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Research Article

DEVELOPMENT AND VALIDATION OF A ROBUST RP-HPLC METHOD FOR THE SIMULTANEOUS QUANTIFICATION OF LIDOCAINE AND HYALURONIDASE IN PHARMACEUTICAL FORMULATIONS

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ABSTRACT

A Simple and Robust Approach as a new development and validation, RP-HPLC method was established for simultaneous determination of lidocaine and hyaluronidase. Analytical separation was performed on C18 column (eg. Spursil C18-EP, 250 × 4.6 mm, 5 µm) using mobile phase consisting of potassium dihydrogen phosphate buffer, pH 3.5: methanol (30:70, v/v) at flow rate of 1.0 mL/min. The detection was done at UV 254 nm. Retention time was found to be 2.264 min (lidocaine) and 3.032 min (hyaluronidase) within 8.0 min runtime. The tailing factors were 1.25 and 1.22, USP plate count was 3302 and 3232, respectively and resolution factor was found to be 6.2. Validation Parameters Aspects Validation criteria Lidocaine Hyaluronidase Specificity Method was specific for each component Linearity (0.1 to 0.5) 10-50 ppm, r^2 = 0.999 5-25 ppm, r^2 = 0.999 Precision (%RSD) 1.5% 0.6% Accuracy (%Recovery) 98-102%Robustness LOD 0.05 and 0.11 ppm LOQ 0.18 and 0.39 ppm The proposed method can be employed for routine quality control analysis of the combination formulations.

Keywords: RP-HPLC, Lidocaine, Hyaluronidase, Method validation, ICH guidelines, Pharmaceutical analysis.

INTRODUCTION

Lidocaine, a widely used local anaesthetic, blocks voltage-gated sodium channels to inhibit nerve impulse transmission, providing rapid numbness for surgical and medical procedures (Butterworth & Strichartz, 2011). Approved by the FDA in 1948, it appears in diverse forms like injections, creams, patches, and sprays, and serves as a Class Ib antiarrhythmic for ventricular arrhythmias (U.S. Food and Drug Administration, 1948 & 2005; Duran-Reynals, 1929). Hyaluronidase, a "spreading factor," hydrolyzes hyaluronic acid in the extracellular matrix to enhance tissue permeability, improving absorption of co-administered drugs and fluids (Zenk, J., *et al.* 2008; National Center for Biotechnology Information, n.d.; Sharma *et al*., 2015). FDA-approved recombinant versions (e.g., Hylenex) launched in 2005 for subcutaneous hydration and local anaesthesia (Frost, 2007; Kumar *et al*., 2018; Patel *et al*., 2020). Combined lidocaine-hyaluronidase formulations (e.g., Amphadase®) enhance anesthetic diffusion in infiltration blocks and subcutaneous procedures, reducing dosage and improving outcomes. However, their physicochemical differences lidocaine (Entaz Bahar and Hyonok Yoon 2021) (small lipophilic molecule, logP ~2.4, MW 234 Da) versus hyaluronidase (large glycoprotein, ~60 kDa) challenge simultaneous analysis. Prior methods focus on individual analytes: UV/fluorescence (Ibrahim & Rashid, 2026) HPLC for lidocaine achieves good sensitivity but ignores hyaluronidase (Shinde & Chandarana, 2024; Hanif *et al*., 2023; Gowekar, M., & Wadher, S. J. 2017). ELISA or SEC-HPLC quantifies hyaluronidase proteins but requires long runtimes (>20 min) and specialized columns (Mehmood *et al*., 2022; Nalkiashary *et al*., 2020). Few reports address combinations one LC-MS/MS study reports (Ahmed *et al*., 2025) adequate separation but demands costly MS detection and complex sample prep unsuitable for routine QC (Navya Sri, Sujatha, *et al*., 2025;). RP-HPLC attempts often suffer poor hyaluronidase peak shape (tailing >2.0), low resolution (<3), or extended runtimes (>15 min) due to inadequate pH/mobile phase control (Barzani, Omer, Abdulrahman, Jawhar, & Sulaiman, 2026). No validated (Khan *et al*., 2025; Bandla, Rao, & Maddipati, 2025), green (low-solvent, short-runtime) RP-HPLC method exists for

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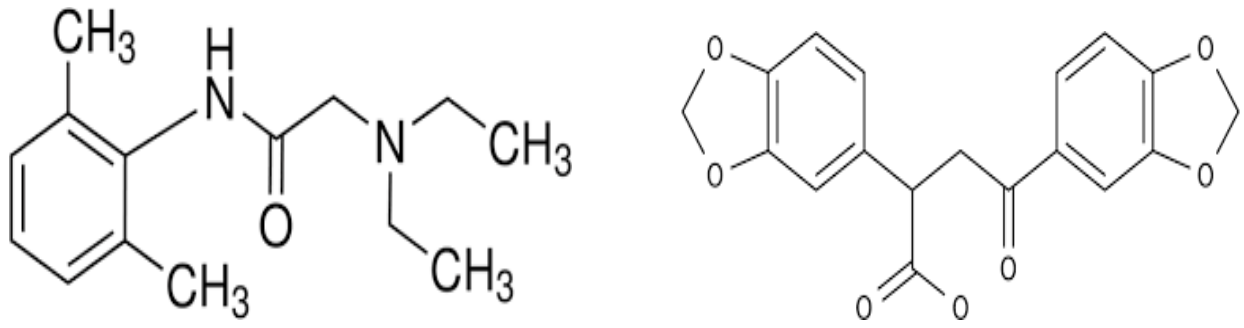
routine pharmaceutical QC of these combinations per ICH Q2(R1). This study fills the gap with a simple RP-HPLC method using C18 column and pH 3.5 buffer: methanol (30:70, v/v), achieving baseline separation (resolution 6.2), sharp peaks (tailing 1.22-1.25), and 8 min runtime. Validated per ICH Q2(R1) for all parameters, it enables efficient quality control.



(A) (B)

Figure 1. Chemical Structure of Lidocaine(A) [and] Hyaluronidase(B).

MATERIALS AND METHODS

Potassium dihydrogen phosphate KH_2PO_4 and formic acid, both of HPLC grade and obtained from Qualigens, were employed in the estimation of lidocaine and hyaluronidase. HPLC grade water supplied by Qualigens, along with acetonitrile of HPLC grade from Qualigens and methanol of HPLC grade from Rankem, was used as the solvent system for the quantitative analysis of all drugs.

Instruments

The study was performed using the following instruments and equipment: an electronic balance (SAB2032, Scaletech) for accurate weighing of chemicals, an ultra-sonicator (SE60US, Labman Scientific India) for sonication, and a thermal oven (i-THERM A17782, Dwaraka Scientific) for drying. pH measurements were carried out using an Orion Star A111 pH meter (Thermo Scientific). Filtration was performed using 0.45-micron Millipore filter papers, and HPLC analysis was conducted on a Waters 2690 Separation Module (WATERS).

Preparation of Solutions

Preparation of Potassium Dihydrogen Phosphate Buffer

Weigh 1.7 g of potassium dihydrogen phosphate and dissolve in about 150 mL of HPLC water in a 250 mL volumetric flask. Make up the volume to 250 mL with HPLC water, mix well, adjust pH if required, then filter through a 0.45 µm membrane and sonicate before use.

Preparation of mobile phase

Mix a mixture of 300buffer 300ml (30%), 700ml methanol (70%) and degas in ultrasonic water bath for 5 minutes. Filter through 0.45µ filter under vaccum filtration.

Preparation of Stock solution

Accurately weigh and transfer 10mg of Lidocaine and 5mg Hyaluronidase working standard into a 10ml clean dry volumetric flask add Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution).

Preparation of working standard solution

Accurately weigh and transfer 10mg of Lidocaine and 5mg Hyaluronidase equivalent weight of the sample into a 10ml clean dry volumetric flask add Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent.

Method Development

A validated RP-HPLC method was developed for the simultaneous estimation of Hyaluronidase and Lidocaine. The analysis was conducted using a waters HPLC system with an Spursil C18-EP, column (150×4.6mm, 3(m). The optimized mobile phase consisted of 70%: 30% (Methanol: KH2PO4) and Potassium dihydrogen phosphate buffer (pH-3.5), delivered at a flow rate of 1.0 ml per min. Detection was carried out at 254nm using a UV detector. Standard and sample solutions were prepared using mobile phase as a diluent. The method was validated as per ICH guidelines for Precision, Accuracy, Limit of Quantification, Specificity, Linearity, Range, Ruggedness, Robustness under the stress conditions, including acidic, alkaline, oxidative, thermal and photolytic degradation. The method exhibited excellent reproducibility and Selectivity, making it suitable for routine pharmaceutical analysis of Hyaluronidase and Lidocaine.

Method Validation

The analytical method was validated according to the ICH Q2 (R1) guidelines, focusing on parameters such as system suitability, specificity, accuracy, precision, linearity, robustness, limit of detection (LOD), and limit of quantification (LOQ).

System suitability

System suitability is a quick check done in the lab to make sure instruments like HPLC or GC are giving accurate and reliable results before testing actual samples. It involves checking the parameters like resolution, tailing factor, %RSD, theoretical plates and retention time.

Accuracy

Accuracy means how close your measured or experimental value is to the actual true value. For accuracy determination, three different concentrations were prepared separately i.e. 50%, 100% and 150% for the analyte and chromatograms are recorded for the same. Accurately weigh and transfer 10mg of Lidocaine and 5mg Hyaluronidase working standard into a 10ml clean dry volumetric flask add Diluents and sonicate to dissolve it completely and make volume up to the mark to prepare stock solution. Further pipette 0.3ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluents.

Precision

Precision means how close repeated measurements of the same sample are to each other when tested under the same conditions. Accurately weigh and transfer 10mg of Lidocaine and 5mg Hyaluronidase working standard into a 10ml clean dry volumetric flask add Diluents and sonicate to dissolve it completely and make volume up to the mark to prepare the stock solution. Further pipette 0.3ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluents.

Limit of Detection

Limit of Detection (LOD) means the smallest amount of a substance that can be detected by the method but not measured accurately. Accurately weigh and transfer 10mg of Lidocaine and Hyaluronidase working standard into a 10ml clean dry volumetric flask add Diluents and sonicate to dissolve it completely and make volume up to the mark to prepare the stock solution. From this stock pipette 1ml, 1ml, 1ml and 0.5ml (Lidocaine) and 1ml, 1ml and 0.11ml (Hyaluronidase) of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluents.

Limit of Quantification

Limit of Quantification (LOQ) means the smallest amount of a substance that can be measured accurately and precisely using the method. Accurately weigh and transfer 10mg of Lidocaine and Hyaluronidase working standard into a 10ml clean dry volumetric flask add Diluents and sonicate to dissolve it completely and make volume up to the mark to prepare the stock solution. From this stock pipette 1ml, 1ml and 0.18ml (Lidocaine) and 1ml, 1ml and 0.39ml (Hyaluronidase) of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluents.

Range

Range means the concentration limits within which the method gives accurate, precise, and linear results**.**

Specificity/ Selectivity

Selectivity (specificity) means the method can accurately measure the target substance even when other components are present without any interference.

Linearity

Linearity means the method gives results that increase proportionally with the concentration of the substance within a certain range. Accurately weigh and transfer 10mg of Lidocaine and 5mg Hyaluronidase working standard into a 10ml clean dry volumetric flask add Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution).

Robustness

Robustness means the method can still give reliable results even when small changes are made to the experimental conditions. The flow rate varied from 0.8 ml/min to 1.2ml/min. Standard solution 30 µg/ml of Lidocaine and Hyaluronidase prepared and analysed using the varied flow rates along with method flow rate. The Organic composition in the Mobile phase varied from 30% to 70% Standard solution 30 µg/ml of Lidocaine and Hyaluronidase was prepared and analysed using the varied Mobile phase composition along with the actual mobile phase composition in the method.

Ruggedness

Ruggedness means the method can give consistent results even when conditions change, like different labs, analysts, instruments, or days.

RESULTS AND DISCUSSION

The system suitability results show that the method is working smoothly, with good peak shape, proper retention, and reliable detector response for both drugs. All parameters like tailing factor, plate count, and resolution meet the limits, confirming the method is efficient and suitable for accurate analysis.

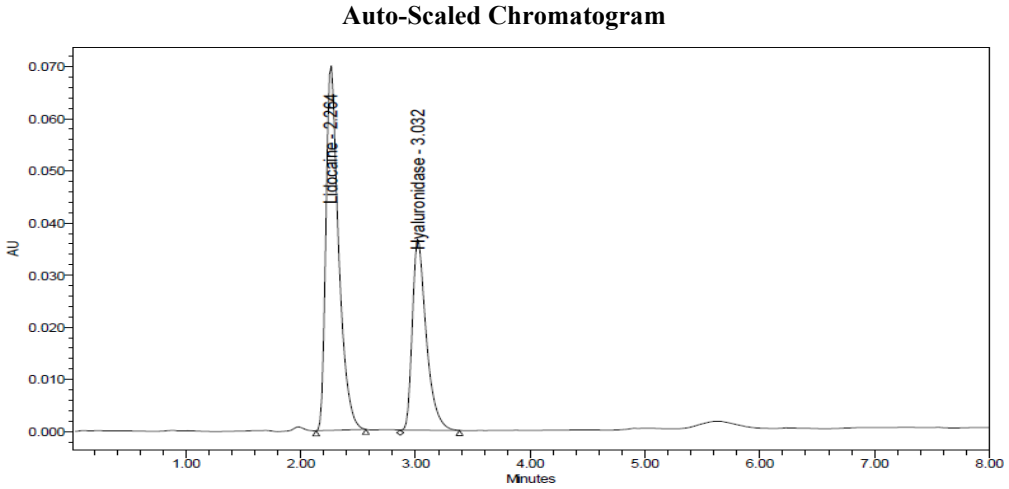


Table 1. System Suitability Results for Lidocaine and Hyaluronidase.

S. No	Parameter	Lidocaine	Hyaluronidase	Acceptance Criteria
1	Retention Time (min)	2.264	3.032	—
2	Peak Area	2540780	224115	—
3	Peak Height	248834	28834	—
4	USPFDA Tailing Factor	1.25	1.22	NMT 2.0
5	USPFDA Plate Count	3302	3232	NLT 2000
6	Resolution	—	6.2 (vs Lidocaine)	NLT 2.0

The tailing factor for both drugs is less than 2, indicating good peak symmetry. The plate count is above 2000, confirming good column efficiency. The resolution (6.2) between the two peaks is well above the required limit (2), showing excellent separation. All system suitability parameters comply with acceptance criteria. The results of specificity show clear and well-separated peaks for both Lidocaine and Hyaluronidase, with no interference observed. Overall, the method demonstrates good specificity and reliability for accurate analysis**.**

Table 2. Results of Specificity for Lidocaine and Hyaluronidase.

S. No	Name (STD)	RT (min)	Area (µV sec)	Height (µV)	Tailing Factor	Resolution	Theoretical plate count
1.	Blank	--	---	--	--	--	-
2.	Lidocaine	2.262	2540780	248902	1.02	6.2	3342
3.	Hyaluronidase	3.039	224115	28842	1.12		3232

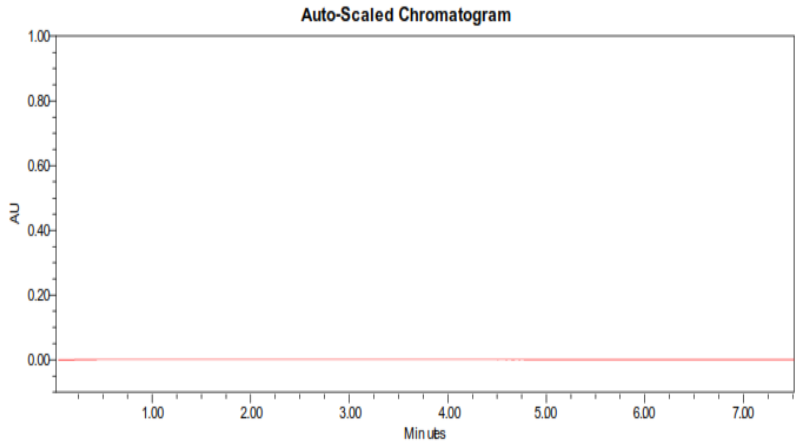


Figure 2a. Chromatogram for Blank.

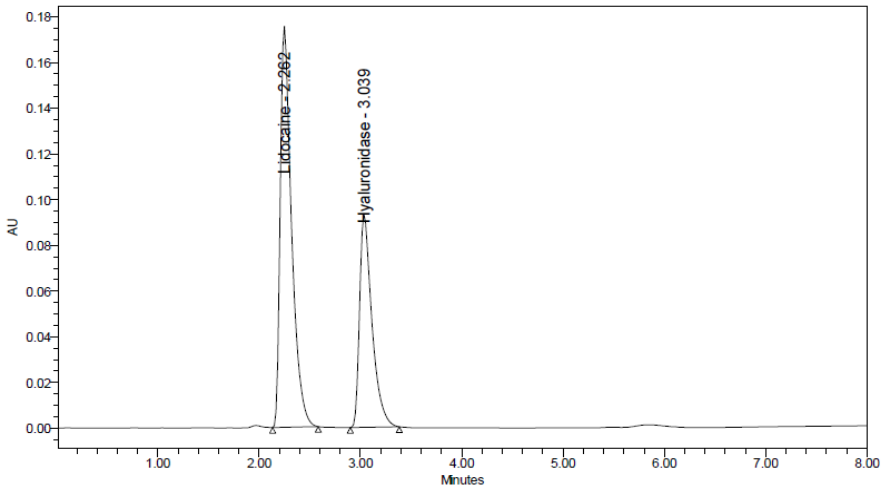


Figure 2b. Chromatogram for Standard.

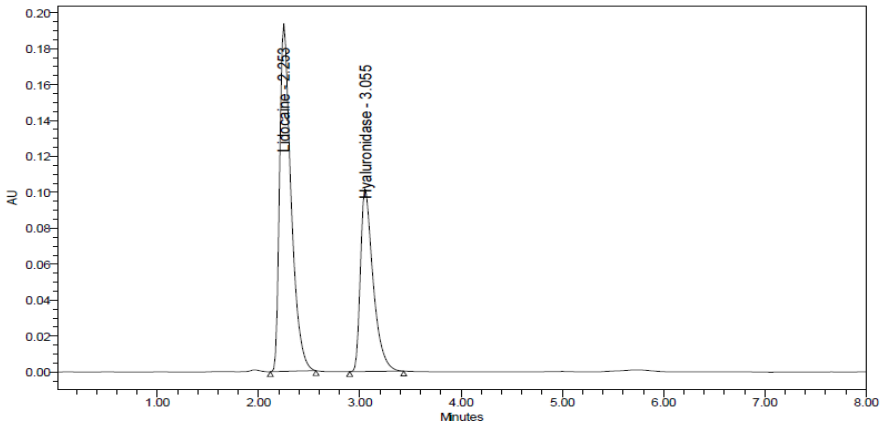


Figure 2c. Chromatogram for Sample.

The calibration data show that the peak area of Lidocaine and Hyaluronidase increase proportionally with concentration in the range of 20µg/ml to 100µg/ml, indicating good Linearity of the method.

Table 3a. Area of different concentration of Lidocaine.

S. No	Lidocaine	
	Concentration (ppm)	Area
1	10	813573
2	20	1527288
3	30	2440781
4	40	3254374
5	50	4067968

Table 3b. Area of different concentration of Hyaluronidase.

S. No	Hyaluronidase Concentration (ppm)	Area
1	5	74925
2	10	139910
3	15	224115
4	20	296820
5	25	372525

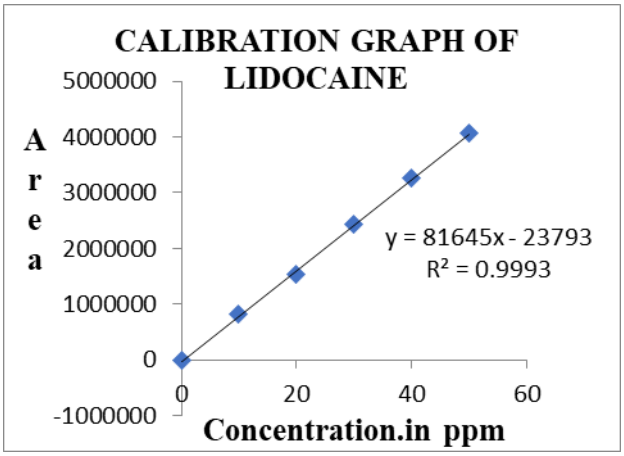


Figure 3a. Calibration graph for Lidocaine

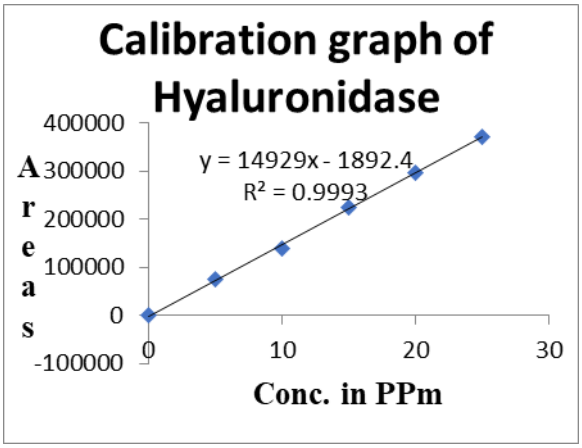


Figure 3b. Calibration graph for Hyaluronidase

Table 4a. Results of Precision for Lidocaine and Hyaluronida.

Injection	Area of Lidocaine	Area of Hyaluronidase
Injection-1	2440782	224116
Injection-2	2440783	223121
Injection-3	2460721	224[13]3
Injection-4	2450752	225141
Injection-5	2540772	226256
Injection-6	2440763	227367
Average	2462428.83	225022.33
Standard Deviation	39200.6	1565.9
%RSD	1.5	0.6

The system intermediate precision results show consistent peak areas for Lidocaine with a % RSD of 0.1% and Hyaluronidase with a %RSD of 0.1%, indicating good repeatability and precision of the analytical method.

Table 4b. Results of Intermediate precision for Lidocaine and Hyaluronidase.

Injection	Area of Lidocaine	Area of Hyaluronidase
Injection-1	2440758	224125
Injection-2	2440773	223133
Injection-3	2440725	224142
Injection-4	2450713	224156
Injection-5	2440751	224172
Injection-6	2440744	224189
Average	2442410.66	223986.166
Standard Deviation	4067.3	418.5
%RSD	0.1	0.1

The signal-to-noise (S/N) ratio for Lidocaine was found to be 2.89 and Hyaluronidase was found to be 2.89, indicating the detectable signal obtained in comparison with the baseline noise.

Table 5a. Results of LOD.

Drug name	Baseline noise(µV)	Signal obtained (µV)	S/N ratio	Conc. in ppm
Lidocaine	76	220	2.89	0.05
Hyaluronidase	76	220	2.89	0.11

The signal-to-noise (S/N) ratio for Lidocaine was found to be 9.93 and Hyaluronidase was found to be 9.93.

Table 5b. Results of LOQ.

Drug name	Baseline noise(µV)	Signal obtained (µV)	S/N ratio	Conc. in ppm
Lidocaine	76	755	9.93	0.18
Hyaluronidase	76	755	9.93	0.39

The accuracy study for Lidocaine shows recoveries ranging from 96.85% to 98.91% and Hyaluronidase shows recoveries ranging from 98.11% to 99.21% across 50%, 100% and 150% concentrations, with a mean recovery of Lidocaine is 98.13% and Hyaluronidase is 98.84%, demonstrating the method's reliability.

Table 6a. Accuracy (recovery) data for Lidocaine.

%Concentration (at specification Level)	**Area* of Lidocaine**	**Amount Added (mg)**	**Amount Found (mg)**	**% Recovery**	**Mean Recovery**
50%	1255392	5	4.93	98.62	
100%	2465781	10	9.69	96.85	98.13%
150%	3777171	15	14.8	98.91	

Table 6b. Accuracy (recovery) data for Hyaluronidase.

%Concentration (at specification Level)	**Area* of Hyaluronidase**	**Amount Added (mg)**	**Amount Found (mg)**	**% Recovery**	**Mean Recovery**
50%	110156	2.5	2.45	98.11	
100%	222815	5	4.96	99.22	98.84%
150%	334172	7.5	7.44	99.21	

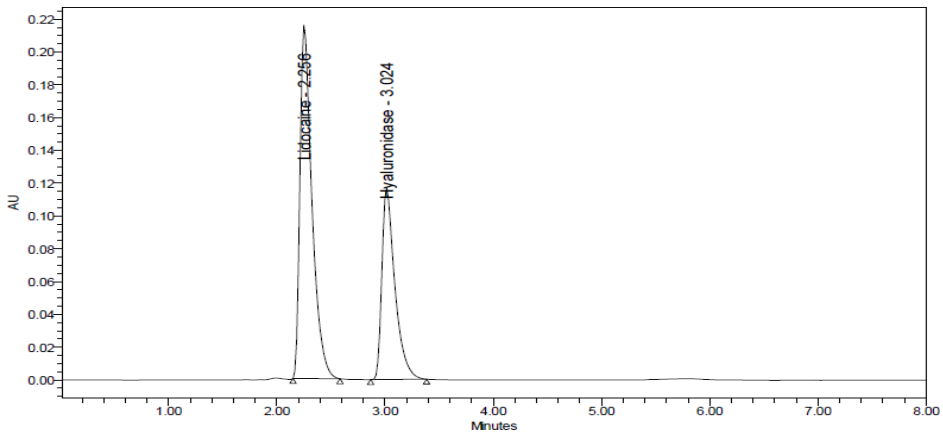


Figure 4a. Chromatogram for Accuracy 50%.

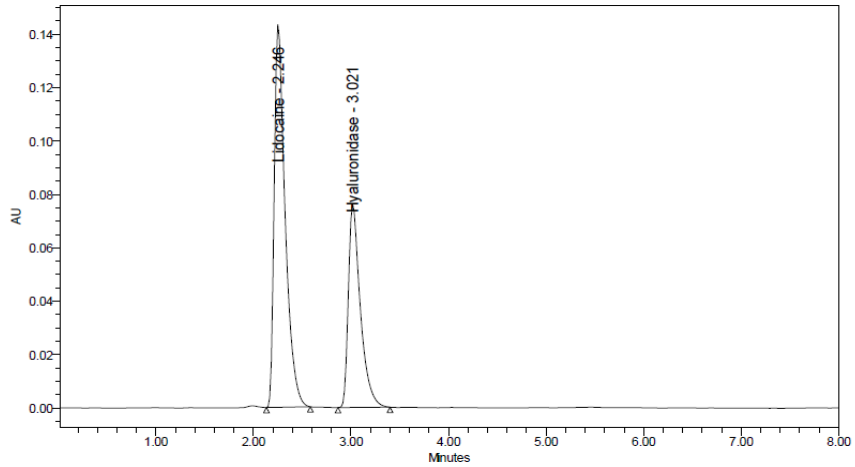


Figure 4b. Chromatogram for Accuracy 100%.

The robustness study for the Lidocaine shows that varying the flow rate from 0.8 to 1.2ml /min and Hyaluronidase flow rate from 0.8 to 1.2ml/min resulted in Theoretical plate counts of Lidocaine is 3299-3312 and tailing factors of 1.21 to1.25 and Hyaluronidase is 3231 to 3235 and tailing factors of 1.10 to1.26, demonstrating that the method remains reliable under small deliberate changes.

Table 7a. Results of Robustness of Lidocaine.

S. No	Flow Rate (ml/min)	System Suitability Results of Lidocaine	
		Theoretical Plate Count	Tailing
1	0.8	3299	1.21
2	1.0	3302	1.25
3	1.2	3312	1.23

Results for actual flow (1.2ml/min) have been considered from assay standard.

Table 7b. Results of Robustness of Hyaluronidase.

S. No	Flow Rate (ml/min)	System Suitability Results of Hyaluronidase	
		Theoretical Plate Count	Tailing
1	0.8	3231	1.10
2	1.0	3232	1.22
3	1.2	3235	1.26

Results for actual flow (1.2ml/min) have been considered from assay standard.

Table 7c. Results for variation in mobile phase composition for Lidocaine and Hyaluronidase.

S. No	Change in Organic Composition in the Mobile Phase	System Suitability Results Lidocaine	
		Theoretical Plate Count	Tailing
1	10% less	3299	1.21
2	*Actual	3302	1.25
3	10% more	3312	1.23
S. No	**Change in Organic Composition in the Mobile Phase**	**System Suitability Results Hyaluronidase**	
		Theoretical Plate Count	**Tailing**
1	10% less	3231	1.10
2	*Actual	3232	1.22
3	10% more	3235	1.26

Results for actual Mobile phase composition have been considered from Accuracy standard.

CONCLUSION

In conclusion, the optimized HPLC method was proven to be reliable and effective for the simultaneous determination of Lidocaine and Hyaluronidase. It meets all regulatory requirements for system suitability and validation parameters, ensuring its applicability for routine analysis in pharmaceutical quality control. This validated method provides a robust tool for ensuring the quality and efficacy of these critical therapeutic compounds.

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CONFLICT OF INTERESTS

The authors declare no conflict of interest

ETHICS APPROVAL

Not applicable

FUNDING

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AI TOOL DECLARATION

The authors declares that no AI and related tools are used to write the scientific content of this manuscript.

DATA AVAILABILITY

Data will be available on request

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