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Development and Validation of Estimation of Genotoxic Impurity (Benzimidamidecontent) in Leflunomide by Using RP-HPLC Technique

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

The measurement of Genotoxic contaminant, a simple, selective, linear, accurate, and specific reverse phase high-performance liquid chromatographic (RP-HPLC) process was proposed. A Benzimidamide impurity in the medication Leflunomide has been discovered. Separation and analysis were carried out on Zorbax SB phenyl (4.6 mm x 250 mm) with a particle size of 5.0 µm. with 0.1 % Triethylamine in purified water with a pH of 7.0 and a buffer of phosphoric acid (20% in water). The mobile phase is a 40:60 mixture of buffer and Acetonitrile with degassing. Isocratic program mode was used. The elution was carried out at a rate of 1.0 mL/min with UV detection at a wavelength of 289 nm. The temperature of the selected column oven is 25°C. The linearity and accuracy of Benzimidamide are covered in this approach, with a LOQ limit of 150 percent (i.e.0.03 to 0.45 ppm). The observed correlation coefficient is 0.99994, with a range of 100.01 to 104.8 for recovery. The measured percent RSD of six spiked test preparation is below 5.0 percent in procedure precision (i.e. repeatability) and intermediate precision (IP). When maintained at room temperature, the standard and sample remained stable for three days. System appropriateness characteristics such as tailing factor and percent RSD do not exhibit significant changes in robustness experiments. For the detection of Benzimidamide the present RP-HPLC method is act as selective, robust, linear and precise.

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Keywords: Benzimidamide; RP-HPLC; stability indicating; genotoxic impurity.

1. INTRODUCTION

Leflunomide (Fig. 1) is a Immune suppressive, anti-rheumatic (DMARD) drug [1] that is primarily used to treat active moderate-to-severe rheumatoid arthritis [2,3] and psoriatic arthritis. 4-Isoxazolecarboxamide, 5-Methyl-N-[4-(triflurомethyl)-phenyl] is the chemical name for the drug Leflunomide. It functions as a pyrimidine synthesis inhibitor by inhibiting dihydroorotate dehydrogenase. Leflunomide has the chemical formula C12H9F3N2O2 and a molecular weight of 270.21 Leflunomide [4] is made from the starting materials 4-(triflurомethyl) aniline (TFMA) and 5-Methylisoxazole-4-carboxylic acid (5-MIA) in the production method. This Benzimidamide impurity is probable impurities and reveals the potential genotoxic agent in the essential starting material TFMA. As a result, the presence of this Benzimidamide impurity in Leflunomide must be identified and investigated. There is no specific method for determining the amount of Benzimidamide in Leflunomide in the literature. The goal of this research is to create a sensitive, and validated RP-HPLC method for determining the level of Benzimidamide impurity in Leflunomide.

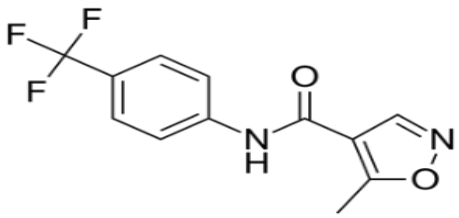

Fig. 1. Leflunomide Chemical structure

2. MATERIALS AND METHODS

Emcure pharmaceuticals ltd, R & D, Hinjawadi, Pune provided a sample of Leflunomide and its impurity for development and validation. For mobile phase and diluent preparations, analytical grade ortho phosphoric acid, trimethylamine, acetonitrile, and purified water (HPLC grade) were employed. Gradient acetonitrile and triethylamine were used. Make-metler Toledo and waters HPLC with UV/ PDA detector and data acquisition, computation with Chromeleon software are used as analytical balances. Before they were utilised, all of the instruments were calibrated.

2.1 Preparation of Mobile Phase

Dilute Ortho phosphoric acid (OPA).
Prepare 20 % of OPA in purified water. Mix well

2.2 Buffer Preparation

Mix 1 ml of triethylamine with 1000 ml of water, mix it and Degas. PH 7.0 (± 0.05) is adjusted by addition of dilute ortho phosphoric acid.

2.3 Mobile Phase Preparation

Prepare homogeneous mixture of buffer preparation and acetonitrile in ratio (40:60) mix and degas.

2.4 Diluent Preparation

Use acetonitrile as diluent.

2.5 Blank Preparation

Diluent is used as blank preparation.

2.6 Standard Stock Preparation

Prepared 0.3 ppm of Benzimidamide standard solution in diuent.

2.7 Test Preparation

Prepared 20000 ppm of Benzimidamide sample solution in diuent.

2.8 Method Development

Leflunomide and its impurities are polar, a reversed phase chromatography method for detecting genotoxic Benzimidamide was established. In RP-HPLC, non-polar stationary phases such as phenyl, C4, C8, and C18 are used, while polar mobile phases such as water, acetonitrile, or buffer solution are used. Other parameters such as column compartment temperature, diluents, wavelength, and pH play a significant role during this evolution in terms of stationary and mobile phases. During stationary phase screening, Hypersil BDS C18 and YMC Triart, both C18 with (4.6 mm x 150 mm) and particle size3 were used. In addition, all display availability in 150 mm and 250 mm lengths. When the Zorbax SB Phenyl (4.6 x 250 mm) 5 was utilised, superior impurity

separation, peak sharpness, and system suitability (tailing factor, column efficiency) were discovered.

Here the mobile phase is made up of triethylamine (TEA). Thus, a homogenous combination of 0.1 % triethylamine in pH 7.0 water with H_3PO_4 added and degassed, and degassed acetonitrile for diluent. The total analysis time is 30 minutes. Different trial runs of standard preparation are used to select the optimal gradient programme, flow rate, and column oven temperature. The concentration limit in ppm of genotoxic impurity (Benzimidamide) in drug substance derived from the TTC (Threshold of Toxicological Concern) can be calculated based on the expected daily dose to the patient using equation:

Concentration limit (ppm) = TTC [µg/day] /dose (g/day] = 1.5/0.100 = 15 ppm

(Maximum daily dose of 100 mg/day and duration of treatment more than 10 years, the TTC limit for Benzimidamide impurity 1.5).

The chromatographic conditions are detailed in Table 1.

Furthermore, the HPLC analysis was performed using the isocratic programme, and the mobile phase was prepared using a 40:60 mixture of buffer and acetonitrile.

3. RESULTS AND DESCUSION

The IP, BP, USP, and Q2 (R1) [5-8] of the ICH guideline were used in this validation and development study. For the finalisation of the specified limit based on treatment duration and dose, the ICH guideline M7 (R1) [9] was used. The validation [10-16] parameters are explored in more detail below.

3.1 Specificity

By injecting Blank (diluent), standard (0.3 ppm Benzimidamide), and sample solution, the selectivity research parameter was done (20000 ppm). The chromatograms are analysed at the same wavelength as the method specifies. Table 2 contains the specificity data, as well as a chromatogram in Fig. 2. Blank (diluent) has no effect on the retention period of the Benzimidamide peak. All recognised and unknown peaks in the sample solution are well isolated from one another. Peak purity is more than 950, indicating that the peak is pure.

Table 1. Content of Benzimidamide impurity chromatographic condition for RP-HPLC

Component	Specification
Apparatus	HPLC with UV/PDA detector, injector, pump, and recorder
Detector	UV/PDA detector
Column	Zorbax SB Phenyl (4.6 mm x 250 mm), 5.0 µ
λmax	289 Nano meter
M.P flow	1.0mL/min
Volume of Injectior	25 micro liter
Column oven temp	25°C
Auto sampler temp	25ºC
Run time	30 min.
Needlewash Solvent	Water: Acetonitrile (10:90)

Table 2. Data of specificity of Benzimidamide hydrochloride in Leflunomide

Impurities Name	Individual solution		Spiked test preparation	
	Retention time (minutes)	Peak purity	Retention time (minutes)	Peak purity
Benzimidamide	12.350	999.9	12.400	1000
Impurity A	4.633	-	4.650	-
Impurity B	1.983	-	1.983	-
Impurity C	5.467	-	Merged with	-
Impurity E	5.367	-	leflunomide	
5-methylisoxazole-4-carboxylic acid	ND	-	-	-

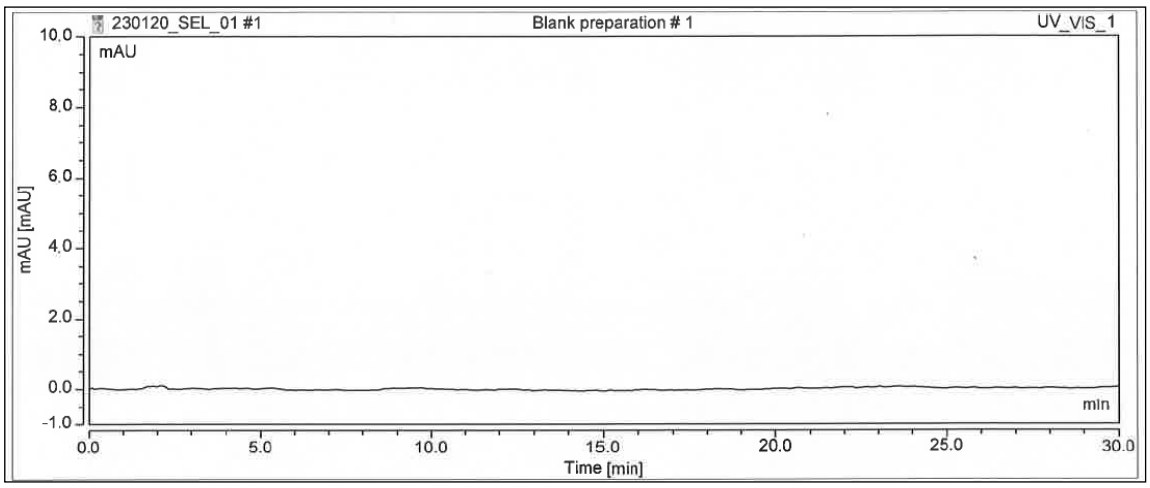

Fig. 2. Specificity: Blank preparation

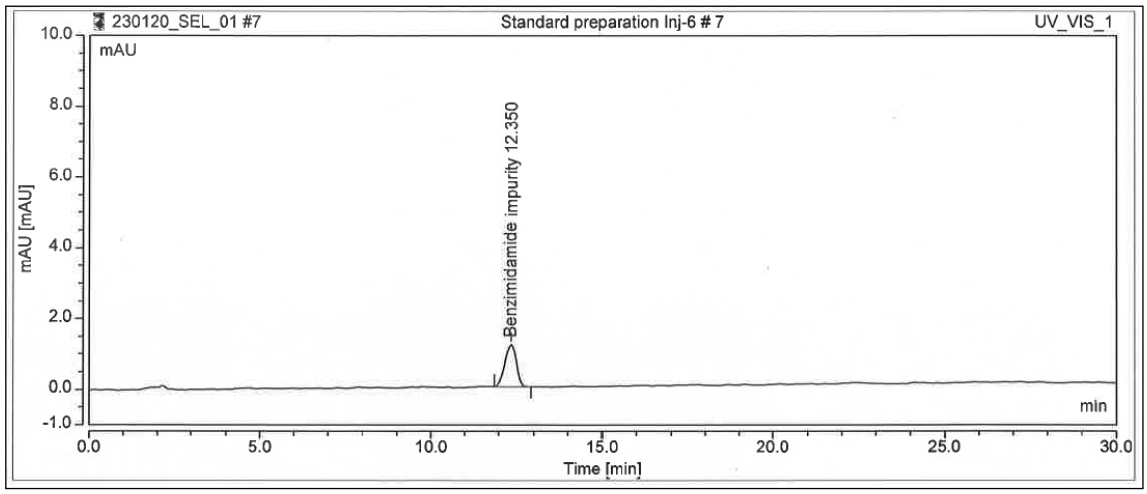

Fig. 3. Specificity: Standard preparation

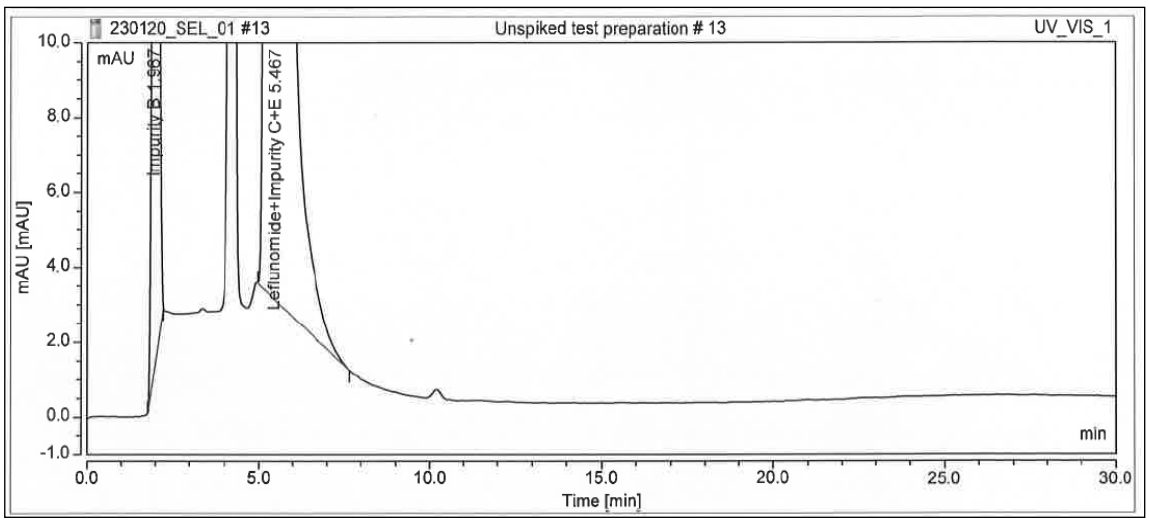

Fig. 4. Specificity: Unspiked Test preparation

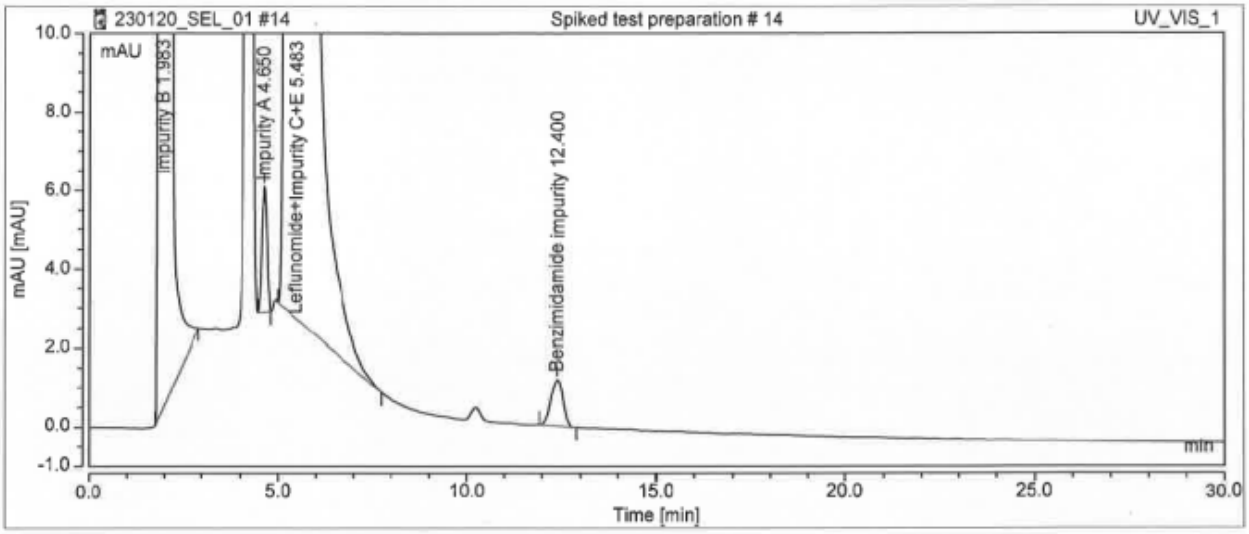

Fig. 5. Spiked Impurities in Leflunomide Typical chromatogram for Selectivity

Table 3. LOD and LOQ data in hydroxylamine hydrochloride

Name of Impurity	Conc. w.r.t test (in ppm)		s/n ratio	
	LOQ level	LOD level	LOQ level	LOD level
Benzimidamide impurity	1.50	0.49	18.0	6.3

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

The signal-to-noise ratio approach was used to calculate the LOD and LOQ conc. of Benzimidamide impurity in Leflunomide. Injecting various concentration levels (between 10 and 100 percent) of standard solutions of hydroxylamine hydrochloride limit level concentrations to determine the projected LOD and LOQ concentrations. 1.50 ppm was the predicted LOQ concentration value for Benzimidamide impurity. The LOD concentration is calculated by multiplying the predicated LOQ concentration by a factor of 0.33. Table 3 shows the predicted LOD and LOQ values.

3.3 Linearity and Range

The capacity of a method to produce test findings that are proportionate to the concentration of analyte in a given test sample is known as linearity. Standard solutions of Benzimidamide impurity with LOQ Level to 150 percent specified limit (including 50, 80, 100, 120, and 150 percent) of concentration were used in the linearity investigation.

Table4 shows the correlation coefficient, slope, concentrations, and intercept of linearity data, and Figure 6 shows the linearity graph. Least squares linear regression analysis was used to examine the peak area versus concentration data. The Benzimidamide impurity has a correlation coefficient of 0.999940, which is higher than 0.999.

3.4 Precision

As stated in the technique of analysis, system precision was achieved by injecting five replicates of the standard preparation. For replicate injections, the observed percent RSD is 0.72, and the tailing factor is 1.0. For method precision, six distinct samples were prepared and analysed; for intermediate precision, six separate samples were created and analysed on various days, systems, and columns. The observed percent RSD in procedure precision and intermediate precision is 2.13 and 3.45, respectively. Overall percent RSD is 2.76, which is less than 5.0 percent, for twelve test preparations (six from procedure precision and six from intermediate precision). Table 5 provides the outcomes of method precision and intermediate precision.

Table 4. Linearity data for the Benzimidamide impurity (LOQ to 150 % Concentration)

Sr. No.	Conc. w.r.t. standard Conc. in %	Concentration (In ppm w.r.t. test conc.)	concentration (in ppm)	Average area (n = 3)
1	LOQ	1.500	0.0300	2657
2	50	7.500	0.1500	13400
3	70	10.500	0.2100	18560
4	80	12.000	0.2400	21250
5	100	15.000	0.3000	26500
6	120	18.000	0.3600	32268
7	140	21.000	0.4200	37420
8	150	22.500	0.4500	40244
Slope				89453.4188
Intercept				-115.048077
Correlation coefficient				0.999940551

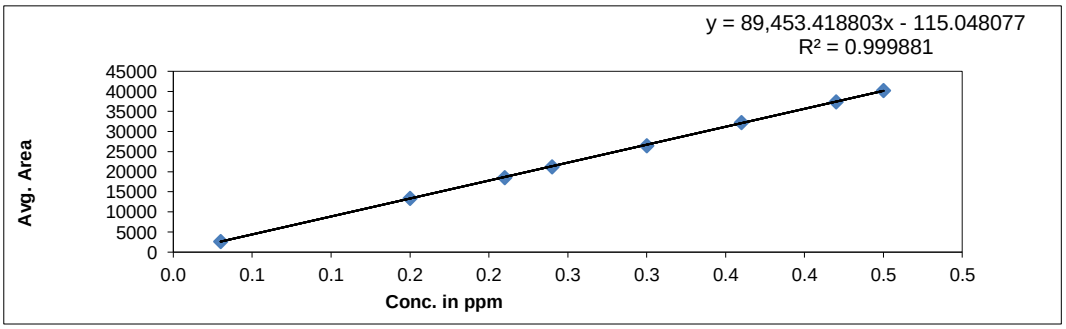

Fig. 6. Linearity graph for the Benzimidamide impurity content from LOQ to 150 % concentration range

Table 5. Benzimidamide impurity result of method and intermediate precision

Spiked sample solutions	% of Benzimidamide impurity (in ppm)	
	Method Precision	Intermediate Precision
Preparation 1.	15.12	15.44
Preparation 2.	15.62	15.82
Preparation 3.	15.44	15.11
Preparation 4.	15.82	16.20
Preparation 5.	15.88	15.06
Preparation 6.	15.12	16.30
Mean	**15.5000**	**15.6550**
SD	0.33	0.54
RSD	**2.13**	**3.45**
Overall Mean (n=12)	15.5775	
Overall SD **(n=12)**	0.43	
Overall% RSD(n=12)	**2.76**	

Table 6. The percent accuracy data of Benzimidamide impurity

Tests	LOQ Level	50% Level	100% Level	150% Level
Preparation -1	105.41	103.48	100.65	100.48
Preparation -2	101.68	100.88	103.45	101.41
Preparation -3	100.01	104.8	103.59	104.2
Mean	102.3667	103.05	102.56	102.03
SD	2.76	1.99	1.66	1.94
% RSD	2.70	1.93	1.62	1.90

Table 7. Retention time, Tailing factor, and RSD of Robustness study for the Benzimidamide impurity

System suitability parameters	Benzimidamide impurity			
	Mobile phase Flow rate		Column oven temperature	
	1.1mL/ min	0.9mL/ min	30 °C	20 °C
Retention time	12.036	12.445	12.018	12.398
Tailing factor	1.15	1.09	1.01	1.08
% RSD (n=6) replicate of standard preparation	2.50	1.99	1.23	2.01

3.5 Accuracy

Spiking test preparation with impurity at LOQ level, 50% level, 100 and 150 percent of specification limit concentrations was used to establish method accuracy. Table 6 shows the percent accuracy data for the Benzimidamide impurity. The percent accuracy observed at the LOQ level and 50% level, 100 and 150 percent, is between 100.02 and 104.8 percent, which is within acceptable limits. (An accuracy of 70 to 130 percent is recommended.).

3.6 Robustness

The method's resilience was tested by altering the flow rate by±10%. The flow rate is changed from 1.0 mL/min to 1.1 mL/min and 0.9 mL/min. In the actual procedure, the column oven temperature is varied by± 5 °C from 25 °C to 30 °C and 20 °C. Table 7 displays the observed area, standard deviation, and percent RSD. The retention times in all of the studies above differed by ±0.2 minutes from the original retention times. Tailing factor 1.01 to 1.15 was used to calculate the system appropriateness parameter. For robustness studies, the percent RSD ranges from 1.87 to 2.26. Changes in method parameters (flow rate and column oven temperature) had no significant impact on system suitability criteria tailing factor and percent RSD, according to Table 7. The values obtained are considerably within the acceptable range.

3.7 Solution Stability

The solution stability of the test preparation was tested at 25°C on a day-by-day basis for up to three days. Up to 3 days, the cumulative percent RSD values of the Benzimidamide impurity are substantially below acceptable limits. This implies that when stored at 25°C temperature, Analytical test preparations are stable for 3 days.

3.8 Mobile Phase Stability

The mobile phase was prepared according to the method of analysis, and the analysis was completed. After you've finished your analysis, keep a mobile phase at room temperature and show that it's stable. Initial system suitability parameter analysis and mobile stability research analysis were compared and checked. In standard Benzimidamide impurity, the percent RSD and change in retention time are within criteria, and no haziness, precipitation, or appearance of mobile phase is seen up to 60 hours. As a result, mobile phase stability at room temperature is 60 hours.

4. CONCLUSION

The RP-HPLC method for Benzimidamide impurity content determination of Leflunomide is very exact, selective, accurate, and stable, and follows ICH criteria. Q2(R1) has been accurately developed and validated. The specificity demonstrates that the Benzimidamide impurity peak can be fully resolved from both known and unknown impurities. With LOQ at 150 percent level w.r.t. specification concentration, the method is linear, and the observed Correlation coefficient is 0.99994. Between 100.01 and 104.8 percent of Benzimidamide impurity was recovered. System appropriateness, such as tailing factor and percent RSD, has no substantial impact on robustness. The observed outcomes were deemed to be within acceptable bounds. For all of the technique parameters that have been examined, the validated method has shown satisfactory results. As a result, the current method is particular, linear, selective, precise, robust, and stable, and can be used well in analysis.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our

area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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