methanol was used but obtained results show that Peak broadening effect observed. The estimation and separation of drugs in the presence of its degradants was done using isocratic mode on zorbax eclipse plus C18 (250 mm x 4.6 mm x 5 µm) reverse phase column with 1.0 mL/min flow rate, mobile phase having composition of 10mM potassium dihydrogen phosphate and methanol in ratio of 60:40 v/v was the optimized and resulted in well resolved and sharp peak in presence of degradation products. The 10 µl sample solution was injected using autosampler. The retention time of vericiguat was 6.9 min. The various system suitability parameters were observed and found within specified limits as per ICH guidelines.

Table: 1 Optimized chromatographic condition for method development

Parameters	Specification
Column	Zorbax eclipse plus C18 (250 mm x 4.6 mm x 5 µm)
Mobile phase	10mM Potassium dihydrogen phosphate: Methanol (60:40v/v)
Flow rate	1.0 mL/min
Detection wavelength	256 nm
Injection volume	10 µL
detector	Photodiode array detector

Sample Preparation

Vericiguat tablets available from local market with brand name of Verquvo (2.5 mg,5 mg and 10 mg, Bayer AG, Germany) taken tablet that contain label claim is 5 mg. Marketed formulation assay was performed under the optimized chromatographic condition. 10 tablets of vericiguat triturate and taken equivalent weight of 50 mg powder into 50 mL volumetric flask. All sample and standard solution prepared in 10mM Potassium dihydrogen phosphate: methanol (60:40 v/v).1 mL of sample solution taken into 10 mL volumetric flask to 100 µg/mL of sample solution was prepared and injected into HPLC system and calculate % Assay using noted peak area of sample.

Table: 2 Assay of formulation

Drug	Label claim	Amount found	% Label claim ±SD
Verquvo Tablet (Vericiguat)	5 mg	4.94 mg	98.8 ± 0.79

RESULTS

The developed method validated in terms of linearity, System suitability, Accuracy, Precision, Robustness in accordance with specification of ICH Q2(R2) guideline.

Specificity

Ability of method that assess analyte peak from other interference peak. Determine by recording chromatogram of 100 µg/mL of vericiguat standard and diluent. Diluent contain buffer solution: Methanol (60:40 v/v). Peaks were not detected in retention time of analyte peak that indicate there is no any interference in analyte peak. Analyte peak is separated from impurity peak or interference peak that indicates this method has specific.

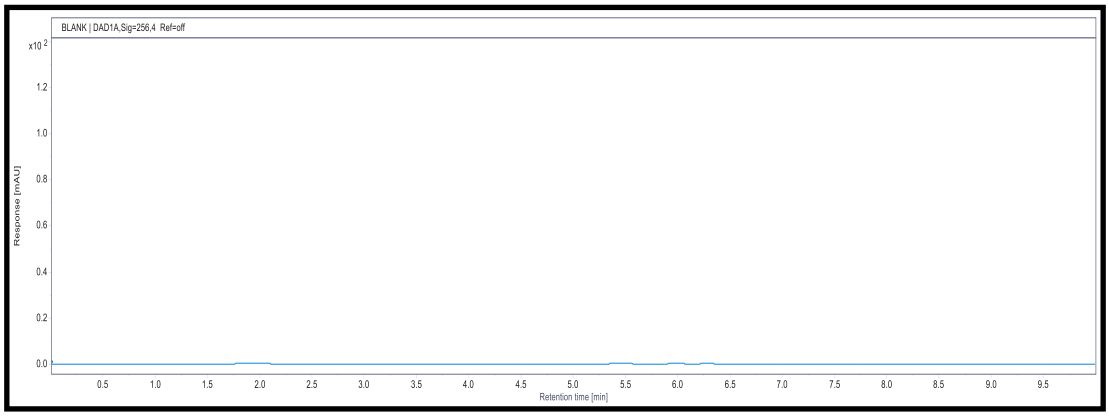

Figure 2: Chromatogram of Blank

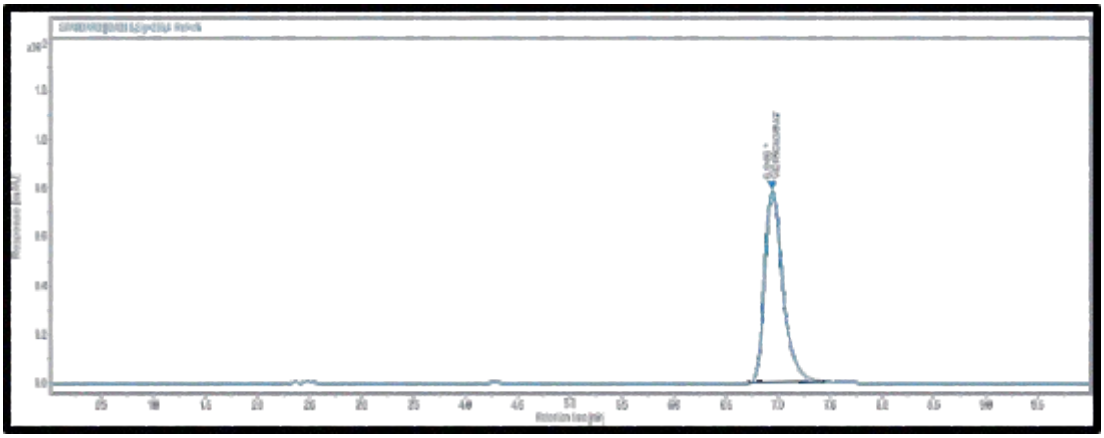

Figure: 3 Chromatogram of Vericiguat standard 100 µg/mL

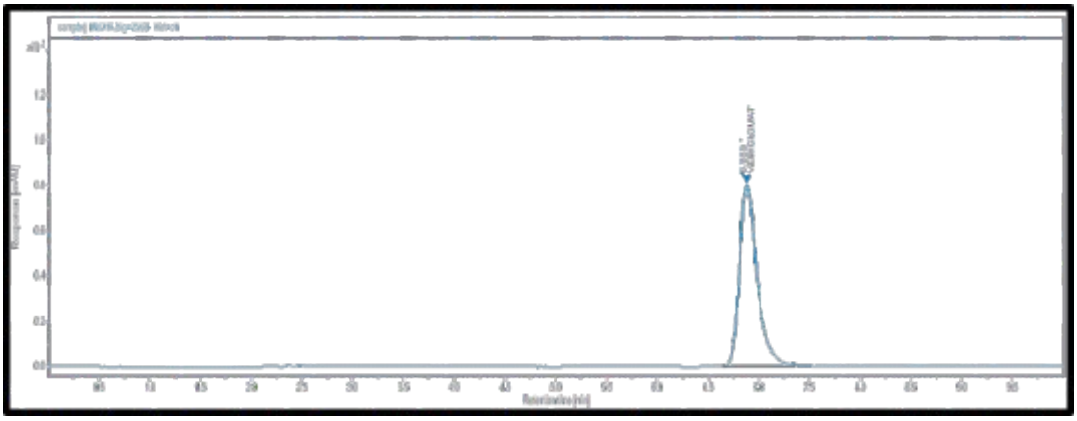

Figure: 4 Chromatogram of vericiguat sample 100µg/mL

System Suitability Testing

System suitability testing is performed to determine the column efficiency, resolution, and repeatability of a chromatographic system in order to confirm its ability to conduct a certain analysis. SST was done by injecting six replicates of standard solution 100 µg/mL. Table 3 summarizes the estimated peak area, tailing factor, theoretical plates and their average, SD, and %RSD.

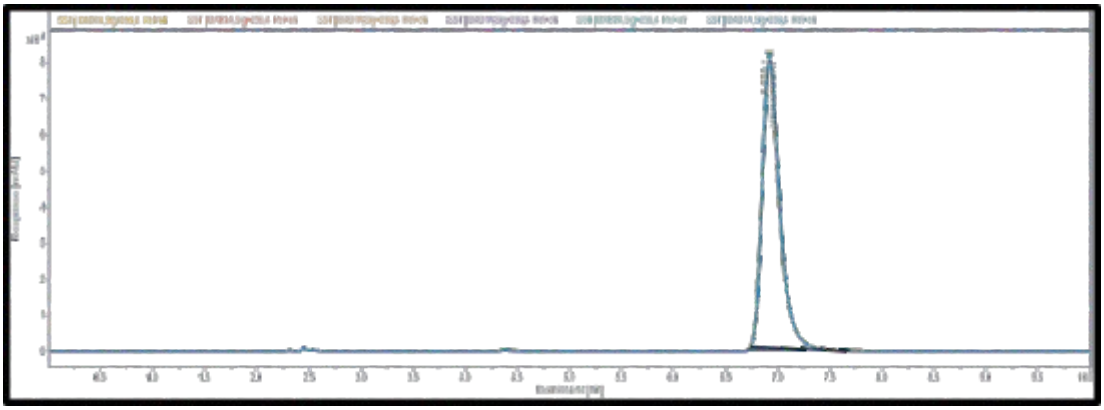

Figure: 5 Chromatogram for system suitability of vericiguat

Table: 3 System suitability testing data for vericiguat

Parameter	Results of Vericiguat
%RSD of Peak Area	0.52%
%RSD of Retention time	0.06%
Mean of Theoretical plates	8646
%RSD of Theoretical plates	0.35%
%RSD of Tailing factor	0.86%

Linearity

Linearity describe as the response is directly proportional to concentration of standard solution. From the standard solution series of the concentration in the range is 50 µg/ml to 150 µg/mL was prepared and injected each standard concentration. On the basis of peak area, the calibration curve was plotted by taking concentration on the x axis and peak area on the y axis. The results of linearity R^2 =0.9995 as shown in Figure 6.

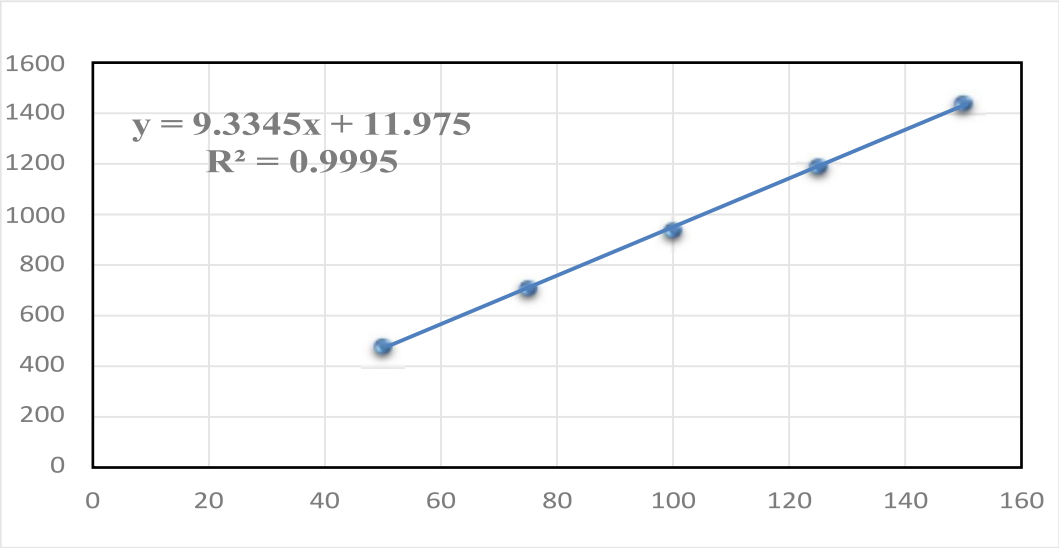

Figure : 6 Calibration curve of vericiguat

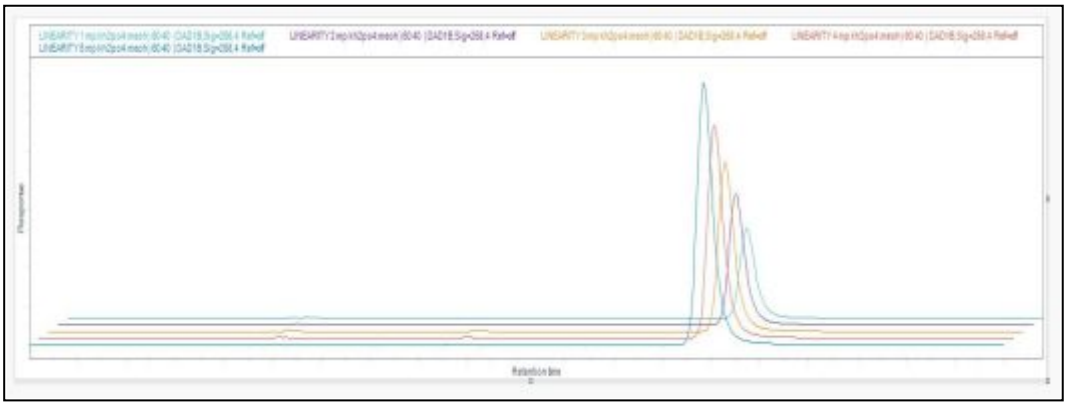

Figure :7 linearity overlay Chromatogram of vericiguat

Table: 4 Precision Data for Vericiguat

Concentration (µg/mL)	Peak Area
50	477.30
75	709.52
100	936.59
125	1187.75
150	1437.73
Correlation Coefficient(R^2)	0.9995

Table: 5 Precision Data for Vericiguat

Intraday Precision		Intraday Precision	
Sample No.	**% Assay**	**Sample No.**	**%Assay**
1	99.19	1	99.73
2	98.35	2	100.19
3	99.05	3	99.89
4	99.38	4	98.96
5	100.07	5	99.59
6	99.73	6	99.38
Mean	99.29	Mean	99.62
±SD	0.59	±SD	0.42
%RSD	0.60	%RSD	0.43

Accuracy

For an accuracy Study, performed by Placebo taken individually into various volumetric flasks and spiked in replicate with known amounts of Vericiguat at three different levels. 50% (Level 1), 100% (Level 2), and 150% (Level 3) of the standard concentration have been used to determine accuracy. The concentration was examined using the suggested procedure, and the amount of recovered Vericiguat was estimated (acceptance criteria: between 98 and 102%). As % Recovery is found within the acceptance criteria, the method is accurate. Results are summarized in Table 7.

Table :6 Accuracy results of vericiguat

Level	Amount added	Amount recovered	% Recovery	Average % Recovery(n=3)	±SD	%RSD
50%	50.3	50.6	101.2			
	50.6	50.3	100.6	100.2	0.69	0.69
	50.4	49.7	99.4			
100%	100.3	99.5	99.5			
	100.6	101.2	101.2	99.7	0.72	0.72
	100.5	99.8	99.3			
150%	150.4	149.9	99.7			
	150.3	150.7	100.2	99.8	0.36	0.36
	150.5	149.8	99.5			
Mean			99.9			
±SD			0.58			
%RSD			0.58			

Limit of Detection (LOD) and limit of Quantitation (LOQ)

The term "limit of detection" refers to the lowest amount of analyte present in a sample that can be detected but not measured.

LOD =3.3 x Standard/Slope

The lowest concentration of analyte in the sample that can be identified and quantified utilizing chromatographic conditions of an analytical technique with sufficient precision and accuracy is referred to as the limit of quantitation.

LOQ =10 × Standard/Slope

Limit of detection and limit of quantification performed by preparing a series of standard preparation of different concentration 10 µg/mL, 5 µg/mL, 2 µg/mL, 1 µg/mL, 0.5 µg/mL, 0.3 µg/mL of vericiguat standard then injected into HPLC system to determine LOD and LOQ was

Table: 7 LOD and LOQ Data

Injection (n=3)	10 µg/mL	5 µg/mL	2 µg/mL	1 µg/mL	0.5 µg/mL	0.3 µg/mL
1	75.05	37.15	15.89	7.24	4.09	ND
2	74.98	37.28	16.75	7.14	4.23	ND
3	74.87	36.96	15.96	7.30	4.17	ND
Mean	74.96	37.13	15.96	7.22	4.15	-
±SD	0.09	0.16	0.08	0.08	0.06	-
%RSD	0.12	0.43	0.50	1.12	1.56	-

Robustness

The robustness of an analytical technique is a measure of its ability to be unaffected by small but deliberate variations in method parameter and provides an indication of its reliability during use. Robustness defines as change in small parameter that should not affect to method for estimation of vericiguat. Robustness performed by changes in parameter like Flow rate (±

0.2 mL/min), Wavelength (± 2 nm), Column temperature 25°C and 35°C.

Change in flow rate ± 0.2 mL/min: This procedure performed by changing flow rate proportion ± 0.2 mL (0.8 mL/min and 1.2 mL/min) for 100 µg/mL concentration of standard solution was injected three times into HPLC system by varying the flow rate conditions.

Change in column temperature: This procedure performed by changing column temperature 25°C and 35°C.Sample solution was injected in three times by varying conditions. Calculated SD and %RSD of peak area of the response.

Change wavelength ± 2 nm: This procedure performed by changing wavelength 100% concentration of sample solution was injected three times by varying wavelength. Noted and calculated peak area, Average, SD and %RSD.

Table 8: Robustness data of vericiguat

Method parameter	Theoretical plates	Tailing factor	%RSD of peak Area	%Assay of Vericiguat
Variation in Wavelength (254 nm)	8140	1.40	0.45	97.53
Variation in Wavelength (258 nm)	8165	1.40	0.54	96.45
Variation in Column oven temperature (25°C)	8817	1.38	0.39	97.34
Variation in column oven temperature (35°C)	8207	1.39	0.46	97.67
Variation in Flow rate (0.8 mL/min)	9488	1.40	0.34	96.79
Variation in Flow rate(1.2 mL/min)	7947	1.36	0.36	97.52

Forced degradation studies

Forced degradation studies include degradation of drug substance and drug product at the condition more severe than accelerated condition. This studies further facilitate the development of stable formulation with suitable storage condition. ICH guidelines demonstrate forced degradation condition such as acidic, alkali, photolytic, thermal and oxidative degradation. Stability data to understand the how the quality of drug substance and drug product change under the influence of various environmental factors. Performed acid degradation (5N HCL), Alkali degradation (5N NaOH), Oxidative degradation (3% H2 O2) Thermal degradation and Photolytic degradation. Representative chromatograms of these evaluations are shown in the following Figures 8,9,11,12,13.

Solution preparation

5N HCL preparation: 42.5mL of HCL taken into 100 mL volumetric flask and volume make up with milli-Q water.

5N NaOH preparation: 20gm of sodium hydroxide pellets are taken into 100 mL volumetric flask and volume make up to mark with milli-Q water.

3% H2 O2 preparation: 3mL of H2 O2 is pipetted out into 100ml volumetric flask and volume make up to mark using milli-Q water.

Procedure

Acid degradation studies: Accurately equivalent weight 5 mg vericiguat tablet

powder taken into 50 mL volumetric flask. Add 30 of diluent that contain 10mM Potassium dihydrogen phosphate: Methanol (60:40 v/v) for the dissolve sample and add 1mL of 5 N HCL kept Room temperature for 30 hrs after the completion of 30 hrs then add 1N NaOH for neutralized the solution and make up to the mark with diluent. Filter the solution and inject into HPLC system at optimized chromatographic condition. Observe the result that how much % of drug degradation.

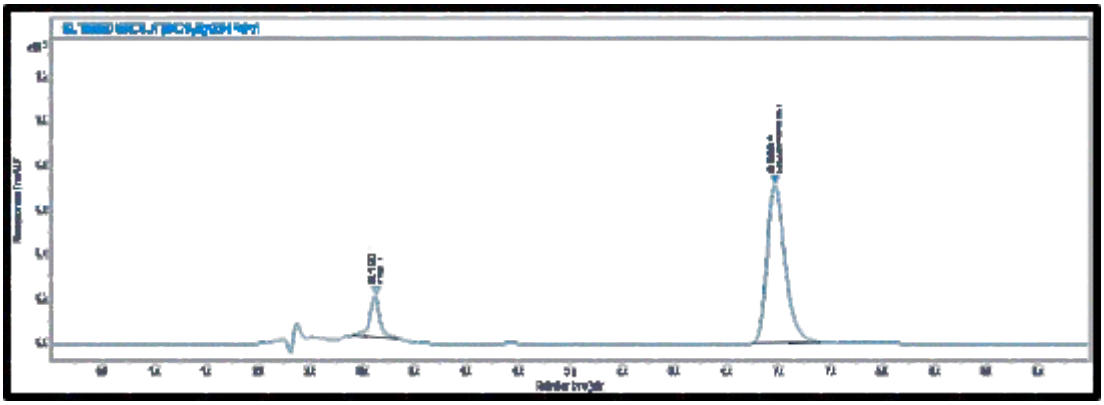

Figure: 8 Chromatogram of sample under 1mL of 5N HCL at Room Temperature for 30 hrs

Alkali degradation studies: Accurately equivalent weight 5 mg of vericiguat tablet powder taken into 50 mL volumetric flask. Add 30mL of diluent that contain 10mM Potassium Dihydrogen phosphate: Methanol (60:40v/v) for dissolve sample. Add 1mL of 1N NaOH kept at Room Temperature for 30 hrs. After completion of 30 hrs. add 1N of HCl for neutralized the solution and make up to mark with diluent. Filter the solution and inject into HPLC system at optimized chromatographic condition. Observe result peak area that how much % of drug degradation.

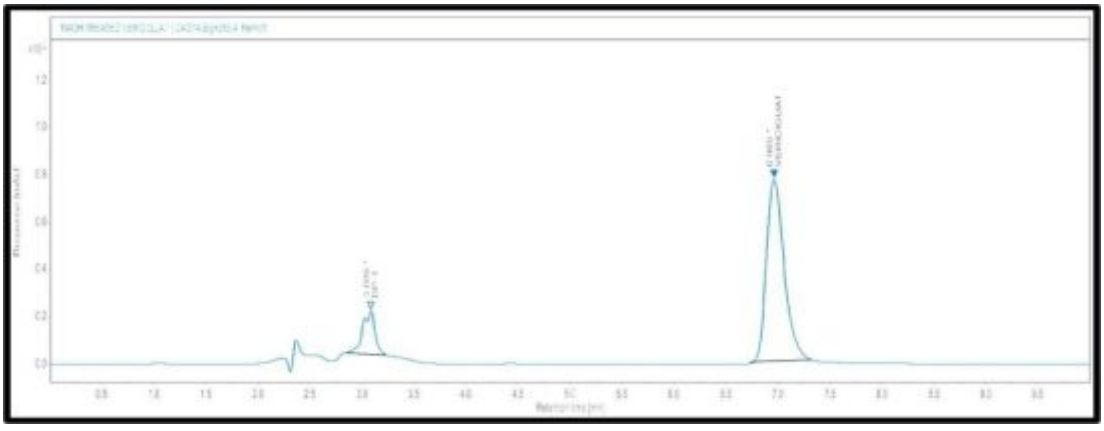

Figure: 9 Chromatogram of sample under 1ml of 5N NaOH at Room Temperature for 30 hrs

Oxidative degradation: Accurately equivalent weight 5 mg of vericiguat tablet powder taken into 50 mL volumetric flask. Add 30 mL of diluent for dissolve the sample. Add 1mL of prepared 3%H_2O_2 and then kept at Room Temperature for 48 hrs. Make up the volume to the mark with diluent. Check peak area under the optimized condition.

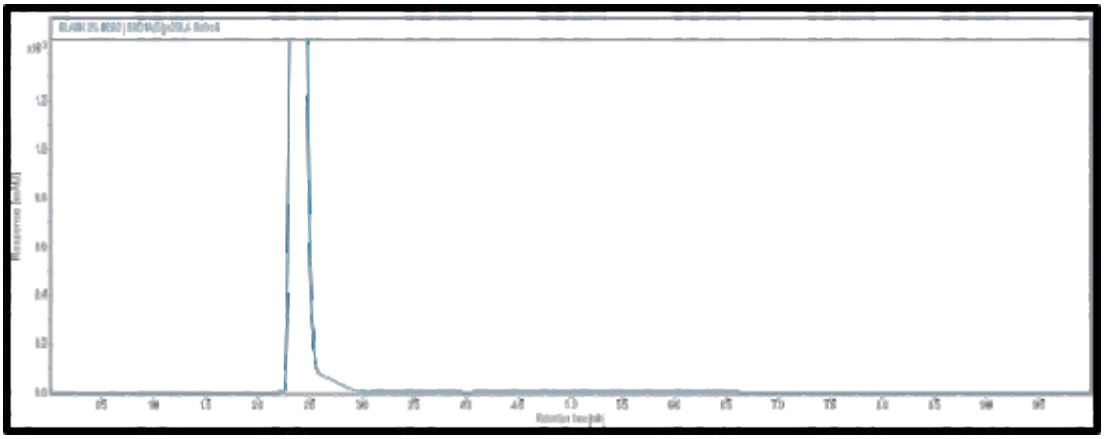

Figure: 10 Chromatogram of blank 3% H_2O_2

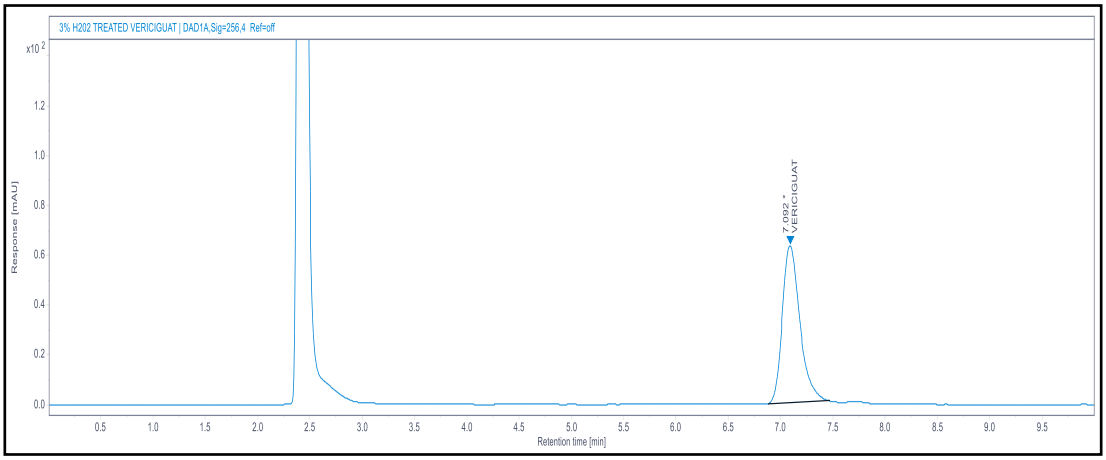

Figure: 11 Chromatogram of sample under 1mL of 3% H_2O_2 at Room Temperature for 48 hrs

Thermal degradation: Accurately weight the 10 tablets of vericiguat and triturated by using mortar and pestle to form fine powder. Taken 5 mg equivalent weight of vericiguat into 50 mL volumetric flask. Make up to volume with diluent. Flask placed on 80 °c heated water bath for 2 hrs. and after 2 hrs. inject the sample solution injected into HPLC system and observe the results.

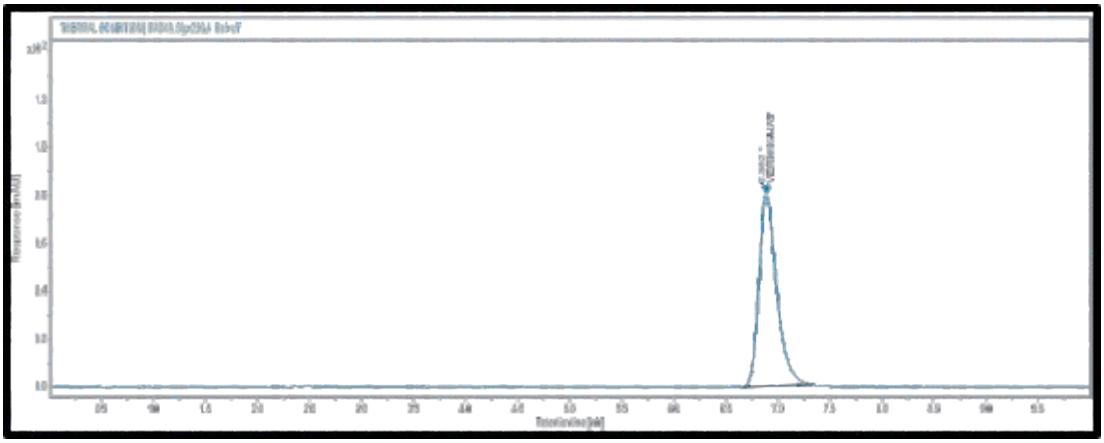

Figure: 12 Chromatogram of sample under at 80°C for 2hr

Photolytic Degradation: Taken 5 mg equivalent powder of sample put it directly in sunlight for 6 hrs. Make up the 50 mL volume with diluent and injected into HPLC system. Check the peak area at optimized condition show in Figure 13.

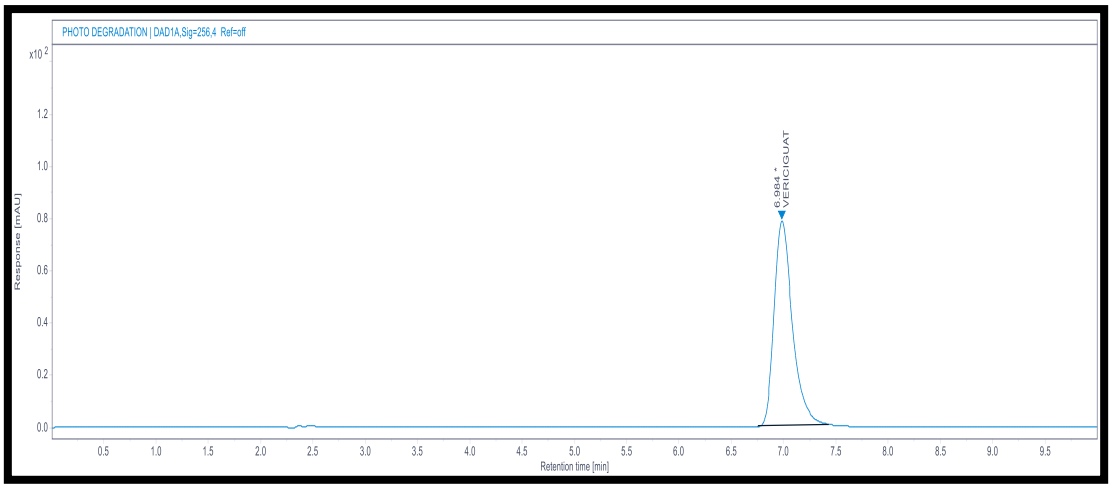

Figure: 13 Chromatogram of sample under directly in sunlight for 6 hrs

Table: 9 Results for forced degradation studies of vericiguat

Mode of Degradation	Condition	Retention time of Impurity	% Assay	% Degradation	Peak Purity
Sample as such	No Treatment	-	99.83	-	998.90
Acid Degradation	1mL of 5N HCL at Room Temperature for30 hrs	3.12	81.60	18.20	998.94
Alkali degradation	1mL of 5N NaOH at Room Temperature for 30 hrs	3.08	79.42	20.57	999.96
Peroxide Degradation	1mL of 3% H2O2 At Room Temperature for 30 hrs	-	97.59	2.41	998.93
Thermal degradation	80°C for 2 hrs	-	99.80	2.37	998.96
Photolytic degradation	Direct sunlight placefor 6 hrs	-	99.39	-	999.91

DISCUSSION

In this developed method the drug substance with various physicochemical characteristics is used in this created approach with the use of a photodiode array detector, the ideal analytical wavelength was found for vericiguat estimation. For effective drug separation in the presence of degradants, various mobile phases were tried. So that the chromatographic conditions could be adjusted, numerous chromatographic factors including wavelength detection, stationary phase, pH of the mobile phase and its composition, and mobile phase

flow rate were assessed. There were several different mobile phases tested in this study, including water, methanol, and 0.1% formic acid in water, but peak broadening was seen. As a result, the mobile phase, which consisted of 10mM potassium dihydrogen phosphate: methanol (60:40 v/v), demonstrated effective and superior separation of the drug and the degradation products produced during the forced degradation study. Several system suitability parameters were used to evaluate the method's system appropriateness. Zorbax Eclipse Plus C18 (250 mm x 4.6 mm x 5 µm) column was used for the project work, which has greater repeatability. According to ICH criteria, the findings of the system suitability parameter were determined to be within acceptable limit values. The determination of the detection and quantification limit values verified that the approach was sensitive enough to identify the drug even when degradants were present. Also, it was noted that the drug's recovery percentage was within the intended range. By injecting a blank, a standard, and a sample, along with completing a forced degradation study, the method's specificity was assessed. The statistical results from the robustness parameter demonstrate the resilience of the presented approach. It was discovered during the stress degradation investigation that vericiguat degrades under acidic, alkaline, and oxidative conditions. There were no degradation products seen under thermal and photolytic conditions. It was determined from the above that the degradants peak does not interfere with the Drug peak. LOD and LOQ was found to be 0.5 µg/ml.

CONCLUSION

For the purpose of determining the amount of vericiguat in commercial formulations, a Specific, Accurate, Robust, and Reliable RP-HPLC technique was established. The validation of the developed method indicates that the Drug and its degradation products were successfully separated. Active ingredient was profitably resolved with good resolution and quantified. Hence, the suggested validated stability-indicating method was successfully employed to work out Vericiguat in the bulk and pharmaceutical dosage form. Analysis of the pharmaceutical dosage form using developed method produced highly repeatable, reliable and high-quality agreement with the label claim of the drug. This study's findings support the assertion that the RP-HPLC technique for determining vericiguat in a tablet formulation is practical and efficient for use in research investigations, quality control, and routine vericiguat analysis in tablet dosage forms.

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REVIEW ARTICLE

EBOLA VIRUS DISEASE: A DISEASE WITH NO SPECIFIC CURE

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ABSTRACT

Ebola virus disease is an infectious virus-based disease that spreads through human-to-human contacts. The first human case was found in 1967, in Germany, the patient was a scientist working in a lab with monkeys that were imported from the Uganda region. The first outbreak of this disease was found in 1976 in the Africa region near the Ebola River, Republic of Congo. Major symptoms of this disease are normal "flu-like" in its primary stage such as fever, coughing, headache, and diarrhea. As the disease progresses the symptoms get worsen. The treatment options for this disease are very limited. Many vaccines are found to be effective against the Ebola virus disease such as the chad3 vaccine, rVSV-ZEBOV vaccine, MVA-BN-Filoã vaccine, ERVEBO vaccine, there are many treatment options therapies such as Janssen Ebola prevention vaccine therapy, combination therapy (2014), EBANGA treatment, etc. Recently in 2019 US-FDA has approved another vaccine as the treatment option for Ebola virus disease. The basic supportive care system Is also found to be very helpful in the treatment of the disease. Some medications are still under investigation for their effectiveness against the Ebola virus such as Remdesivir, Favipiravir, Quinacrine, Tilorone. Some studies were done to compare the effectiveness of newly found drugs against Ebola virus disease which were known as PALM studies. Here In this review, we are going to look at all the possible treatment options and vaccines that are effective against Ebola virus disease.

Keywords: Ebola virus disease, Treatment options, Vaccines, Medications, Supportive care

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INTRODUCTION

Ebola is a human-to-human transmitted viral disease that was recognized as Ebola virus disease when the outbreak of this viral infection took place near the Ebola River in 1976 in the Republic of Congo. Afterward many outbreaks have been reported but the most severe one was between 2013- 2016 and it was a major outbreak that originated from the middle Africa region affecting many countries in the West Africa region[1]. In 1976 the underlying reason for the outbreak was found to be the use of the contaminated syringe in the hospitals.[2] This disease is fatal with a mortality rate varying from 50% to 90% in different regions of affected population areas.[3] This disease is mainly caused by the "**Ebola virus**" Which is a negatively charged single-stranded RNA virus belonging to order- *mononegaviral* and family *filoviridae* and genus *ebolavirus* [4]. This disease can infect all ages from the elderly to infants and adults as well.[5]

The family of *filovir*idae includes mainly genera such as Ebolavirus, Marburgvirus, Striavirus, Dianlovirus, and Thamnovirus. There are mainly 5 species of Ebola virus that have been identified till now: Bundibugyo Ebolavirus, Reston Ebolavirus, Sudan Ebolavirus, Tai Forest Ebolavirus, Zaire Ebolavirus.[6] Among these 5 species major 3 species are associated with human-to-human transmitted Ebola virus disease: Bundibugyo Ebolavirus and Sudan Ebolavirus (South Sudan and Uganda), Zaire Ebolavirus (Republic of Congo and Gabon). The highest infections in humans are seen by the Zaire Ebolavirus (80%) followed by Sudan Ebolavirus (50%) and Bundibugyo Ebolavirus (25%) [7]. EBOLA (EBOV) and MARBURG (MARV) are the viruses of the *filoviridae* family.

EBOV virus is said to be more dangerous and lethal than HIV virus. It is noted that these 2 viruses produce hemorrhagic fever and death within few days of their entry into host cells. Within the range of 60-90% deaths of infected persons.[8] in 1989, in the USA another species of Ebola virus was found which was named as *Reston ebolavirus* after it was found in Reston, Virginia, USA.[9] after that outbreak the major outbreak of REBOV was seen in 2008-2009 in the Philippines.[10]

Ebola virus disease shows the major symptoms as high fever, headache, vomiting, anorexia, diarrhea, unexplained bleeding from the eyes, nose, gut and gums. This disease is regarded as endemic in some areas of central Africa.[11]. Many vaccines and treatment options have been developed till now. Vaccines such as chad3, MVA-BN-Filoã, rVSV-ZEBOV has been found among these rVSV-ZEBOV is found to be most effective till now which was found in 2016.[3] Many treatment options are found and approved to date such as combination therapy of 3 drugs was approved in 2014 by USFDA and another EBANGA treatment was approved recently in 2020 by USFDA. There are

many studies are going on such as PALM 2019 and 2020 which focus on the comparison of different potentially useful drugs.

In April 2019 Remdesivir prodrug was under investigation for its potential use in Ebola virus disease treatment. The major mechanism to be thought behind this drug is said to be the inhibition of the Ebola virus RNA-dependent RNA- polymerase.[12] There are majorly 2 types of immune system parts involved in response to controlling viral replication: innate and adaptive immune system. The innate system response to EBOV is done in the manner of "cytokines storm" as various pro-inflammatory cytokines such as IL-1β, IL-6, IL-8, CCl_2, CCl_3, and CCl_4.[13] the interactors for Nucleoproteins; RUVBL1 and RUVBL2 play a role in transcription/replication of the virus and these also affect the minigenome activity.[14] In 2002 Japan found a drug favipiravir which was an anti-viral drug against the influenza virus but it has been now found to be a potential treatment option for Ebola virus disease as well.[15]

HISTORY OF EBOLA VIRUS DISEASE

In 1967 the first case of the illness was recorded in a laboratory in Germany. Then ultimately 37 people were found to be infected and from which 9 people died. The virus to be responsible for this was found to be from the *filoviridae* family.[16]

The first laboratory worker, Porton down accidentally got infected by the virus when he mistakenly pricked himself with an infected syringe.[17] After 9 years of this incident Ebola virus disease first major outbreak was noted in the Democratic Republic of Congo (DRC) and Sudan in 1976.[18] In that outbreak 284 cases were recorded in Sudan while 318 cases were recorded in DRC. As the outbreak happened near the Ebola River in DRC this disease was named as Ebola virus disease.[19] And 2 species were acknowledged from this outbreak were: EBOV-Zaire and EBOV-Sudan. [11]

The outbreak of 1976 in DRC started when a 42 years old male Yambuku appeared at the clinical outpatient ward at YMH, Zaire (DRC). The virus already spread to many people who have been in close contact with him due to which 13 of YMH staff became infected and 11 of them died.[20]

In 1989, another species of Ebola virus was found after an outbreak was observed in cynomolgus macaques in Reston, Virginia, USA. This was seen in a primate facility care center, where the new species *Reston ebolavirus* (REBOV) was found.[9] the major non-human primate outbreak of this virus was also seen in the same year of 1989. And as the scientist tried to find the main origin of this outbreak it was found to be Philippines.[10]

To this date, there are major more than 20 outbreaks have been reported since the first outbreak in 1976. During the outbreak in 2014, there was panic and fear in people

that this disease can spread to other countries like North America and Europe.[21] As the number of cases was rising worldwide WHO on 8th august 2014 declared Ebola endemic as public health emergency of international concerns.[8] During the 2014-16 outbreak there were almost 30,000 people infected with the EBOLA virus in the West Africa region and the mortality rate reached 70% initially.[22]

The outbreaks from 2014 to 2021 are recorded in major areas as below: 2014-2016 west Africa; 2017 BAS-Uele and DRC; 2018 Equateur and DRC; 2018-2020 North Kivu/Ituri and DRC; 2020 Equateur and DRC; 2021 North Kivu and DRC.[23] Based on the severity of the virus infection and the mortality rate of the disease this virus can also be misused as bioweapon.[24]

The data in the chart (Fig 1) below shows the number of cases and number of deaths observed in each year in the regions of Africa due to the outbreaks of Ebola virus disease.

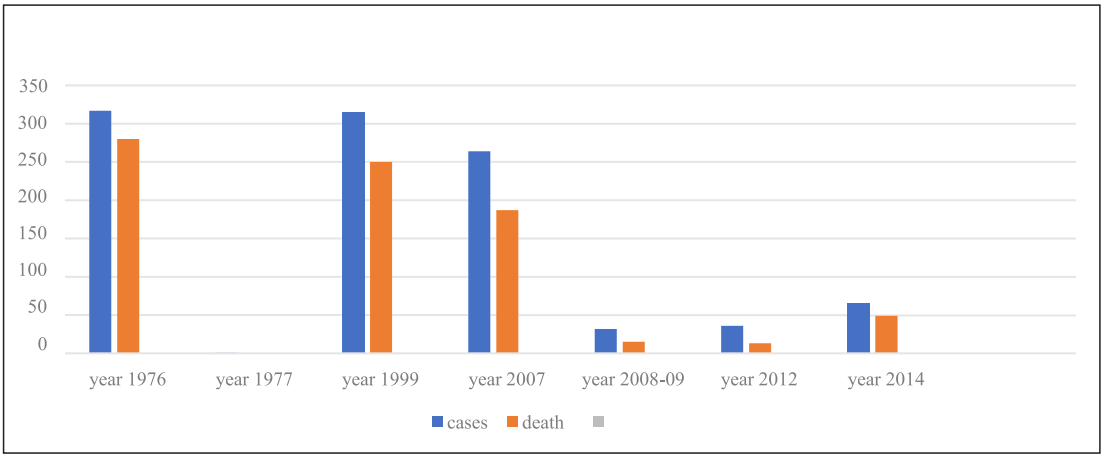

Fig 1: chart of ratio of cases observed each year in Africa region and number of deaths observed

Structure of the virus

Ebola virus structure is mainly a single strand negatively charged ssRNA genome. The main part of the structure of the Ebola virus is the glycoprotein (GP)envelop that is encoded by the 4th gene in the viral genome. This GP is responsible for the entry of the virus into a host cell. There are 2 subunits defined in the GP membrane which are the GP1 andGP2. GP1 associates with the cell membrane receptors while the GP2 facilitates the entry of the virus into the cell cytoplasm [25].

The Ebola virus virions are naturally 80-90 nm in diameter, 19-kb, and 14000nm in long [26,27]. Ebola virus is composed of 7 genes that code at least 10 proteins from 3' head to 5' end which is essential for virus

integrity and its functioning and its work. These 10 proteins are: Nucleoprotein {NP}; Viral protein 35 {VP35}; Viral protein 40 {VP40}; viral protein 24 {VP24}; viral protein 30 {VP30}; soluble glycol protein {SGP}; glycol protein {GP}; small soluble glycol protein {ssGP}; "-peptide; and RNA-dependent viral polymerase (L)[28].

Many types of glycoproteins are present on the cell surface. The VSV (vesicular stomatitis virus) has been used in one study to know about the replication and assembly of RNA viruses that are enveloped. In the study, the VSV virus was used as recombinant containing GRP gene, and thus it is not infectious until the envelope is provided which contains the protein which is responsible for the binding of receptor and fusion of membrane.[29]

The secreted GP is a non-residual 364-residue encoding protein present in all the ebolavirus GP. It is not known till now what role dose sGP plays in the infection but during the infection by EBOV high amount of sGP is observed in patient blood. The pre-sGP undergoes proteolytic cleavage by furin Prost-transitionally which gives a yield of mature sGP and a small, nonstructural peptide "-peptide which is heavy O-glycosylated and it is also a secreted protein. The major difference between GP and sGP is that they both have different C- terminals which is a result of the insertion of extra adenosine nucleotide in 20% of the transcription process. GP and sGP have similar N-terminals. The final GP is a trimer while the starting sGP is a dimer. Due to the difference in GP in various stands of proteins, there is more difference in their infection as well e.g., ZEBOV GP causes destruction of endothelial cells in monkeys and humans both, while the REBOV GP causes cytotoxicity in only monkeys. It is observed that the music-like domain is highly responsible for the cytotoxicity caused by GP.It is still not clear how the EBOV enters the host cells but it is believed that the host cell entry mechanism is through receptor-mediated endocytosis mechanism.[30]

It is confirmed in many studies that there are specific interactions of host-pathogen in the EBOV virus lifecycle. To date, the known host interaction partners to EBOV virus proteins are VP30, VP35, VP40, GP1, GP2 and VP24. The studies also confirmed that the host proteins RBBP6 compete with nucleoprotein for binding with VP30 to inhibit the EBOV virus RNA synthesis. Nucleoproteins are playing a major role in transcription and capsid assembly. The major nucleoprotein-host interaction impactor protein is Hsp70 which is a chaperone protein that binds to and impacts nucleoprotein stability.

The structural similarity of all 5 species is very distant and it is also fascinating to know that the 2 most dangerous species Sudan ebolavirus and Zaire ebolavirus are the most distant in case of structural similarities.[32] The structure of the virus is shown in the figure below (fig 2,3).

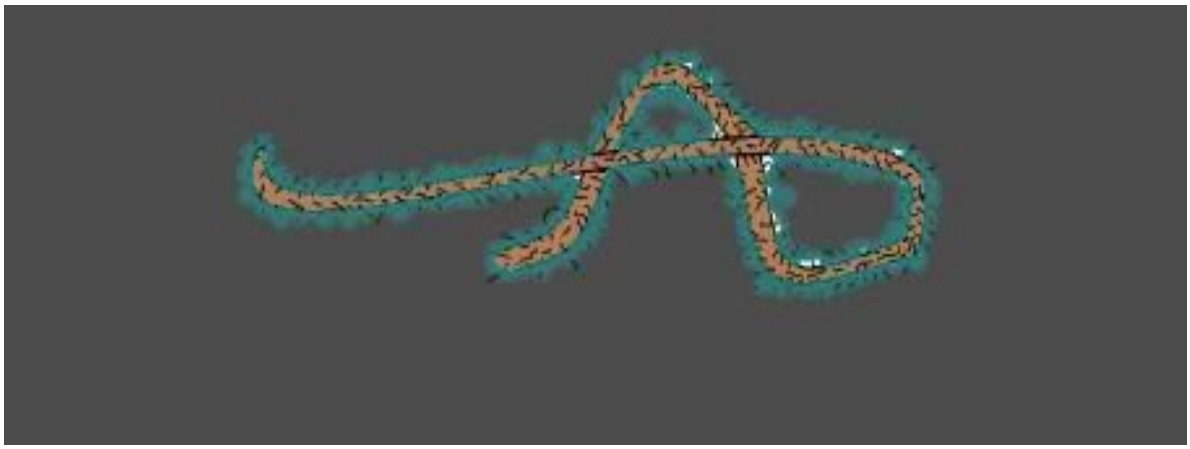

Fig 2: Structure of Ebola virus under microscope

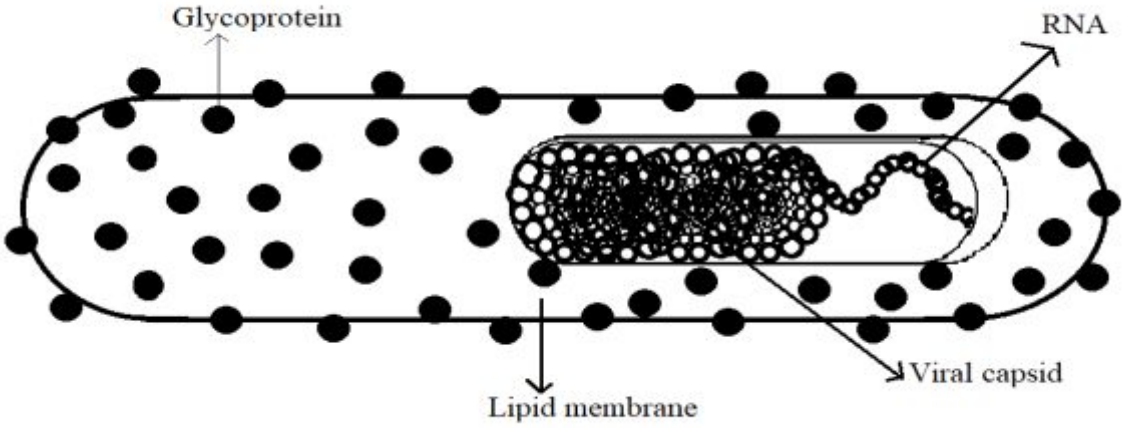

Fig 3: Structure of Ebola virus with RNA, Lipid membrane, Viral capsid, Glycoprotein

Transmission of the virus through different pathways

The human genome sequence of 5% of patients of all the population infected showed the record of spread in the country. The main entry point into a human body is through the mucous membranes such as the eye, nose and mouth[5]. Ebola virus disease is known to be an animal to human disease. Natural hosts of the Ebola virus are thought to be the fruit bats belonging to the family of *pteropodidae.*[7] The infection spreads when a healthy human being comes in contact with any blood or body fluid secreted from an infected animal or bat.[33]

After the entry of the Ebola virus into the human body, it spreads into the body through first striking the macrophages, monocytes, and the dendritic cells, then it goes into the lymph nodes where the replication of the virus starts and then

before the onset of symptoms the virus begins to spread and it immediately starts to infect a variety of cells such as hepatocytes, adrenocortical cells, endothelial cells, fibroblast, and many others.[5]

Then after the first infection, the secondary transmission seen here is direct or indirect human-to-human contact either by blood or any other body secretions contacts.[19] The virus has been detected to be present in human breast milk, saliva, semen and blood. Studies have also reported that airborne transmission is possible between the pigs and other primates but further transmission through the air is not reported after that and one of the reasons being concluded is the lack of virus in the human lungs and other parts of the body giving no chances of transmission. The pigs with EVD are having a high concentration of virus in their respiratory tract than in their bloodstream so they can transmit the disease through droplet form in the air [27].

It is seen that the EBOV virus can remain present in some human sites such as the brain, testis, and eyes which are known as immune privilege sites since they can protect the virus from rejection by host animals or humans [34]. Infectious EVOB virus has been observed in breast milk, semen, blood, saliva, urine, tears, skin, amniotic fluid, stool and CSF From 1976 to 2014 there were 65 EBOV GP has been collected from isolates which showed that the temporal evaluation of EBOV is mostly due to neutral genetic drift [35] It is observed that non-human primates show more close conditions to human infections than the animal models (in immunocompetent mice, Syrian hamsters, domesticated hamsters). Viral pathogenesis data has been largely studied by experiments on the wild-type EBOV predominant in the crab-eating macaques and rhesus monkey[35]. After the 1976 outbreak in DRC and Sudan, it was said that the EBOV-Zaire virus is autochthonic and pestilent within the so-called Zaire virus triangle.[20]

In the 2014 outbreak, the major seen mode of transmission was the nosocomial transmission. This transmission is the one that was responsible for disease progression in the population of healthcare workers. In west Africa in 2014 outbreak of majorly 665 cases was reported due to the nosocomial transmission from which 375 deaths occurred.[36]

A study in Texas biomedical research institute shows that one of the mechanisms of entry of the Ebola virus into host cells is “Autophagy” which means the self-eating mechanism of the cell. This is observed that the cell enters the host cell through a process called “micropinocytosis”. So basically, the process of this entry of the Ebola virus into the cell starts when the plasma membrane ruffles over the virus attached to the cell surface, and then this plasma membrane forms a vesicle over the virus. And then this vesicle is enclosed with virion being inside it and then it gets internalized into the cell.[37]

Transmission of immunization infection is a hypothetical chance. Even after 14 days of vaccination Antibody of infectious RNA has been identified in the body fluids of the person. The time period of shedding isn't known, but the sample after 28 days of vaccination tested negative for the presence of a virus. The antibody of infectious RNA has been recognized in secretions from the skin that showed up after vaccination.[19]

There is a comfortable mathematical model has been adopted for predicting the spread of the Ebola virus. This system is known as the SIRDP model which governs the problem. This system was developed after the 2014 major outbreak in the West Africa region.[38]

During a recent outbreak in 2019 in the West Africa region, it was observed that there was an increase in transmission of disease due to violent incidents that took place as people were angry as the number of cases were rising and thus, they were attacking the hospitals and the isolation ward where Ebola virus disease patients were kept.[39]

SIGNS AND SYMPTOMS OF EVD

Symptoms of this disease are seen after the incubation period of 2-21 days.[5] The major symptoms observed in the current 2021-outbreak are a severe decrease in neutrophile count and an increase in liver enzyme and hematological changes (leukopenia and lymphopenia). It is observed in disease pathophysiology studies that the recovery from this disease is majorly dependent on the immune response against the virus.[40]

In children, the symptoms are hard to be identified as in children the symptoms are more common and undifferentiated from other disease symptoms. It is seen that in some cases the major symptom of fever is also not present in children.[18]

The symptoms of this disease are normal at the first stage 'flu like' (headache, coughing, weakness) and then as the disease progresses it becomes more severe (internal and external bleedings, rhinorrhea) which makes this disease more and more unidentified at the initial stage and when it gets identified it has already reached to a bigger stage.[41] After the onset of the disease, the most common symptoms prevalent at 5 -7 days period are fever with anorexia, asthenia, and maculopapular rash. In the last stage of disease, the death of a patient occurs due to shock, hemorrhage, and multi-organ failure conditions [42].

It is observed that this disease mainly starts with rather unidentified symptoms such as fever, myalgia, and arthralgia [43]. It is also observed that not all patients in the 2014 outbreak showed hemorrhagic symptoms.[36]

TREATMENT OPTIONS FOR EBOLA VIRUS DISEASE

VACCINE

The first vaccine was made of the whole virus deactivated by formalin, heat and

radiations and it was ineffective to all humans and animals.[41]

For future treatment development, an inhibitor of viral cell entry will be a better option. As in a study done, it has revealed that immunity transfer by transfusing the blood of survival of Ebola virus disease to a patient suffering from Ebola virus disease is not much of help and not always effective.[41] Till 2016 there were no specially licensed or approved vaccines or antiviral treatment approved by U.S.FDA. China, Russia and European regions have approved 3 EBOV vaccines.

1. The chad3-EBO non-replicating vaccine vector based on the chimpanzee adenovirus type 3 (chad3)

2. The heterologous prime-boost Ebola vaccine regimen based on recombinant adenovirus type 26 vector (Ad26.ZEBOV) priming followed by boosting with modified vaccinia Ankara vector (MVA-BN- Filoã)

3. The recombinant Vesicular Stomatitis Virus (VSV) vector-based Ebola vaccine (rVSV-ZEBOV) [44].

In 2016 most promising and most effective treatment was by rVSV-ZEBOV which is a vesicular stomatitis virus-based vaccine.[45] But this vaccine was approved to be used in emergency conditions only. Till 30 November 2019 total of 274,871 individuals were vaccinated with this vaccine between the clinical trials and emergencies within the DRC region [43].

Although many vaccines have been designed for the treatment and just a year ago USA-FDA in December 2019 has approved another vaccine called ERVEBO for the prevention of disease caused by the *Zaire ebolavirus* in 18 years old or older than that. But this vaccine is only useful in infection by *Zaire ebolavirus* and not against any other infection caused by other species of Ebola virus[46].

INTRODUCTION TO MECHANISM AND BASICS OF VACCINES

rVSV-ZEBOV VACCINE

In the response mechanism of this vaccine both innate and adaptive immune systems take part. Yet the mechanism of contribution from these responses in the protective action after primary exposure for the short or long term is still unknown.[47]

After the intravenous introduction of a lethal dose of ZEBOV, monkeys were vaccinated with rVSV-ZEBOV showed cerebral and humoral immune responses with no evidence of virus replication. The rVSV-ZEBOV was observed to be present in synovial fluid and skin tissues have confirmed viral replication in the peripheral tissues [44].

During the phase 1 trial, the serological investigation showed that EBOV-GP specific IgG antibody responses were detected in almost all participants, with significantly high EBOV specific IgG concentrations in high-dose treatments. It has also been recently shown that ZEBOV-

specific circulating follicular T helper cells (ctfh) correlate with antibody titers and with the Tfh17 subset [44].

Many MVA vectors initiated a strong innate immune response in human monocytes cell lines with the production of interferon β, proinflammatory cytokines, and chemokines. In the rVSV-ZEBOV vaccine, VSV GP has been substituted by the GP from EBOV Kikwit-96 variants, having pathogenicity of the VSV and enabling infection of cells and replication using the EBOV GP. Chad3-EBO-Z also encodes the EBOV GP in the monovalent or bivalent form, and its impregnability and susceptibility have been tested in many human clinical trials[26].

As of April 2019 WHO stated that the rVSV-ZEBOV vaccine in total 97.5% effective. To check the duration of effectiveness of this vaccine rodent models were also used and the result showed that this vaccine is quite effective for a long time after first dosing.[48]

ERVEBO VACCINE

The ERVEBO vaccine is given in a dose of 1ml intramuscularly only. It is to be given in a non-dominant arm in the hastate area of the arm.[49]The adverse events occurrence rate after the administration of ERVEBO is relatively low and the events noted till now are basic systemic adverse effects such as headache, nausea, abnormal sweating, rash, and muscle/joint pain. This vaccine is contraindicated in patients who has a history of anaphylaxis type hypersensitivity reactions.[46]

This vaccine works on the immune system response of person. The vaccine contains proteins of the virus. When this vaccine is injected to the person it activates the immune system and triggers a response against the virus protein, thus when after receiving the vaccine person comes in contact with the disease virus it will not affect the person. There was study done for the clinical development program in which open-label and placebo control groups were observed for the adverse effects' occurrence. The results of this study are given below:

Study group	Major injection site adverse effect observed (% of the population)	The major systemic adverse effect observed (% of the population)
Open-label trial group	Swelling (12%) redness (17%) Pain at injection site (70%)	Joint pain (18%) Vascular lesions (2%) Arthritis (5%) Rash (4%)
Placebo control group	Pain at site of inj ection (3 4.0%), redness/swelling (2%)	Headache (37%), Fever (34%), Muscle pain (33%), Nausea (8%), Rash (4%), Sweating (3%)

JANSSEN EBOLA PREVENTION VACCINE THERAPY

In May 2020, Zabdeno and Mvabea 2 component vaccine authorization were recommended by the European medicine agency. This vaccine is given in 2 parts when given to the patient; first Zabdeno is given and after 8 weeks Mvabea is given, both are given intramuscularly. In times of an outbreak situation when immediate action is needed this 2 dose vaccine is not much of use.[50] this vaccine therapy is known as Janssen Ebola prevention vaccine therapy.[51] This Janssen therapy was found by Johnson and Johnson's Janssen division. The Zabdeno vaccine is a monovalent vaccine while the Mvabea vaccine is a multivalent vaccine. The major component of Zabdeno vaccine is Zaire ebolavirus Mayinga variant GP. This variant stimulates the immune response and it is expressed locally.

Clinical studies were done to check the safety and immunology of the vaccines and these vaccines were observed to be well-tolerated and un-harmful to healthy volunteers. There were no such serious adverse effects observed in the patients who received both the doses and only a minor headache was observed. The Mvabea vaccine is designed to provide immunity against the Sudan virus, Ebola virus, Marburg virus, and the Tai Forest virus. [51] These studies also showed that the 1st dose of Ad26.ZEBOV induced more robust initial antibody binding and cell response than the 2nd dose of MVA-BN-Filo vaccine.[52]

The Zabdeno vaccine is mainly designed to provide active specific immunity against the Ebola virus and this vaccine is based on the Ad26 vector expressing the Ebola virus mayinga variant's glycoprotein. [51]

DNA BASED VACCINE

Vaccination by plasmid DNA delivery is very practical and advantageous for the future development of a more effective and safe vaccine based on the Ebola virus DNA vaccine concept. A study on 21 healthy participants showed that 3- plasmid DNA candidate Ebola virus vaccine is secure and well accepted.[24]

A study was conducted in Africa in which the immunogenicity and safety of two DNA-based vaccines were checked: first encoding Zaire and Sudan ebolavirus glycoprotein and second encoding Marburg virus glycoprotein. This study showed that both these vaccines were well accepted and gave antigen-specific antibody-mediated and cellular immune responses.[53]

The DNA vaccines were designed from Zaire ebolavirus and Sudan ebolavirus transmembrane-deleted glycoproteins with viral nucleoproteins. In 2006 during the first clinical trials of these types of vaccines, the vaccine was given by intramuscular injection but as time changes and technologies have advanced now this vaccine is given by a novel biojector 2000

needle-free injection management system.[43] A study has shown that the protective efficacy and immunogenicity of DNA based vaccines can be enhanced and improved by the use of Nanoplasmid vectors.[54]

DRUG THERAPIES

COMBINATION THERAPY OF ATOLTIVIMAB, MAFTIVIMAB AND ODESIVIMAB-EBGN

In October 2014 U.S.FDA approved a mixture of 3 monoclonal antibodies atoltivimab, maftivimab, and odesivimab-ebgn. This mixture mainly targets the glycol protein present in the virus which facilitates the virus entry into the cell by binding to the cell membrane receptor. The mixture binds with this glycoprotein and inhibits its activity and thus inhibits the entry of the virus into the host cell. [43]

Atoltivimab, maftivimab, and odesivimab-ebgn together is a combination used to treat infection of Zaire ebolavirus and this is a mixture of Zaire ebolavirus glycoprotein-directed human monoclonal antibodies used in adult and juvenile or infant patients, including neonates given birth by a mother who is RT-PCR positive for infection. This combination is of Zaire ebolavirus glycoprotein (GP) directed recombinant human IgG1 human monoclonal antibodies of similar structure.[55]

The potential for this combination to repress replication of a live vaccine virus indicated for avoidance of *Zaire ebolavirus* infection and conceivably decrease the efficacy of the vaccine, avoid the simultaneous administration of a live vaccine during treatment with this combination. The time difference between vaccination after the initiation of the combinational therapy should be in according to current vaccination guidelines.[55]

This combination is an infusion. It is given one time by a healthcare provider straight into the vein (intravenous imbuement). The amount of the drug combination to be administered is calculated by the patient's weight. It may cause serious side effects including severe and life-threatening allergic reactions during and after the infusion. The most commonly observed adverse effects of this are body temperature rise, chills, rapid heartbeats with heavy breathing, and puke.[56]

EBANGA TREATMENT

In 2020 December U.S. FDA has approved a monoclonal antibody named "EBANGA" for treatment of Ebola virus disease caused by the *Zaire Ebola virus* strain.[57]

EBANGA is also known as ansuvimab. The major side effects associated with the use of this monoclonal antibody are: fever, tachycardia, tachypnoea, hypoxia, chills, hypotension, vomiting and diarrhea.[58] It binds to a certain part of the EBOV virus and prevents it from entering into any host cells. The major mechanism of its effect is by inhibiting receptor binding and thus it inhibits the virus entry into the host cells.[59]

EBANGA is a human monoclonal antibody directed by Zaire ebolavirus glycoprotein for treatment against Ebola virus disease caused by Zaire ebolavirus. But this can only be used in adults and pediatric patients as geriatric use data have not been supported yet.[57]

PALM STUDIES

After the 2018 outbreak in the West Africa region WHO decided to start and research and development department for research on Ebola virus disease treatment options.[60]

The PALM studies compared 4 drug options which were possibly the most effective ones for the treatment of Ebola virus disease: ZMapp, Remdesivir, MAB114, and REGN-EB3. At the end of the study, it was found that both MAB114 and REGN-EB3 are more effective in comparison to ZMapp drugs and they have superiority in case of mortality rates than ZMapp and Remdesivir both. Here Remdesivir is a nucleotide analog RNA polymerase inhibitor, MAB114 is a single human monoclonal antibody derived from EBOLA survivor while REGN-EB3 is a mixture of 3 IgG1 monoclonal antibodies.[61]

In June 2020 another study called PALM studies were randomized control trials of 3 investigational agents which are under investigation compared with the control group including patients of EVD (infected with the filoviridae: ebolavirus) treated with ZMapp drugs. ZMapp is comprised of 3 chimerical monoclonal antibodies under development for EVD.[62]

The safety and efficacy of ERVEBO in people younger than 18 years old have not been done yet. For the development of treatment and prevention against Ebola virus disease, the year 2019 was seen to be very useful as many treatment options and vaccines were approved by U.S. FDA. [63]

A study was also done to identify some antibodies which might be useful in giving protective action. These antibodies mAbs were isolated from the survivors of Ebola virus disease. There were 2 antibodies mab110 and mab114 were the most potent and gave the most neutralizing effect and has shown ADDC (antibody-dependent cell-mediated cytotoxicity) activity as well.[64]

OTHER MEDICATIONS

In 2016, one small novel molecule GS-5734 was found which was effective against many different strains of the Ebola virus and it was investigated in rhesus monkey species and has an effective antiviral activity. This drug is an adenosine analog monophosphoramidate prodrug and it showed inhibition of virus replication in many human cell types such as human endothelial cells and primary macrophages[65].

The molecule GS-5734 was later named “Remdesivir”. The clinical data of the efficacy and safety of Remdesivir were generated in 2016 by studies done using rhesus monkeys. Remdesivir acts by inhibition of virus replication by interference with RNA polymerase.[66]

Currently there are 7 clinical trials involving Remdesivir which are ongoing and registered under clinicaltrial.gov.[60]

One of the most advanced technologically manufactured treatment options for Ebola virus disease was done in 2019 when isolated lectin from banana was engineered into H84T banlec molecule which was found to be very effective against EBOV virus in cell cultures. This molecule was found to be very much effective against the strain which was responsible for the 2013-2016 outbreak of the Makona strain.[67]

A mechanical-based study identified a potent antiprotozoal drug "quinacrine" to might be useful against the Ebola virus disease. The basic mechanism of this drug is that it enters the cells and cell organelles and rises the pH which interferes with the cathepsin B activity which is important for Ebola virus entry into the host cell. Thus, it is safe to say that this medication works by inhibiting virus entry into the host cells.[68]

Some data shows that quinacrine showed good effectiveness in studies as an anti-viral agent against dengue and zika virus. Quinacrine is used in salt form as quinacrine hydrochloride. As a small-molecule inhibitor of the Ebola virus "Tilorone" was acknowledged by the Bayesian machine learning model. It is the most potent one of all the tested drugs. It is also proven to be effective in-vitro against the Ebola virus.[69]

SUPPORTIVE CARE SYSTEM

Once the disease is confirmed, the patient is required to be hospitalized in an isolation ward and closely monitored and the supportive care is started. Supportive care includes fluid resuscitation, electrolyte and glucose monitoring, correction and treatment of possible co-infections.[70]

When GIT problems arise during Ebola virus disease, the highly oral bioavailable drugs will be of less use thus use of supportive care systems will be playing a major role in monitoring and treatment of the patient with these complications.[6] In five Ebola treatment units (ETU) a retrospective cohort study was conducted on Ebola virus disease patients to check the effect of multivitamins supplements as supportive care during treatment of Ebola virus disease patients. The result from these studies showing low facility-based mortality in patients suggested that multivitamin supplements should be used as supportive care during the treatment of Ebola virus disease treatment.[71]

As a part of the supportive care If the patient suffers from high fluid loss it may result in hypovolemia; so, to prevent such an event patient should be provided with oral rehydration solutions, and if required anti-diarrheal and anti-emetic should also be given.[3]

In a study, it was also seen that antimicrobials used in the supportive care systems have reduced mortality due to

bacterial infection associated with Ebola virus disease. And for this purpose, antibiotic therapy is also said to be useful as a supportive care treatment option. Broad-spectrum antibiotics to be used in this therapy.[72]

After the 2014-2016 outbreak clinicians found that basic supportive care is very helpful in controlling the mortality rate of Ebola virus disease. Analgesic therapy recommendation was made in a guideline that follows GRADE methodology. According to this therapy option, analgesics should be used for the patient having high abdominal pain and parenteral opioid therapy can also be given in some cases.[71]

CONCLUSION

Based on the available studies it can be concluded that the vaccines are more effective then the drug treatment options which are available. Based on clinical trials data and results of different triala it is concluded that rVSV-ZEBOV and the Janssen therapy are most promising and effective options for Ebola virus disease treatment. The functioning of glycoprotein must be evaluated in further studies as it may give a potential new theory of binding and fusion of the Ebola virus into the host cell from which a target can be generated to formulate aa medicine or therapy which can inhibit the entry of the Ebola virus into the host cell. Multi-organ failures are associated with Ebola virus disease progression for which the supportive care system with advanced machines and technology might be useful for better treatment. DNA-based vaccines should be considered as potential candidates for Ebola virus disease treatment. Clinical trials should be done for the efficacy and safety of the use of the drug quinacrine in Ebola virus disease treatment.

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REVIEW ARTICLE

3D BIOPRINTING - RECENT ADVANCES IN TISSUE REGENERATION

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ABSTRACT

The scarcity of organs for transplantation has been a significant problem for the healthcare sector for years. Despite technical advances, too many patients are still waiting for organ transplants compared to the number of qualified donors. Numerous lives are lost; as a result, every year worldwide. Successful organ transplantation is further restricted because of the expense of the procedure and the possibility of immunological rejection. Fortunately, 3D bioprinting technology has become a viable answer to this urgent need. 3D bioprinting technology can completely transform healthcare systems by making it possible to produce tissues and organs unique to each patient. These technologies make it feasible to construct complex, useful biological structures, opening the door to the production of tissues and organs that closely resemble the anatomy and physiology of the human body. With an emphasis on tissue engineering and synthetic organ printing, we examine the most recent advancements in 3D bioprinting technology and their applications in healthcare systems in this review paper. This review summarises the work of many scientists who have made significant contributions to the field of 3D Bioprinting, examining their methods for designing and printing intricate biological structures, the biomaterials they use, and the strategies they use to improve cell viability and functionality.

INTRODUCTION

The applications of 3D Bioprinting are varied and have the potential to significantly impact various fields. These includes creation of artificial organs, which could potentially eliminate the long waiting list for organ donation, wound healing by creating artificial skin cells, as well as developing neurons, and hepatocytes for use in therapeutic procedures. Additionally, the bioprinted tissues can be used in place of animal testing to provide a more ethical and

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cost-effective solution for drug discovery process. Finally, 3D Bioprinting can be used to develop "Organs-on-Chips," which combine microfluidic technology with 3D Bioprinting to create 3D extracellular environments that mimic native extracellular matrix. These models can be used for disease modeling, drug discovery, and high-throughput assays. 3D Bioprinting also has the potential to develop a body-on-a-chip.

Extrusion-Based Bioprinting, Inkjet-Based Bioprinting, Pressure-Assisted Bioprinting, Laser-Assisted Bioprinting, and Stereolithography are some of the 3D bioprinting technologies that have received a lot of attention. Extrusion-based Bioprinting is a popular method for producing three-dimensional models and structures. Materials that can be converted into filaments are used in extrusion-based Bioprinting and are temperature controlled before being dispensed onto a stage via nozzles that move in all three dimensions (X, Y, and Z axes). Multiple head nozzles may aid in the deposition of different types of biomass, such as cells, over a single scaffold in order to facilitate the development of complex tissues or organs. To achieve desirable results in 3D Bioprinting, two primary mechanisms are used: semi-solid extrusion (SSE) and fused deposition modelling (FDM). SSE employs pressurised air or rotating screw gears to create a streamlined input of semi-solid mass from one or more nozzles, depositing biomass layer by layer. In contrast, high temperatures are used in FDM to melt thermoplastic filaments, which are then extruded from the nozzle and deposited as multiple layers on the platform. As a result, 3D printers are made up of two major components: the extrusion system and the positioning system, which collaborate to produce visually and geometrically structured models and structures.

The Inkjet-Based Bioprinting technique, also known as Drop-on-Demand Bioprinting, has gained significant popularity in the field of 3D printing for both biological and non-biological applications. Originally developed for 2D ink-based printing on paper, this technique has been modified to include electronically-controlled rising stages and the use of biologically relevant materials in the printer cartridge. However, it is important to note that the biomaterials must be in liquid state in order to form droplets. Inkjet-based bioprinter operates by ejecting precise liquid droplets onto a substrate using thermal or acoustic forces. With the help of an electrically heated print head, thermal inkjet bioprinting creates enough pressure to push droplets out of the nozzle. Contrarily, Acoustic Inkjet Bioprinting uses a piezoelectric crystal to create an acoustic wave inside the printer head, which separates the liquid into the tiniest droplets possible. The appropriate choice of inkjet bioprinting technology depends on the specific requirements for tissue or organ development, as well as the availability and expertise of the operator.

Using pressure to force biomaterials out of a nozzle, pressure-assisted bioprinting creates 3D bio-compositions. This method employs a variety of hydrogels, viable cells, nutrients such as proteins, ceramics, collagen, and the biodegradable polymer chitosan. The primary benefit of this method is that it allows for room temperature processing, which is advantageous for thermolabile materials, as well as direct integration of homogeneous cells onto the substrate. The printer speed, on the other hand, is limited, resulting in approximately 40% to 80% cell viability after the process. Using pneumatic pressure from a plunger or a screw-based pressure generator, the appropriate amount of pressure can be generated, resulting in a continuous filament. The biomaterials are then deposited in multiple layers on a stationary platform.

Laser Assisted Bioprinting is a method of spraying biomass over a platform that makes use of a laser gun as an energy source. This technology, which was previously used for metal bioprinting, has been updated for biomass such as cells, DNA, peptides, and others. A pulse of a unique laser-targeted beam, a device to focus, and donor transport support in the form of a ribbon, a thin layer of biomaterial suspended in liquid, and a receiving substrate for the upside-down projection are all part of the process. The biomass is made up of hydrogel, culture growth media, incubating cells, proteins, and ceramic materials. The laser propels the biomass forward over a solid platform, irradiating laser light as a ribbon over the linear structure, causing the liquid biomass to evaporate as droplets over the receiving substrate, which is a cell culture medium with the ability to adhere.

Stereo-Lithography (SLA) is a nozzle-free 3D bioprinting technology that allows for the precise printing of biological and non-biological models. This technology makes use of light-sensitive hydrogels that are deposited layer by layer on a platform at a rate of approximately 40,000 mm/s while maintaining a high cell viability rate of up to 90%. The liquid photosensitive polymer is solidified by being exposed to light during the process. SLA is a promising technology for various applications in tissue engineering and regenerative medicine due to its ability to process a wide range of materials and the high accuracy of the printing process. The most extensively researched bioprinting techniques are laser-assisted Bioprinting, stereolithography, and extrusion bioprinting.

The demand for organ transplantation far outnumbers the supply of available organs. Bioprinting, which allows researchers to create functional artificial organs that can be transplanted into patients, could provide a solution. Although this technology is still in its early stages, significant progress has been made. Tel Aviv University researchers successfully 3D printed a heart using human cells and materials compatible with the patient's immune system. While the heart is not yet functional, this breakthrough represents a significant advancement in the development of artificial organs.

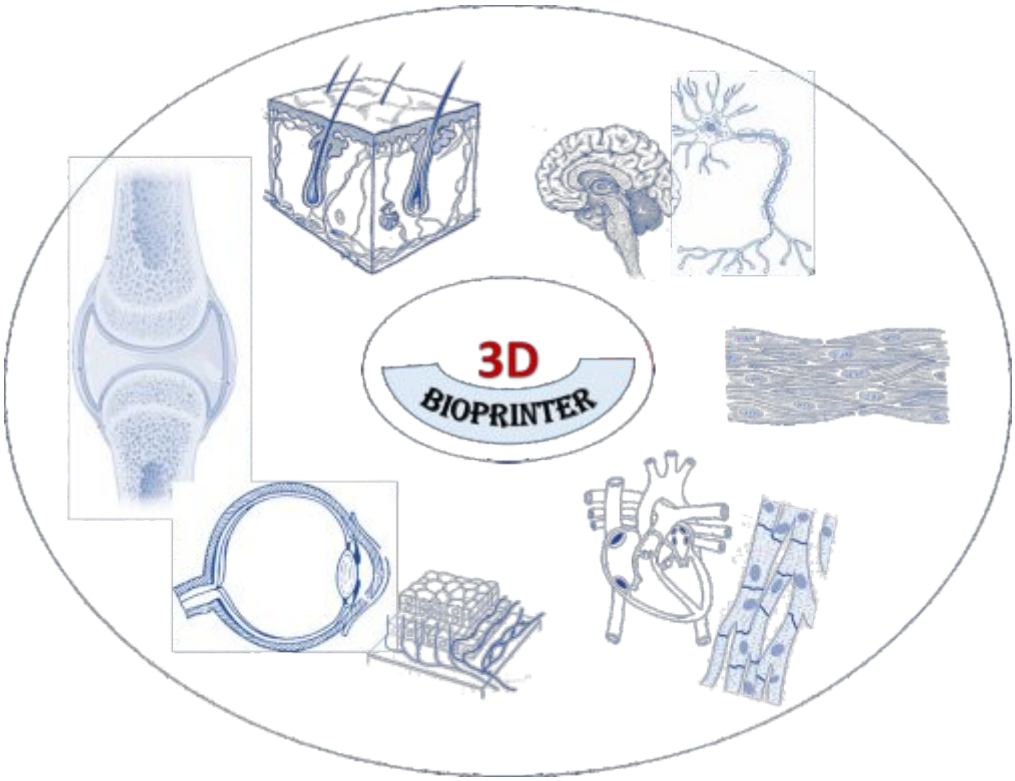

Figure 1: Applications of 3D Bioprinting Technologies in Tissue Engineering

The selection of appropriate scaffold fabrication technique plays an important role in developing structures that can support cell growth as well as promotes tissue regeneration. Traditional conventional techniques like salt-leaching, solvent casting, and gas foaming are simple and economical; however variable pore size and interconnectivity are major challenges. On the other hand, advanced techniques like 3D bioprinting and electrospinning can resolve the above issues with greater precision in scaffold design and cell placement; however, it lacks scalability due to the complex and time-consuming approaches. Hence, the appropriate selection of fabrication techniques needs a lot of consideration. Traditional approaches like salt-leaching, solvent casting, and thermally induced phase separation have proven moderate scalability in scaffold fabrication by increasing batch sizes; whereas Lyophilization being a well-established industrial technique can offer higher scalability. However, 3D bioprinting, electrospinning, and electrohydrodynamic jetting are yet more in research exploration by scientists and large-scale tissue engineering applications may be expected after developing proof-of-concept. Biological variations like donor variability, tissue-specific differences, and patient-

specific factors are the most critical factors that may significantly influence scaffold design and tissue engineering due to variable cell behavior and tissue integration. Standardizing cell sourcing, appropriate processing, tailoring scaffold designs as per specific tissue requirements, and customizing treatment plans to address individual patient characteristics are the need of an hour. Preclinical testing must establish the impact of biological variables on clinical outcomes, as well as validate safety and efficacy of the product. Thorough quality checkpoints should be employed during the fabrication process and focused pharmacovigilance for further enhancement of product performance may offer a detailed understanding and management of biological variations in tissue engineering approaches.

CARTILAGE AND BONE TISSUE

Recent advances in 3D Bioprinting have demonstrated enormous potential for transforming treatment approaches for bone and cartilage injuries. Bioprinted constructs have emerged as a promising solution for tissue engineering by allowing for the rapid and long-term regeneration of damaged tissue. Bionics principles are being used by researchers to create ideal scaffolds that closely resemble native tissues. Furthermore, bioinks such as polylactic acid are being used to improve the mechanical viability and cell growth of printed constructs. 3D Bioprinting's future appears to be even brighter, with the potential to transform surgical procedures, imaging techniques, and drug delivery technologies and thus improving the quality of the treatment and life of the patients. [1,2]

Inkjet-based bioprinting techniques are also studied for cartilage and bone tissues engineering. Xu et al. utilised rabbit articular chondrocytes dispersed in a hydrogel and printed them on a polycaprolactone matrix, which resulted in a scaffold with improved mechanical properties. The printed cells showed good viability, and the scaffolds developed dense, well-organised collagen after implantation in immunodeficient mice. [3]

The use of extrusion bioprinting techniques for bone tissue regeneration has seen significant progress in recent years. Fedorovich et al. printed a complex porous bone like tissue construct using this technique.[4] Hung et al. conducted a study on composite scaffolds for craniofacial regeneration using polycaprolactone. The incorporation of hydroxyapatite in the composite scaffold exhibited superior bone regeneration.[5] Schuurman et al. and Kundu et al. have conducted studies showcasing the development of scaffolds through the utilisation of diverse biomaterials and cell types to promote cartilage tissue regeneration. The structural support to the scaffold was provided by gelatin methacryloyl (GelMA) implanted with chondrocytes and polycaprolactone. Kundu et al. employed a multi-head deposition system for the development of polycaprolactone -alginate gel scaffolds incorporated with chondrocytes. After that, the scaffolds were introduced into the

dorsal subcutaneous area of mice resulting into a generation of cartilaginous tissues after four weeks with a noteworthy 85% cell survival rate. [6,7]

The studies of Dhariwala et al., Lu et al., and Zhou et al. demonstrated potentials of stereolithography bioprinting technique for bone and cartilage tissue engineering. Dhariwala et al. fabricated scaffolds with live CHO cells by utilising poly(ethylene oxide) as a biomaterial and 3D printing technology with a print resolution of 250 µm. Despite the limited mechanical strength observed in the produced scaffolds, the study demonstrated promise for further investigations with the incorporation of another polymer to enhance its strength.[8] Lu et al. conducted a study in which the scaffold exhibited significant mineralisation, after a period of 2 and 4 weeks, indicating that the seeded cells had undergone osteogenic differentiation.[9] Zhou et al. utilised a stereolithographic printer commonly used in small-scale production to fabricate a 3D structure loaded with cells for the purpose of studying breast cancer in bone tissue. Their findings revealed that scaffolds with lower concentrations of GelMA exhibited greater cell viability, implying that higher GelMA concentrations may impede nutrient transportation. Furthermore, the study demonstrated the feasibility of constructing intricate biological systems for investigating disease interactions at a tissue level.[10] Kim et al. accomplished the reconstruction of maxillary bone through the utilisation of a PCL/β-TCP scaffold manufactured by micro-extrusion-based 3D Bioprinting. After an 8-month postoperative period, the study observed a successful reconstruction, marking the first instance of successful maxillary bone defect restoration through a 3D-printed structure.[11]

Several studies have been explored to study the use of Laser-assisted bioprinting (LAB) technique in bone tissue engineering, including the development of biopapers to enhance cell viability. Galbraith et al. conducted a study where they fabricated a self-assembling biopaper composed of osseous tissue using hASCs that had been induced to differentiate into osteogenic cells. This biopaper showed increased levels of ALP activity and matrix mineralisation compared to biopapers made from stromal cells, implying that hASCs may have advantages over stromal cells for specific applications.[12] In their study, Kawecki et al. employed laser-assisted methods to accurately position human umbilical vein endothelial cells (HUVECs) on biopapers, resulting in the production of viable cells and tube-shaped endothelial structures after seven days. The results imply that laser-assisted techniques can augment scaffold regeneration by aiding the formation of a suitable capillary network in the implanted scaffold.[13] Catros et al. explored the consequences of 3D cell positioning within polycaprolactone (PCL) biopapers, demonstrating that scaffolds with internal cell placement exhibited markedly increased proliferation rates

relative to those with cells deposited solely on the scaffold surface.[14]

SKIN TISSUE

Skin bio-printing is an innovative technology with potential applications in various fields. Developing functional skin that has appropriate vascularity, innervation, and can perform functions like touch sensation and perception is the primary obstacle. However, skin bio-printing has the potential to address the increasing demand for skin bio-fabrication for clinical and research applications, such as regenerative medicine, modeling physiological/pathological conditions, and the cosmetic/pharmaceutical industry. Furthermore, the use of skin bioprinting technology may assist in creating tissue that is physiologically relevant, resulting in improved and consistent functional outcomes for burn victims, as well as contributing to faster healing, less scarring, and improved aesthetic results. In conclusion, skin bio-printing technologies have the potential to significantly enhance wound healing and patient outcomes.

Skin tissue production using inkjet-based bioprinting methods has been studied by Lee et al., Rimann et al., and Skardal et al. Adult human dermal fibroblasts and epidermal keratinocytes were grown inside of a collagen gel scaffold by Lee et al. using a microvalve-based bioprinting technique. The printed scaffolds were dimensionally stable, keeping their original shape and allowing three compact layers of keratinocytes to develop on the scaffold's surface. After being exposed to an air-liquid contact, keratinocytes formed a thick epidermal layer.[15] The bioprinted scaffold used in Rimann et al.'s alternative polyethylene glycol based (PEG) skin model showed vascularisation and the highest histological similarity to native skin.[16] In-situ printing was employed by Skardal et al. to apply mesenchymal stem cells (MSCs) and amniotic fluid stem cells (AFS) to complete skin lesions. The results show that inkjet-based bioprinting techniques are promising for producing skin tissue for mending wounds.[17]

Extrusion bioprinting approaches for skin tissue have generated excellent results in recent study. Printing multilayered skin-like tissue constructs with superior shape stability and cell survival was shown by Lee et al. and Kim et al.[15,18] Using a combination of extrusion and inkjet printing technologies, Lee et al. prepared a multilayered scaffold with fibroblasts and keratinocytes suspended in a culture medium. For mechanical stability, the cells were printed between crosslinked layers of a hydrogel. The printed cells showed high vitality after an 8-day culture period, with fibroblasts having a higher cell density than keratinocytes. Kim et al. used an extrusion-based bioprinting method, and samples revealed biological characteristics comparable to native human epidermis after 7 days of culture.

Koch et al. evaluated the possibility of laser-assisted Bioprinting (LAB) for creating skin tissue from a range of skin cells and discovered that LAB resulted in

good cell viability with no change in phenotypic, indicating that the transfer process did not compromise the cells' integrity.[19]

NEURAL TISSUE

Bioprinting has shown promise in the field of neural tissue engineering, allowing for the quick fabrication of physiologically appropriate brain constructions for cell therapy and drug testing. Controlling brain area development, vascularisation, and optimising printing parameters for cell viability are significant challenges. The combination of Bioprinting and organoid technologies could result in complex neurological networks. This method may reduce the requirement for animal models, hence boosting biomedical research in the field of neurological disorders.

The use of inkjet-based bioprinting techniques for neural tissue engineering is a fast-expanding field with huge potential for the development of new therapeutics for a wide range of neurological illnesses. Lorber et al. conducted a study that demonstrated that shear pressures did not induce significant cell distortion or damage during the droplet ejection process. Additionally, the printed cells showed comparable survival rates.[20] In another study, Tse et al. printed a combination of neuronal and supportive glial cells, which exhibited high cell viability and faster neurite growth compared to non-printed cells. These findings hold great promise for the potential use of Bioprinting in the treatment of extensive, full-thickness skin burns.[21]

The use of extrusion bioprinting technique for neural tissue engineering has been shown to be a promising alternative to traditional autologous grafts. Hsieh et al. conducted a study on zebrafish traumatic brain injury model and demonstrated the effectiveness of polyurethane (PU) scaffolds embedded with neural stem cells (NSCs) in repairing neural tissue.[22] Meanwhile, Owens et al. developed nerve grafts made up of mouse bone marrow stem cells and Schwann cells using a multichannel extrusion-based bioprinter. These nerve grafts were implanted in rats and resulted in the restoration of motor and sensory functions at 40 weeks. The use of extrusion bioprinting allowed for the fabrication of precise and complex structures that closely resemble autologous grafts, which are currently the best option for nerve rehabilitation.[23] The research of Hsieh et al. and Owens et al. demonstrates the potential of extrusion bioprinting for neural tissue engineering and could lead to new treatment options for neural injuries.

Zhu et al. employed the Stereolithography bioprinting to transform neural stem cells into neurons. The study discovered that low doses of GelMA were efficient in increasing hNSC proliferation and encouraging neuronal differentiation. After 14 days of culture, GFAP and -tubulin III expression, as well as neurite elongation, suggested effective neural differentiation. The use of nanoplatelets of graphene to induce hNSC differentiation into neurons

is a groundbreaking and promising strategy in the field of tissue engineering.[24]

CARDIAC AND VASCULAR TISSUE

The potential of 3D bioprinting technology in managing myocardial infarction and its complications by creating clinically viable cardiac constructs is evident. The injection of hydrogels derived from decellularised extracellular matrix into damaged cardiac tissue has proven to be effective in enhancing function after ischemia. The use of bioprinting technology presents promising prospects for scaffold micropatterning and scaffold-free systems to reproduce the histological makeup of native cardiac tissue. Numerous bioink formulations based on various biomaterials and composite bioinks have been created for 3D printing of heart tissue engineering, such as heart patches, tissue-engineered cardiac muscle, and other bionic structures.

Inkjet-based Bioprinting has shown great promise in the printing of cardiac and vascular tissue. Xu et al. have successfully fabricated a heart-like structure with two ventricles using primary feline adult and human H1 cardiomyocytes encapsulated in alginate hydrogel. The printed structure possessed porosities that facilitated cell viability, while electrical stimulation triggered rhythmic beating of the entire construct.[25] In a similar vein, Boland et al. introduced a novel technique for producing microscopic porosity in alginate/gelatin gel by printing a crosslinking agent into the solution. This approach allowed the formation of different types of tubes, which served as a scaffold for endothelial cells to grow.[26]

Extrusion bioprinting has proven to be a promising technique in the fabrication of cardiac tissue constructs for regenerative medicine, as demonstrated by Duan et al. In their study, human aortic valvular interstitial cells were encapsulated in a hybrid hydrogel that was 3D bioprinted. According to their findings, it is possible to develop functional cardiac tissue using this technique because the cells have a high level of viability and the capacity to remodel the hydrogel and contract spontaneously.[27] Similarly, Gao et al. used extrusion bioprinting to create a bio-blood-vessel (BBV) by combining atorvastatin-loaded PLGA microspheres and decellularized extracellular matrix derived from vascular tissue. In their study. They found that this technique significantly increased the formation of capillaries and arterioles.[28]

MUSCULAR TISSUE

Musculoskeletal tissue engineering has seen many applications for three-dimensional bioprinting. One important use of bioprinting is the capacity to produce implantable tissues that can restore or replace tissue that has been lost or damaged as a result of an illness, an injury, or aging-related degeneration. Bioprinting stimulates tissue regeneration by creating scaffolds with exact control over their architecture, mechanical characteristics, and cell distribution. For instance, the use of bio-printed muscle

tissue in the treatment of musculoskeletal injuries or degenerative diseases like muscular dystrophy has great potential. Creating complex models of musculoskeletal tissues, like the bone-muscle-tendon interface, using bioprinting enables more precise drug testing and disease modeling. Bioprinting enables the creation of interface tissues that are essential for the proper functioning of musculoskeletal systems. With Bioprinting, the precise control of material and cell placement necessary for the fabrication of interface tissues is achievable. Examples of interface tissues include the bone-cartilage interface and the tendon-bone interface, both of which are implicated in injuries and diseases such as osteoarthritis and rotator cuff tears.

Miri et al. devised a novel method to create intricate hierarchical structures that resemble muscular tissues. They have employed a hybrid approach, utilising microfluidic chips that have been fabricated through pneumatic extrusion and crosslinked with UV light. Various bioinks, such as PEGDA and GelMA, that contain different types of cells, have been printed into specific patterns. After seven days, the researchers observed sufficient proliferation rate. Remarkably, the printed structures maintained their patterns and interfaces even after being implanted in rats for 30 days. The researchers deduced that an optimal concentration of 10% GelMA for the prints is consistent with the studies conducted by Zhou et al.[29] In a summary, this research work showcases the immense potential of extrusion bioprinting for creating complex and hierarchical structures that bear a striking resemblance to muscular tissue. The study offers valuable insights into the use of multiple bioinks and their effects on cell proliferation rates and print patterns, opening up new avenues for in vitro and in vivo studies.

Bajaj et al. conducted a study that sheds light on applications of stereolithography bioprinting for muscular tissue generation. They employed dielectrophoresis and SLA in PEGDA hydrogels to pattern and encapsulate the cells.[30]

CORNEAL TISSUE

The use of bio-printing technology holds significant promise in the field of ophthalmology. One of the most exciting potential applications is the creation of corneal tissue through tissue engineering. This could help address the shortage of donor corneas available for transplant and reduce the risk of rejection by creating personalised corneas. Further, the pace of drug development process could be increased by using bio-printed corneal models for controlled drug testing. This would eliminate the need for animal testing. Moreover, this will help to understand the ophthalmic disease model and the underlying mechanisms in a better way, which in turn will help to develop new methods of treatment. Last but not least, printing of retinal tissues is also made possible, which can help patients with degenerative retinal diseases to regain

their vision.

In order to overcome the shortcomings of corneal transplantation, Isaacson et al. created a cornea made of collagen with corneal keratinocytes using extrusion-based technology. It was observed that this can be a workable substitute for the conventional Penetrating Keratoplasty procedure. In their study, they found 90% viability rate on the second day and 83% after a week.[31]

Sorkio et al. studied laser-assisted bioprinting for repairing corneal tissue. In the study, human adipose derived stem cells and limbal epithelial stem cells were printed on three different scaffolds and adequate cell proliferation was observed.[32]

CONCLUSION

Regeneration of cartilage and bone, skin, neural, cardiac, vascular, muscular, and corneal tissue has been successfully studied by numerous scientists using a variety of bioprinting techniques and promising results are obtained. These studies offer a strong foundation for the advancement of tissue engineering and 3D printing of artificial organs. The advancement in this area will facilitate the improvement in regenerative medicine and drug discovery process and reduce the need for animal testing. These bioprinted models can be an ideal substitute for researching drug efficacy and toxicity because they can mimic the mechanical and biochemical characteristics of native tissues. These models can also be used to research diseases and personalized medicines. After the success of 3D bioprinting, a growing trend toward 4D bioprinting has been observed in recent years. 4D bioprinting adds a time factor in 3D printing to build a base structure that can be reshaped or reconfigured over time to take on the desired form. Various hydrogels, shape-memory polymers, and other responsive materials are being studied in combination with 4D bioprinting to develop multifunctional tissues and organs with ability to respond to environmental changes.

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REVIEW ARTICLE

COMPREHENSIVE REVIEW OF SIMHANADA GUGGULU: A FORMULA FOR AMAVATA (RHEUMATOID ARTHRITIS)

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ABSTRACT

Amavata is a disease characterized by gatrastabdhata caused by the combined effect of the formation of ama and prakupita vata per trikasandhi. When Ama and Vata simultaneously vitiate and enter Trika and Sandhi, it ultimately leads to stabdhata (stiffness), which is known as Amavata. The disease Amavata can be presented as very similar to Rheumatoid Arthritis. The sickness of Rheumatoid Joint pain is ongoing in nature and influences mostly the moderately aged people. It is one of the common crippling sicknesses due to its chronicity and effects. The beginning of infection is successive during the middle age with 80% of patients fostering the illness between 30 to 50 years old. Several studies show that female is more victim than male and the proportion of event between them is 3:1. It is also noted that frequency is often associated with remission of the disease in last trimester with subsequent relapse after delivery. However, in present era no appropriate medication is available that can give everlasting benefit by permanent remission of the symptoms. The treatment which are available can only give symptomatic relief for time being but their continuous use causes various adverse effects. Thus, people are looking for a safe and effective drug that gives permanent relief. The Ayurvedic system is having its pride in providing such a treatment to cure all these types of chronic ailments. Among them, Simhanada Guggulu is a well-known Guggulu preparation indicated in Amavata. It was first mentioned by Acharya Chakrapani in Chakradatta. Action of this drug works to offer pain relief and detoxification and combats loss of appetite. An attempt is made to compile the review of Ayurvedic literature of

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Simhanada Guggulu.

Key words : Simhanada Guggulu, Amavata, Rheumatoid Arthritis

INTRODUCTION

Amavata with extraordinary reference to Rheumatoid Joint pain is one of the feared infections that the humanity faces today. This repulsive infection starts as joint solidness eventually opens up as a reason for some others. It can influence numerous features of a patient's life, viz. his family, social and professional life. It influences not just the social and financial place of the individual and his family, however it prompts the depleting of public asset because of the work hours lost, bringing about reduced creativity and output. The current medications cannot fulfill the goals of an optimal treatment and cause several side effects like gastrointestinal ulceration and bleeding. The Ayurvedic system is having its pride in providing such a therapy to address all these types of chronic ailments.

Vati are essentially a tamper proof dosages form, therefore there are no chances of adulteration or mixing, having accuracy of dosage, unit doses forms and offer the greatest dose precision and the least content variability, cost is lowest of all oral dosage forms and offers great ease of administration, as compared to other dosage forms.

Simhanada Guggulu is also one of the vati kalpana and offers above mentioned advantages as medicament.

MATERIALS AND METHODS

All the literature regarding the use, doses, Anupana, Rasa Panchaka, pharmacological actions, probable mode of action of Simhanada Guggulu were compiled from different classical treatise and available sources of information.

Different formulation of Simhanada Guggulu in different classical texts :

Formula 1: Ayurvedic Formulary of India [1]

Sr. No.	Drug	Quantity of drug
1	Haritaki	48g
2	Bibhitaka	48g
3	Amalaki	48g
4	Eranda taila	192g
5	Shuddha Gandhaka	48g
6	Shuddha Guggulu	48g
7	Water for Decoction	576ml

Procedure : Decoction of triphala is prepared by diluting it four times with water and then reducing it to a quarter. Eranda oil was added to this kwatha and then heated. When its concentrated enough, fine powders of Shuddha gandhaka and Shuddha guggulu were added and combined thoroughly.

Dose :- 3g

Anupana :- Warm water

Formula 2 : Ayurveda Sara Sangraha [2]

Sr. No.	Drug	Quantity of drug
1	Shu.Guggulu	3.75 part
2	Shu.Gandhaka	1.25 part
3	Triphala	11.25 part
4	Eranda taila	1 part

Eranda oil is added to the mixture and after that Shuddha guggulu is dissolved in hot water, added to above mixture, then it is pounded in lauha ullukhal yantra. Then pills weighing 3 ratti each are made and dried.

Formula 3: Vangasena[3], vatarakta adhikara, Rasa Tantra Sar and Siddha Prayoya Sangraha[4], Bhrihat nighantu Ratnakara[5], chakradatta6, Bhaisajya Ratnavali[7], Bhava Prakash[8], Yoga ratnakara[9]

Sr. No.	Drug	Quantity of drug
1	Triphala decoction -separate	3 pala
2	Eranda taila	1 kudava
3	Shuddha gandhaka	1 pala
4	Shuddha guggulu	1 pala

The method of preparation is same as for the acharyas described above.

Formula 4: Yoga ratnakara[10], Vrihat yoga ratnakara[11]

Sr. No.	Drugs	Quantity of drug
1	Haritaki	1 prastha
2	Bibhitaka	1 prastha
3	Amalaki	1 prastha
4	Guggulu	1 prastha
5	Water for decoction	Sardha rashi
6	Devadaru	1/2 pala
7	Triphala	1/2 pala
8	Trikatu	1/2 pala
9	Musta	1/2 pala
10	Chitraka	1/2 pala
11	Vidanga	1/2 pala
12	Guduchi	1/2 pala
13	Ajavayan	1/2 pala
14	Mankanda	1/2 pala
15	Bhallataka	1/2 pala
16	Shavari (chitrak mula)	1/2 pala
17	Trivrit	1/2 pala
18	Shudhha parade	1/2 pala
19	Shudhha gandhaka	1/2 pala
20	Dantibeeja	1000 no.
21	Kutajabeeja	8 pala

Procedure : kwatha preparation (from 1-4 drugs in said quantity of water and reduced to half), filtered kwatha heated and allowed to concentrate, then churna (16-17, 20-21 drugs and kajjali) added and medicine prepared.

Therapeutic indication : amavata

Formula 5: Bhaishjya Ratnavali[12], Bhava Prakash

Sr. No.	Drugs	Quantity of drug
1	Haritaki	2 prastha
2	Bibhitaka	2 prastha
3	Amalaki	2 prastha
4	Guggulu	8 pala
5	Katu taila	8 pala
6	Water for decoction	Sardha drona
7	Devadaru	2 karsha
8	Triphala	2 karsha
9	Trikatu	2 karsha
10	Musta	2 karsha
11	Chitraka	2 karsha
12	Vidanga	2 karsha
13	Guduchi	2 karsha
14	Chavya	2 karsha
15	Mankanda	2 karsha
16	Surankand	2 karsha
17	Trivrit	2 karsha
18	Danti	2 karsha
19	Shuddha suta	2 karsha
20	Shuddha gandhaka	2 karsha
21	Dantibeeja	1000 no.

Procedure : the procedure is same as mentioned above .

Dose :2 ratti

Anupana : warm water

Therapeutic indication : Amavata , Sandhivata , Ashmari , Pandu shirovata , Kamla

The quantities of guggulu and triphala in Yoga Tarangini13 are different, yet they are the same .

Guggulu	1 prastha
Triphala	1 prastha (each)

Vrihat Nighantu Ratnakara differs from the previous formulation in the following ways :

Formula 6: Vrihat Nighantu Ratnakara[14]

Sr. No.	Drugs	Quantity of drug
1	Triphala (each)	1 prastha
2	Guggulu	1 prastha
3	Katu tail	1 pala
4	Ghrita	1 pala

Dose : 2 shana

Formula 7: Vangasena [15]

Sr. No.	Drug	Quantiity of drug
1	Haritaki	1 prastha
2	Bibhitaka	1 prastha
3	Amalaki	1 prastha
4	Guggulu	8 pala
5	Water for decoction	2 drona
6	Kraunch	1/2 pala
7	Triphala	1/2 pala
8	Trikatu	1/2 pala
9	Musta	1/2 pala
10	Chitraka	1/2 pala
11	Vidanga	1/2 pala
12	Guduchi	1/2 pala
13	Vacha	1/2 pala
14	Mankanda	1/2 pala
15	Trivrit	1/2 pala

16	Danti	1/2 pala
17	Shuddha parada	1/2 pala
18	Shuddha gandhaka	1/2 pala
19	Kanaka beeja	1000 no.

Table 1 :

Sr. No.	Name of the Dravya	Rasa Panchaka	Doshaghanata	Rogaghanata	Chemical Constituents	Pharmacological Action
1	Amalaki	Rasa - Kashaya, Amla, Madhur Guna - Laghu Veerya - Sheeta Prabhava - Pittasamak rasayan	Tridosha shamaka, especially pittashamaka	Shotha, Paittikashirahshula, Kustha, Trishna, Arsha, Daha, Drishtimandya, Udavarta, Charmaroga, Aruci, Kshaya, Jeernajwara, Agnimandya,Yakridvikara, Khalitya, Amlapitta, Parinamashula, Hridroga, Raktapitta	Fruit contains vitamin C, carotene, nicotinic acid, riboflavin, myoinositol and a pectin, X-glucosyl, D-Glucose, D-fructose, D-mannosyl, Indole acetic acid, phyllembic acid, phyllembin tannins, polyphenolic compound, terchebin, corialgin, ellagic acid, alkaloids, phyllantidine and phyllantine.	Antioxidant,Immuno modulatory, Antipyretic, Analgesic, Cytoprotective, Antitussive and Gastroprotective actions
2	Vibhitaki	Rasa- Kashaya Guna - Laghu,Ruksha Veerya - Ushna Vipaka - Madhura Prabhava – kaphapiita samak	Tridoshashamaka, especially kaphashamaka	Shotha-vedanayuktavikara, Vatavyadhi, Agnimandya, Charmaroga, Ashmari, Palitya, Shwasa, Klaibya, Hridroga, Vrana, Anidra, Adhmana, Trishna, Netraroga	Chebugalic acid, Chebulasic acid Sitosterol, Egalic acid, Galic acid, Galloylglucose, Oxalic acid,ramnose	Purgative, Anti - helminthic, Anti-biotic

3	Haritaki	Rasa - Panchras (lavanvarjt) Guna - Laghu Veerya - Ushna Prabhava – Tridoshahara	Tridoshashamaka, especially vata shamaka	Shotha-vedanayuktavikara, Vatavyadhi, Shula, Raktavikara, Udararoga, Mukharoga, Nadidaurbalya, Agnimandya, Anaha, Gulma, Vibandha, Arsha, Shwasa, Ashmari, Kustha, Vishamajwara, Mutraghata		Rejuvenating, Brain tonic, Appetizer.
4	Guggulu	Rasa - Katu, Tikta, Kashaya Guna - Laghu, Sukshma, Ruksha, Picchala Prabhava - Vatabalasjeet	Vata Kapha Samaka	Amavata, Kustha, Prameha, Vatavyadi, Granthi, Sopha, Gandamala, Medoroga	Diterpenoids, triterpenoids, steroids, long-chain aliphatic tetrols, aliphatic esters, ferulates, lignans, carbohydrates, minor amounts of sesamin	Anti-inflammatory, Diuretics, Antioxidant, manages high cholesterol.
5	Eranda	Rasa - Madhur, Tikta, Kashay, Katu Guna - Tikshna (taila) Veerya - Ushna (taila) Prabhava - Vedan, Rechan (veej), Vataghna (mul), Shothahara (Patra)	Balances kapha and vata dosha	Udavartahara ,pleehaghna,gulmahara,bastishoolahara,antravruddhinut,shonitavikara , shoshahara, shoolaghna ,margashodhana ,shwasahara,kasaha5ra, anahahara, kati - basti rujahara ,shiroruji ,mehahara, amavatahara	Steroids, flavonoids, saponins, glycosides, and alkaloids	Anti-inflammatory, Spasmogenic, Hepatoprotective, CNS depressant, Anti-fertility, Purgative, Immunizing, and spasmolytic
6	Gandhaka	Rasa -Madhura Guna -usna Veerya -ushna Vipaka -katu Prabhav - deepana ,pachana,anti -		Kandu (itching), kushta(skin diseases),visarpa (herpes),dadru (ringworm)	pure sulphur, sulphides and sulphates	Antifungal, antibacterial,keratolytic

PRACTICABLE USES OF SIMHANADA GUGGULU FOR COMPREHENSIVE HEALTH

Rheumatoid arthritis

Rheumatoid arthritis is an autoimmune condition in which the body attacks healthy cells, which in turn may weaken our bones. Dallavi et al.[16] did a study in 2018 on the clinical efficacy of simhanada guggulu on rheumatoid arthritis. The study showed that simhanada guggulu might be used in the management of rheumatoid arthritis. Further studies are necessary to prove these findings.

Systemic sclerosis

Systemic sclerosis is an autoimmune disease that causes fibrosis (damage or injury) of blood vessels, skin and other tissues, along with organs like the lungs, heart, kidneys and gastrointestinal tract. Sreenithya et al. in 2021, carried out a study on the ayurvedic management of systemic sclerosis[17]. Results indicated that simhanada guggulu could be possibly used along with other ayurvedic medicines for the management of systemic sclerosis. Further studies are necessary to prove its pharmacological effects.

Spondylitis

Ankylosing spondylitis is a condition that causes bones in the spine (vertebrae) to fuse, which may result in reduced flexibility and a hunched-back posture. Singh et al. in 2020 did a study that showed that the administration of simhanada guggulu, along with other ayurvedic medicines, may be used for ankylosing spondylitis.[18] However, further work is necessary to evaluate the complete effect of simhanada guggulu in ankylosing spondylitis.

Lung function

Bronchial asthma is a lung condition in which the airways become narrow, and breathing becomes difficult. A study was done by Byadgi et al. in 2008 to evaluate the effect of simhanada guggulu on bronchial asthma.[19] The results concluded the potential use of simhanada guggulu in the management of asthma. However, more research is required to prove the efficacy of simhanada guggulu in managing asthma.

Anti-inflammatory agent

Panda et al. in 2011, conducted a study to evaluate the anti-inflammatory activity of simhanada guggulu in rats.[20] The study results concluded that simhanada guggulu might have anti-inflammatory properties. However, studies are needed to evaluate the efficacy of simhanada guggulu in humans.

Diabetes

A study done by Sharma et al. in 2015 on herbal medicine showed that amalaki (*Emblica officinalis*), a constituent of simhanada guggulu, may have an anti-diabetic property. Therefore, simhanada guggulu may be used as an anti-diabetic agent. However, more work is required to

prove the effect of simhanada guggulu in diabetes.[21]

OTHER PRACTICABLE USES OF SIMHANADA GUGGULU

- Simhanada guggulu contains *Emblica officinalis,* which may be used for eyesight disorders.
- It may also be used as an anti-microbial agent.
- Simhanada guggulu also contains *Commiphora mukul,* which has potential uses for managing obesity.
- It may be used in the management of gastric disorders like acidity and peptic ulcers.

PROBABLE MODE OF ACTION OF DRUGS

The essentials of Ayurvedic pharmacology are skilled to give a superior logical lead in method of medication activity. Pharmacology of Ayurveda depends on the hypothesis of Rasa, Guna, Virya, Vipaka and Prabhava which were the most straightforward boundaries in those days to learn the activity of the medication. Ayurvedic classics provides a clear therapeutic guidence for the treatment of Amavata. Normally, langhana, swedana, Tikta-katu-Deepana drugs, virechana etc. were found. The treatment is based on Ama pachana and amelioration of vitiated vata. Simhanada Guggulu is havingTikta - Kshaya rasa, laghu ushna guna, ushna virya, katu vipaka. As the current infection is brought into the world out from the vitiated vata and kapha. Primary pathology in the illness is Ama nirmiti. Simhanada Guggulu had positive activity on vitiated vata and kapha dosha. The vast majority of the medications utilized in this detailing were Katu, Tikta in Rasa and Ushna Virya which have the opposing properties to that of Ama and Kapha which are the main causative variables in this illness. Because of this Katu rasa and Ushna Virya prompts increment stomach related power which additionally processes Àmarasa. In view of Tikshna, Ushna and Ruksha Guna assuage the vitiated Vata and Kapha and do not permit the Ama to remain at the site of pathogenesis and to make Srotorodha. Most of the drugs in the Simhanada Guggulu have Agnideepaka and pachana property, which play important role in Amapachana of Rasa Dhatu and helps to reduce the formation of Amarasa.

Most of the drug in the formulation had Amanashana, Yogvahi and adhered Dosha from the Dushita Srotas. Due to formation of Amarasa, a large portion of the medication in the detailing had Amanashana, Yogvahi and Vatanulomana. Tikta Rasa eliminate stuck Dosha from the Dushita Srotas. Because of Srotovivronoti, Agnideepana and Pachana property of Katu Rasa helps in Strotashodhana and do not permit the Ama to stick at the site of pathogenesis.

DISCUSSION

Madhavakara was the first to give the detailed description of Amavata.

Numerous clinical cases would have been found in that period making the author to deal it separately. He has devoted a separate chapter in Madhava Nidana to narrate its aetio-pathology, clinical features and types according to Dosha Pradhanya and Sadhya Asadhyata of Amavata. Àmavata is one of the most challenging joint disorders to the human being, because of its chronic & life-threatening nature. Change in life style like sedentary & stressful situation and fast-food dietetic pattern are responsible for the manifestation of disease. In Allopathy the treatment therapy for Rheumatoid Arthritis is experiential and mainly emphasizes on relief of pain along with management of inflammatory condition, enhancing working capacity of the joints and preventing deformity. Therefore, there is a necessity for the development of newer and more potent Anti-Inflammatory drugs with lesser side effects. After extensive literature review, 15 references of Simhanada Guggulu were found. Evaluation of these references, indicates that main ingredients are same as quoted in A.F.I, some variations appear in the quantity of drug.

Simhanada Guggulu is a combination of drugs which are mostly Katu, Tikta, Kasaya in Rasa, Laghu, Ruksha Tikhsna, Sukshma in Guna, Ushna in Virya, Madhura in Vipaka and Kaphavatahara Tridoshahara in action. This combination is also Vibandhahara as it contains Eranda Taila and is Vedanahara due to Guggulu. Due to its Laghu-Ruksa Gunas and Katu-Tikta Kashaya Rasa it could have palliated the Kapha and could have checked the production of Ama. The combination is Vatahara due to its Madhura Vipaka and Ushna Virya of Guggulu.

But on the other hand, Simhanada guggulu is in semisolid form, if prepared with actual classic reference. Due to palatability, it was generally not taken by many patients. Easy administration and fixed dosage form are the plus points of pills which are lacking in this semisolid Simhanada guggulu. Thus, due to more quantity of eranda tail and Guggulu it offers better results. In market we get pill form Simhanada Guggulu which didn't provide the benefits of Eranda tail so delaying in relief. So to give better results to patients we have to prepare the medicine as mentioned in our classics or if we modify the SOP according to feasibility it will not give proper results in recommended dose of each ingredient in the formulation with anupana or sahapana.

CONCULSION

Simhanada Guggulu is a Complementary medicine which are in practice for eons for Rheumatoid Arithritis due to its known anti-inflammatory and anodyne action. This incredulous herbal formulation coupled with properties, like Deepan, Pachan ,Vatanulomana and Vedanahara becomes a promising therapy.

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RESEARCH ARTICLE

IN SILICO SCREENING OF EFAVIRENZ AND EFAVIRENZ NICOTINAMIDE COCRYSTAL FOR ITS ACTIVITY AGAINST HIV AND SARS COV-2

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ABSTRACT

Recently many antiviral agents are under screening for the management of COVID-19 caused by SARS CoV-2. The *in silico* screening provides the insights of drug – receptor binding and efficacy of molecule to treat the diseases. In this work, the efficacy of an antiviral agent efavirenz and efavirenz nicotinamide cocrystal (ENCOC) against the SARS CoV-2 was determined using *in silico* techniques using AutoDock PyRx. Interestingly EFV shows the binding affinity with both HIV and SARS CoV-2 whereas newly synthesized ENCOC have prominent binding affinity with HIV and leading protease inhibitor of SARS CoV-2. The screening results confirms the activity of EFV and ENCOC against HIV-1 and SARS CoV-2 and warrants *in vivo* study for estimation of activity against SARS CoV-2

Key words: Efavirenz, Nicotinamide, Cocrystal, Co-formers, *in silico* screening, SARS CoV-2, acting antiviral agent.

INTRODUCTION

Human Immunodeficiency Virus (HIV) and viral infections like severe acute respiratory syndrome coronavirus 2 (SARS CoV-2) have become a serious public health issue globally.

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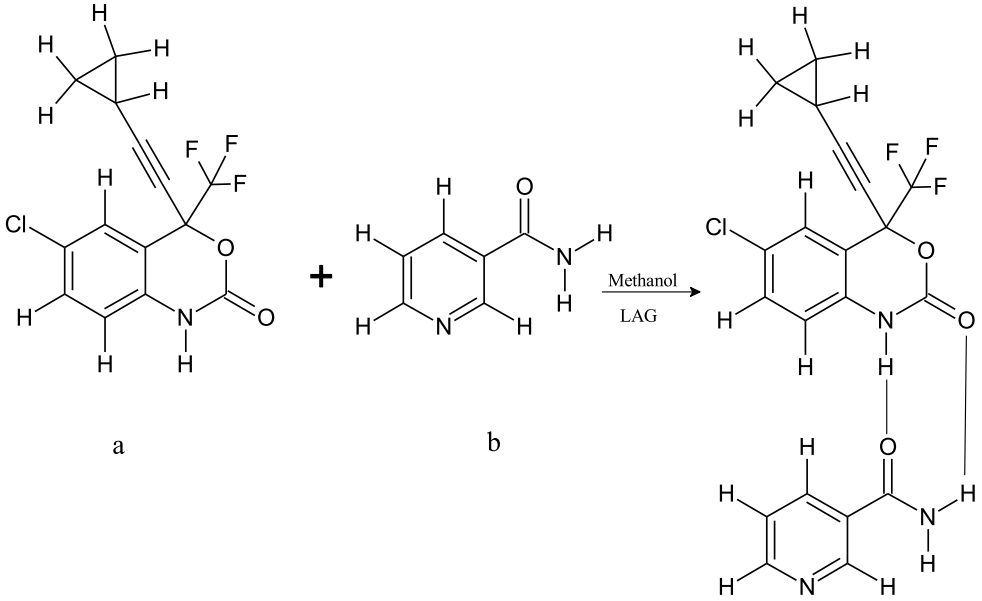
According to the World Health Organization (WHO), till 14 July 2021, 36.3 million people lost their lives, and 37.7 million individual were infected with HIV (1). In the current SARS CoV-2 pandemic 4.5 million human deaths and 217.55 million people were infected till the end of Aug 2021(2). To treat such deadly infections antiviral agents are the choice of treatment, Food and Drug administration (FDA) approved many antiviral agents like Veklury (Remdesivir) as therapeutic agents for treatment of SARS CoV-2. (3). The literature based evidences exhibits the utility of non-nucleoside reverse-transcriptase inhibitors (NNRTI) for blocking the RNA-dependent RNA polymerase (RdRp) and suppress the infection of SARS CoV-2 (4–9). Hence Efavirenz (EFV) a NNRTI used in the treatment of HIV in combination therapy was selected as model drug in this study. An approximate dose of EFV is 200 – 600 mg and it comes under BCS –II and having poor aqueous solubility, due to low aqueous solubility it exhibits low oral bioavailability (10–12). EFV is part of high activity antiretroviral therapy (HAART) and considered as best suitable API for treating HIV (13). This fact of EFV leads to be the suitable drug molecule for solubility enhancement. In this study nicotinamide (NICO) or vitamin B3 was used as coformer because it consisting both hydrogen bond donor (-NH2) and acceptor (C=O) group in its chemical structure which favors to formation of hydrogen bond. The expected molecular arrangement is depicted in Scheme 1.

To enhance the solubility, stability and pharmacokinetics various pharmaceutical approaches like Solid dispersion(14), Co-crystallization (15,16) Hot melt extrusion(17), nanotechnology(18), cyclodextrin based tactics (19,20) are utilized. In this study attempt was made for solubility enhancement of an antiviral agent EFV through pharmaceutical cocrystallization technique.

The pharmaceutical cocrystal approach is gaining attention from pharmaceutical industries because of its scalability, uninterrupted manufacturing process, economy and applicability(21).

In this work we synthesized pharmaceutical cocrystal (ENCOC) of EFV and NICO through liquid assisted grinding (LAG) method to enhance the solubility and physicochemical properties. Further we performed *in silico* screening of EFV and ENCOC for screening of its antiviral activity and study the effectiveness against SARS Co-2. The AutoDock PyRx, Auto dock Vina, open Bable and Discovery Studio software were utilized for molecular modeling and computational studies. The main focus of present work is to study the effect of pharmaceutical cocrystallization on solubility of an antiviral agent EFV and it's *in silico* screening for anti- HIV and SARS CoV-2 activity.



a b c

Scheme I. Schematically representation of formation of EFV (a), NICO (b) Pharmaceutical cocrystal (ENCOC) (c)

MATERIALS AND METHODS

EFV was received as a gift sample from the Emcure R&D center Gandhinagar, Ahmedabad. NICO was purchased from the central drug house, solvents such as Methanol, water (HPLC) were purchased from Finar chemicals.

Synthesis of EFV Nico Cocrystal

The cocrystal of EFV was prepared through the Liquid Assisted Grinding (LAG) method. Accurately weighed EFV and NICO were triturated for 1.30 hrs. with slow addition of few drops of methanol. The formulated cocrystals were kept in a vacuum desiccator for 12 hrs. at room temperature and further used for characterization (22,23)

EXPERIMENTAL

In Silico Molecular Interaction

Platform For Molecular Modelling

Auto Dock python prescription 0.8 (PyRx)(24) were used for computational studies of EFV and ENCOC with HIV-1 reverse transcriptase protein (PDB ID: 1FK9)(25) and SARS CoV-2 main protease inhibiter (PDB ID: 5R81 and 5R82)(26). The Protein data base (PDB) file of proteins were downloaded from RCSB PDB (https://www.rcsb.org/). Discovery Studio Visualizer (v 21.1. 0.20298) was used for preparation of input

file for PyRx and post docking analysis (molecular interaction, Ligand- protein binding, H- bond , hydrophobicity, charges, ionizability and Aromaticity). The ChemDraw ultra v 12.0.2.1076 2D was used for elucidation of structure of ligand (EFV and ENCOC). The protein molecule file was uploaded to PyRx software and macromolecule was prepared through AutoDock and converted in to pdbqt format. Whereas the 3D ligand was uploaded through Open Bable and converted in to pdbqt file after energy minimization with uff force field. Further for the selection of binding site at HIV Protein (1fk9) macromolecule at reported residues(25) and its interaction with ligand after preparation of grid was studied through Auto Dock Vina software. Further the stability of EFV and ENCOC in the active site of concern macromolecule was determined through Root-Mean-Square Deviation (RMSD) and binding affinity was observed through AutoDock Vina(27).To study the intermolecular interaction and selection of amino acid residue of SARS CoV-2 main protease inhibitor the blind docking was performed.

Docking of Sars Cov-2 Using Autodock Vina

The main protease inhibitor of SARS CoV-2 5r81 and 5r82 were reported binding affinity with EFV with docking score -6.5 and -6.6 respectively(28). To confirm the binding affinity and selection of interacting residue the blind run of AutoDock Vina was carried out at Vina search space at center (x, y, z) 12.02, 0.67,4.52, and dimensions (Å) (x, y, z) 37.36, 64.30, 61.26.(29). The residues were selected based on the interaction between the macromolecule and ligand (Table2) and same residues were selected for docking of newly synthesized formulation ENCOC.

RESULTS AND DISCUSSION

In silico Molecular Interaction

AutoDock Vina reproduced the ten orientation for intermolecular interaction between macromolecule and ligand, Amongst that the orientation with higher negative binding affinity was selected(30) and further results of binding of EFV and ENCOC with HIV and SARS CoV-2 protein were mentioned in table no 2 and 3 and shown in fig 9 to 14. Approved antiviral agent EFV shows the intermolecular H- bonding (Lys-101) and Pi –Alkyl interaction (Leu-100, Val-106) with amino acid residue of HIV protein with binding score -6.7 kcal/mol. The newly synthesized ENCOC exhibits the conventional H- Bonding (Thr-139, Glu-28, and Lys 32) and Pi- Hydrogen interaction (Ile 31) with amino acid residues with bond length 3.22 to 3.69 Å. This molecular simulation results indicates the better *in silico* interaction of ENCOC with HIV protein (PDB ID 1fk9) and exhibits the enhanced anti HIV activity than EFV. Furthermore, we did the *in-silico* screening of EFV for the activity against the SARS CoV-2 and we reported here EFV exhibits the binding affinity with main protease inhibitor of SARS CoV-2 and exhibits the potential activity (*in*

silico) against SARS CoV-2. The EFV binds with amino acid residue of main protease inhibitor of SARS CoV-2 (PDB ID 5r81 and 5r82) with binding affinity -7.1 and -6.6 and forms the intermolecular H-bond (Thr-111, Arg-298), halogen (Asn-151) and Pi- Alkyl (Phe-294) bond. We also studied *in silico* binding affinity of ENCOC with main protease inhibitor of SARS CoV-2 and found that ENCOC exhibits the better intermolecular interaction with binding affinity -7.2 and -7.7 kcal/mol. (Table 2, Fig 14). ENCOC forms the H- bond (Asp-289, Leu-287, Tyr-239, Pro-108, Asn-203 and His-246 with amino acid residues.

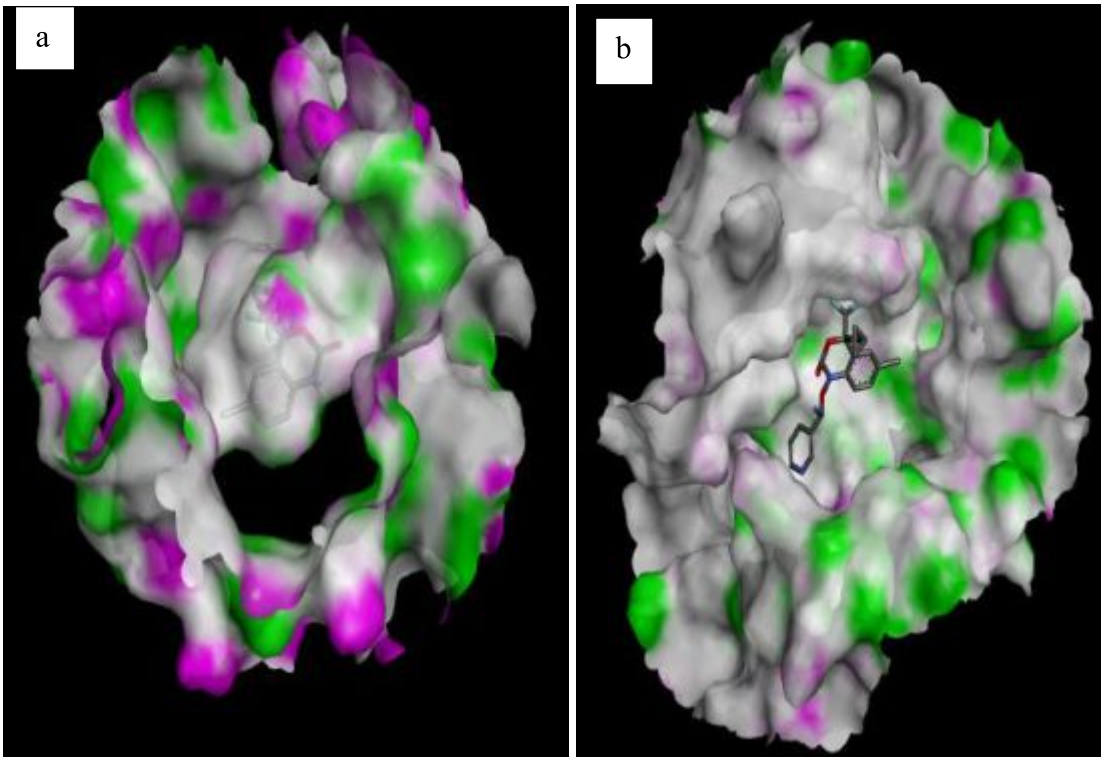


Fig.1. Binding Pocket of EFV (a) and ENCOC (b) in active site of HIV protein 1k9

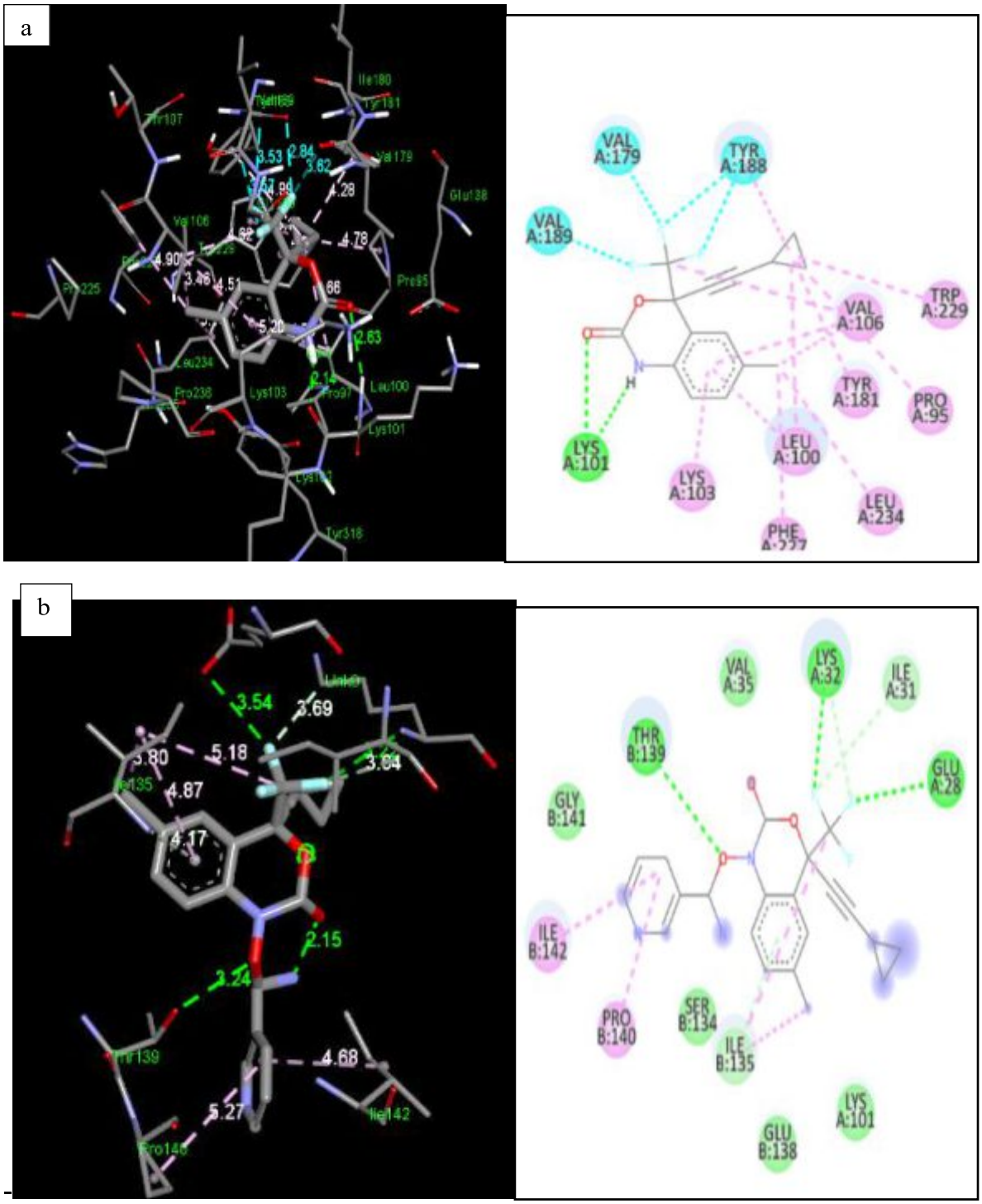


Fig.2. Docked poses of EFV (a) and newly synthesised pharmaceutical cocrystal ENCOC (b) in active site of HIV protein (1fk9)

Table 1. Ligand Protein binding interaction of EFV and ENCOC with HIV protein

Name of Ligand	HIV Protein	Binding affinity (kcal/mol)	Interacting Moieties	Type of Bond/ Interaction	Amino acid residue	Bond length (Å)
EFV	1FK9	-6.7	C=O	Conventional H-Bond	Lys 101	2.63
			NH_2	Conventional H-Bond	Lys 101	2.14
			CF_3	Halogen (F)	Val 189	3.57
			Ar-Ring	Pi- Alkyl	Leu-100 Val-106	4.75 4.51
ENCOC	1FK9	-7.6	C=O	Conventional H-Bond	Thr-139	3.24
			CF_3	Conventional H-Bond Pi donor H- Bond	Glu-28 Lys-32 Ile -31	3.22 3.54 3.69
			Ar-Ring	Pi- Donor H- Bond Pi- Alkyl Alkyl	Ile-135 Ile-135 Ile -142	4.17 4.87 4.68

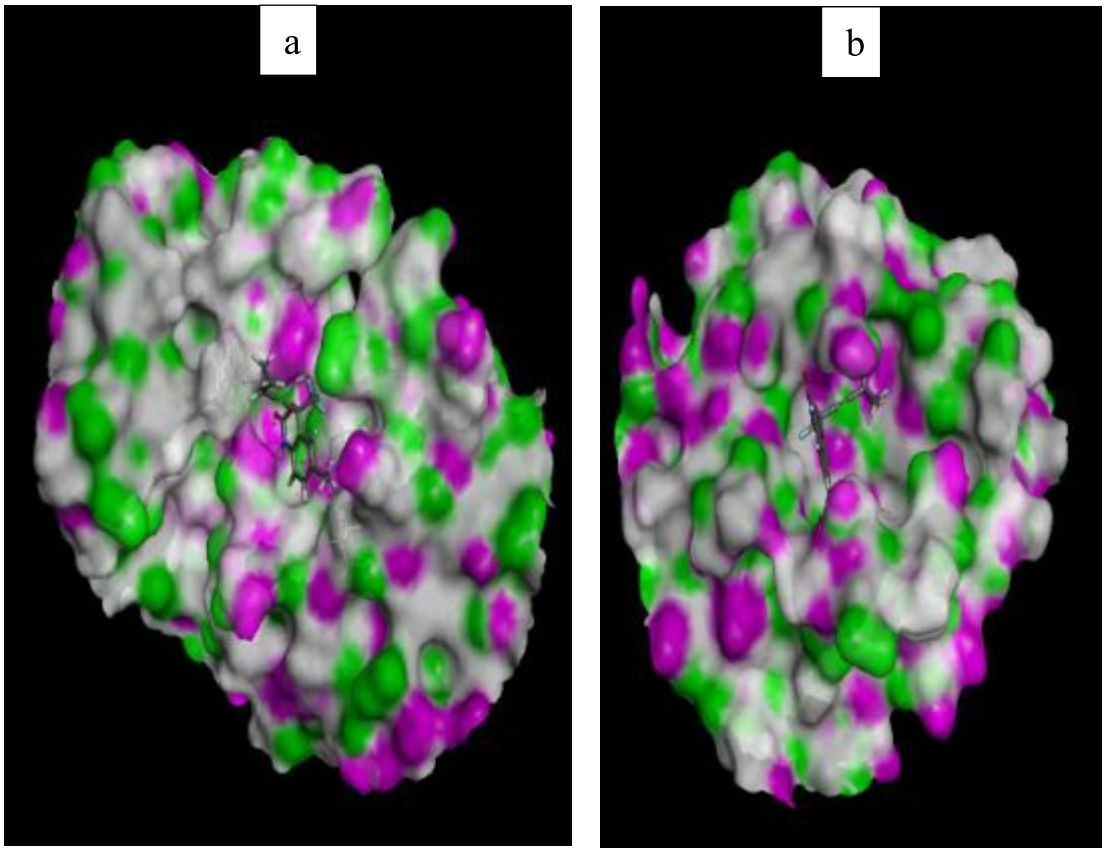


Fig.3 Binding pockets of EFV with SARS CoV-2 protein (a) 5r81 and (b) 5r82

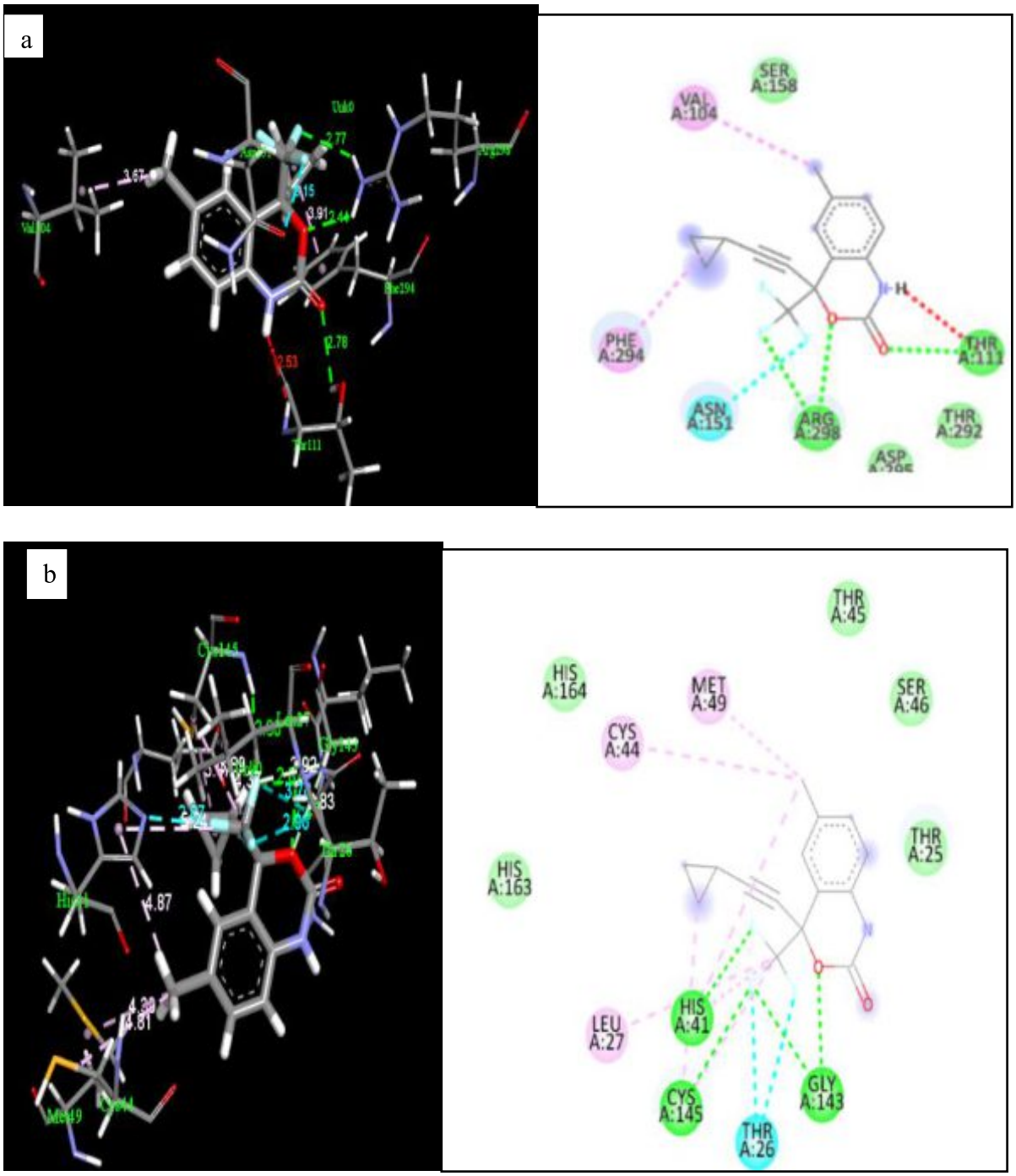


Fig.4. Docking poses of EFV in active site of SARS CoV-2 protein 5r81 (a) and 5r82 (b)

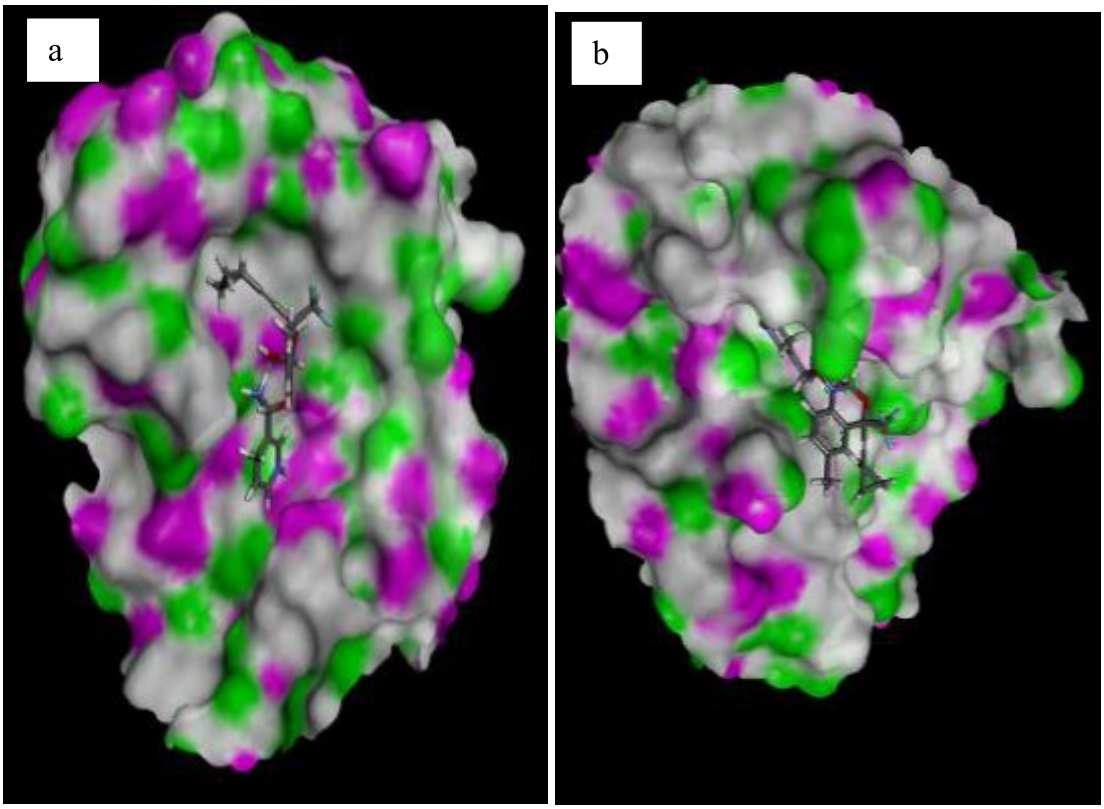


Fig.5. Binding pockets of newly synthesised pharmaceutical cocrystal ENCOC with SARS CoV-2 protein (a) 5r81 and (b) 5r82

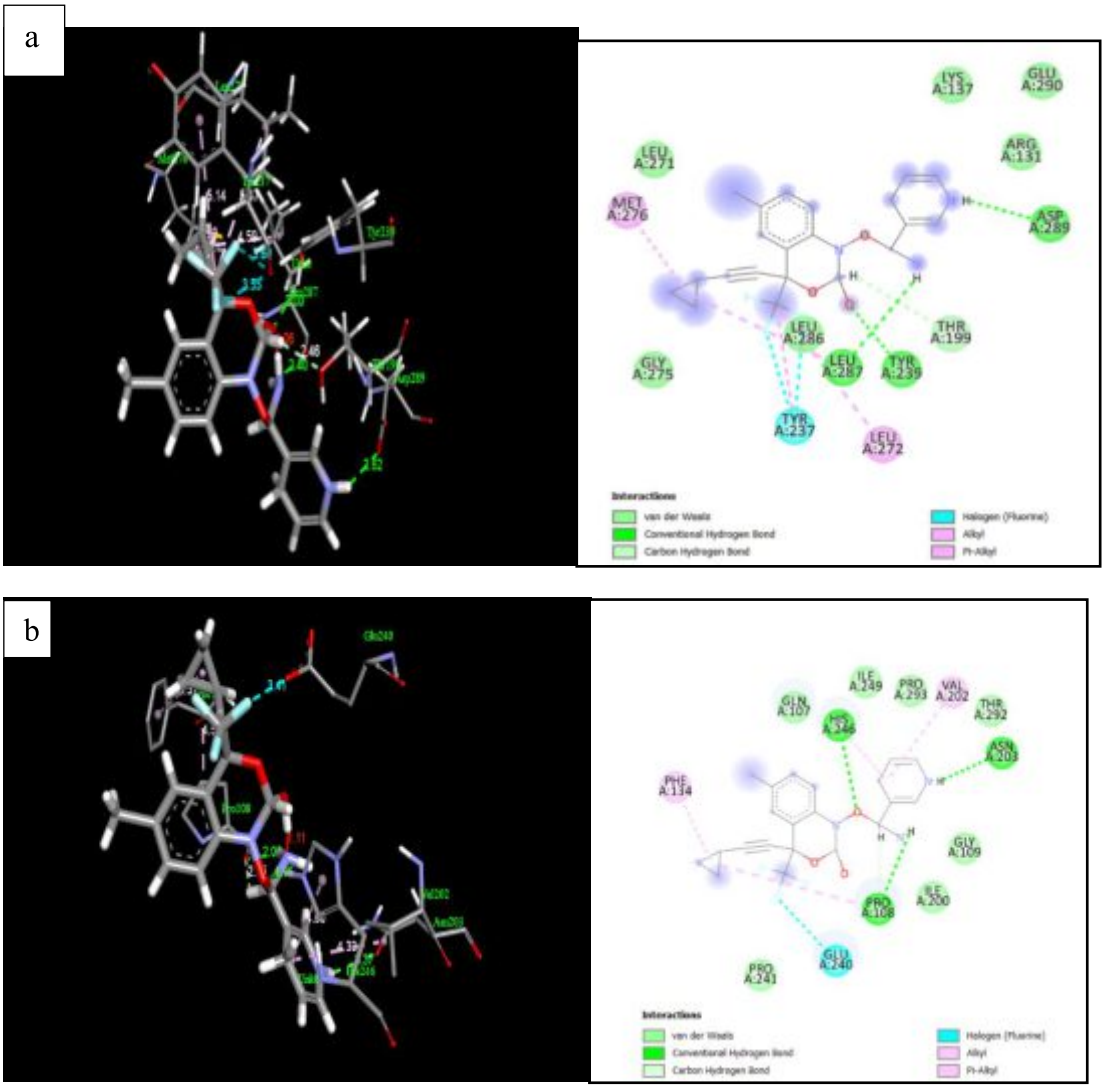


Fig.6. Docking poses of newly synthesised pharmaceutical cocrystal ENCOC with SARS CoV-2 protein (a) 5r81 and (b) 5r82

Table.2. Ligand Protein binding interaction of EFV and ENCOC with SARS CoV-2 protein

Name of Ligand	COVID 19 protein	Binding affinity (kcal/mol)	Interacting Moieties	Type of Bond/ Interaction	Amino acid residue	Bond length (Å)
EFV	5r81	-7.1	C=O	H- Bond	Thr-111	2.78
			C-O	H- Bond	Arg-298	2.44
			C-F	H- Bond Halogen (F)	Arg-298 Asn-151	2.77 3.15
			C-H	Pi- Alkyl	Phe-294	3.99
	5r82	-6.6	C-O	H- Bond	Gly-143	2.47
			CF_3	H- Bond Halogen (F) Halogen (F)	Cys-145 Gly-143 His-41 Thr-26	2.80 2.87 2.87 2.90
			C-H	Pi- Alkyl Alkyl	His-41 Met-49 Cys-44	4.87 4.39 4.81
ENCOC	5r81	-7.2	NH	H-Bond	Asp-289	2.62
			NH_2	H- Bond	Leu-287	2.40
			C=O	H- Bond	Tyr-239	2.00
			CH	Van der Waals	Thr-199	2.46
			CF_3	Halogen (F)	Tyr-237	3.53 3.60
			CH	Alkyl Alkyl	Leu-287 Met-276	4.59 4.93
	5r82	-7.7	NH	H- Bond	Pro-108 Asn-203	2.06 2.37
			C=O	H- Bond	His-246	1.95
			Ar- Ring	Alkyl Pi- Alkyl	Val-202 His-246	4.33 4.90
			CF_3	Halogen (F)	Glu-240	3.41
			CH	Alkyl	Pro-108	4.55

The *in silico* screening of EFV and ENCOC shows the potential activity against both HIV and SARS CoV-2. The carboxylic acid (C=O) and amino (NH_2) group of coformer NICO used in synthesis of ENCOC plays the crucial role in H-bonding of ENCOC with HIV and SARSCoV-2 protein. Hence, we recommended further studies on the application of pharmaceutical co-crystallization for enhanced *in vivo* pharmacokinetic activity of approved antiviral agent against SARS CoV-2.

CONCLUSION

The antiviral drug EFV's cocrystal was created in the current work employing liquid assisted grinding with methanol as the solvent. In order to increase the solubility of EFV, the vitamin B3 nicotinamide, which is easily water-soluble, was utilized as a coformer. *in silico* screening we determined the strong binding affinity of EFV and ENCOC with both HIV-1 reverse transcriptase and main protease of SARS CoV-2 that confirms the activity of EFV and ENCOC are highly active against HIV-1 and SARS CoV-2 and warrants *in vivo* study for estimation of activity against SARS CoV-2.

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RESEARCH ARTICLE

VALIDATION AND ASSESSMENT OF STABILITY INDICATING RP-HPLC METHOD FOR SALBUTAMOL SULFATE IN INHALATIONAL SOLUTION

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Abstract

RP-HPLC SIAM was developed and validated for the assessment of salbutamol sulfate in inhalational solution on an adopted compendial method. The method was tested for its feasibility and specificity to separate the principal component, related substances and impurities from the prepared in-house formulation undergoing stability studies. The studies elaborate feasibility testing of adopted method optimization of forced degradation studies to achieve 10-30% degradation of API as such and in formulation. The API and formulation were exposed to stressful condition for 7 days and they were analyzed using a waters symmetry C_8 (150mm X 3.9mm, 5µm) column at 25°C on a flow rate of 1.0mL/min for over 50 minutes. The mobile phase was prepared by mixing 220 mL acetonitrile and 780 mL phosphate buffer (pH 3.65). Detection wavelength used was 220nm. The optimized method was validated as per the ICH guidelines(Q2A).

Keywords: Salbutamol sulfate, RP-HPLC, SIAM, Forced degradation

INTRODUCTION

Salbutamol sulfate is a beta-2 adrenergic receptor agonist class of drug used as bronchodilator in treatment of asthma. The IUPAC name of salbutamol is 4-[2-(*tert*-butylamino)-1-hydroxyethyl]-2(hydroxymethyl)phenol-sulfate (Figure 1).

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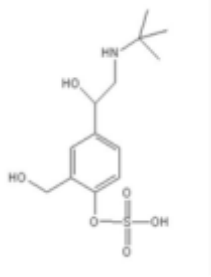

Figure 1. Structure of Salbutamol

It is used as a bronchodilator in management of asthma and various chronic obstructive respiratory disorders. R-isomer show potent broncho-dilatory activity whereas S-isomer increases reactivity of bronchi. It stimulates beta-2 adrenergic cyclase which catalyzes enzyme adenylate cyclase which catalyzes ATP conversion into cAMP which stimulates cAMP resulting into relaxation of bronchial muscles and reliving spasm. Salbutamol sulfate is administered either by oral or nasal route. It is available in tablets, capsules, metered dose inhalers and nebulizer solution dosage form in varying strength of 0.5mg/mL to 5mg/mL with pH of inhalational formulations between 3 to 4 [1][2].

EXPERIMENTAL

Materials

Salbutamol Sulphate (99% purity) was gifted by Aculife healthcare Pvt. Ltd. (Ahmedabad, India). HPLC grade acetonitrile was procured from Finar Chemicals (Ahmedabad, India). Analytical reagent grade sodium hydroxide, hydrochloric acid, hydrogen peroxide was procured from Rankem (Mumbai, India). HPLC grade sodium heptane sulphonic acid and potassium dihydrogen ortho phosphate was procured from Finar Chemicals (Ahmedabad, India). MilliQ water purified by Milli Q water purification system (Merck Germany) was used for the preparation of various solutions.

Instrumentation

The HPLC system LC-2010 HT with SPD-M20A diode array detector (Shimadzu, Kyoto, Japan) was used. A reversed phase Waters Symmetry C_8 (150mm x 3.9mm; 5µm) column was used for separation of compounds. The chromatographic data was recorded on Lenovo computer system with lab solution data acquiring software (Shimadzu, Kyoto, Japan). Photo stability chamber (Thermolab Scientific equipment, Palghar, India) was used for the experiment.

Forced degradation study

Forced degradation study of salbutamol sulfate API and inhalation formulation was carried under conditions such as oxidative, photolytic, hydrolysis and thermal degradation. Oxidation was carried out by employing 3% hydrogen peroxide solution, for thermal degradation, samples were kept at 70°C. For photo degradation, samples were exposed to ultra violet light for 20 watt hours and for 1.2 million lux hours. Hydrolysis of the samples was carried for acid hydrolysis and base hydrolysis by using 0.1M HCl solution for acid and 0.1M

NaOH solution for base hydrolysis respectively. The studies were carried out for 7 days for oxidation, thermal and hydrolysis degradation whereas the samples were exposed to photo degradation until the programmed exposure ended in photostability chamber. The optimized forced degradation conditions are outlined in Table 1 [3] [4][5][6].

Table1: Stress conditions employed in forced degradation studies

Degradation type	Condition	Study duration
Hydrolysis	0.1M Acid	7 Days
	0.1M Base	7 Days
Oxidative	3% H_2O_2	7 Days
Photo	200-watt hours & 1.2 Million lux hours	Until completion of both the exposure parameters (approx. 6 days)
Thermal	70°C	7 Days

Chromatographic parameters

Waters symmetry C_8 (150mm x 3.9mm, 5µm) column was used for the elution of the analytes. Elution was carried out at 25°C oven temperature, with mobile phase flow rate 1.0 mL/min. 20 µL sample was injected into the system and run time of chromatographic elution was 50min. The column eluent was monitored at 220nm [7][8].

Solution preparation

Buffer was prepared by taking 2.87 gm of sodium heptane sulphonate and 2.5gm of potassium dihydrogen ortho phosphate and dissolving it in 1000 mL water. The content was mixed well and pH was adjusted to 3.65 with 2M orthophosphoric acid. Mobile phase was prepared by taking mixture of 220mL acetonitrile and 780 mL of buffer. The mobile phase mixture was sonicated to degas before use. Mobile phase was also used as a diluent for various solution preparation. Standard solution of drug was prepared by taking 20.0 mg of working standard of salbutamol sulphate and transferring it into a 50 mL volumetric flask. Few mL of mobile phase diluent was added to it and drug was dissolved completely using sonication. 3.0 mL of this solution was taken and diluted up to 20 mL with diluent. From this 20 mL solution, 1.0 mL was taken and diluted up to 10 mL with diluent. The final solution was labelled as standard solution.

Drug sample preparation was carried out by taking 5 mL of sample solution and diluting it up to 20 mL using diluent.

Placebo / blank preparation was carried out by accurately transferring 5mL diluent to various 20 mL volumetric flasks and adding each of degrading solutions i.e. 0.1 M HCl, 0.1M NaOH, 3% H_2O_2 in an individual flask. The solutions were kept for 7 days in the same experimental conditions. For photo and thermal degradation blank samples, the diluent solution was kept as such in a flask under defined experimental conditions.

Method validation

System suitability

To check the system suitability of the method a standard solution of 3 µg/mL of the salbutamol sulphate was prepared as per the method mentioned above six replicate injections were injected in HPLC.

Specificity

Specificity was carried out by injecting 3 µg/mL standard salbutamol solution, placebo and sample solution as per the experimental method. The peaks were analyzed using PDA detector. Standard impurities were spiked in the standard solution and peaks due to the impurities and solution of principle component were identified.

Linearity

Linearity of the method was check at 5 levels 25% to 150% for concentration range from 1.250 µg/mL to 3.750 µg/mL. Linearity solutions were prepared as per table 2. A linearity curve was plotted for concentration Vs peak area at each level. A linearity stock solution was prepared by diluting 7.5 mL of principal standard solution to 30 mL using diluent.

Table2. Linearity solution preparation

Linearity level	Volume of linearity stock	Dilution	Concentration of solution (µg/mL)
Level-1 (25%)	2	10	1.250
Level-2 (50%)	3	10	1.875
Level-3 (100%)	4	10	2.500
Level-4 (125%)	5	10	3.125
Level-5 (150%)	6	10	3.750

Precision

For method precision standard, sample and placebo solutions were prepared as per the method and injected in HPLC system. Six replicates of standard solutions were injected to establish system % RSD, NTEP, tailing factor and difference between retention time. %RSD of six results for each individual impurity and total impurity were calculated.

Accuracy

Accuracy of the method was studied at 3 levels from 50% to 100% for concentration range from 0.25% to 1.0 %. Each set was injected as triplicate and % recovery of impurity D, mean % recovery and %RSD for each level was calculated.

LOD and LOQ

Three sets of LOD and LOQ at 0.025% and 0.050 % concentrations were prepared and injected and S/N ratio of 3 and 10 were determined for LOD and LOQ respectively.

Robustness

Robustness study was performed for variation in column oven temperature, variation in flow rate, variation in pH of buffer in mobile phase and variation in composition by ±5% change in above all conditions [9].

RESULTS AND DISCUSSION

An ICH guideline Q2A was followed for validation and assessment of the method. To validate the method all recommended parameters such as system suitability, specificity, linearity, limit of detection, limit of quantification, robustness, precision and accuracy of the method were analyzed and evaluated. Which gave a documented evidence that the developed method is accurate and give precise results being robust to ±5% variation in methods parameters such that they do not affect the specificity of the method and gives accurate and precise results within the range. The linearity of the method lies in range of 1.254-3.762 µg/mL with regression coefficient to be 0.99 and equation of linearity be y = 36852x + 776 (Figure 2).

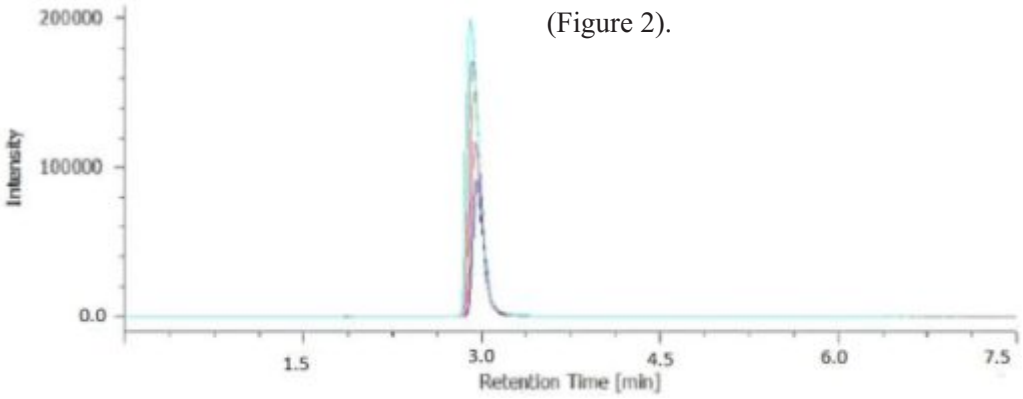

Figure 2. Linearity chromatogram for salbutamol sulphate (1.254-3.762 µg/mL)

The method was found to be accurate with recovery range of 98.2-100.4% calculated on results obtained in triplicate injection of three accuracy level at 50, 100 and 150% accuracy levels. The method passes intraday precision with %RSD for the peak area of the impurity D, max unknown impurity and total impurities is found to be 0.10%, 0.00% and 0.20% respectively and intermediate precision with %RSD for the peak area of the impurity D, max unknown impurity and total impurities is found to be 0.37%, 1.04% and 0.38% respectively. Mass balance and total impurity for API and sample is given in Table 3 and Table 4 respectively.

Table 3. Total impurities and mass balance for API

Conditions	Assay %	Total Impurity %	Total Assay %	As Such Assay %	Mass Balance %
Acid	102.2	0.069	102.27	99.4	2.87
Alkali	91.0	2.8.1	93.80	99.4	-5.60
Peroxide	100.5	0..92	100.59	99.4	1.19
Photo	101.4	0.081	101.48	99.4	2.08
Thermal	101.8	0.108	101.91	99.4	2.51

Table 4. Total impurities and mass balance for sample

Conditions	Assay %	Total Impurity %	Total Assay %	As Such Assay %	Mass Balance %
Acid	100.3	0.207	100.51	100.4	0.11
Alkali	98.1	2.104	100.20	100.4	-0.20
Peroxide	98.4	0.593	98.99	100.4	-1.14
Photo	98.9	0.356	99.26	100.4	-1.14
Thermal	97.7	0.355	98.06	100.4	-2.34

Range of the method rom the results obtained in linearity and accuracy study, it is concluded that the analytical procedure is precise, accurate and linear from 0.13% to 0.752% for determination of related substances of salbutamol sample solution.

CONCLUSION

Safety and efficacy of any drug substance or product highly depends upon the chemical stability of the pharmaceutical molecule which is of great concern in

terms of impurity and its self-life. A method indicating stability of the preparation capable of separating all the process and product related impurities from the principle component is developed and validated for the cause. The study shows that our previous method is successfully extended to determine salbutamol in aqueous solution as pharmaceutical preparation. The method was found to be robust for the experimental operating conditions over range of ±5% of the optimized conditions. The method is precise and accurate as on repeated performance it is giving reproducible results precisely and accurately. From the results obtained in validation studies it can be said that method is fit for use in quality control department for routine analysis and as method for analysis of sample in batch processing. The forced degradation study of API and finished product shows that there is no such potential impurity generated in the samples which shows that the formulation is well optimized and stable and also withholds with the stressful conditions exposed. The method is employed in separation and detection gives feasible results and from the forced degradation study and results obtained in validation it can be concluded that the method is stability indicating and separates analytes accurately.

CONFLICTS OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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