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RESEARCH ARTICLE

Method Development and Validation of HPTLC Method for Simultaneous Estimation of Atropine, Piperine and Vasicine in *Ayurvedic* Formulation

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Abstract

Background: *Dhumayoga*, an *Ayurvedic* formulation is used for the management of respiratory disorders, including dyspnea and asthma. **Aim:** To develop and validate a High-Performance Thin-Layer Chromatography (HPTLC) method for the simultaneous estimation of atropine, vaccine and piperine in *Dhumayoga Ayurvedic* formulation. **Methods:** The three biomarkers were resolved using precoated silica gel $60F_{254}$ as stationary phase, dioxane: toluene: methanol: ethyl acetate: Aqueous ammonia (4:7:3:5:1v/v/v/v/v) as mobile phase and detected and quantified at 254nm. **Results:** The R_f values of atropine, vasicine, and piperine were found to be 0.406 ± 0.02, 0.56 ± 0.02, 0.87 ± 0.02, respectively. The calibration plot was linear in the range of 500-2500ng per zone with a correlation coefficient (r^2) of atropine, vasicine and piperine was 0.994, 0.9986 and 0.9935, respectively. **Conclusion:** The developed method was applied for the quantitation of biomarkers in *Dhumayoga*. The method was validated in terms of linearity, precision, specificity, accuracy and robustness as per International Council for Harmonisation (ICH) guidelines.

Keywords: Atropine, HPTLC, Piperine, Vasicine

1. Introduction

Ayurveda, an ancient Indian medical system, is recognised globally as a complementary and alternative medicine approach. It focuses on preventing diseases and promoting overall well-being through lifestyle choices and therapeutic interventions aimed at revitalising the body. *Dhumayoga* is a polyherbal powder recommended by *Ayurvedic* practitioners for treating respiratory disorders such as dyspnea and asthma and aiding in smoking cessation through medicated smoking. The formulation comprises five components: *Adusa* (*Adhatoda zeylanica*), *Dhattura* (*Datura metel*), *Bhanga* (*Cannabis sativa*), *Cavya* (*Piper retrofractum*) and *Khurasani ajwain* (*Hyoscyamus niger*). Equal proportions of each ingredient are blended to create the powder[1].

Vijaya, Sanskrit for cannabis, is scientifically identified as *Cannabis sativa* Linn. and belongs to the Cannabinaceae family. This plant exhibits various pharmacological effects, including bronchodilation, immune system modulation, anti-inflammatory properties, anti-fibrotic activity against SARS-CoV, antiviral effects and antioxidant capabilities[2-4].

Kala Datura, as referred to in Sanskrit, is scientifically known as *Datura metel* Linn. and belongs to the Solanaceae family. This plant contains tropane alkaloids, including scopolamine, hyoscyamine and atropine. Alkaloids like these contribute to its bronchodilator effects and its antispasmodic and antiemetic properties. Additionally, *Kala Dhatura*

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exhibits antioxidant properties and actions against bacteria and fungi and demonstrates antioxidant activity[5-8].

Parasika yavani (Sanskrit name), also known as *Hyoscyamus niger* Linn. (scientific name) from the Solanaceae family, contains components like hyoscyamine, apoatropine, hyoscine, skimmianine, scopolamine and belladonines. It is a valuable medicinal substance used to address several conditions such as insomnia, psychological disorders, epilepsy, swelling, pain and breathlessness. It contains chemicals and extensive experimentation has demonstrated the drug's efficacy in displaying properties such as analgesic, anti-inflammatory, sedative, antihistaminic, anti-allergic, antimicrobial and antispasmodic[9].

The plant *Cavya* is scientifically known as *Piper retrofractum* Vahl. and termed as "*Cavya*" in Sanskrit, belongs to the Piperaceae family. Its pharmacological activities stem from prime alkaloids like piperine and guineesine, which collectively induce potent bronchodilation and regulate thermogenesis, aiding in energy balance maintenance. Piperine effectively addresses asthma through anti-histaminic actions, inhibiting eosinophil infiltration and suppressing T cell activity. Guineesine acts as an anti-inflammatory by inhibiting Anandamide reuptake and functions as a bronchodilator. Moreover, computational studies highlight their potential against SARS-CoV-2, showing significant binding affinities to crucial viral targets, suggesting a promising role in combating COVID-19[10].

Vasa, known as *Adhatoda vasica* Nees. in scientific terms, belongs to the Acanthaceae family. This plant contains various alkaloids such as vasicine, vasicinone, anisotine and vasicoline. These alkaloids contribute to a range of pharmacological properties, including bronchodilation, expectorant effects, anti-inflammatory actions, anxiolytic and nootropic effects and anti-proliferative properties. Moreover, there is evidence suggesting anti-COVID-19 properties[11-17].

HPTLC represents an advanced instrumental method building on the extensive potential of thin-layer chromatography. Its strengths lie in automation, scanning, comprehensive optimisation, selective detection principles, minimal sample preparation and hyphenation, among other features. These attributes collectively render it a potent analytical tool, particularly valuable for obtaining chromatographic insights into intricate mixtures of inorganic, organic and biomolecules.

In pharmaceutical analysis, HPTLC demonstrates remarkable versatility as an analytical method. Its utility extends to qualitative and quantitative determinations of compounds and impurities, assay procedures, semi-quantitative limit tests and investigations into the degradation behaviour of pharmaceutical substances.

An instrumental chromatographic approach, HPTLC is utilised for effectively separating, detecting and quantifying the active components found within plant extracts and their associated formulations.

HPTLC exhibits enhanced proficiency in separating multiple components and offers ease in analysing various coloured chemical constituents. Moreover, it accommodates the utilisation of sensitive colour reagents, facilitating the detection of specific spots with high sensitivity[18-20].

2. Materials and Methods

2.1 Experimental Materials and Reagents

All materials and reagents used in our study were carefully selected from reputable suppliers. Atropine, vasicine and piperine, crucial standards for our research, were acquired from Yucca Enterprises, renowned for high-quality chemical compounds. Methanol, an essential solvent, was sourced from Astron Chemicals, known for its purity and consistency. Ammonia, another key solvent, originated from Aatur Laboratories, recognised for producing top-tier laboratory-grade chemicals. Ethyl acetate and toluene, vital solvents in various experimental stages, were obtained from Sisco Research Laboratories Pvt Ltd., acknowledged for reliable chemical supplies. Dioxane, an important solvent was sourced from Advent Chembio Pvt Ltd, known for maintaining stringent quality standards. Our selection of these materials and reagents was aimed at ensuring precision, reliability and adherence to high-quality standards throughout our experimental procedures.

2.2 Instruments and Equipment

- CAMAG Thin Layer Chromatography (TLC) scanner 4 for HPTLC, accompanied by Win-CATS software.

- LINOMAT V sample applicator with nitrogen gas continuous pressure assistance.
- CAMAG twin trough chamber with dimensions of 10cm × 10cm and 20cm × 10cm
- Pre-coated TLC aluminium sheets with silica Gel 60 F254F254 TLC aluminium sheets measuring 20cm×20cm, sourced from Merk (Germany).
- Digital electronic balance from Reptech (India).
- Ultraviolet cabinet(UV-1800) by Shimadzu, a Japanese manufacturer.
- Ultrasonic bath produced by Inco, based in Dubai.
- 100µL Linomat syringe provided by CAMAG.

2.3 Solvent Selection

Methanol was decided as a solvent for both standards and the sample as all three standards were properly estimated in methanol.

2.4 Sample Solution Preparation

To create the sample solution, 1 gram of *Dhumayoga* was accurately weighed and subjected to maceration with 10ml of methanol over 24 hours. Subsequently, the mixture underwent thorough filtration, after which the filtrate was completely dried in a water bath. Following the process of drying, approximately 1mg of the dried sample was gathered and subsequently dissolved in 1ml of methanol.

2.5 Stationary Phase

Aluminium precoated TLC plates containing silica gel 60G F_{254} (10cm *10cm) were selected as the stationary phase.

2.6 Preparation of Mobile Phase

Dioxane, toluene, methanol, ethyl acetate and aqueous ammonia in the ratio of 4:7:3:5:1 v/v/v/v/v was selected as the optimised mobile phase for simultaneous estimation of all three standards.

2.7 Method Validation

Method validation is the systematic process of collecting and presenting evidence to ensure that a specific procedure or activity, conducted during testing and production in research, consistently meets predetermined standards. It involves thorough documentation to verify the reliability and accuracy of the method, establishing confidence in its consistent application throughout the research study as per ICH guideline Q2(R1).

Validation Parameters are given below.

2.7.1 Accuracy

An analytical method accuracy signifies the degree of closeness between a determined value and a recognised reference or conventional true value. This alignment, often referred to as trueness, articulates the extent of agreement between the conventional true value and the value derived from the analytical methodology.

2.7.2 Precision

The precision of an analytical technique assesses the consistency or closeness of measurements obtained from multiple samples of the same substance under specified conditions. The study assessed the repeatability, intermediate precision and reproducibility of the method.

2.7.3 Robustness

The robustness of an analytical procedure serves as a quantification of its resilience in the face of deliberate and slight variations in method parameters. This evaluation provides insight into the method's dependability under normal conditions.

2.7.4 Detection Limit

The detection limit of a particular analytical method is characterised as the minimum concentration of an analyte within a sample that can be recognised, not necessarily precisely quantified.

2.7.5 Quantitation Limit

The quantitation limit of a specific analytical method refers to the minimum quantity of an analyte in a sample that can be accurately and precisely determined. This parameter is crucial in quantitative assays targeting low levels of compounds within sample matrices, particularly in the analysis of impurities and degradation products.

2.7.6 Specificity

The ability to definitively evaluate the analyte in the presence of anticipated components, such as impurities, degradants, matrix and similar elements.

2.7.7 Linearity

The analytical linearity is characterised by the capacity, in a defined range, for the result of tests that exhibit a direct proportionality to the concentration or amount of the analyte present in the sample.

2.7.8 Range

The range of an analytical method refers to the span between the upper and lower concentrations or amounts of the analyte within a sample, encompassing these specific concentrations. This range is established through a demonstration that the analytical procedure maintains an appropriate level of precision, accuracy and linearity within this defined interval.

3. Results and Discussion

3.1 Development of Analytical Method

After going through various literature surveys and TLC trials a mobile phase was finalised using dioxane: toluene: methanol: ethyl acetate: aqueous ammonia in a ratio of 4:7:3:5:1 (v/v/v/v/v) with other developed parameters. Using this parameter HPTLC trial was also conducted to see whether proper separation and of standards was obtained or not. The stationary phase comprised plates coated with silica gel 60 F_{254}, while the mobile phase consisted of a mixture of dioxane, toluene, methanol, ethyl acetate and aqueous ammonia in a ratio of 4:7:3:5:1 (v/v/v/v/v). Saturation time was set at 20 minutes, with detection performed at a wavelength of 254nm. The R_f values obtained were as follows: Atropine (0.406 ± 0.02), vasicine (0.56 ± 0.02), and piperine (0.87 ± 0.02) (Table 1 and Figures 1-3).

Table 1. Sample spotting

Track No.	Sample Spotting	Volume (µg)
T1	Atropine	2.0
T2	Vasicine	2.0
T3	Piperine	2.0
T4	Biomarker Mixer	2.0
T5	Biomarker Mixer	4.0
T6	Biomarker Mixer	6.0
T7	Biomarker Mixer	8.0
T8	Biomarker Mixer	10.0
T9	Formulation	6.0
T10	Formulation	8.0
T11	Formulation	10.0
T12	Formulation	12.0
T13	Formulation	14.0

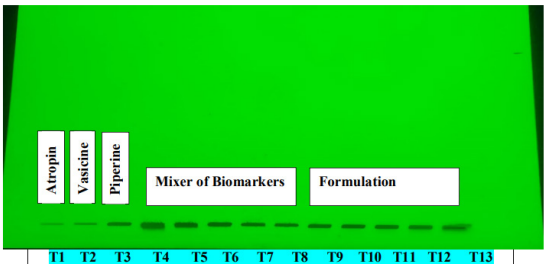


Figure 1. Spotted HPTLC plate of standard and samples.

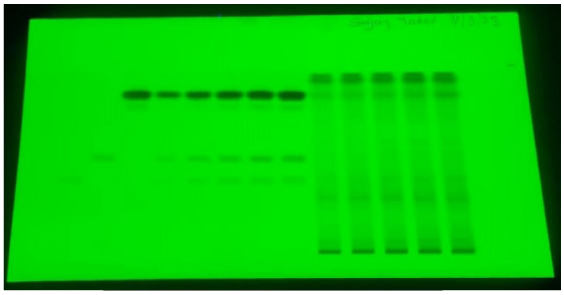


Figure 2. Developed HPTLC plate of standard and samples (track numbers per Table 1).

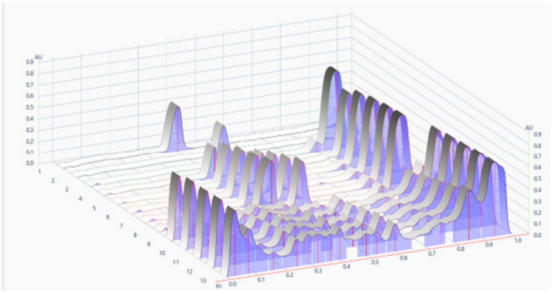


Figure 3. HPTLC 3D densitogram for the mentioned spotting as per Table 1.

3.2 Method Validation

3.2.1 Specificity

Specificity was determined by analysing standard and test samples in the developed mobile phase. All HPTLC densitograms for blank methanol spots, standards and formulations are represented in Figures 4, 5, 6 and 10 respectively.

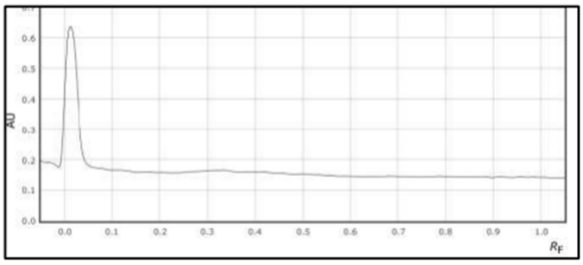


Figure 4. HPTLC densitogram of blank methanol.

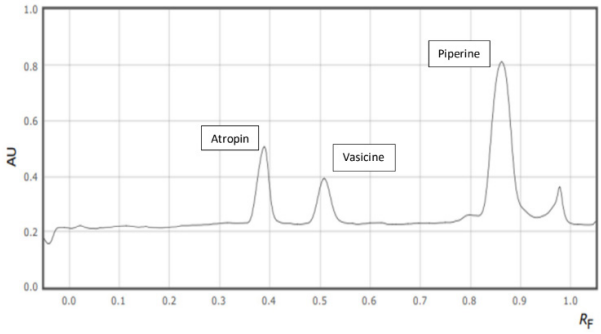


Figure 5. HPTLC densitogram of atropine, vasicine and piperine.

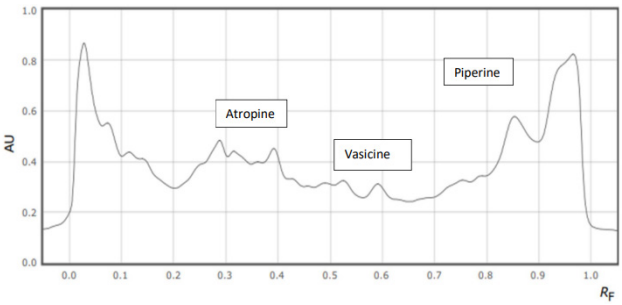


Figure 6. HPTLC densitogram of the formulation.

3.2.1.1 *Discussion*

The identification of each standard spot in the sample was validated through the verification of Retention Factor (R_f) values and UV spectra corresponding to the distinct bands of the standard peaks. Consequently, the method was affirmed to be specific.

3.2.2 *Linearity*

Spotting of a mixture of standards was done on HPTLC plates. The linearity range was decided by the concentration of markers found in the formulation. Spotting of 1000 µg/ml initial solution was applied as spots on a 10*10 dimension stationary plate within the specified volume of 1-5µl according to the range decided. The linearity of atropine, vasicine and piperine is shown in Figures 7, 8 and 9 respectively. The calibration curve was then calculated with the help of the areas obtained for atropine, vasicine and piperine present.

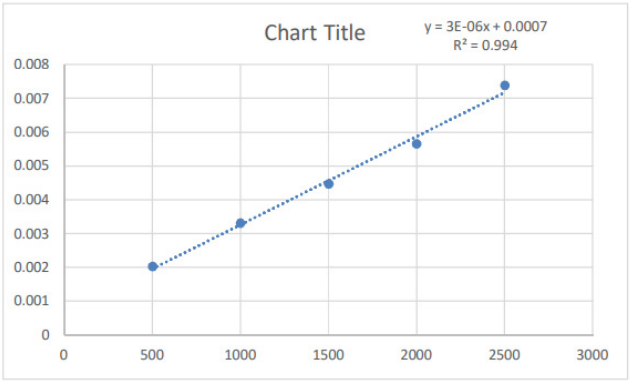


Figure 7. Linearity of atropine.

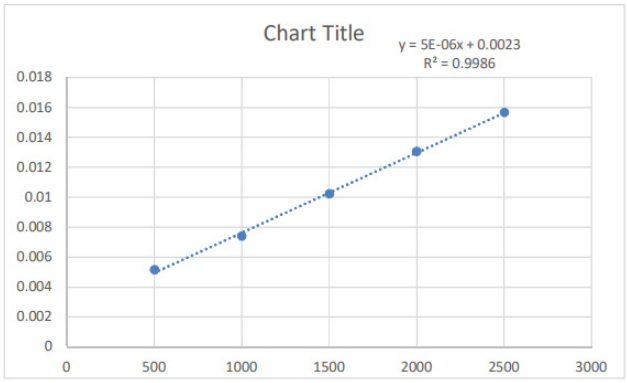


Figure 8. Linearity of avsicine.

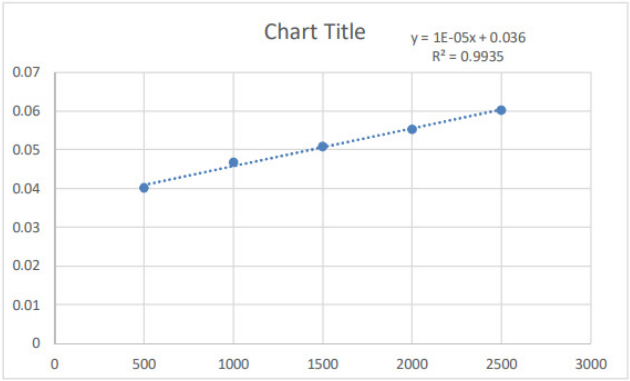


Figure 9. Linearity of piperine.

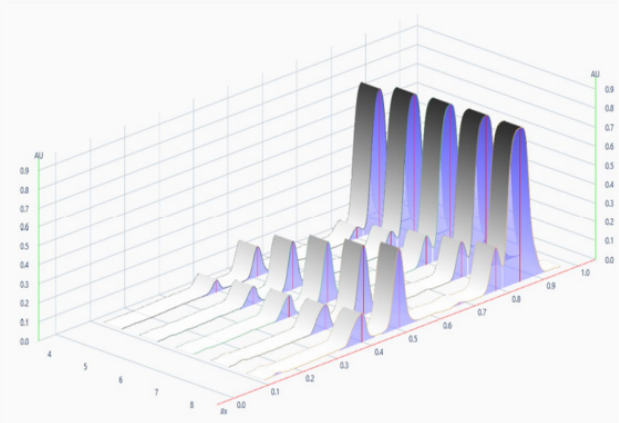


Figure 10. 3D HPTLC densitogram of linearity.

3.2.2.1 Discussion

In consideration of the concentration within the formulation, a range was established to determine the linearity of all three markers, the range of 500-2500ng was selected to determine the linearity study. By obtaining the result of the linearity study, the regression equation and R^2 value of atropine were 'Y = 3E-06x + 0.0007' and 0.994, regression equation and R2 value of vasicine value were 'Y = 5E-06x + 0.0023' and 0.9986 and that of piperine was found to be 'Y = 1E-05x + 0.036' and 0.9935.

3.2.3 Accuracy

With the help of the regression equation found from the linearity study, the concentration of all three biomarkers was found in the sample and by spiking the formulation sample in three distinct concentration levels (80%, 100% and 120%), accuracy study was performed and the average per cent recovery was calculated as shown in Table 2.

3.2.3.1 Discussion

The average per cent recovery of atropine, vasicine and piperine was found to be 101%, 98% and 99% respectively.

3.2.4 Precision

The precision study was been performed using a mixture of standards (1mg/ml) for the same concentration to see agreement of closeness between the measurements. Inter-day precision study was performed in intervals of days as shown in Table 3 and % RSD was calculated.

3.2.4.1 Discussion

Precision study was performed and closeness of measurements were found. % RSD calculated was found to be less than 2.

Table 2. Accuracy

Accuracy of Atropine						
Spike %	**Concentration in sample (ng)**	**Concentration added (ng)**	**Concentration recovered (ng)**	**(%)Recovered**	**SD**	**%RSD**
80	1800	800	1797	99.83	5.56	0.309664
100	2000	1000	2006	100.3	3	0.149551
120	2200	1200	2198	99.9	8	0.363967
Accuracy of Vasicine						
Spike %	**Concentration in sample (ng)**	**Concentration added (ng)**	**Concentration recovered (ng)**	**(%)Recovered**	**SD**	**%RSD**
80	1800	800	1769	101.75	6.32	0.339175
100	2000	1000	2003	99.85	7	0.349476
120	2200	1200	2190	100.45	5	0.228311
Accuracy of Piperine						
Spike %	**Concentration in sample (ng)**	**Concentration added (ng)**	**Concentration recovered (ng)**	**(%)Recovered**	**SD**	**%RSD**
80	1800	800	1753	102.68	7.54	0.430681
100	2000	1000	2007	99.66	6	0.298954
120	2200	1200	2184	100.73	10.53	0.482402

Table 3. Intra-day and Inter-day precision

Phytoconstituent	Study Type	Parameter	Mean Conc. (ng/spot)	Mean Area	SD	%RSD
Atropine	Inter-day	Area	2000	-	0.000028	0.451
Intra-day	Area	2000	-	0.000026	0.451	
Repeatability	Area	2000	0.00565	0.000025	0.451	
Vasicine	Inter-day	Area	2000	-	0.000036	0.254
Intra-day	Area	2000	-	0.000035	0.254	
Repeatability	Area	2000	0.01306	0.000033	0.254	
Piperine	Inter-day	Area	2000	-	0.000030	0.049
Intra-day	Area	2000	-	0.000028	0.049	
Repeatability	Area	2000	0.055316	0.000027	0.04	

Table 4. LOD and LOQ

Standards	Atropine	Vasicine	Piperine
LOD	35.2	18.0566	9.47
LOQ	106.66	54.716	28.7

Table 5. Robustness for wavelength change

Change in wavelength		
Wavelength	**Mean Area*±SD**	**%RSD**
Atropine		
252	0.00737 ± 0.000032	0.43967
254	0.00447 ± 0.000029	0.652232
256	0.00565 ± 0.000025	0.451241
Vasicine		
252	0.01306 ± 0.000033	0.253953
254	0.01565 ± 0.000020	0.127796
256	0.007386 ± 0.000043	0.587043
Piperine		
252	0.055316 ± 0.000027	0.048683
254	0.05082 ± 0.000021	0.041742
256	0.0553 ± 0.000029	0.047809

3.2.5 LOD and LOQ

Utilising the linearity study, the LOD and LOQ were computed employing the standard deviation and slope method, as outlined in Table 4.

3.2.6 Robustness Study

The evaluation of the robustness parameter was conducted by altering the optimised parameters of wavelength and saturation time within a range of (±2), as depicted in Table 5 and Table 6 respectively.

Table 6. Robustness for saturation time change

Change in chamber saturation time.		
Saturation (min)	**Mean Area*±SD**	**%RSD**
Atropine		
18	0.002026±0.000018	0.896639
20	0.00331±0.000029	0.880808
22	0.00447±0.000029	0.652232
Vasicine		
18	0.005132±0.000028	0.540703
20	0.007386±0.000043	0.587043
22	0.010232±0.000019	0.187992
Piperine		
18	0.04016±0.000033	0.082585
20	0.046736±0.000030	0.065083
22	0.05082±0.000021	0.041742

Table 7. Quantification of standards

Standards	Concentration in sample (ng/spot)
Atropine	1797
Vasicine	2006
Piperine	2198

3.2.6.1 Discussion

The robustness study was done by changing developed parameters like saturation time and wavelength change where % RSD was less than 2. Thus, the developed method was robust.

3.2.7 Quantification of Standards

Quantifying the formulation containing standard compounds for analysis was a crucial incorporation

Table 8. Summary of method validation parameters

Parameters	Atropine	Vasicine	Piperine
R_f value	0.406±0.02	0.56±0.02	0.87±0.02
Scanning Wavelength (nm)	254	254	254
Linearity Range (ng)	500-2500	500-2500	500-2500
Regression Equation	Y = 3E-06x +0.0007	Y = 5E-06x +0.0023	Y = 1E-05x + 0.036
R^2	0.994	0.9986	0.9935
Intercept	0.0006568	0.002279	0.0360244
Saturation Time (min)	20	20	20
Specificity	Specific	Specific	Specific
Robustness	Robust	Robust	Robust
LOD (ng)	35.2	18.0566	9.47
LOQ (ng)	106.66	54.716	28.7

formulation study. The quantification of standards in the provided sample was determined using the regression equation and the area of standards in the formulation, as outlined in Table 7.

3.2.7.1 Discussion

A summary of validation parameters for the developed method for the estimation of atropine, vasicine and piperine is been mentioned in Table 8.

4. Conclusion

A precise method was established for the quantification of atropine, vasicine and piperine. in *Dhumayoga* by HPTLC. Validation procedures were conducted for the developed method to detect three main standards by HPTLC. The suggested HPTLC method is straightforward and accurate and exhibits precision, specificity and robustness. It can be effectively utilised for routine quantification or separation of the previously mentioned components standards in any raw plant material or formulations simultaneously.

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