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Original Article

NEW VALIDATED METHOD FOR THE ESTIMATION OF ALLANTOIN AND PERMETHRIN USIGN RP-HPLC IN BULK AND PHARMACEUTICAL DOSAGE FORM

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ABSTRACT

Objective: Special, effective high pressure liquid chromatography method has been developed for the simultaneous quantification of Allantoin and Permethrin.

Methods: By using Waters HPLC e-2695 quaternary pump with a PDA detector of 2998 instrument the chromatographic separation of Allantoin and Permethrin was achieved on the column of Symmetry C_{18} (150x4.6 mm, 3.5 µm) using an isocratic elution with a buffer containing 0.1percent ortho phosphoric acid and acetonitrile at a rate of 40:60 as a mobile phase with a flow rate of 1 ml/min at ambient temperature. A detector wavelength of 226 nm utilizing the PDA detector were given in the instrumental settings. The linearity was studied between the concentration range of 1-15 µg/ml of Allantoin and 25-375 µg/ml of Permethrin were injected with a run time of 6 min. As a part of method validation the parameters like specificity, linearity, accuracy, ruggedness, robustness were determined and the results were found to be within the allowable limit. Validation of the proposed method was carried out according to an International Conference on Harmonization (ICH) guidelines.

Results: LOD and LOQ for the two active ingredients were established with respect to test concentration. The plotted calibration curves were linear with a regression coefficient of R^2>0.999, indicates that the linearity was with in the limit. As a part of method validation the parameters like specificity, linearity, accuracy, ruggedness, robustness were determined and the results were found to be within the allowable limit.

Conclusion: The method developed was found to be applicable to routine analysis and to be used for the measurement of both active pharmaceutical ingredients (i. e, Allantoin and Permethrin). Since, there is no HPLC method reported in the literature for the estimation of Allantoin and Permethrin, there is a need to develop quantitative methods under different conditions to achieve improvement in specificity, selecivity etc.

Keywords: Allantoin, Permethrin, HPLC, Development, Validation

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INTRODUCTION

The chemical compound Allantoin has the formula C4H6N4O3. It's also known as glyoxyldiureide or 5-ureidohydantoin. It's a glyoxylic acid diureide [1, 2]. In most species, including mammals, plants, and bacteria, allantoin is a major metabolic intermediate. Urate oxidase (uricase) converts uric acid [3], which is a degradation product of nucleic acids [4], to uric acid [5-7]. It's popular in toothpaste [8], mouthwash [9], and other oral health items [10], as well as shampoos, lipsticks, anti-acne products [11], sun care products, and clarifying lotions, as well as other cosmetic and pharmaceutical products [12]. Since uric acid is the end product of purine metabolism in humans [13], allantoin can only be produced by non-enzymatic processes involving reactive oxygen species, making it a good biomarker for oxidative stress in chronic illnesses [14] and ageing [15, 16].

Permethrin is a drug and insecticide that is marketed under various brand names, including Nix [17]. It is used to treat scabies [18] and lice [19] as a drug. It's used as a cream or lotion on the skin. It can be sprayed on clothing or mosquito nets [20] as an insecticide to destroy any insects that come into contact with them. Rashes and annoyance at the application site are common side effects. It tends to be safe to use during pregnancy. It's safe to use on and around people who are at least two months old. Permethrin belongs to the pyrethroid [21] drug class. It functions by interfering with the activity of lice and scabies mite neurons. Permethrin is available as a cream or lotion for topical application. It's used to treat and avoid head lice in people who have been exposed to them, as well as to treat scabies. So, we are interested in developing a new validated method for the simultaneous quantification of Allantoin and Permethrin using RP-HPLC.

MATERIALS AND METHODS

Chemicals

Acetonitrile, HPLC-grade ortho phosphoric acid, water were purchased from Merck India Ltd, Mumbai, India. APIs of Allantoin and Permethrin standards were procured from Glenmark, Mumbai.

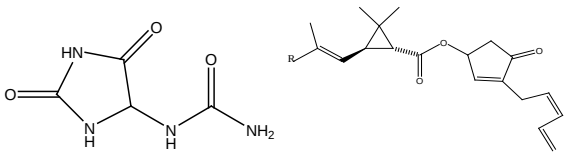


Fig. 1: Chemical structure of (A) Allantoin and (B) Permethrin

The ınstrumentation

Waters alliance liquid chromatography (model e-2695) monitored with empower 2.0 data handling system and a detector of photo diode array (model 2998) was used for this study [22].

Preparation of buffer

1 ml of ortho phosphoric acid is dissolved in 1 lt of HPLC grade water and filter through 0.45 µ filter paper.

Method optimization

To optimize the chromatographic conditions, different ratios of phosphate buffer and the acetonitrile in the mobile phase with isocratic mode was tested. However the mobile phase composition was modified at each trial to enhance the resolution and also to achieve acceptable retention times. Finally 0.1% ortho phosphoric acid buffer and acetonitrile with isocractic elution was selected because it results in a greater response of active pharmacy ingredients. During the optimization of the method various stationary phases such as C_8, C_{18} phenyl and amino, inertsil ODS columns were tested. From these trials the peak shapes were relatively good with a Symmetry C_{18} column of 150 x 4.6 mm, 3.5 µm with a PDA detector. The mobile phase flow rate has been done at

226 nm in order to obtain enough sensitivity. By using above conditions we get retention times of Allantoin and Permethrin were about 3.882 and 2.453 min with a tailing factor of 1.02 and 1.05. The number of theoretical plates for Allantoin and Permethrin were 6351, 3576 which indicate the column's successful output the % RSD for six replicate injections was around 0.27% and 0.11%, the proposed approach suggests that it is extremely precise. According to ICH guidelines, the established method was validated.

Till today there are no HPLC methods reported in the literature, So, it has more interested to develop a novel and reliable HPLC strategy for simultaneous establishment of Allantoin and Permethrin.

Chromatographic conditions

The HPLC analysis was performed on reverse phase HPLC system with isocratic elution mode using a mobile phase of acetonitrile and 0.1% ortho phosphoric acid and Symmetry C_{18} (150x4.6 mm, 3.5 µm) column with a flow rate of 1 ml/min.

Diluent

Mobile phase was used as diluent.

Preparation of the standard stock solution

For standard stock solution preparation, add 70 ml of diluents to 10 mg of Allantoin and 250 mg of Permethrin taken in a 100 ml volumetric flask and sonicate for 10 min to fully dissolve the contents and then make up to the mark with diluent.

Preparation of standard solution

5 ml of solution is drawn from the above normal stock solution into a 50 ml volumetric flask and diluted up to the level.

Validation procedure

The analytical parameters such as system suitability, precision, specificity, accuracy, linearity, robustness, LOD, LOQ, forced degradation and stability were validated according to ICH Q2 (R1) guidelines [23-25].

System suitability

System suitability parameters were measured to verify the system performance. The parametrers including USP plate count, USP tailing and % RSD are found to be within the limits.

Accuracy

Accuracy is the closeness of the test results obtained by the method to the true value. It was assessed by the recovery studies at three different concentration levels. In each level, a minimum of three injections were given and amount of the drug present, percentage recovery and related standard deviation were calculated.

Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of other components (impurities, degradates or excipients), which may be expected to be present in the sample and standard solution. It was checked by examining the chromatograms of blank samples and samples spiked with Allantoin and Permethrin.

Precision

Precision of an analytical method is the degree of agreement among individual test results. It was studied by analysis of multiple sampling of homogeneous sample. The precision of the present method was assessed in terms of repeatability, intra-day and inter day variations. It was checked by analyzing the samples at different time intervals of the same day as well as on different days.

Linearity and range

Linearity of an analytical method is its ability to obtain results directly proportional to the concentration of the analyte in the sample within a definite range. The seven series of standard solutions were selected for assessing linearity range. The calibration curve was plotted using peak area versus concentration of the standard solution and the regression equations were calculated. The least squares method was used to calculate the slope, intercept and correlation coefficient.

LOD and LOQ

LOD is the lowest amount of analyte in a sample that can be detected while LOQ is the lowest amount of analyte in a sample that can be determined with acceptable precision and accuracy. LOD and LOQ was separately determined based on the calibration curve. The LOD and LOQ for Allantoin and Permethrin were determined by injecting progressively low concentrations of standard solutions using the developed RP-HPLC method. as per ICH guidelines.

Stress degradation

Stress degradation should be no interference between the peaks obtained for the chromatogram of forced degradation preparations. Stress degradation studies were performed as per ICH guidelines Q_1A (R2). The degradation peaks should be well separated from each other and the resolution between the peaks should be at least 1.0 and the peak purity of the principle peaks shall pass. Forced degradation studies were performed by different types of stress conditions to obtain the degradation of about 20%.

Robustness

The robustness of an analytical procedure is a measure of its ability to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage. Robustness study was performed by injecting standard solution into the HPLC system and altered chromatographic conditions such as flow rate (±0.2 ml/min), organic content in the mobile phase (±10%). The separation factor, retention time and peak asymmetry were calculated by determining the effect of the modified parameters.

Stability

Analytical solution was prepared and injecting into the HPLC system at periodic intervals of 0 h to 24 h at 6 h intervals depending on the instrument utilization and sequence of injection.

RESULTS AND DISCUSSION

The main analytical challenge during development of a new method was to separate active Pharma ingredients. In order to provide a good performance the chromatographic conditions were optimized.

Method validation

The optimized RP-HPLC validated method according to ICH guidelines in terms of system suitability, linearity, accuracy, precision and robustness.

System suitability

Device suitability was performed by injecting standard solution containing 10 µg/ml of Allantoin and 250 µg/ml of Permethrin in six replicates. The results show that the machine fitness parameter is within the limit provided by ICH. The results were shown below.

Specificity

In this test method placebo, sample and standard solutions were analyzed individually to examine the interference. The below fig. shows that the active ingredients were well separated from blank and their excipients and there was no interference of placebo with the principal peak. Hence the method is specific.

Linearity

Linearity was calculated by plotting a calibration curve of the peak area against its respective concentration, linearity was determined. From this calibration curve, it was noticed that the curve was linear between the range of 1-15µg/ml of Allantoin and 25-375µg/ml of Permethrin. The regression equations for calibration curve was Y=84225.07x+16194.97 (R^2=0.9993) for Allantoin and Y= 10542.71x+93791.93 (R^2=0.99922) for Permethrin respectively.

Table 1: Results of system suitability

System suitability parameter	Acceptance criteria	Drug name	
		Allantoin	Permethrin
USP Plate count	NLT 2000	6325	3127
USP Tailing	NMT 2.0	1.05	1.01
USP Resolution	NLT 2.0	-	10.27
% RSD	NMT 2.0	0.27	0.11
Retention Time	NLT 2.0	2.453	3.882

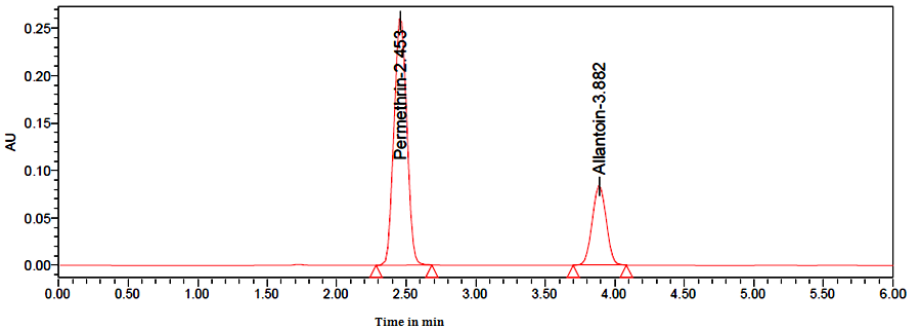


Fig. 2: Chromatogram of standard

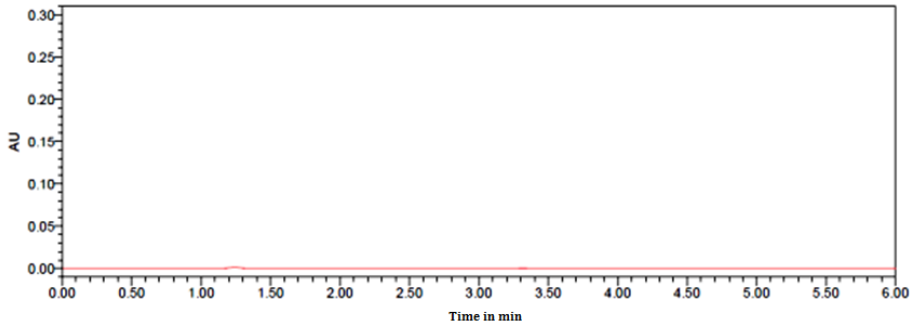
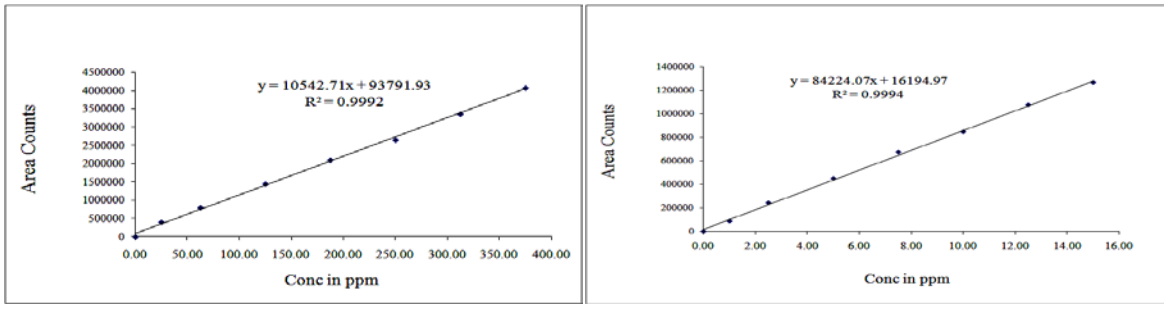


Fig. 3: Chromatogram of blank

Table 2: Linearity results of allantoin and permethrin

Linearity	Allantoin		Permethrin	
	Conc. (µg/ml)	Area	Conc. (µg/ml)	Area
Linearity-1	1.00	85632	25.00	401203
Linearity-2	2.50	241874	62.50	798654
Linearity-3	5.00	449857	125.00	1452643
Linearity-4	7.50	672543	187.50	2103564
Linearity-5	10.00	845263	250.00	2653140
Linearity-6	12.50	1075648	312.50	3365472
Linearity-7	15.00	1264784	375.00	4076534
Slope	84225.07		10542.71	
Intercept	16194.97		93791.93	
CC	0.9993		0.9992	



A **B**

Fig. 4: Calibration plots of (A) Permethrin and (B) Allantoin

Accuracy

The accuracy of the system was achieved by measuring the recovery experiments at three stages (50 percent, 100 percent and 150 percent). APIs with concentrations of 5, 10 and 15µg/ml of Allantoin and 125, 250 and 375µg/ml of Permethrin were prepared. For each spike stage, the test solution was injected three times and the test was performed according to the test process. The recovery results were similar to 100% and also the RSD values were less than±2%. The percentage recovery, mean and relative standard deviations were determined. Recovery values shown within the desired range were correct. The results are summarized below. Accuracy findings have been shown in table 3.

Table 3: Results of accuracy

S. No.	% Level	Allantoin % recovery	Permethrin % recovery
1	50	99.52	99.45
2	100	99.84	100.24
3	150	98.93	100.21
Mean		99.43	99.97
Std Dev		0.462	0.448

mean±SD (n=3)

Precision

The precision of the analytical technique is the degree of proximity of the sequence of measurements obtained from multiple homogeneous mixture samplings. The accuracy of the process of the drugs were calculated by injection of six individual determinations of Allantoin (10 µg/ml) and Permethrin (250µg/ml). Method precision results were shown in table 4 and sample chromatogram was shown in fig. 5.

Intraday precision

Six replicates of a sample solution containing Allantoin (10µg/ml) and Permethrin (250µg/ml) were analysed on the same day. Peak areas were calculated, which were used to calculate mean, SD and %RSD values.

Intermediate precision

Six replicates of the sample solution were analyzed by different researchers and different tools were checked on separate days. The peak regions used to assess the average percent of RSD values have been determined. The findings are shown in the table below.

Interday precision

Six replicates of a sample solution containing Allantoin (10µg/ml) and Permethrin (250µg/ml) were analysed on a different day. Peak areas were calculated which were used to calculate mean, SD and % RSD values. The present method was found to be precise as the RSD values were less than 2% and also the percentage assay values were close to be 100%. The results are given in table 5 [26].

Table 4: Intraday precision results of allantoin and permethrin

S. No.	Allantoin			Permethrin		
	Conc. (µg/ml)	Area	% Assay	Conc. (µg/ml)	Area	% Assay
1	10	845632	100.21	250	2647593	100.24
2		845721	100.15		2632015	100.16
3		846320	100.06		2645178	99.78
4		847596	100.27		2632958	99.95
5		843265	100.11		2675412	100.07
6		846217	99.98		2694301	99.63
% RSD	0.17			0.94		
Mean	100.13			99.97		
Std Dev	0.104			0.233		

mean±SD (n=6)

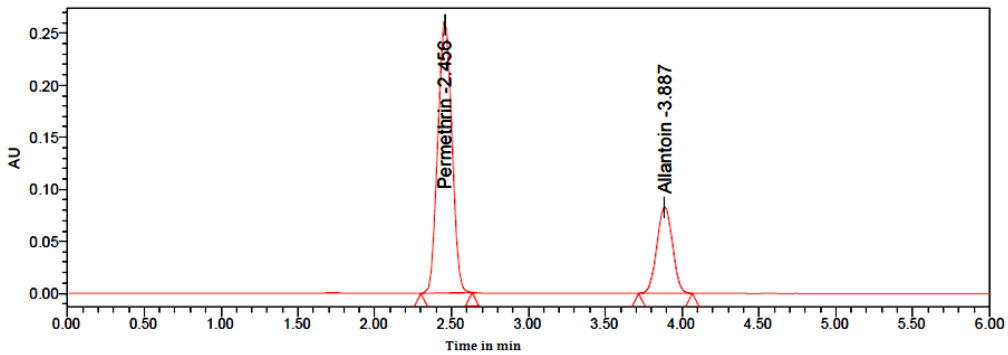


Fig. 5: Chromatogram of sample

LOD and LOQ

LOD and LOQ were determined separately using the calibration curve technique. The LOD and LOQ of the compound were measured using the developed RP-HPLC method by injecting lower and lower concentrations of the standard solution. The LOD and LOQ concentrations and their s/n values of Allantoin and Permethrin were represented in the following table.

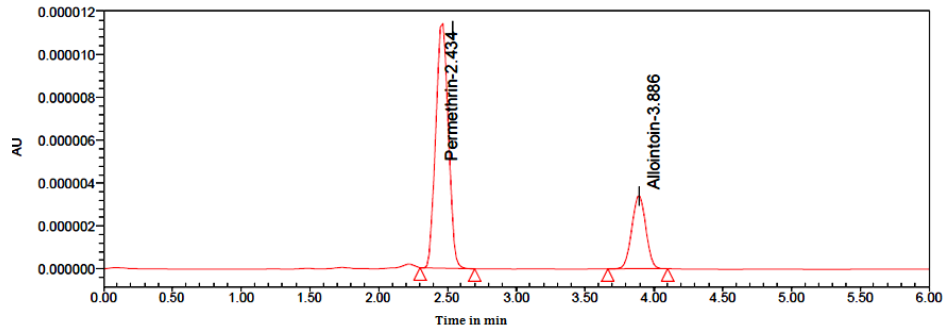
Table 5: Inter-day precision results

S. No.	Allantoin			Permethrin		
	Conc. (µg/ml)	Area	% Assay	Conc. (µg/ml)	Area	% Assay
1	10	845261	99.63	250	2634957	100.01
2		847575	99.57		2615478	100.12
3		846327	99.42		2635925	100.45
4		846321	100.15		2685964	100.63
5		846237	100.43		2685471	100.28
6		849563	100.11		2685694	101.07
%CV	0.178			1.205		
Mean	99.89			100.43		
Std Dev	0.400			0.384		

mean±SD (n=6)

Table 6: Results of LOD and LOQ

Allantoin				Permethrin			
LOD		LOQ		LOD		LOQ	
Conc. (µg/ml)	s/n	Conc. (µg/ml)	s/n	Conc. (µg/ml)	s/n	Conc. (µg/ml)	s/n
0.013	3	0.0.043	24	0.313	5	1.033	27



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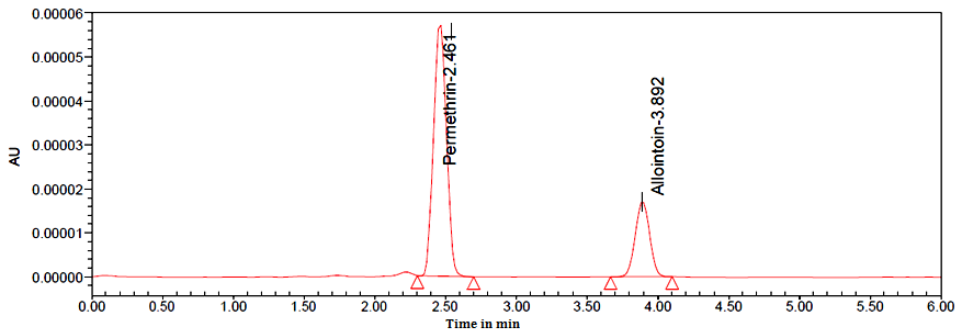

B

Fig. 6: Chromatogram of (A) LOD and (B) LOQ

Table 7: Robustness results of allantoin and permethrin

Parameter name	% RSD	
	Allantoin	Permethrin
Flow rate (0.8 ml/min)	0.63	1.24
Flow rate (1.2 ml/min)	1.15	0.96
Org Plus (66:34) (+10%)	1.27	0.72
Org Minus (54:46) (-10%)	0.39	0.65

Robustness

The conditions of the experiment was designed to measure the robustness of the intentionally changed conditions such as flow rate, mobile phase in organic percentage in all these varied conditions. Robustness results for Allantoin and Permethrin were found to be within the limit and results were tabulated in table 7 [27].

Stability

Normal solution was kept at room temperature and 2-8 °C for up to 24 h. These solutions were then pumped into the system and the percent deviation from the initial to 24 h [28, 29] was measured. No major variations were found and verified that the solutions were stable up to 24 h percentage of the assay was not quite 2%. There is

no effect in storage conditions for Allantoin and Permethrin drugs. Stability results were tabulated in table 8.

Degradation studies

Allantoin and Permethrin standard was subjected to various conditions of forced degradation in order to induce partial degradation of the compound. Forced degradation experiments have been performed to establish that the process is acceptable for degradation materials [30, 31].

In addition the studies include information on the condition under which the drug is unstable, such that the steps are also taken during formulation to prevent possible instabilities.

Table 8: Stability results of allantoin and permethrin

Stability	Allantoin		Permethrin	
	Purity	% deviation	Purity	% deviation
Initial	99.99	0.01	99.99	0.01
6 h	99.25	0.75	99.53	0.47
12 h	98.96	1.04	99.24	0.76
18 h	98.72	1.28	98.95	1.05
24 h	98.31	1.69	98.56	1.44

Acid degradation

Acid degradation was done by using 1N HCl and 14.2% Allantoin and 13.9% Permethrin degradation was observed.

Alkali degradation

Alkali degradation was done by using 1N NaOH and 14.7% of Allantoin and 13.1% of Permethrin degradation was observed.

Peroxide degradation

Peroxide degradation was done by using 30% peroxide and 13.6% of Allantoin and 12.4% of Permethrin degradation was observed.

Reduction degradation

Reduction degradation was done by using 30% sodium bisulphate solution and 13.3% of Allantoin and 11.2% Permethrin degradation was observed.

Thermal degradation

Thermal degradation was done at 105 °C and 12.9% of Allantoin and 10.3% Permethrin degradation was observed.

Hydrolysis degradation

Hydrolysis degradation was done by using HPLC water and 11.3% Allantoin and 10.7% Permethrin degradation was observed.

Table 9: Forced degradation results of allantoin and permethrin

Degradation condition	Allantoin	Permethrin
	% deg	% deg
Acid deg	14.2	13.9
Alkali deg	14.7	13.1
Peroxide deg	13.6	12.4
Reduction deg	13.3	11.2
Thermal deg	12.9	10.3
Hydrolysis deg	113	10.7

CONCLUSION

This method described the quantification of Allantoin and Permethrin in bulk and pharmaceutical formulation as per ICH guidelines. The evolved technique was found to be accurate, precise, linear and reliable. The advantage lies in the simplicity of sample preparation and reproducibility data are satisfactory. The evolved chromatographic method can be effectively applied for regular investigation in drug research.

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AUTHORS CONTRIBUTIONS

All authors have contributed equally.

CONFLICTS OF INTERESTS

Author declares that there have been no conflicts of interest.

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