IJCRT.ORG ISSN : 2320-2882

INTERNATIONAL JOURNAL OF CREATIVE RESEARCH THOUGHTS (IJCRT)

An International Open Access, Peer-reviewed, Refereed Journal

DEVELOPMENT AND VALIDATION OF HPLC METHOD FOR ESTIMATION OF PIMOBENDAN IN TABLET DOSAGE FORM

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ABSTRACT:

The primary aim of this study was to develop simple, rapid, isocratic, precise and accurate high performance liquid chromatographic method (RP-HPLC) for the quantitative estimation of Pimobendan in tablet dosage form. The chromatographic separation was achieved by using column Reprosil BDS C18 (250mm x 4.6mm, 5µm) and mobile phase containing ratio of 0.1% triethylamine (TEA) in water (pH adjusted to 3.0 with orthophosphoric acid): acetonitrile (60:40% v/v), at a flow rate 0.7 ml/min using UV detector at 330 nm. Diluent used was acetonitrile: water in the ratio of 80:20% v/v. The retention time of Pimobendan was found to be 7.37 minutes. The reliability and analytical performance of proposed HPLC method was validated as per ICH guidelines with respect to linearity, range, accuracy, precision, robustness, limit of detection and quantification. Linearity for Pimobendan was observed in the range of 2-20 µg/mL, with correlation coefficient 0.9998. All the other parameters were found to be within prescribed limits. The developed HPLC method was found to be rapid, sensitive and precise for estimation of Pimobendan in tablet dosage form.

KEYWORDS: Pimobendan, Method development, Method validation, RP-HPLC.

INTRODUCTION:

Pimobendan is an orally administered veterinary drug used as an inodilator. It is used in management of congestive heart failure in canines due to atrioventricular valvular insufficiency. Pimobendan is calcium sensitizer and a selective inhibitor of Phosphodiesterase 3 (PDE3) with positive inotropic and vasodilator effects. Chemically it is 3-[2-(4-methoxyphenyl)-3*H*-benzimidazol-5-yl]-4-methyl-4,5-dihydro-1H-pyridazin-6-one. Pimobendan is available in the market as tablet dosage form for the treatment of congestive heart failure caused by dilated cardiomyopathy.

Literature survey reveals that the only one RP- HPLC method is available for the estimation of pimobendan but lacks of all SST parameters and also, they have used ODS column instead of BDS column based on molecular structure of the compound and they haven't described it or give any particular reason [1]. Therefore, present method aims to develop and validate simple, robust, rapid, selective, accurate, precise, economical and reproducible RP- HPLC method for the estimation of pimobendan in tablet dosage form.

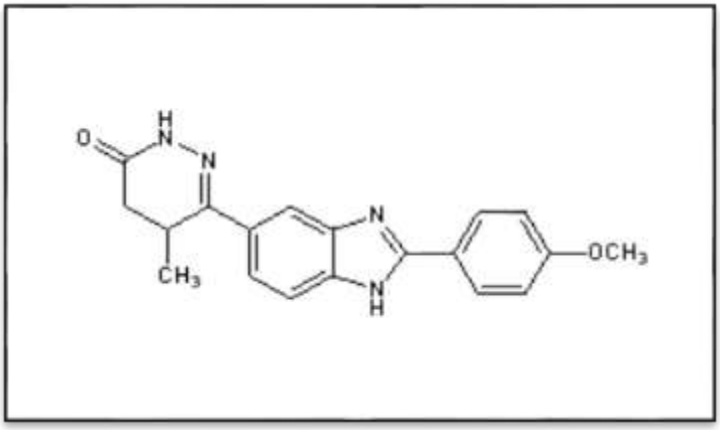

Figure no. 1: structure of pimobendan

MATERIAL AND METHODS:

Chemical and Reagents:

Pimobendan working standard with defined purity of 99.03% was obtained from Central Drug Testing Laboratory, Mumbai. Cardisure tablet 1.25mg (Pimobendan tablet) was also obtained from Central Drug Testing Laboratory, Mumbai. Acetonitrile of HPLC grade from Merck life science Pvt. ltd., Triethylamine AR grade from Sisco chem laboratory and Ultrapurified HPLCgrade water was obtained from the Milli - Q® system were used during method development. Nylon membrane filter (0.45µ) from milipore (USA) was used.

Instrumentation:

For all spectrophotometric measurement Perkin Elmer UV/VIS spectrophotometer Lambda 45 having Perkin Elmer UV WinLab software was used. The Chromatography separation was performed on the Thermo Scientific Ultimate 3000 HPLC system equipped unit chromeleon7. 4. 2 software. All weighing were carried out using Sartorius analytical balance. Different types of apparatus like waterbath, vacuum filter pump, Millipore filtration kit, sonicator, sample filtration assembly and glassware were used throughout the study.

Determination of wavelength:

10 mg pimobendan standard was weighed accurately and transferred into the 100 ml volumetric flask and volume was made up to the mark with diluent (100 µg/ml). The aliquot portion of pimobendan standard stock solution was diluted to obtained solution of 12.5 µg/ml concentration. Then the above solution was scanned in UV visible spectrophotometer in the range of 400.0 nm to 200.0 nm and diluent used as blank solution. Pimobendan showed maxima at 330 nm as shown in Fig. 2. Hence this wavelength was approved for the analysis of the pimobendan.

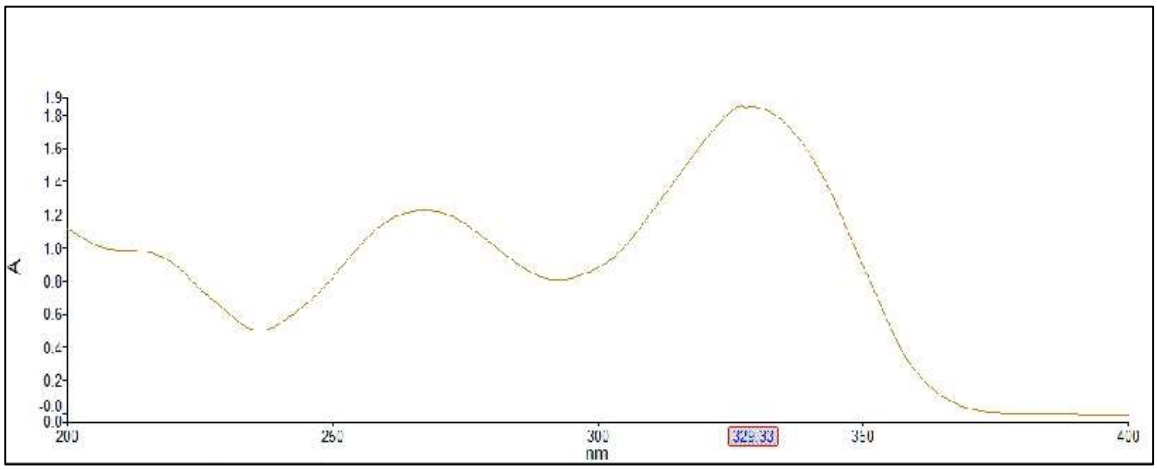

Figure 2: UV spectra of pimobendan

Chromatographic condition:

The chromatographic separation was performed using column Reprosil BDS C18 (250mm x 4.6mm, 5µm) and mobile phase containing 0.1% triethylamine in water pH adjusted is 3.0 with OPA and acetonitrile in the ratio of (60:40% v/v) was used. The diluent consisting acetonitrile and water in the ratio of (80:20% v/v) was used. The flow rate was adjusted to 0.7 ml/min. The injection volume was 10 µl and column temperature was 40^0 c. The total run time was 10 min. and detection were carried out at 330 nm.

Preparation of buffer for mobile phase:

0.1% triethylamine was prepared by dissolving 1ml triethylamine into 1000 ml of HPLC grade water and sonicated for few minutes using sonicator. After that pH was adjusted to pH 3.0 with orthophosphoric acid (OPA) and the mobile phase was filtered using 0.45 µ nylon membrane filter.

Selection of diluent:

Based on the solubility and chemical nature of pimobendan, acetonitrile and water in the ratio of (80:20% v/v) were chosen as diluent for the preparation of standard and sample solutions.

Preparation of Mobile phase:

0.1% triethylamine (pH 3.0) and acetonitrile in the ratio of 60:40% v/v were used as a mobile phase for the present study. The mobile phase was sonicated and then degassed using an ultra sonicator.

Preparation of Standard solution:

The standard solution of pimobendan was prepared by dissolving exactly 10 mg standard into 100 ml of volumetric flask. The solution was sonicated and the volume was made up with diluent and further dilutions were made to get a concentration of about 12.5 µg/ml of pimobendan.

Preparation of Sample solution:

Ten tablets of Cardisure® (1.25 mg) were weighed and average weight was calculated. The tablets were then crushed to get fine powder and weight equivalent to 1 tablet (233.2mg) was transferred in 100ml volumetric flask and sufficient amount of diluent was added. The content was sonicated for 10minutes and the final volume was made up to the mark with diluent (12.5 µg/ml).

Method Development:

The pKa of pimobendan is 11.17 indicating that the molecule is basic. Hence base deactivated (BDS) column was first choice for the retention of the drug. Different types/brands of BDS columns with different proportion of mobile phase containing water and organic solvents were studied. Initial trials on HPLC were carried out on Sepachrome C18 column, with mobile phase containing 0.1% triethanolamine buffer (pH 3): Acetonitrile (60:40% v/v) at a flow rate of 1ml/min. But poor peak shape and lower retention time was observed. Further trials were conducted with 0.1% TEA buffer (pH 3) and acetonitrile on Hemochrome C18 column. Finally, better peak shape with acceptable system suitability (SST) parameters were found with mobile phase comprising 0.1% TEA buffer (pH 3) with acetonitrile in the ratio of (60:40% v/v) on Reprosil BDS C18 (250mm x 4.6mm, 5µm) column. The chromatographic conditions are summarized in Table no.1.

Table 1: chromatographic conditions.

	Chromatographic condition
Column	Reprosil BDS C18 (250mm x 4.6mm, 5µm)
Mobile phase	0.1% Triethylamine: Acetonitrile (60:40% v/v)
Flow rate	0.7 ml/min.
Run time	10 min.
Column Temperature	40^0 C
Injection Volume	10 µl
Detection wavelength	330 nm

Method validation:

Validation of the optimized RP-HPLC method was done according to ICH Q2 (R2) (International Council for Harmonisation) guidelines concerning various parameters like specificity, linearity, precision, accuracy and recovery, detection and quantification limit (LOD, LOQ) and robustness.

System Suitability Testing:

System suitability test was developed and used to verify system performance by injecting six replicates of pimobendan standard solution containing concentration of 12.5 µg/ml into the HPLC system. The chromatograms were recorded to assess the SST parameters such as retention time, theoretical plates, tailing factor and %RSD.

Linearity:

The linearity of pimobendan was determined by preparing appropriate aliquots of standard stock solution of pimobendan to obtain samples in the concentration range of 2-20 µg/ml. The linear calibration graph was created by analysing the concentration over the selected range versus the peak area of sample solutions. Pimobendan was found to be linear in the given concentration range.

Accuracy and Recovery:

Accuracy is the measurement of closeness of experimental results obtained by a certain method to the true value. Recovery studies were done by the standard addition method of known amount of pimobendan standard solution added in pre-analysed sample solution at three different level such as 110%, 120%, 130%. At each concentration level three determinations were performed and mean % recovery was calculated and reported. The result of mean % recovery was within the acceptable limits and is shown in Table no. 4.

Precision:

System Precision

System precision studies were performed by injecting six injections of pimobendan standard solution (12.5µg/ml) in HPLC system. The mean, SD, % RSD of peak areas of six replicate injections were calculated and are reported in Table no. 5.

Method Precision (Assay repeatability)

Method precision was carried out by injecting six replicate injections of standard solution of pimobendan (12.5µg/ml) and six sample solution of pimobendan (12.5µg/ml) in triplicates into the HPLC system. The mean, SD, % RSD and % assay was calculated and is reported in Table no. 6.

Intermediate Precision

This was carried on two different days by injecting six replicates of standard solution (12.5µg/ml) and six sample solution of pimobendan (12.5µg/ml) in triplicates into the HPLC system. The mean, SD, %RSD, and % assay was calculated and is reported in Table no. 7.

Robustness:

Robustness is performed by small or deliberate change in the parameters that should not affect any method. For HPLC method, robustness was established by making changes include different wavelength (±2nm), change in mobile phase composition (± 2%), change in flow rate (± 0.2ml/min.), change in column temperature (± 2°C) and the results was calculated and reported in Table no. 8.

Limit of Detection (LOD):

The pimobendan limit of detection value was detected and quantitated by using following formula,

LOD = 3.3 x σ/s

Where,

σ = standard deviation,

s = slope of the calibration curve

Limit of Quantification (LOQ):

The pimobendan limit of quantification value was quantified by using following formula,

LOQ = 10 x σ/s

Where,

σ = standard deviation,

s = slope of the calibration curve

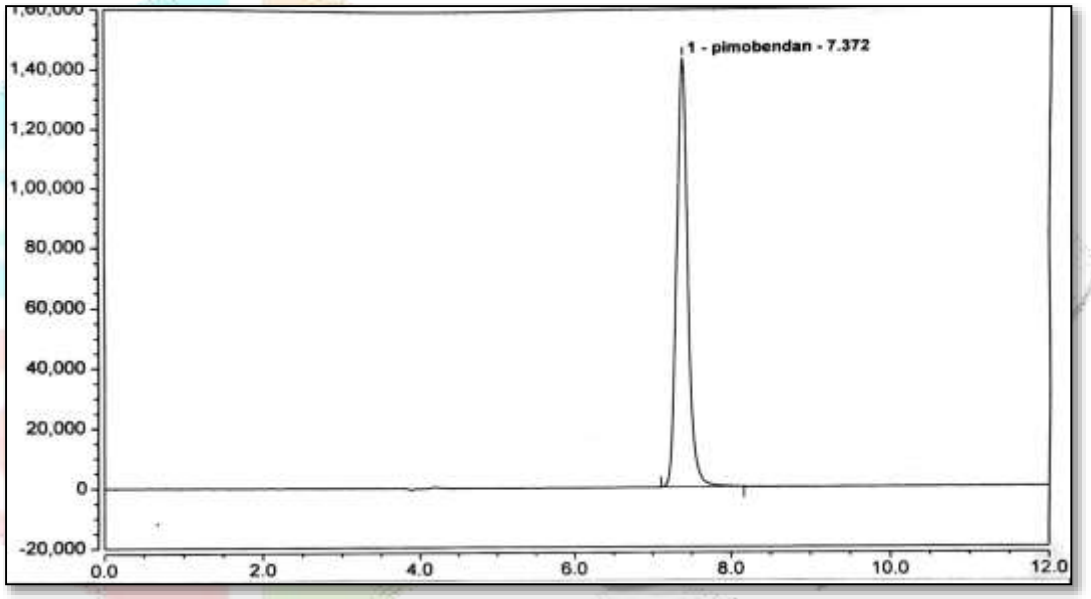

Figure 3: chromatogram of standard solution of pimobendan

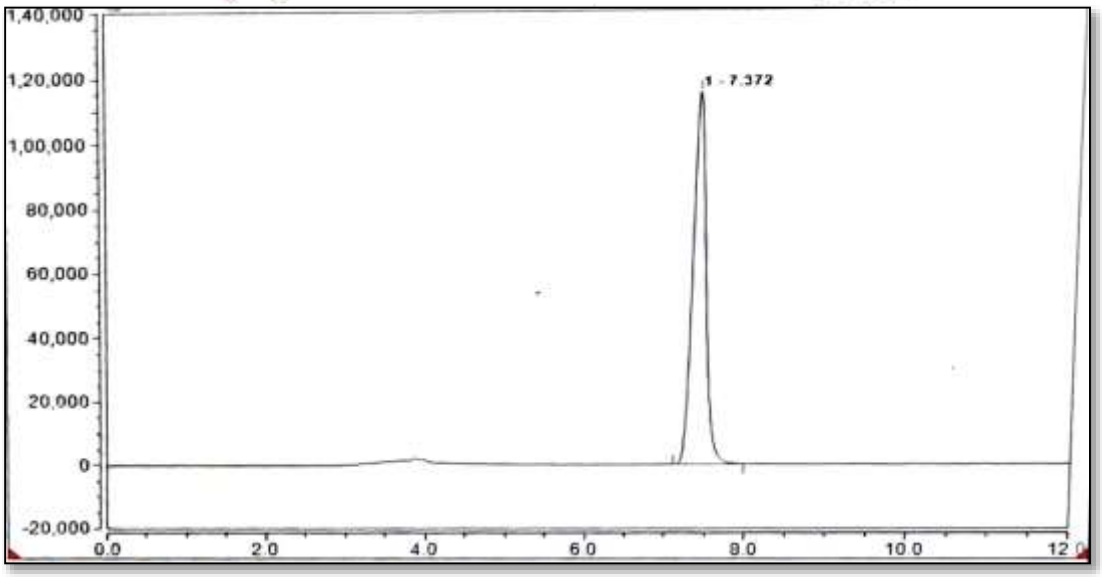

Figure 4: chromatogram of sample solution of pimobendan

Table no. 2: system suitability studies of pimobendan

Sr no.	Peak Area	Retention Time	Tailing Factor	Theoretical Plates
1	23965	7.38	1.21	13965
2	23736	7.38		
3	23726	7.37		
4	23968	7.37		
5	23895	7.37		
6	23825	7.37		
Mean	23853	7		
SD	107.827	0.00516		
%RSD	0.452	0.070		

Table no. 3: linearity data of pimobendan

Linearity Level	Concentration µg/ml	Peak Area
1	2	3759
2	5	9740
3	10	19124
4	12.5	23894
5	15	28559
6	17.5	33787

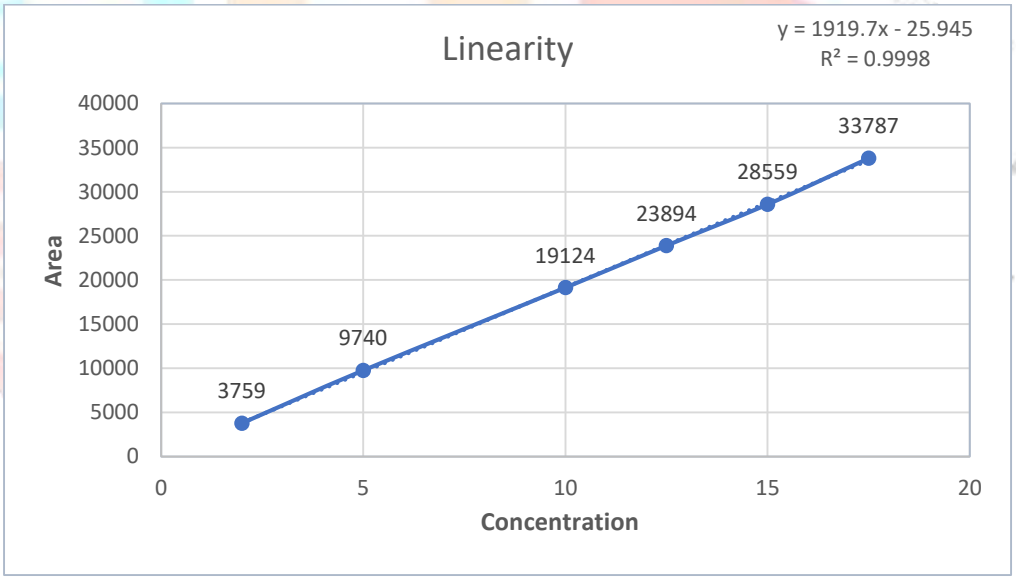

Figure 5: linearity graph of pimobendan

Table no. 4: accuracy study data of pimobendan

%Level Added	STD Spiked (ml)	Amount Recovered (mg/tab)	% Recovery	Mean % Recovery	SD	%RSD
110	0.5	1.412	102.77	102.74	0.07571	0.07370
110	0.5	1.412	102.79			
110	0.5	1.410	102.65			
120	1	1.519	101.29	101.34	0.04582	0.04522
120	1	1.519	101.35			
120	1	1.520	101.38			
130	1.5	1.662	102.78	102.76	0.01732	0.01685
130	1.5	1.662	102.75			
130	1.5	1.662	102.75			

Table no. 5: system precision (standard) data

Injection no.	Area at 330nm	Limit
1	23965	NMT 2.0 %
2	23736	
3	23726	
4	23968	
5	23895	
6	23825	
Mean	23853	
SD	107.827	
%RSD	0.452	

Table no. 6: method precision (sample) data

Injection no.	Area at 330nm	Limit
1	21618	NMT 2.0 %
2	21886	
3	21602	
4	21707	
5	21718	
6	21792	
Mean	21721	
SD	106.995	
%RSD	0.493	

Table no. 7: intermediate precision/interday precision

Injection no.	Day 1-Analyst A (%)	Day 2-Analyst 2 (%)	Limit
1	99.97	99.85	NMT 2.0 %
2	101.21	99.98	
3	99.90	99.71	
4	100.39	99.42	
5	100.44	100.4	
Mean	100	100	
SD	0.52227	0.36107	
%RSD	0.520	0.362	

Table no. 8: robustness data of pimobendan

Parameters	Change in Parameters (±)	% Estimation	Mean	SD	%RSD	Limit
Wavelength	328nm	99.16	99.76	1.036356	1.038814	NMT 2%
	330nm	100.96				
	332nm	99.17				
Temperature	38°C	98.99	99.77	1.047043	1.049457	
	40°C	100.96				
	42°C	99.36				
Flow Rate	0.5ml	98.79	99.42	1.341007	1.34883	
	0.7ml	100.96				
	0.9ml	98.51				
Mobile Phase	62:38	99.11	99.68	1.11072	1.114286	
	60:40	100.96				
	58:42	98.97				

Table no. 9: limit of detection and limit of quantification (lod & loq)

Parameters	Pimobendan
Linearity Range (µg/ml)	2-17.5
Regression Equation	1919.7x – 25.945
Slope	1919.7
Y-Intercept	-25.945
Correlation Coefficient	0.99989
Standard Error	180.9562
Observations	6
Limit of Detection (µg/ml)	0.3110
Limit of Quantification (µg/ml)	0.9426

Table no. 10: assay result of pimobendan

Sample no.	Weight of Standard (mg)	Sample weight (233.2mg contains 1.25mg	Mean Area of Standard at 330nm	Area of Sample at 330nm	%Assay
1	10.08	233.1	23858	21618	99.97
2		233.2		21886	101.21
3		233.1		21602	99.90
4		233.3		21707	100.39
5		233.4		21718	100.44
6		233.2		21792	100.78
Mean					100.4483
SD					0.49459
%RSD					0.492

RESULTS & DISCUSSION:

A simple, rapid, isocratic method was developed for determination of pimobendan in pharmaceutical tablet dosage form. Optimization of method was done by choice of mobile phase composition, selection of column, selection of wavelength, injection volume, temperature, flow rate. The observed chromatogram of pimobendan standard and sample solution gives sharp peak with retention time 7.37 min.

The SST parameters were applied to standard chromatogram and parameters like theoretical plates were observed to be greater than 7000, tailing factor was less than 2. RSD of peak area and retention time were also determined.

The concentration for pimobendan was linear over the range of 2-20 µg/ml. The linear calibration curve was plotted by analysing the concentration over the selected range versus peak area of reference solution and R^2 value was found to be 0.9998 from calibration curve.

For the accuracy studies the average of percent mean recovery at the level of 110%, 120%, 130% was found to be 102.28% and mean percent RSD was found to be 0.7950% which is within the limit, hence the method is accurate.

For the system precision percent RSD value was found to be 0.452 and for method precision percent RSD value was found to be 0.493 which are within the acceptable limit of 2.0%. Intermediate precision was evaluated in term of interday precision by considering two different day interval. The % assay, average, SD, RSD was found within the limit for pimobendan.

The robustness was achieved by varying one parameter at a time. The resulting values were found to be within the limit thus, the developed method was found to be robust.

The optimized method can detect and quantify the analyte at a lower concentration. The limit of detection and quantification values for pimobendan were 0.3110 µg/ml and 0.9426 µg/ml respectively. The observed assay of pimobendan in Cardisure tablet dosage form is 100.44%

CONCLUSION:

The present RP-HPLC method was developed for quantitative and qualitative estimation of pimobendan in pharmaceutical tablet dosage form. The proposed method was successfully validated for parameters such as system suitability, linearity, accuracy, precision, robustness, LOD & LOQ and assay as per the ICH Q2(R2) guidelines. The results for all validation parameters were within the acceptable limits. The developed RP-HPLC method was found to be simple, selective, precise and accurate. Also, this method uses lesser mobile phase, simple reagents with minimum preparations and shorter duration for analysis. The present RP-HPLC method can be used for routine analysis and quality control of pimobendan in tablet dosage form in the pharmaceutical industry.

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