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METHOD DEVELOPMENT AND VALIDATION FOR THE QUANTITATIVE ESTIMATION OF LENALIDOMIDE IN BULK FORM AND MARKETED PHARMACEUTICAL DOSAGE FORM BY USING RP-HPLC

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Abstract:

A new, simple, rapid, precise, accurate and reproducible RP-HPLC method for estimation of Lenalidomide in bulk form and marketed formulation. Separation of Lenalidomide was successfully achieved on a Symmetry ODS C_{18} (4.6 x 250mm, 5µm) column in an isocratic mode of separation utilizing Acetonitrile: Methanol in the ratio of 80:20% v/v at a flow rate of 1.0 mL/min and the detection was carried out at 272nm. The method was validated according to ICH guidelines for linearity, sensitivity, accuracy, precision, specificity and robustness. The response was found to be linear in the drug concentration range of 10-50mcg/mL for Lenalidomide. The correlation coefficient was found to be 0.999 for Lenalidomide. The LOD and LOQ for Lenalidomide were found to be 1.1µg/mL and 3.2µg/mL respectively. The proposed method was found to be good percentage recovery for Lenalidomide, which indicates that the proposed method is highly accurate. The specificity of the method shows good correlation between retention times of standard solution with the sample solution. Therefore, the proposed method specifically determines the analyte in the sample without interference from excipients of pharmaceutical dosage forms.

Keywords: *Lenalidomide, RP-HPLC, Accuracy, Precision, Robustness, ICH Guidelines.*

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INTRODUCTION:

Lenalidomide (previously referred to as CC-5013) is an immunomodulatory drug with potent antineoplastic, anti-angiogenic, and anti-inflammatory properties [1]. It is a 4-amino-glutamyl analogue of thalidomide 3 and like thalidomide, Lenalidomide exists as a racemic mixture of the active S (-) and R (+) forms. However, Lenalidomide is much safer and potent than thalidomide, with fewer adverse effects and toxicities. Thalidomide and its analogues, including Lenalidomide, are referred to as immunomodulatory imide drugs (also known as cereblon modulators), which are a class of immunomodulatory drugs that contain an imide group[2]. Lenalidomide works through various mechanisms of actions that promote malignant cell death and enhance host immunity. Lenalidomide is a dicarboximide that consists of 1-oxoisoindoline bearing an amino substituent at position 4 and a 2, 6-dioxopiperidin-3-yl group at position 2. Inhibits the secretion of TNF-alpha. It has a role as an angiogenesis inhibitor, an antineoplastic agent and an immunomodulator [3]. It is a member of isoindoles, a dicarboximide, a member of piperidones and an aromatic amine. The IUPAC Name of Lenalidomide [3] is 3-(7-amino-3-oxo-1H-isoindol-2-yl) piperidine-2, 6-dione. The Chemical Structure of Lenalidomide is as follows

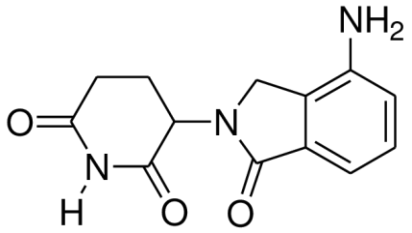

Fig-1: Chemical Structure of Lenalidomide

EXPERIMENTAL METHODS:

Instruments Used

Table-1: Instruments Used

S.No.	Instruments and Glass wares	Model
1	HPLC	WATERS Alliance 2695 separation module, Software: Empower 2, 996 PDA Detector.
2	pH meter	Labindia
3	Weighing machine	Sartorius
4	Volumetric flasks	Borosil
5	Pipettes and Burettes	Borosil
6	Beakers	Borosil
7	Digital ultra sonicator	Labman

Chemicals Used:

Table-2: Chemicals Used

S.No.	Chemical	Brand Names
1	Lenalidomide (Pure)	Synpharma Research Lab, Hyderabad
2	Water and Methanol for HPLC	LICHROSOLV (MERCK)
3	Acetonitrile for HPLC	Merck

HPLC Method Development:

Preparation of Standard Solution:

Accurately weigh and transfer 10 mg of Lenalidomide working standard into a 10ml of clean dry volumetric flasks add about 7ml of Methanol and sonicate to dissolve and removal of air completely and make volume up to the mark with the same Methanol. Further pipette 0.3ml of the above Lenalidomide stock solutions into a 10ml volumetric flask and dilute up to the mark with Methanol.

Preparation of Sample Solution:

Take average weight[4] of the Powder and weight 10 mg equivalent weight of Lenalidomide sample into a 10mL clean dry volumetric flask and add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. Further pipette 0.3ml of the above Lenalidomide stock solutions into a 10ml volumetric flask and dilute up to the mark with Methanol.

Procedure:

Inject the samples[5] by changing the chromatographic conditions and record the chromatograms, note the conditions of proper peak elution for performing validation parameters as per ICH guidelines.

Mobile Phase Optimization:

Initially the mobile phase tried was methanol: Water and ACN: Water with varying proportions. Finally, the mobile phase was optimized to ACN: Methanol 80:20% v/v) respectively.

Optimization of Column:

The method[6] was performed with various C18 columns like Symmetry, Zodiac and Xterra. Symmetry ODS C18 (4.6 x 250mm, 5µm) Column was found to be ideal as it gave good peak shape and resolution at 1ml/min flow.

Preparation of Mobile Phase:

Accurately measured 800 ml (80%) of HPLC[7] Acetonitrile and 200 ml of Methanol (20%) were mixed and degassed in a digital ultra sonicater for 15 minutes and then filtered through 0.45 µ filter under vacuum filtration.

Diluent Preparation:

The Mobile phase[8] was used as the diluent.

Validation Parameters

System Suitability

Accurately weigh and transfer 10 mg of Lenalidomide working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)**.** Further pipette 0.3ml of the above Lenalidomide stock solutions[9] into a 10ml volumetric flask and dilute up to the mark with Methanol.

Procedure:

The standard solution was injected for five times and measured the area for all five injections in HPLC. The %RSD for the area of five replicate injections was found to be within the specified limits[10].

Specificity:

Preparation of Standard Solution:

Accurately weigh and transfer 10 mg of Lenalidomide working standard into a 10ml of clean dry volumetric flasks add about 7ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.3ml of the above Lenalidomide stock solutions into a 10ml volumetric flask and dilute up to the mark with Methanol.

Preparation of Sample Solution:

Take average weight of the Powder and weight 10 mg equivalent weight of Lenalidomide sample into a 10mL clean dry volumetric flask and add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. Further pipette 0.3ml of the above Lenalidomide stock solutions into a 10ml volumetric flask and dilute up to the mark with Methanol.

Procedure:

Inject the five replicate injections of standard and inject the three replicate injections sample solutions and calculate the assay [11-13] by using formula:

$$\%ASSAY = \frac{\text{Sample area}}{\text{Standard area}} \times \frac{\text{Weight of standard}}{\text{Dilution of standard}} \times \frac{\text{Dilution of sample}}{\text{Weight of sample}} \times \frac{\text{Purity}}{100} \times \frac{\text{Weight of tablet}}{\text{Label claim}} \times 100$$

Linearity:

Accurately weigh and transfer 10 mg of Lenalidomide working standard into a 10ml of clean dry volumetric flasks add about 7ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent[14]. (Stock solution)

Preparation of Level – I (10ppm of Lenalidomide):
Take 0.1ml of stock solution in to 10ml of volumetric flask and make up the volume up to mark with diluent.
Preparation of Level – II (20ppm of Lenalidomide):
Take 0.2ml of stock solution in to 10ml of volumetric flask and make up the volume up to mark with diluent.
Preparation of Level – III (30ppm of Lenalidomide):
Take 0.3ml of stock solution in to 10ml of volumetric flask and make up the volume up to mark with diluent.
Preparation of Level – IV (40ppm of Lenalidomide):
Take 0.4ml of stock solution in to 10ml of volumetric flask and make up the volume up to mark with diluent.
Preparation of Level – V (50ppm of Lenalidomide):
Take 0.5ml of stock solution in to 10ml of volumetric flask and make up the volume up to mark with diluent[15].

Procedure:
Inject each level into the chromatographic system[16] and measure the peak area. Plot a graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) and calculate the correlation coefficient[17].

Precision:
Repeatability:
Preparation of Lenalidomide Product Solution for Precision:
Accurately weigh and transfer 10 mg of Lenalidomide working standard into a 10ml of clean dry volumetric flasks add about 7ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Take 0.3ml of stock solution in to 10ml of volumetric flask and make up the volume up to mark with diluent.

The standard solution was injected for six times and measured the area for all six injections in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits.

Intermediate Precision:
To evaluate the intermediate precision (also known as Ruggedness) of the method, Precision was performed on different days by maintaining same conditions.

Procedure:
Analyst 1:
The standard solution was injected for six times and measured the area for all six injections in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits.
Analyst 2:
The standard solution was injected for six times and measured the area for all six injections in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits.

Accuracy:
For Preparation of 50% Standard Stock Solution:
Accurately weigh and transfer 10 mg of Lenalidomide working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)**.** Take 0.15ml of stock solution in to 10ml of volumetric flask and make up the volume up to mark with diluent.
For Preparation of 100% Standard Stock Solution:
Accurately weigh and transfer 10 mg of Lenalidomide working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)
Take 0.3ml of stock solution in to 10ml of volumetric flask and make up the volume up to mark with diluent.
For Preparation of 150% Standard Stock Solution:
Accurately weigh and transfer 10 mg of Lenalidomide working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)**.** Take 0.45ml of stock solution in to 10ml of volumetric flask and make up the volume up to mark with diluent.

Procedure:
Inject the Three replicate injections of individual concentrations (50%, 100%, 150%) were made under the optimized conditions. Recorded the chromatograms and measured the peak responses. Calculate the Amount found and Amount added for Lenalidomide and calculate the individual recovery and mean recovery values.
Limit of Detection (LOD) and Limit of Quantification (LOQ):
Preparation of 0.597µg/ml solution (LOD):

Accurately weigh and transfer 10 mg of Lenalidomide working standard into a 10ml of clean dry volumetric flasks add about 7ml of Methanol and sonicate to dissolve and removal of air completely and make volume up to the mark with the same Methanol.

Further pipette 0.00597ml of the above Lenalidomide stock solutions into a 10ml volumetric flask and dilute up to the mark with Methanol.

Preparation of 1.811µg/ml solution (LOQ):

Accurately weigh and transfer 10 mg of Lenalidomide working standard into a 10ml of clean dry volumetric flasks add about 7ml of Methanol and sonicate to dissolve and removal of air completely and make volume up to the mark with the same Methanol. Further pipette 0.01811ml of the above Lenalidomide stock solutions into a 10ml volumetric flask and dilute up to the mark with Methanol.

Robustness:

The analysis was performed in different conditions to find the variability of test results. The following conditions are checked for variation of results. .

For Preparation of Standard Solution:

Accurately weigh and transfer 10 mg of Lenalidomide working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Take 0.3ml of stock solution in to 10ml of volumetric flask and make up the volume up to mark with diluent.

Effect of Variation of Flow Conditions:

The sample was analyzed at 0.9ml/min and 1.1ml/min instead of 1ml/min, remaining conditions are same. 20µl of the above sample was injected and chromatograms were recorded.

Effect of Variation of Mobile Phase Organic Composition:

The sample was analyzed by variation of mobile phase i.e. ACN: Methanol was taken in the ratio and 75:25, 85:15 instead of 80:20, remaining conditions are same. 20µl of the above sample was injected and chromatograms were recorded.

RESULTS AND DISCUSSION:

Method Development:

Optimized Chromatographic Conditions:

Column	:	Symmetry ODS C18 (4.6 x 250mm, 5µm)
Column temperature	:	Ambient
Wavelength	:	272 nm
Mobile phase ratio	:	ACN: Methanol (80:20% v/v)
Flow rate	:	1.0mL/min
Injection volume	:	20 µl
Run time	:	8 minutes

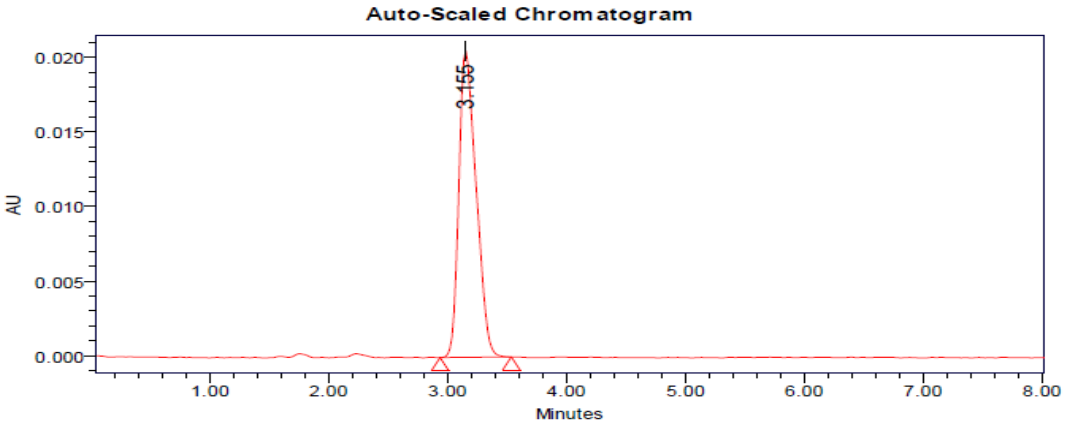

Fig-2: Optimized Chromatographic Condition

Validation of Analytical Method:

The optimized method [18] for determination of Lenalidomide has been validated as per International Conference of Harmonisation (ICH) guidelines[32,33] Q2 (R1) for evaluating system suitability, specificity, precision, accuracy, linearity, limit of detection (LOD), limit of quantitation (LOQ) and robustness.

System Suitability:

System suitability parameters with respect to tailing factor, repeatability, number of theoretical plates and resolution between Lenalidomide peak was assessed [19] by injecting a five replicates of Lenalidomide (30 µg/ml).

Table-3: Results of System Suitability for Lenalidomide

S.No.	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Lenalidomide	3.192	225645	20584	6286	1.38
2	Lenalidomide	3.146	225847	20965	6358	1.39
3	Lenalidomide	3.123	228656	20758	6285	1.41
4	Lenalidomide	3.167	228547	20859	6278	1.40
5	Lenalidomide	3.158	229658	20968	6395	1.42
Mean			227670.6			
Std. Dev.			1810.899			
% RSD			0.795403			

Specificity:

The ICH documents define specificity as the ability to assess unequivocally the analyte in the presence of components that may be expected to be present, such as impurities, degradation products, and matrix components. Analytical method was tested for specificity[20] to measure accurately quantitates Lenalidomide in drug product.

$$\%ASSAY = \frac{\text{Sample area}}{\text{Standard area}} \times \frac{\text{Weight of standard}}{\text{Dilution of standard}} \times \frac{\text{Dilution of sample}}{\text{Weight of sample}} \times \frac{\text{Purity}}{100} \times \frac{\text{Weight of tablet}}{\text{Label claim}} \times 100$$

= 99.24%

The % purity of Lenalidomide in pharmaceutical dosage form was found to be 99.24%.

Linearity:

Chromatographic Data for Linearity Study:

Table-4: Data for Linearity

Concentration µg/ml	Average Peak Area
10	78683
20	146545
30	213584
40	279895
50	346568

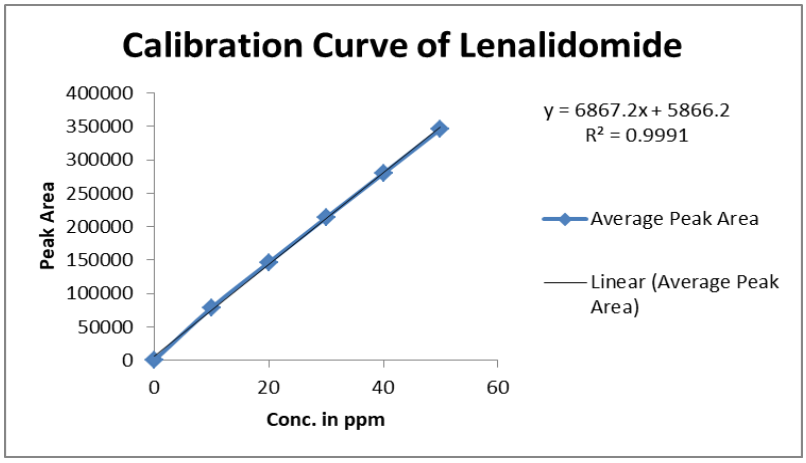

Fig-3: Calibration Curve of Lenalidomide

Linearity Plot:
The plot of Concentration (x) versus the Average Peak Area (y) data of Lenalidomide is a straight line[21].

Y = mx + c
Slope (m) =6867
Intercept (c) = 5866
Correlation Coefficient (r) = 0.99

Validation Criteria: The response linearity[22] is verified if the Correlation Coefficient is 0.99 or greater.

Conclusion: Correlation Coefficient (r) is 0.99, and the intercept is 5866. These values meet the validation criteria.

Precision:
The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions[23].

Repeatability:
Obtained Six (6) replicates of 100% accuracy solution as per experimental conditions. Recorded the peak areas and calculated % RSD [24].

Table-4: Results of Method Precision for Lenalidomide:

S. No	Peak Name	Retention time	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Lenalidomide	3.165	225645	20562	6125	1.36
2	Lenalidomide	3.163	225847	20645	6129	1.36
3	Lenalidomide	3.158	226542	20534	6135	1.35
4	Lenalidomide	3.167	226598	20564	6189	1.36
5	Lenalidomide	3.171	226584	20549	6138	1.35
6	Lenalidomide	3.181	226859	20685	6179	1.37
Mean			226345.8			
Std. Dev			482.1068			
%RSD			0.212996			

Intermediate Precision:
Analyst 1:

Table-5: Results of Ruggedness for Lenalidomide

S.No	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Lenalidomide	3.165	226534	20653	6235	1.35
2	Lenalidomide	3.163	226542	20598	6198	1.36
3	Lenalidomide	30158	225989	20653	6254	1.36
4	Lenalidomide	3.167	226512	20548	6281	1.35
5	Lenalidomide	3.171	226531	20653	6199	1.36
6	Lenalidomide	3.171	225898	20658	6253	1.35
Mean			226334.3			
Std. Dev.			304.2622			
% RSD			0.13443			

Analyst 2:

Table-6: Results of Intermediate Precision Analyst 2 for Lenalidomide

S.No.	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate count	USP Tailing
1	Lenalidomide	3.173	225487	20542	6253	1.35
2	Lenalidomide	3.134	225484	20532	6098	1.36
3	Lenalidomide	3.161	225364	20541	6254	1.35
4	Lenalidomide	3.174	226513	20534	6235	1.36
5	Lenalidomide	3.199	225487	20549	6199	1.36
6	Lenalidomide	3.199	226532	20451	6235	1.35
Mean			225811.2			
Std. Dev.			553.0524			
% RSD			0.244918			

Accuracy:

Accuracy [25] at different concentrations (50%, 100%, and 150%) was prepared and the % recovery was calculated.

Table-7: The Accuracy Results for Lenalidomide

%Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	109283.3	15	15.060	100.40%	100.42%
100%	212732	30	30.124	100.413%	
150%	316263.3	45	45.201	100.446%	

Limit of Detection for Lenalidomide

The detection limit[26] of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value.

$$LOD = 3.3 \times \sigma / s$$

Where

σ = Standard deviation of the response

S = Slope of the calibration curve

Result: = 0.597µg/ml

Limit of Quantitation for Lenalidomide

The quantitation limit[27] of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined.

$$LOQ = 10 \times \sigma / S$$

Where

σ = Standard deviation of the response

S = Slope of the calibration curve

Result: = 1.811µg/ml

Robustness

The robustness [29] was performed for the flow rate variations from 0.9 ml/min to 1.1ml/min and mobile phase ratio variation from more organic phase to less organic phase ratio for Lenalidomide. The method is robust only in less flow condition [30] and the method is robust even by change in the Mobile phase ±5%. The standard and samples of Lenalidomide were injected by changing the conditions of chromatography [31]. There was no significant change in the parameters like resolution, tailing factor, asymmetric factor, and plate count.

Table-8: Results for Robustness

Parameter Used for Sample Analysis	Peak Area	Retention Time	Theoretical Plates	Tailing Factor
Actual Flow rate of 1.0 mL/min	225645	3.155	6125	1.36
Less Flow rate of 0.9 mL/min	236586	3.488	6452	1.38
More Flow rate of 1.1 mL/min	219865	2.877	6098	1.42
Less organic phase	235848	4.705	6126	1.43
More organic phase	241245	2.090	6324	1.39

SUMMARY:

The analytical method was developed by studying different parameters. First of all, maximum absorbance was found to be at 272nm and the peak purity was excellent. Injection volume was selected to be 20µl which gave a good peak area. The column used for study was Symmetry ODS C18 (4.6 x 250mm, 5µm) because it was giving good peak. Ambient temperature was found to be suitable for the nature of drug solution. The flow rate was fixed at 1.0ml/min because of good peak area and satisfactory retention time. Mobile phase is Acetonitrile: Methanol (80:20% v/v) was fixed due to good symmetrical peak. So this mobile phase was used for the proposed study. Run time was selected to be 8.0min because analyze gave peak around 3.155 and also to reduce the total run time. The percent recovery was found to be 98.0-102 was linear and precise over the same range. Both system and method precision were found to be accurate and well within range. The analytical method was found linearity over the range of 10-50µg/ml of the Lenalidomide target concentration. The analytical passed both robustness and ruggedness tests. On both cases, relative standard deviation was well satisfactory.

CONCLUSION:

In the present research, a fast, simple, accurate, precise, and linear HPLC method has been developed and validated for Lenalidomide, and hence it can be employed for routine quality control analysis. The analytical method conditions and the mobile phase solvents provided good resolution for Lenalidomide. In addition, the main features of the developed method are short run time and retention time around 8 min. The method was validated in accordance with ICH guidelines. The method is robust enough to reproduce accurate and precise results under different chromatographic conditions. Hence the proposed RP-HPLC method proved to be simple, accurate and reproducible for the determination of Lenalidomide in a reasonable run time. The method was validated showing satisfactory data for all the method validation parameters tested. The developed method can be conveniently used by quality control laboratories.

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