International Journal of ChemTech Research
CODEN(USA): IJCRGG ISSN : 0974-4290
Vol.5, No.5, pp 2154-2164, July-Sept 2013

Development And Validation Of Methods For Estimation Of Pimobendan In Pharmaceutical Dosage Form

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Abstract: The present research work aims to develop a simple, sensitive, accurate and reproducible method for the estimation of Pimobendan by Spectrophotometric and chromatographic methods. In Spectrophotometric method an absorbance maximum for Pimobendan was found to be at 328 nm using methanol as a solvent and linearity was observed in concentration range of 1-7µg/ml. In RP-HPLC, chromatographic separation was carried out on Shimadzu LC-2010 CHT using Inertsil®ODS-3(4.6 X 100mm,3µm) column as stationary phase and mobile phase containing Buffer(KH_2PO_4 ,pH=2.5): Acetonitrile (80:20 v/v) at flow rate of 1 ml/min using UV detection at 328 nm. The retention time for Pimobendan was found to be 4.2 min. The methods were successfully validated in accordance to ICH guidelines. The drug was found to undergo degradation when exposed to acidic, basic, oxidation, thermal and photo degradation conditions. The developed method can be applied successfully to estimate Pimobendan in tablet dosage form without the interference of common excipients.
Keywords: Pimobendan, UV Spectrophotometry, RP-HPLC, Forced degradation.

INTRODUCTION

Pimobendan (PIMO) is a novel drug with ability to inhibit phosphodiesterase III (PDE3), which play key role in management of the signs of mild, moderate, or severe congestive heart failure due to atrioventricular valvular insufficiency or dilated cardiomyopathy. Chemically it is (Pimobendan,4,5-dihydro-6-[2-(4-methoxyphenyl)-1H-benzimidazole-5-yl]-5-methyl-3(2H)-pyridazinone[1] Fig 1 is a positive inotropic agent with a vasodilatory properties. Pimobendan is available as tablet dosage form in market for the treatment of heart failure, most commonly caused by myxomatous mitral valve disease (also known as endocardiosis), or dilated cardiomyopathy[2-4] . The review of literature reveals that two HPLC methods available for estimation of enantiomers and metabolites of Pimobendan using chiral column[5-6]. However, the literature survey does not reveal any Spectrophotometric, RP-HPLC and stability study method for the estimation of Pimobendan. The present paper presents the development of a simple, sensitive and accurate UV Spectrophotometric, Stability study and RP-HPLC method for estimation of Pimobendan in tablet dosage form.

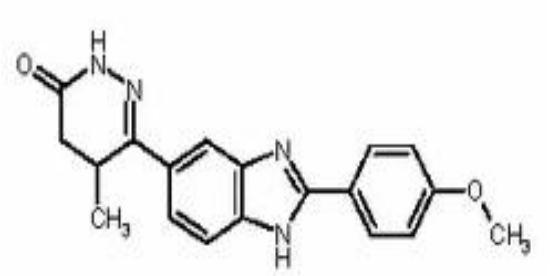

Fig. 1: Structure of Pimobendan

MATERIALS AND METHODS

Chemicals and Reagents

The pure drug PIMO and VETMEDIN Tablets (Pimobendan-5mg) were obtained as a gift sample from Astron Research Pvt. Ltd., Ahmedabad. AR grade and HPLC grade Methanol (SPECTROCHEM), Potassium Dihydrogen Orthophosphate Anhydrous (MERCK), Orthophosphoric acid HPLC grade (SPECTROCHEM), Acetonitrile (SPECROCHEM), 3% H_2O_2 (SPECTROCHEM), Milli-Q water, hydrochloric acid and sodium hydroxide of AR grade were used for the work..

Instrumentation

A UV–Visible double beam spectrophotometer (Shimadzu) model 1800 with spectral slit width of 2.0 nm was used for experiments. Chromatographic separation was carried out on Shimadzu LC-2010 CHT equipped with Inertsil®ODS-3(4.6 X 100MM, 3µm). Water bath, Vacuum oven and UV cabinet were used for Stability study.

EXPERIMENTAL WORK

UV-SPECTROPHOTOMETRY METHOD [6-7]

Selection of solvent

The drug is soluble in methanol. Therefore methanol was selected as solvent.

Preparation of stock solution

Accurately weighed portion of Pimobendan (100 mg) was transferred to a separate 100ml

Volumetric flask and dissolved in diluent and diluted to the mark. Further 10 ml was taken and transferred to 100 ml volumetric flask to give solution containing 100µg/ml.

Selection of analytical wavelength

The solution of Pimobendan was prepared in methanol at a concentration of 6µg/ml. It was scanned in the wavelength range of 200-400 nm. Maximum absorbance was obtained at 328 nm. This analytical wavelength was selected for determination of Pimobendan.

Preparation of Sample Solution From tablet dosage form

Twenty tablets were weighed and powdered equivalent to 50 mg of Pimobendan transferred in to a 50 ml volumetric flask. Methanol was added to it and sonicated for 20 min. The solution was filtered through Whatman filter paper No.41 and the volume was made up to mark with methanol. This solution is expected to contain 50 mg Pimobendan. This will produce sample solution containing Pimobendan 1000µg/ml. Take 10 ml of resulting solution and make up to 100 ml in 100 ml volumetric flask which containing Pimobendan 100µg/ml.(Stock solution A).From the stock solution A, 4 ml was transferred to volumetric flask of 100 ml capacity. Volume was made up to the mark with methanol to give a solution containing 4µg/ml. This solution was used for the estimation of Pimobendan.

Method Validation [8]

Linearity

A calibration curve was plotted over a concentration range of 1-7µg/ml. Accurately measured standard stock solution of PIMO (0.1, 0.2, 0.3, 0.4, 0.5, 0.6 and 0.7 ml) were transferred to a separate series of 10 ml of volumetric flasks and diluted to the mark with methanol. The absorbance of each solution was measured at 328 nm. Calibration curve was constructed by plotting absorbance versus concentrations at 328 nm. Each reading was average of three determinations. (Fig. 2-3)

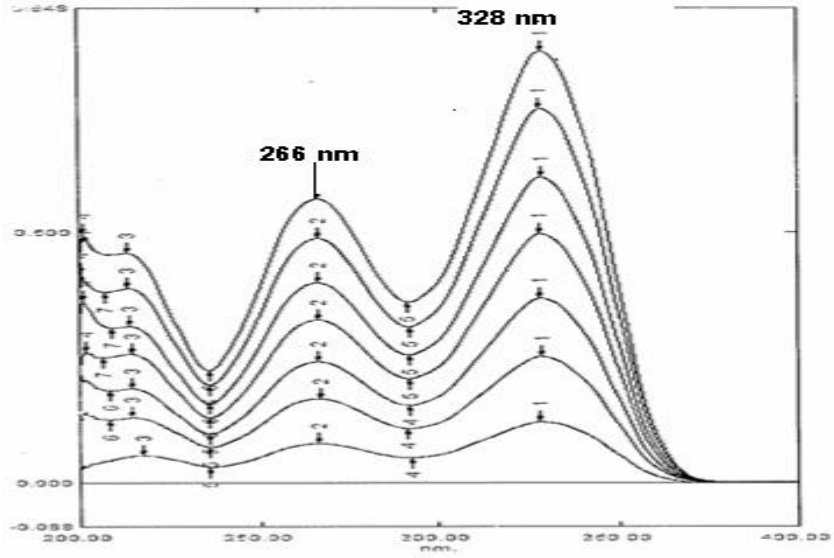

Fig. 2: Overlay spectra of PIMO

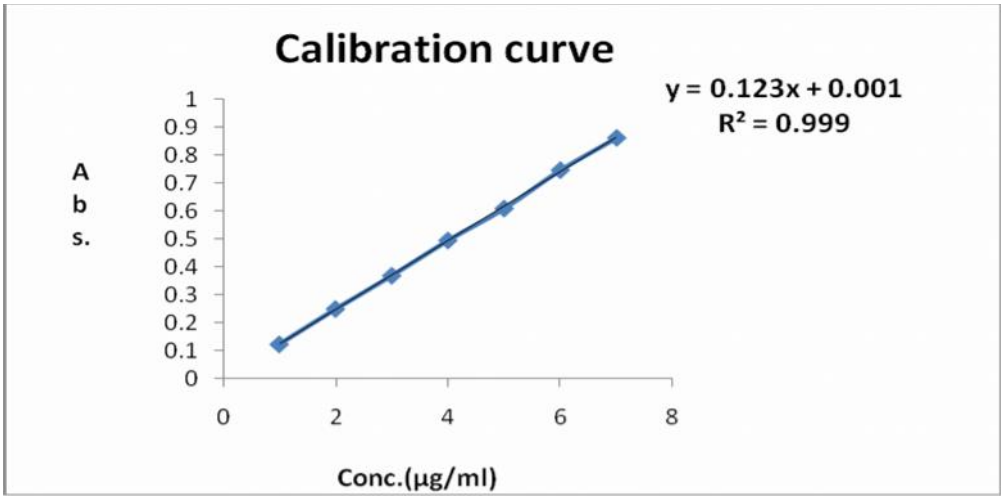

Fig. 3: Calibration curve of PIMO at 328nm

Precision

The intra-day and inter-day variation for determination of PIMO was carried out three times in the same day and three consecutive days and % RSD were calculated. The method was found to be precise due to low values of the %RSD (Table 2).

Accuracy (% Recovery)

Accuracy of the method was determined in terms of % recovery of standard. Recovery studies were carried out by addition of standard drug solution at the level of 50%, 100% and 150% to the pre analyzed sample. In this

method the known concentration of standard drug was added to the assay sample. The average percent recoveries for PIMO are shown in Table 3.

LOD and LOQ

The LOD and LOQ of developed method were studied as per ICH guidelines. Several approaches for determining the LOD & LOQ are possible, depending on the procedure i.e, a non-instrumental or instrumental. Among them here employed method was,

LOD= 3.3σ/S and
LOQ= 10σ/S
Where σ = the standard deviation of response,
S = the slope of calibration curve.

The results obtained are shown in Table 1.

Table 1: Regression characteristics and validation parameters of PIMO

Parameters	Result
Solvent	Methanol
λ_{max} (nm)	328
Regression equation	y = 0.123x + 0.001
Slope	0.123
Intercept	0.001
Correlation coefficient (r)	0.999
Linearity Range (µg/ml)	1-7
LOD (µg/ml)	0.097
LOQ(µg/ml)	0.296

Table 2 : Precision data

Conc. (µg/ml)	Intraday precision(n=3) Mean ± S.D.*	%RSD*	Interday precision(n=3) Mean ± S.D.*	%RSD*
1	0.123±0.0020	1.68	0.128±0.0020	1.60
2	0.251±0.002	0.79	0.252±0.0045	1.78
3	0.363±0.0020	0.57	0.367±0.005	1.36
4	0.493±0.0020	0.42	0.498±0.0055	1.10
5	0.61±0.002	0.32	0.617±0.0060	0.97
6	0.741±0.0035	0.47	0.747±0.0050	0.67
7	0.863±0.0041	0.48	0.872±0.0035	0.40

***** Mean of three estimations

Table 3 : Determination of Accuracy

% Level of spike	Amount Taken (µg/ml)	Amount Added (µg/ml)	Amount Found* (µg/ml)	%Recovery± SD
50%	2	1.5	3.47	99.41±0.0065
100%	2	3	4.96	99.34±0.0080
150%	2	4.5	6.52	100.31±0.0075

***** Mean of three estimations

Table 4: Analysis of Marketed formulation by UV-spectrophotometer

Formulation	Amount Labeled (mg)	Amount found* (mg)	% Recovery ± S.D (n=3)
Vetmedin	5	5.03	100.4±0.045

***** Mean of three estimations

STABILITY STUDY BY UV SPECTROPHOTOMETRIC METHOD [9-11]

Preparation of stock solution (100µg/ml):

An accurately weighed quantity of standard Pimobendan (5 mg) powder was transferred to 50 ml volumetric flask and dissolved in 30 ml of methanol. The flask was sonicated for 5 min and volume was made up to mark with methanol to get 100µg/ml of Pimobendan.

Acid hydrolysis

Transfer 5 ml stock solution of standard Pimobendan (100µg/ml) in 100 ml volumetric flask and 5 ml of 1 N Hydrochloric acid was added. The flask was heated in a water bath at 70ºc for 1 hour and allowed to cool to room temperature. Solution was neutralized with 1 N Sodium hydroxide using pH meter and suitably diluted with diluents to obtain final conc. 5µg/ml of Pimobendan. The UV spectrum was recorded (Fig. 4) & the conc. was calculated using regression equation (Table 5).

Base hydrolysis

Transfer 5 ml stock solution of standard Pimobendan (100µg/ml) in 100 ml volumetric flask and 5 ml of 1 N Sodium hydroxide was added. The flask was heated in a water bath at 70ºc for 1 hour and allowed to cool to room temperature. Solution was neutralized with 1 N Hydrochloric acid using pH meter and suitably diluted with diluents to obtain final conc. 5µg/ml of Pimobendan. The UV spectrum was recorded (Fig. 5) & the conc. was calculated using regression equation (Table 5).

Oxidative condition

Transfer 5 ml stock solution of standard Pimobendan (100µg/ml) in 100 ml volumetric flask and 5 ml of 3% Hydrogen Peroxide was added. The flask was heated in a water bath at 70ºc for 1 hour and allowed to cool to room temperature and suitably diluted with diluents to obtain final conc. 5µg/ml of Pimobendan. The UV spectrum was recorded (Fig. 6) & the conc. was calculated using regression equation (Table 5).

Dry Heat Degradation

Solid drug was exposed in oven at 60º for 24 hour then allowed to cool down at room temperature and 2.5 mg of standard Pimobendan was weighed , transferred to 25 ml vol. flask and dissolved in methanol and volume made up to the mark. Further diluted to get final conc. of 5µg/ml of Pimobendan. The UV spectrum was recorded (Fig. 7) & the conc. was calculated using regression equation (Table 5).

Photo degradation studies

Solid drug was exposed to UV light at 254 nm for 3 days then 2.5 mg of standard Pimobendan was weighed, transferred to 25 ml vol. flask and dissolved in methanol and volume made up to the mark. Further diluted to get final conc. 5µg/ml of Pimobendan. The UV spectrum was recorded (Fig. 8) & the conc. was calculated using regression equation (Table 5).

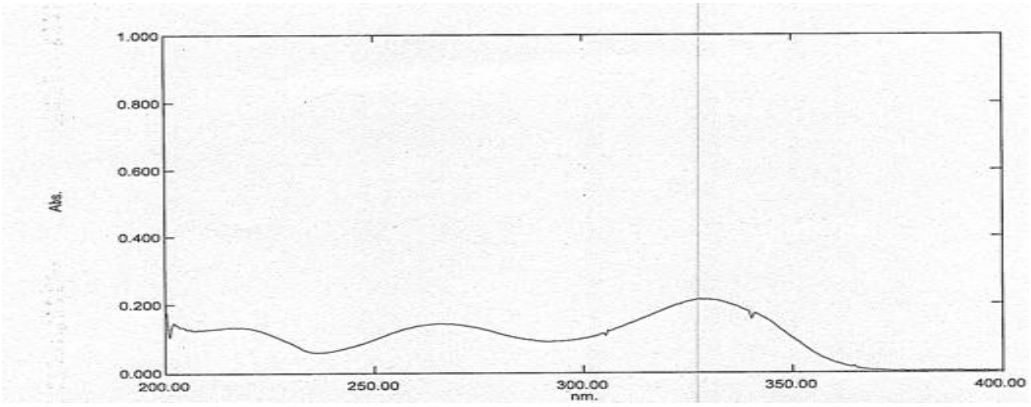

Figure 4: Acid Hydrolysis of Pimobendan (5µg/ml)

Table 5: Results of stability study of Pimobendan

Sr. No.	Condition Applied	Amount taken(µg/ml)	Amount found (µg/ml)	% Degraded
1	Acidic Hydrolysis (1 N HCl)	5	1.62	67.6
2	Alkali Hydrolysis (1 N NaOH)	5	7.23	44.6
3	Oxidative Stress Degradation(H_2O_2 3%)	5	Change in Spectrum Pattern (λmax)	
4	Dry Heat Degradation (60ºC, 24 hrs)	5	2.61	47.6
5	Photo Degradation **(**UV light, 3 days)	5	1.52	69.6

*****Mean of three determinations

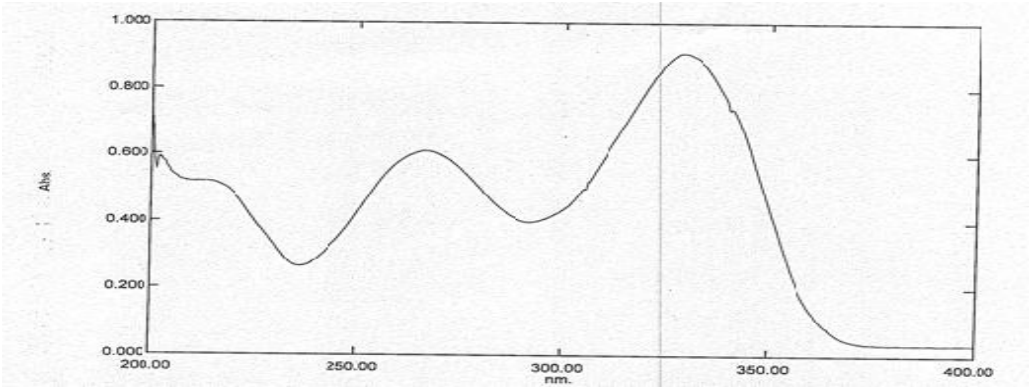

Figure 5: Alkali Hydrolysis of Pimobendan (5µg/ml)

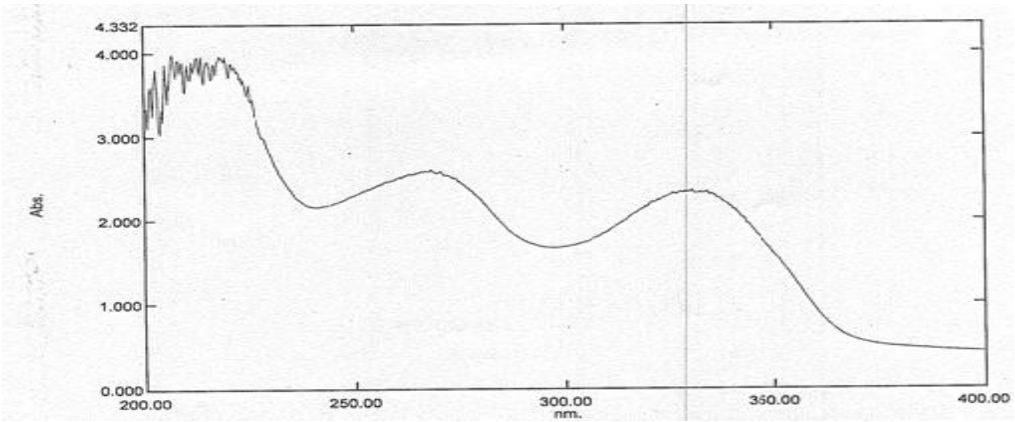

Figure 6: Oxidative Stress Degradation of Pimobendan (5µg/ml)

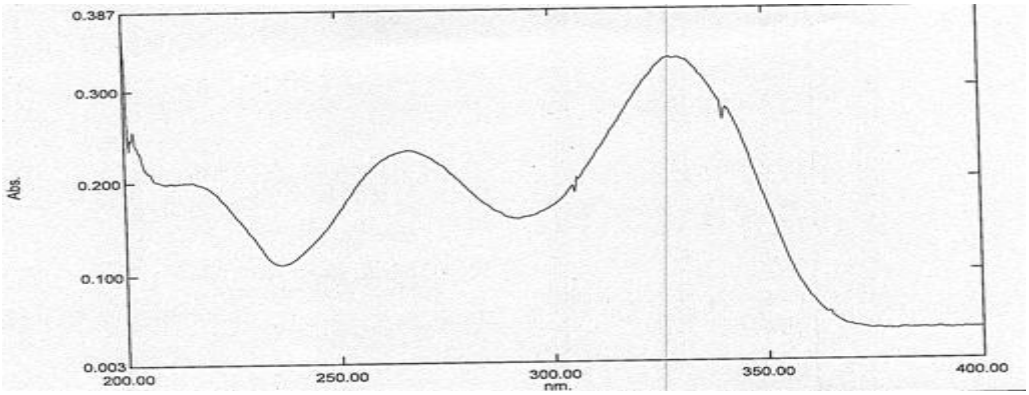

Figure 7: Dry Heat Degradation of Pimobendan (5µg/ml)

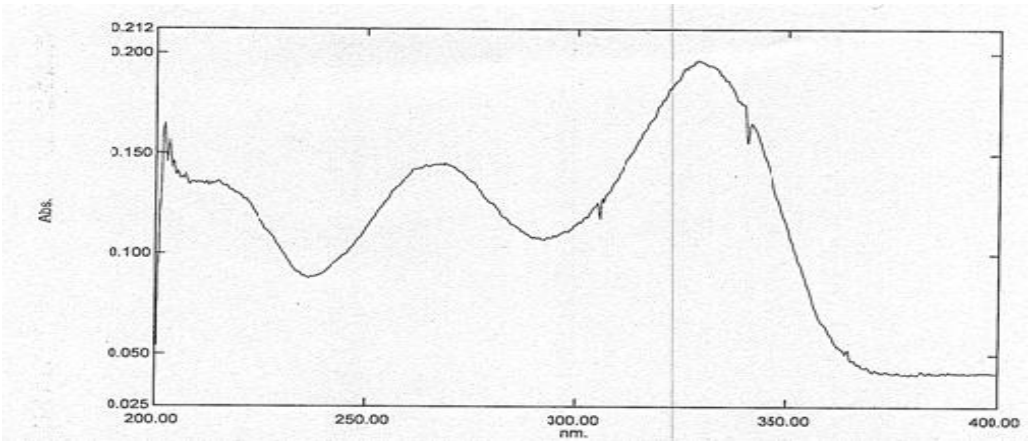

Figure 8: Photo Degradation of Pimobendan (5µg/ml)

RP-HPLC METHOD

Preparation of 0.05 M Phosphate Buffer pH 2.5 adjusted with *o*-phosphoric Acid

Accurately weighed Potassium Dihydrogen phosphate 1.7 g was dissolved in 250ml of HPLC grade water pH was adjusted to 2.5 with the help of *o*-phosphoric acid.

Preparation of mobile phase

A mixture of 20 ml Acetonitrile and 80 ml of 0.05 M Phosphate Buffer pH 2.5 adjusted with Orthophosphoric Acid filtered through 0.45µm filter paper, sonicated for 10 minutes to degas the mixture and used as mobile phase.

Working Standard Stock Solution

An accurately weighed quantity of standard PIMO (5 mg) powder was weighed and transferred to 50 ml volumetric flask and dissolved in 30 ml of mobile phase. The flasks were shaken and sonicated for 15min and volumes were made up to mark with mobile phase to get 100µg/ml. Then 1, 2, 3, 4, 5, 6 ml of PIMO were transferred to 10 ml volumetric flasks made up to mark with mobile phase.

Analysis of marketed formulation (20µg/ml Pimobendan)

Twenty tablets of brand Vetmedin were weighed and average weight determined. The quantity of the powder equivalent to 25 mg of Pimobendan was transferred to a 25 ml volumetric flask. The content was mixed with diluent and sonicated for 5 min to dissolve the drug as completely as possible. The solution was then filtered through a Nylon filter (0.45 µ). The volume was made up with diluent. An aliquot of this solution (10.0 ml) was transferred in to a 100 ml volumetric flask and the volume was made up with diluents to give a solution containing 100µg/ml of the drugs. From this solution, 2 ml was transferred to volumetric flask of 10 ml

capacity. Volume was made up to the mark with methanol to give a solution containing 20µg/ml. This solution was used for the estimation of Pimobendan.

Chromatographic condition

Chromatographic separation was carried out on Shimadzu LC-2010 CHT using Inertsil®ODS-3 (4.6 X 100mm,3µm) as stationary phase and mobile phase Acetonitrile and 0.05 M Phosphate Buffer, pH 2.5(20:80) at flow rate of 1 ml/min using UV detection at 328 nm. All determinations were performed at constant column temperature (25ºc) (Table 6) (Fig. 9).

Method Validation [9]

Linearity

The linearity of the response for PIMO assay method was determined by preparing and injecting standard solutions with concentrations of 10-60µg/ml. The calibration curve (Fig. 10) indicate that the response is linear over the concentration range studied with correlation coefficient (r) value 0.999.

Precision

The precision of the method was demonstrated by inter-day and intra-day variation studies. In the intra-day studies, three repeated injections of standard and sample solutions were made and the response factor of drug peaks and percentage RSD were calculated. In the inter-day variation studies, three repeated injections of standard and sample solutions were made for three consecutive days and response factor of drugs peaks and percentage RSD were calculated. From the data obtained, the developed RP-HPLC method was found to be precise (Table 8).

Accuracy

It was performed at three levels 50%, 100%, 150% by Standard addition method. Each concentration was analyzed 3 times and average recoveries were measured (Table 9).

Limit of Detection and Limit of Quantification

The limit of detection (LOD) and the limit of quantification (LOQ) of the drug were derived by calculating the signal-to-noise ratio (S/N, i.e., 3.3 for LOD and 10 for LOQ) using the following equations as per International Conference on Harmonization (ICH) guidelines.

$LOD = 3.3 \times \sigma/S$

$LOQ = 10 \times \sigma/S$

Where σ = the standard deviation of the response and S = Slope of calibration curve.

The results obtained are shown in Table 7.

Robustness

Robustness of the method was determined by making slight changes in the chromatographic conditions. It was observed that there were no marked changes in the chromatographic parameters (Table 10).

Analysis of PIMO in Dosage Form

Pharmaceutical formulation of PIMO was obtained as a gift sample from Astron research pvt. Ltd. The response of formulation was measured at 328 nm for quantification of PIMO by using RP-HPLC. The amount of drug present in sample solution was determined by fitting the response into the regression equation for PIMO. Result is given in Table 11.

Table 6: Chromatographic conditions

Parameters	**Optimized condition**
Column	Inertsil®ODS-3 (4.6 X 100mm,3µm)
Mobile phase	Buffer : Acetonitrile (80:20 v/v)
Flow rate	1 ml/min
Detection wavelength	328 nm
Injection volume	10 µl
Run time	10 min

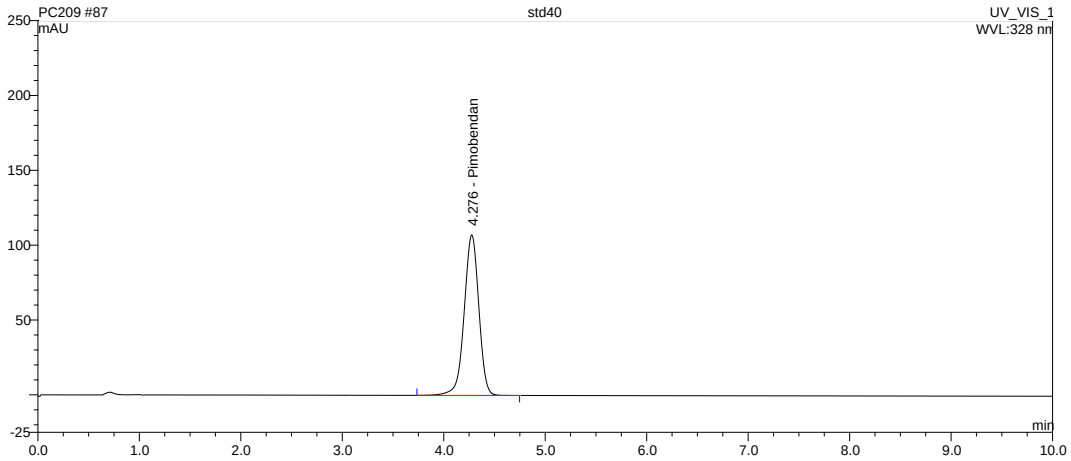

Fig. 9: RP-HPLC chromatogram of PIMO (40µg/ml)

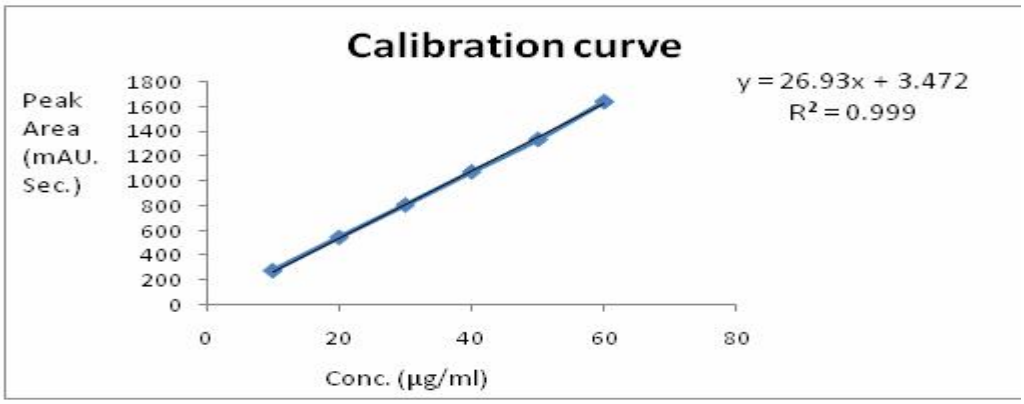

Fig. 10: Calibration curve of PIMO for RP-HPLC method

Table 7: System Suitability Parameters for HPLC method

Parameters	**PIMO**
Calibration range (µg/ml)	10-60
LOD (µg/ml)	0.522
LOQ(µg/ml)	1.584
Regression equation	y = 26.93x + 3.472
Slope	26.93
Intercept	3.472
Correlation coefficient	0.999
Intraday RSD, %	0.28-0.72
Interday RSD, %	0.12-0.94

*****Mean of three determinations

Table 8: Precision data

Drug	Concentration (µg/ml)	Intra-day precision		Inter-day precision	
		Mean ± S.D.(n=3)	% RSD	Mean ± S.D.(n=3)	% RSD
PIMO	10	264.96 ± 0.75	0.28	265.40 ± 0.56	0.21
	20	530.76 ± 2.83	0.53	532.08 ± 3.21	0.60
	30	810.64 ± 4.34	0.53	811.70 ± 4.17	0.51
	40	1103 ± 6.58	0.59	1109.06 ± 1.42	0.12
	50	1369.75 ± 9.86	0.72	1382.04 ± 5.14	0.37
	60	1649.06 ± 4.46	0.27	1646.72 ±15.55	0.94

*****Mean of three determinations

Table 9: Determination of Accuracy

Drug	Amount taken (µg/ml)	Amount added (µg/ml)	Amount found* (µg/ml)	% Recovery ± S.D (n=3)
PIMO	20	10	29.93	99.76±2.92
	20	20	39.78	99.45±2.97
	20	30	49.57	99.13 ±8.95

* Mean of three estimations

Table 10: Robustness (20 µg/ml)

Factor	Value	Area*
Wavelength (nm)	326	529.34
	328	530.76
	330	528.51
	MEAN ± S.D	**529.53±1.59**
	% RSD	**0.30**
Flow rate (ml/min)	0.9	528.92
	1	530.48
	1.1	531.52
	MEAN ± S.D	**530.30± 1.30**
	% RSD	**0.24**
Mobile phase	Buffer: Acetonitrile 85: 15	527.42
	Buffer: Acetonitrile 80: 20	530.76
	Buffer: Acetonitrile 75: 25	532.45
	MEAN ± S.D	**530.21± 2.55**
	% RSD	**0.48**

*Mean of three determinations

Table 11: Assay of PIMO in Dosage Form by RP-HPLC

Formulation	Amount Labeled (mg)	Amount found*(mg)	% Recovery ± S.D (n=3)
Vetmedin	5	4.99	99.8 ± 2.67

* Mean of three estimations

RESULTS AND DISCUSSION

In Spectrophotometry linearity was observed for 1-7µg/ml (Figure 2-3) and correlation coefficient was found to be 0.999 with %RSD below 2% (Tables 1-4). In RP-HPLC method linearity was found in the range of 10-60µg/ml with correlation coefficient of 0.999 and total run time of 10 minutes (Tables 6-11, Figures 4-8). The validations for both the developed methods were performed as per the ICH guideline (Q_2R_1) [9]. The Stability study indicate that appreciable changes were observed by treating the drug with acidic hydrolysis, basic

hydrolysis, oxidative condition, thermal degradation and photo degradation (Table 5 and Figure 4,5,6,7,8). The developed methods were successfully applied to the estimation of the drug in commercially available Vetmedin tablets. The results obtained indicate the additives present in the formulation do not interfere with analysis of Pimobendan. The developed methods can be used in quality control laboratories for analysis of Pimobendan in tablet formulation.

CONCLUSION

The developed and validated spectrophotometric and chromatographic methods for the estimation of pimobendan are accurate, precise, simple, sensitive and rapid. The proposed methods can be successfully applied for routine estimation of Pimobendan in tablet dosage form.

ACKNOWLEDGEMENTS

The authors are grateful to Astron research pvt ltd., Ahmedabad for providing pure API and formulation for project work. We acknowledge Saraswati Institute of Pharmaceutical Sciences, Gandhinagar, Gujarat for provision of facilities for the research work.

REFERENCES

1. Fujino K., Sperelakis N., Solaro R.J., "sensitization of dog and guineapig heart myofilaments to ca^{+2} activation and the inotropic effect of Pimobendan", *Circ. Res.* , 1988, 63(5), 911-22.
2. Fraker LD., Van Eyk J., Solaro RJ., "Reversal of phosphate indused decreases in force by the pyridazinone in myofilaments from human ventricle", *Mol. Cell Biochem*,1997,176 (1-2),83.
3. Kubo SH., Gollub S., Bourge R., Rahko P., Cobb F., Jessup M., Brozena S., Brodsky M.,Kirlin P., Shanes ,"Beneficial effects of Pimobendan on exercise tolerance and quality of life in patients with heart failure" , J., *Circulation* ,1992,85(3) , 942.
4. Sasayama S., Asanoi H., Kihara Y., Yokawa S., Terada Y.,Yoshida S., Ejiri M., Horikoshi I. ,"Clinical effects of long term administration of pimobendan in patients with moderate congestive heart failure", *Heart Vessels*, 1994,9(3),113-20.
5. Asakura M., Nagakura A., Tarui S., Matsumura R.,"Simultaneous determination of the enantiomers of Pimobendan and its main metabolite in rat plasma by high-performance liquid chromatography", *J. of Chrom.*, 1993,614(1),135-41.
6. Kai-Min Chu and Shyh-Ming Shieh, "Enantiomeric Separation of a Cardiotonic Agent Pimobendan and its Major Active Metabolite, UD-CG 212 BS, by Coupled Achiral- Chiral Normal-Phase High-Performance Liquid Chromatography", *J.of Chromatographic Sci.*,1992, 30 (5),171-176.
7. Sethi P.D., HPLC- Quantitative analysis of pharmaceutical formulations, New Delhi, CBS Publication and Distributors, 2001, 215-63.
8. Beckett A.H., Stenlake J.B., Practical Pharmaceutical Chemistry, 1st Edn, Delhi, C.B.S. Publishers, ,1988, 296-300.
9. ICH, Q2 (R1), Harmonised tripartite guideline, Validation of analytical procedures: Text and Methodology International Conference on Harmonization ICH, Geneva, 2005.
10. Ahuja S. and Rasmuseen H. In HPLC method development for pharmaceuticals, 1st Edn, Elsevier academic press, 2007, pp 125-77.
11. ICH guideline Q1A (R2). Stability testing of new drug substances and products. Geneva: 2003.
12. ICH guideline Q1B.Stability testing: photo stability testing of new drug substances and products. Geneva: 1996.

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