Validated Method Development of Levamisole and Inosine pranobex by using UPLC &Characterization of Degradants by LC-MS/MS

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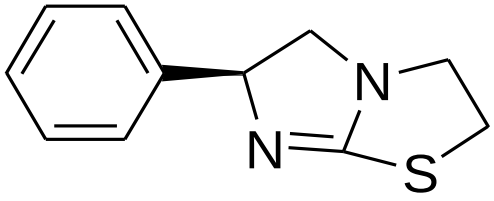
Abstract
In order to simultaneously evaluate Inosine pranobex and levamisole in pharmaceutical formulations using UPLC, a reliable and simple method was developed. The chromatographic separation used in this method a Phenomenox C18 column (50mmx2.1mm, 3.5). % Trifluoroacetic Acid (TFA) and Methanol were employed in a mobile phase with a flow rate of 0.5 mL/min and room temperature. At 223 nm, a UV observation was made. With these settings, we were able to successfully use UPLC to achieve good linearity throughout a range of 12.5-75 g/ml of inosine pranobex and 2.5-15 g/mL of levamisole. The results of other UPLC validation parameters, such as system precision, method precision, accuracy, robustness, and degradation studies, were present within the permitted limit while employing the aforementioned assay method, in accordance with ICH criteria.

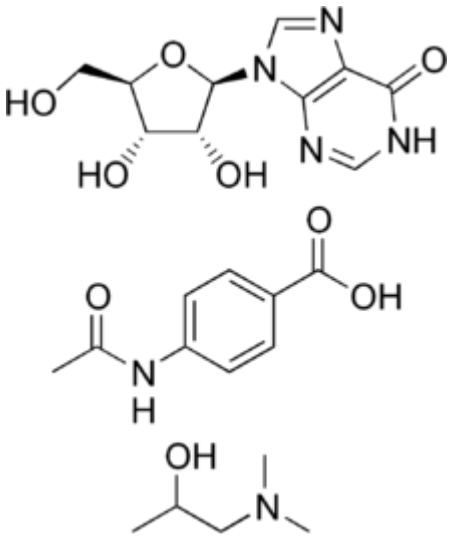
Keywords: *Levamisole, Inosine pranobex, validation, UPLC, LC-MS/MS.*

1. Introduction
Levamisole, an anthelminthic, was first employed to treat both humans and animals for worm infestations. Along with treating specific forms of cancer, it has also been used to treat inflammatory diseases. [1] It is (6S)-6-phenyl2H, 3H, 5H, and 6H-imidazo [2, 1-b][1,3]thiazole chemically. In addition to being used to treat malignant melanoma and head/neck cancer, it is suggested for adjuvant treatment in combination with fluorouracil following surgical resection in patients with Dukes' stage C colon cancer. The Indian Pharmacopoeia, [2] European Pharmacopoeia, [3] British Pharmacopoeia, [4] and American Pharmacopoeia [5] all recognize levamisole as an official medication.



Figur1. Structure of Levamisole

Inosine and dimepranol acedoben, a salt of acetamidobenzoic acid and dimethylaminoisopropanol, are combined in the antiviral medication inosine pranobex, also known as inosine acedoben dimepranol or methisoprinol, in a ratio of 1 to 3. On viral particles themselves, inosine pranobex has no effect. As an immunostimulant, it functions as a synthetic version of thymus hormones. [6] It is most frequently used in conjunction with intrathecal interferon-alpha therapy to treat the uncommon measles side effect subacute sclerosing panencephalitis. [7] It is an immunomodulatory medication that has been licenced for the treatment of viral infections in a number of nations.



Figur2. Structure of Inosine pranobex

2. Materials and Methods

Chemicals and reagents: The following products were purchased from Merck India Ltd., Worli, Mumbai, India: methanol (HPLC mark), trifluoroacetic acid (HPLC mark), and water (HPLC mark). APIs for Inosine Pranobex (purity 99.8%) and Levamisole (quality 99.9%) were purchased from the Mumbai-based Cipla Pharmaceutical Company.

Instrumentation: UPLC conditions: Waters Acquity UPLC with a quaternary pump, a PDA detector, and empower 2.0 software were utilized.

Chromatographic conditions: Use suitable Ultra Performance Liquid Chromatographic equipped with Agilent1290 Infinity II LC System.
Column: Phenomenox C18, 50x2.1mm, 3.5µ.
Wavelength: 223 nm
Injection Volume: 5µL
Column Temperature: Ambient
Flow rate: 0.5 ml/min
Sample Temperature: Ambient
Run time: 3 min
Diluent: Methanol

Preparation of ordinary solution: Inosine pranobex and levamisole were dissolved in enough mobile phase and structure to make 100 mL of standard solution, which contained 50 mg of Inosine pranobex and 10 mg of levamisole per mL. The prepared stock was diluted with diluent from 5ml to 50ml.

3. Results and Discussions

System precision: Inosine pranobex (50 g/ml) and levamisole (10 g/ml) were injected into the UPLC system to create the standard solution, and figure 1 depicts the chromatogram of the UPLC. The peak regions obtained by UPLC were used to determine percent RSD, and the findings were confirmed to be within the acceptable range.

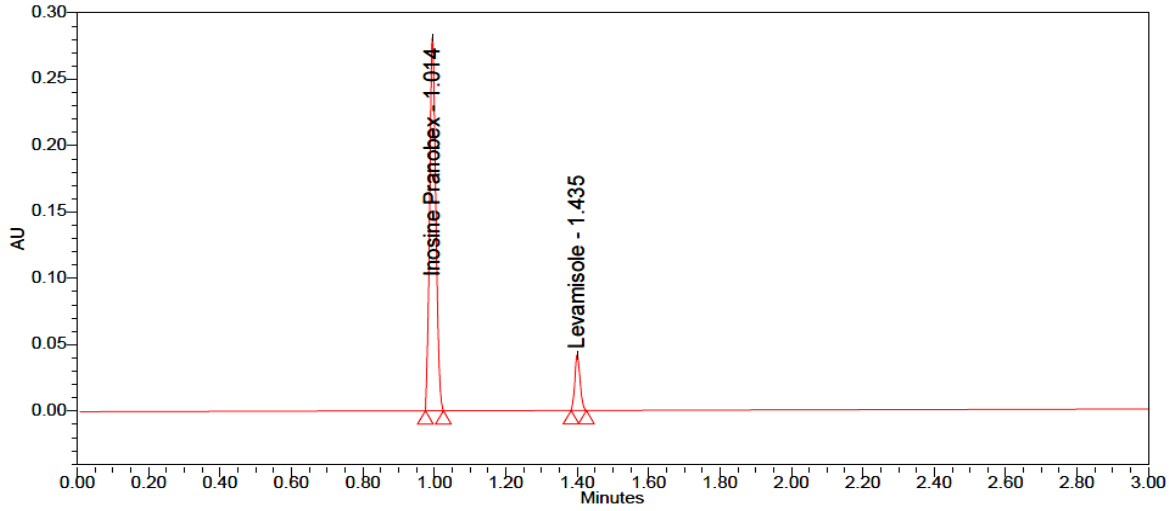


Figure 3. UPLC's baseline chromatogram

Specificity: By explicitly comparing the chromatograms to the blank sample, which is showed in Figure 2, it was possible to determine the specificity of the assay method's ability to completely exclude the effects of any interfering chemicals on the peak results for Inosine pranobex and levamisole. The supported methodology demonstrated that the chosen pharmaceuticals were eluted without the use of peaks produced by the excipients in the commercial items.

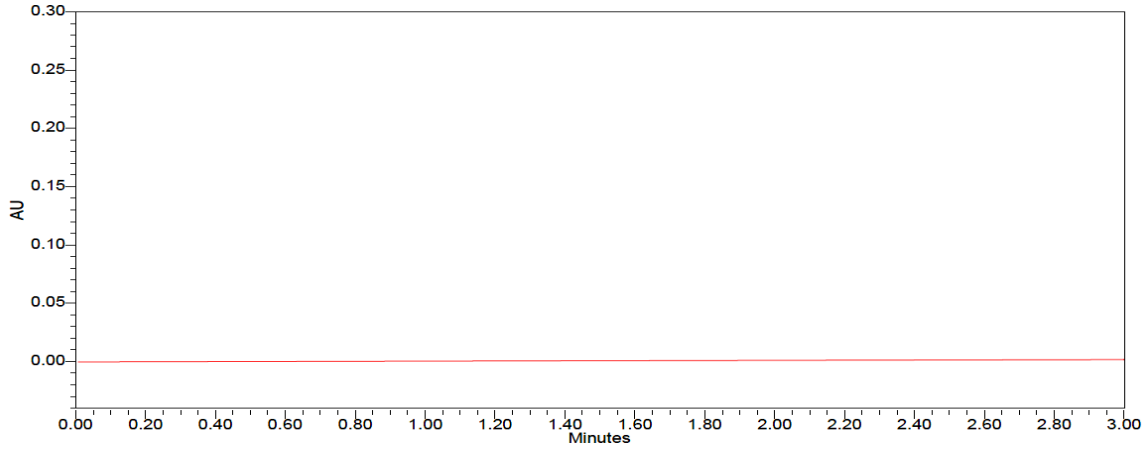
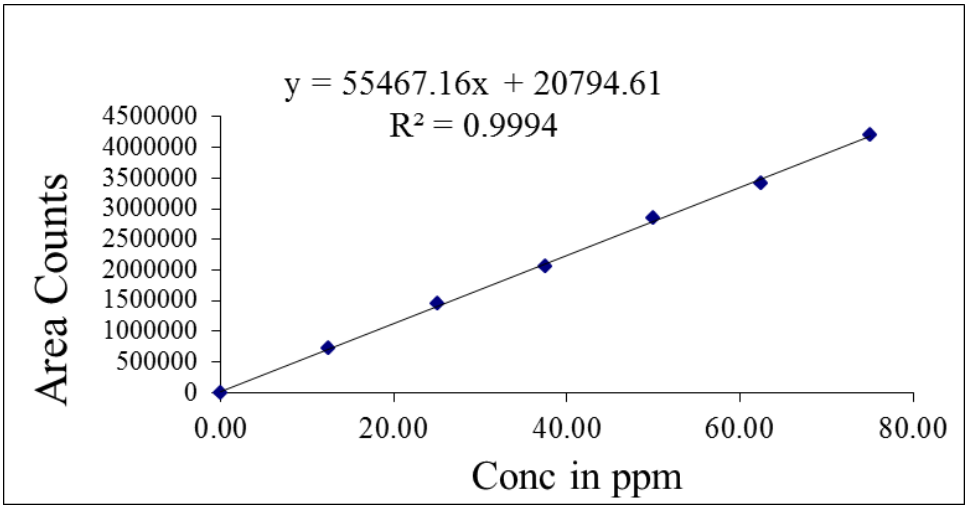


Figure 4. Blank chromatogram of UPLC

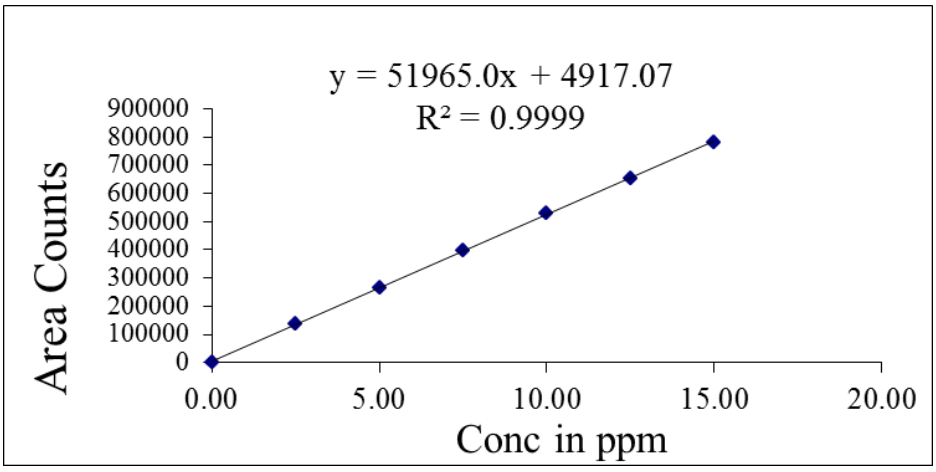
Linearity: By developing a typical solution comprising 50 g/ml of Inosine pranobex and 10 g/ml of Levamisole, the tactic's linearity was assessed. At successive dilutions of 25, 50, 75, 100, 125, and 150 percent of the chosen concentrations, the given dilutions were applied. UPLC received an injection of these. The Levamisole and Inosine Pranobex concentration series calibration curves were linear the entire time. Table 1 contains a summary of the linearity values. These analytes' coefficient of correlation values were 0.999. Figure 3 displays the UPLC calibration curve for inosine pranobex and levamisole (a&b).

Table 1. HPLC results of Linearity

Linearity	Inosine pranobex		Levamisole	
	Conc (µg/ml)	Area	Conc (µg/ml)	Area
Linearity-1	12.50	714642	2.50	136689
Linearity-2	25.00	1462381	5.00	266734
Linearity-3	37.50	2054869	7.50	396658
Linearity-4	50.00	2853049	10.00	528573
Linearity-5	62.50	3410129	12.50	652695
Linearity-6	75.00	4210621	15.00	781233
Slope	55467.16		51965.00	
Intercept	20794.61		4917.07	
CC	0.99941		0.9999	



(a)



(b)

Figure 5. UPLC linearity plots of (a) Inosine pranobex (b) Levamisole

Accuracy and precision: Recover investigations, which were conducted at three distinct concentration levels, determined accuracy (50%, 100% and 150% levels). Inosine pranobex and levamisole APIs with concentrations of 25, 50, and 75 g/mL each were created. The assay was carried out in accordance with the test method after the test solutions were injected into 3 preparations at each spike level. The results are provided in Table 2, which shows that the share recovery values were seen to be between 98 and 102%.

Table 2. UPLC accuracy results of (a) Inosine pranobex and (b) Levamisole

a

S. No	% Level of Accuracy	Average % Recovery
1	50	98.9
2	100	99.5
3	150	99.2

b

S. No	% Level of Accuracy	Average % Recovery
1	50	99.9
2	100	100.1
3	150	99.9

Precision

This approach's accuracy was evaluated in terms of the method and intermediate variants. By performing six further analyses of the sample solution of Inosine pranobex and levamisole on an equivalent day and under an equivalent set of experimental circumstances, the intraday studies were calculated. The approach was delivered with intermediate precision in the same laboratory by analyzing the data using different analysts and tools; RSD values were discovered to be under 2 %. The fact that each attached concentration of the chosen medications had good recoveries (98 to 102%) indicates that the strategy was successful. The outcomes were provided in Table 3.

Table 3. UPLC Precision results of (a) Inosine pranobex (b) Levamisole

a

S. No	Concentration (µg/ml)	Area	% RSD
Method precision			
1	50	2843598	0.5
2	50	2811696	
3	50	2832323	
4	50	2822357	
5	50	2821758	
6	50	2812665	
Intermediate precision results			
1	50	2857759	0.41
2	50	2841695	
3	50	2860724	
4	50	2831596	
5	50	2841691	
6	50	2833213	

b

S. No	Concentration (µg/ml)	Area	% RSD
Method Precision results			
1	10	522735	0.6
2	10	523895	
3	10	528878	
1	10	528481	
2	10	521365	
3	10	525815	
Intermediate precision results			
1	10	524471	0.46
2	10	523722	
3	10	525628	
1	10	524699	
2	10	520816	
3	10	528074	

LOD and LOQ: The calibration curve method was used to individually determine LOD and LOQ. Using the created RP-UPLC method, the LOD and LOQ of the substances were determined by injecting continuous lower accumulation of standard solutions. Levamisole and Inosine pranobex had LOD values of 0.15 g/mL and 0.03 g/mL, respectively, while their s/n values were 3 and 3. The s/n values were 10, 10, and the LOQ values were 0.5 g/mL and 0.1 g/mL, respectively.

Robustness: To test the tactic's ability to remain unaffected, tiny but deliberate changes were applied to method parameters, such as the flow change (10%) and organic content inside the mobile phase (10%), in accordance with ICH standards. Table 4 demonstrates the outcome of the changed parameters on retention duration, tailing factor, and percentage content when utilizing UPLC to assess the robustness of the strategy. The strength of the technique was demonstrated by the degree of reliability of the outcomes that were obtained by modest, intentional adjustments.

Table 4. UPLC results of Robustness

Change in parameter	%RSD of Inosine pranobex	%RSD of Levamisole
Flow (0.9 ml/min)	0.65	0.36
Flow (1.1 ml/min)	0.4	0.15
Org Phase (22:78)	0.6	0.26
Org Phase (18:82)	0.27	0.3

Stability: The stability of the sample solutions was evaluated initially for 24 hours at various time intervals. Since there was no discernible decline during this time period, the mean deviation and mean were both below 5.0 percent. Indicating that the solutions were stable for at least 24 hours, which was enough time for the whole UPLC analytical process.

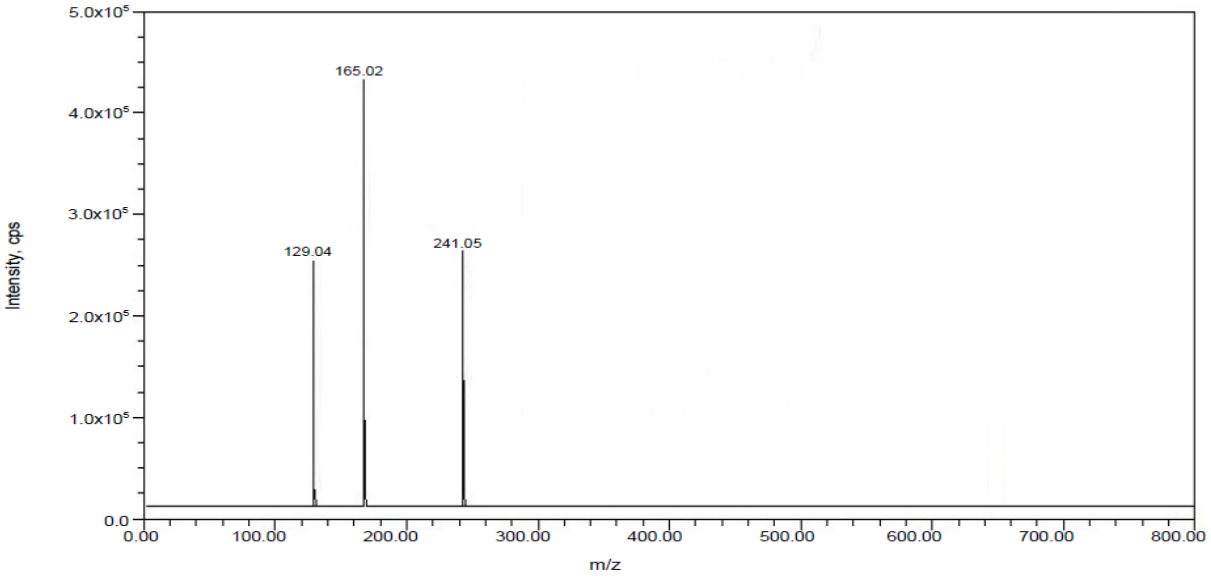
Forced Degradation: To demonstrate that the approach is appropriate for degraded products, forced degradation trials were carried out. In order to prevent potential instabilities, appropriate steps were frequently adopted during formulation. The investigations provide information about the circumstances in which the medicine is unstable. Figure 4 depicts the MS spectra used to characterize these degradation samples using LCMS. Table 5 has the degradant values.

Acid Degradation: When certain medications were investigated for acid degradation in 1N HCl, degradation of Inosine Pranobex (3.7%) and Levamisole (12.7%) was detected in UPLC and one degradation product was generated.
Alkali Degradation: Studying the alkali degradation of a few pharmaceuticals in 1N NaOH led to the detection of one degradation product and UPLC degradation rates of 4.0% for isosine pranobex and 13.0% for levamisole.
Peroxide Degradation: 5.1 % of Inosine Pranobex and 14.1% of Levamisole degradation was found in UPLC and one degradation product was generated during the peroxide degradation of chosen pharmaceuticals in 30 percent hydrogen peroxide.
Reduction Degradation: 3.5 % of Inosine pranobex and 10.3 % of Levamisole showed degradation in UPLC tests after being investigated for reduction degradation in a solution of 30 percent sodium bisulphate. One degradation product was also generated.
Thermal Degradation: Levamisole and inosine pranobex both showed signs of degradation in UPLC after being exposed to a thermal degradation standard at 105°C for six hours. No degradation products were produced.
Photolytic Degradation: After being exposed to sunlight for 12 hours, the standard showed signs of degradation in UPLC of 2.5 percent of inosine pranobex and 4.5 percent of levamisole, but no degradation products were produced.
Hydrolysis Degradation: In 3 ml of HPLC water, the hydrolysis degradation of a few different medications was examined. Inosine pranobex and levamisole both showed degradation in UPLC, but no degradation products were produced.

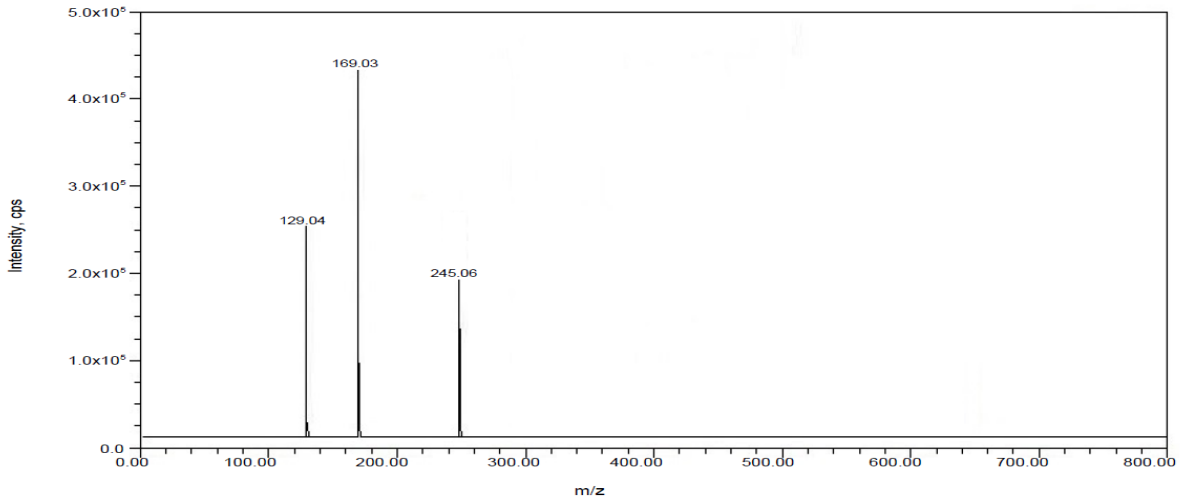
Table 5 Degradation results using UPLC

Deg condition	Time/Temp	Inosine pranobex		Levamisole		Number of DPs formed
		% Deg	% Assay	% Deg	% Assay	
Acid deg	3 hrs, 60°C	96.2	3.7	87.3	12.7	One
Alkali deg	3 hrs, 60°C	95.9	4	87	13	One
Peroxide deg	-	94.8	5.1	85.9	14.1	One
Reduction deg	3 hrs, 60°C	96.4	3.5	89.7	10.3	One
Thermal deg	24 hrs, 105°C	95.6	4.3	96	4	No
Photolytic deg	UV-Vis light	97.4	2.5	95.5	4.5	No
Hydrolysis deg	3 hrs, 60°C	96.8	3.1	95.8	4.2	No

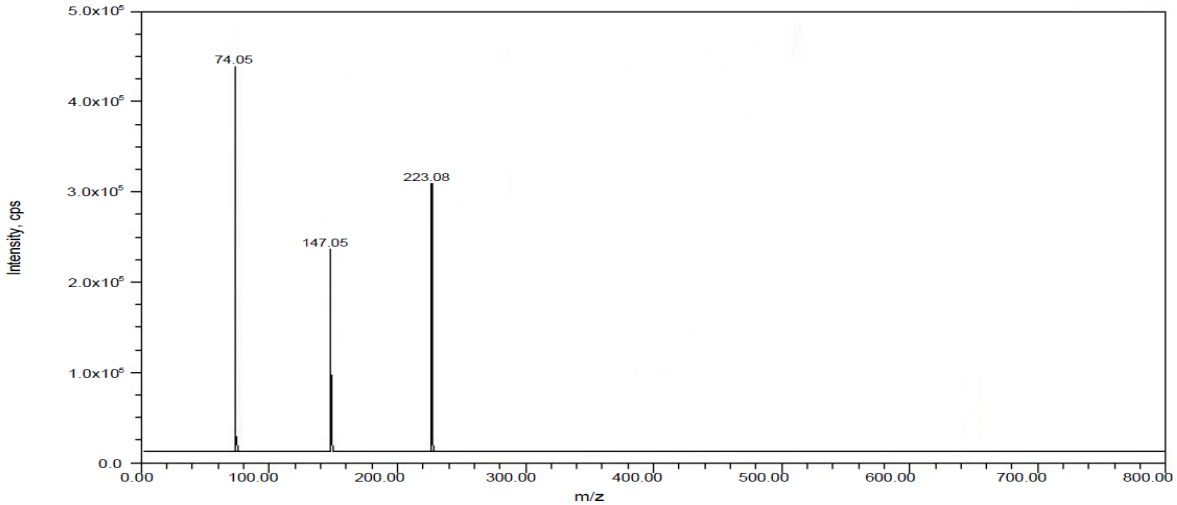
DP-Degradation product
UV-Vis light- (200 W h/m^2) and fluorescent light (1.2 milliion lux-h)


Intensity, cps
5.0x10⁵
4.0x10⁵
3.0x10⁵
2.0x10⁵
1.0x10⁵
0.0
165.02
129.04
241.05
0.00
100.00
200.00
300.00
400.00
500.00
600.00
700.00
800.00
m/z

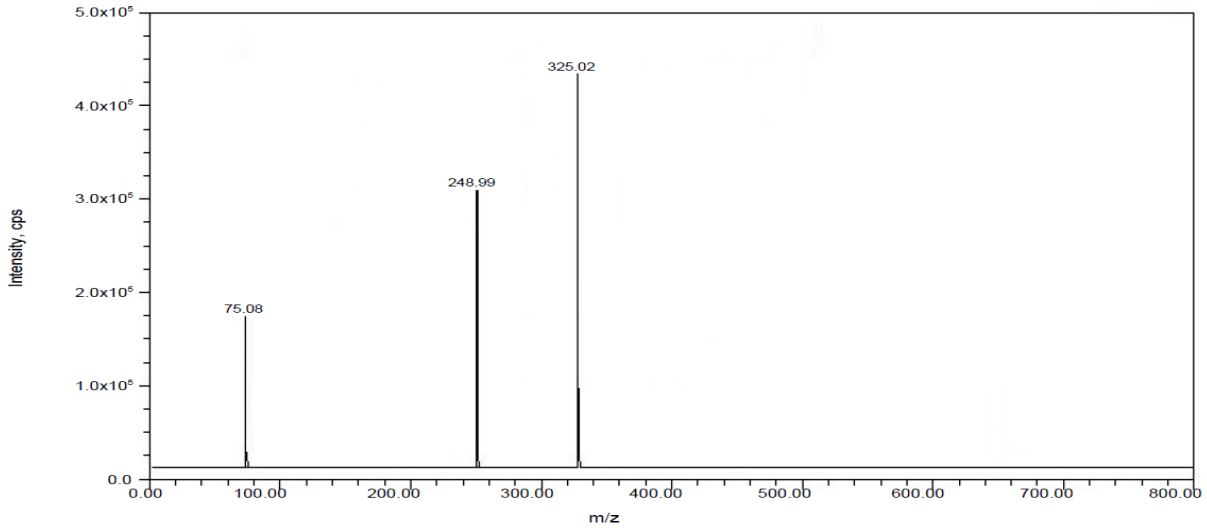
a


Intensity, cps
5.0x10⁵
4.0x10⁵
3.0x10⁵
2.0x10⁵
1.0x10⁵
0.0
169.03
129.04
245.06
0.00
100.00
200.00
300.00
400.00
500.00
600.00
700.00
800.00
m/z

b


Intensity, cps
5.0x10⁵
4.0x10⁵
3.0x10⁵
2.0x10⁵
1.0x10⁵
0.0
74.05
223.08
147.05
0.00
100.00
200.00
300.00
400.00
500.00
600.00
700.00
800.00
m/z

c

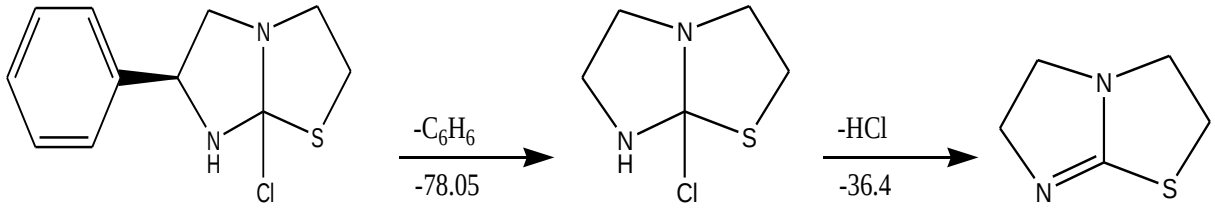


d

Figure 8. MS Spectra of (a) DP1 (b) DP2 (c) DP3 (d) DP4

Dissociation of Levamisole and Inosine Pranobex caused by collision

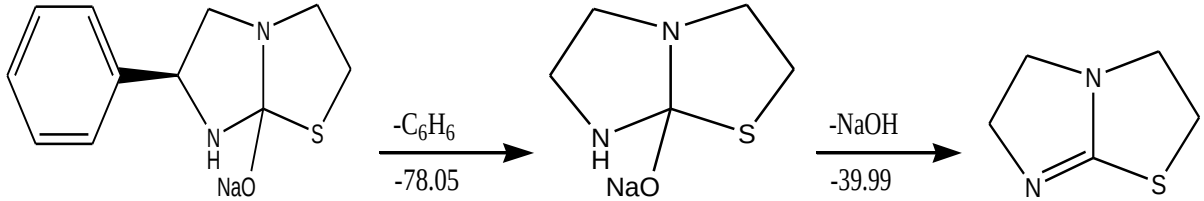
Scheme 1 show the fragmentation mechanism of degradation product 1 of m/z-240.05 which was observed under acid degeneration condition. The spectrum displays abundant product ions at m/z-164.02 (loss of C_6H_6), m/z-128.04 (loss of HCl). The MS/MS experiments combined with accurate mass measurements have confirmed the proposed scheme.



240.05 164.02 128.04

Scheme 1 Proposed fragmentation mechanism for Inosine pranobex (DP1)

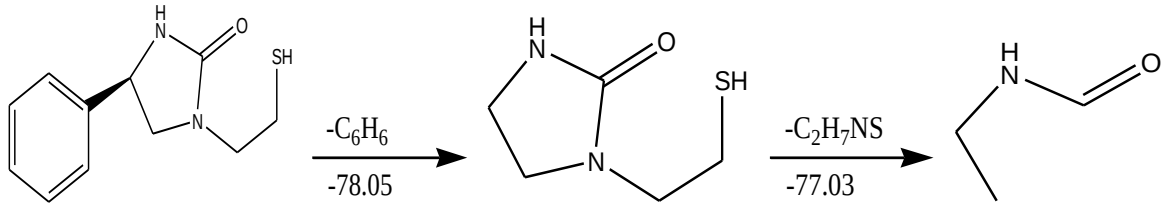
Scheme 2 shows the fragmentation mechanism of Levamisole degradation product 2 of m/z-244.06 which was noticed under alkali condition. The spectrum displays abundant product ions at m/z-168.03 (loss of benzene), m/z-128.04 (loss of NaOH). The MS/MS experiments combine with correct mass evaluations confirmed the proposed scheme.



244.06 168.03 128.04

Scheme 2 Proposed fragmentation mechanism for Levamisole (DP2)

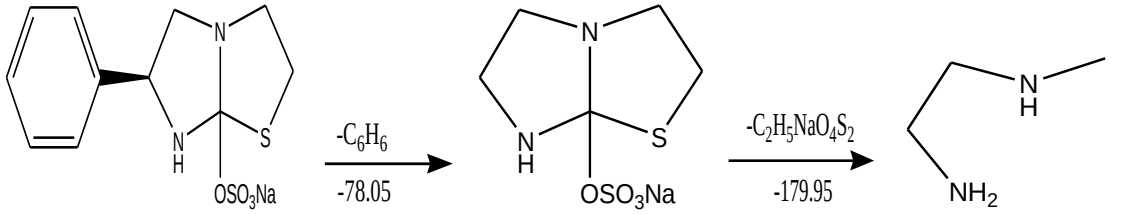
The fragmentation mechanism of the degradation product 3 of m/z 222.08, which was discovered under peroxide conditions, is depicted in Scheme 3. A lot of product ions are visible in the MS spectrum at m/z-146.05 (loss of C_6H_6) and m/z-73.05 (loss of m/z C_2H_7NS). The proposed scheme was validated by the MS/MS measurements and accurate mass assessments.



222.08 146.05 73.05

Scheme 3 Proposed fragmentation mechanism for Levamisole (DP3)

The fragmentation mechanism for the degradation product 4 of m/z-324.02, which was observed under reduction degradation conditions, is shown in Scheme 4. The spectrum shows numerous product ions at m/z 74.08 (loss of m/z $C_2H_5NaO_4S_2$) and m/z 247.99 (loss of m/z C_6H_6). The proposed scheme was validated by the MS/MS tests in conjunction with accurate mass assessments.



324.02 247.99 74.08

Scheme 4 Proposed fragmentation mechanism for Levamisole (DP4)

4. Conclusion

This work developed a completely new, straightforward, quick, affordable, sensitive, and easily accessible UPLC technique for the simultaneous determination of Inosine pranobex and levamisole in API form. The fact that no UPLC methods have been documented is one of this method's greatest benefits. Shorter run times, lower costs, accessibility, dependability, sensitivity, and reproducibility are features of this approach. Under acid, base, neutral, oxidation, reduction, photolytic and thermal stress settings, the medicines' degeneration-inducing effects were studied. In neutral, thermal, and photolytic conditions, there was no degradation. The [M+H]+ ion was used to identify the degradation products, and LC-MS/MS tests in conjunction with accurate mass calculations supported the predicted structures. According to ICH guidelines, the RP-UPLC approach was supported.

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Conflict of Interest:

The authors declare that no conflict of interest for this research.

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