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Low level determination of Genotoxic impurities in Pimobendan drug by RP-HPLC

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ABSTRACT

(6-(3,4-diaminophenyl)-5-methyl-4,5-dihydropyridazine-3(2*H*)-one(GTI-A) and *p*-anisaldehyde (GTI-B)have been highlighted a (GTI's) potential genotoxic impurities (PGIs) of Pimobendan. A sensitive RP-HPLC method was developed and validated for the determination of (6-(3,4-diaminophenyl)-5-methyl-4,5-dihydropyridazine-3(2H)-one and *p*-anisaldehyde in pimobendan using Zodiac C18 (125 X 4.6mm 5µ) column, with UV Detector. The calibration curves showed good linearity over the concentration range of 0.5µg/mL to 1.5µg/mL with respect to the sample. The correlation coefficient was 0.999. Excellent recoveries of 102 % were obtained at the level of 0.5µg/mL.

Keywords: Pimobendan; Genotoxic; (6-(3, 4-diaminophenyl)-5-methyl-4,5-dihydro-pyridazine-3(2*H*)-one and *p*-anisaldehyde; RP-HPLC.

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INTRODUCTION

Pimobendan (PMB) is a novel drug with ability to inhibit phosphodiesterase-III (PDE3), which plays a key role in management of the signs of mild, moderate, or severe congestive heart failure due to atrioventricular valvular insufficiency or dilated cardiomyopathy. Chemically pimobendan is 4,5-dihydro-6-[2-(4-methoxyphenyl)-1*H*-benzimidazole-5-yl]-5-methyl-3(2*H*)-pyridazinone [1] monomer, it acts as a positive inotropic agent with vasodilatory properties and 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 are available for the estimation of enantiomers and metabolites of pimobendan using chiral column [5-6]. However, the literature survey does not reveal RP-HPLC method for the impurities in pimobendan. The present paper presents the development of a simple, sensitive and accurate RP-HPLC method for estimation of pimobendan.

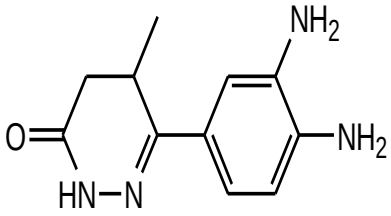
Synthetic starting materials and intermediates are reactive by design and may occur as impurities in the final API. The nature of this chemical reactivity can often be translated into biological reactivity and these materials can often be mutagens or carcinogens. Many times it has been established that due to high chemical relativities the fate of the several genotoxic agents precluded their retention within the final API especially if their formation was separated from the final API by several synthetic steps.

6-(3,4-diaminophenyl)-5-methyl-4,5-dihydropyridazine-3(2*H*)-one and *p*-anisaldehyde are often used during manufacture of pharmaceuticals, as counter-ions to form salt, as acid catalysts or as the result of protecting group removal during the synthesis. In fact (6-(3,4-diaminophenyl)-5-methyl-4,5-dihydropyridazine-3(2*H*)-one (Figure 1a) and *p*-anisaldehyde (Figure 1b) are known genotoxins and is known to be carcinogenic in rats and mice [7]. The potential presence of these genotoxins has attracted the attention of regulatory authorities, although no official guidelines have yet been issued. Draft guidelines [8] from the European agency and feedback from the US Food Drug Administration (FDA) to the pharmaceuticals industry *via* responses to drug applications have enabled the industry to establish interim strategies. Generally, it is accepted that genotoxins will be limited to a daily dose of 1.0 µg/day to 1.5µg/day unless safety studies establish that it is safe to receive a higher dose or that the drug is used for only a short term exposure, e.g. as an antibiotic. As some genotoxicity studies can take up to two years, e.g. carcino-genicity studies, it is preferable for the potential genotoxins to be controlled during the synthesis. In some cases where levels cannot be controlled and no safety data exists, it may be preferable for the pharmaceutical

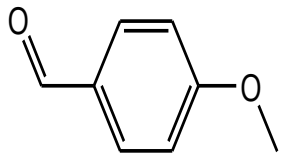
company to change the route of the drug substance or the isolation procedure, though this normally happens only during early development.

Due to the increasing concern from the regulatory perspective in relation to the potential hazards, there has been a general renaissance and increased number of analytical techniques, liquid chromatographic methods utilizing mass spectrometric detectors is reported in literature for the determination of (6-(3,4-diaminophenyl)-5-methyl-4,5-dihydropyridazine-3(2*H*)-one and p-anisaldehyde. But this method is quite expensive as it re-quires MS detector for low level determination.

The proposed HPLC method for determination of (6-(3,4-diaminophenyl)-5-methyl-4,5-dihydropyridazine-3(2*H*)-one and *p*-anisaldehyde is sensitive and robust involving no laborious sample preparation steps. This method has many advantages over the method reported in the literature in terms of cost, specificity, accuracy and reproducibility.



1a.GTI-A [6-(3,4-diaminophenyl)-5-methyl-4,5-dihydropyridazine-3(2*H*)-one)



1b.GTI-B (*p*-Anisaldehyde)

Figure 1: Structures of GTI-A and GTI-B

MATERIALS AND METHOD

Reagents and Chemicals

HPLC-grade methanol, ammonium acetate was purchased from Merck chemicals. Milli-Q water (Millipore). Reference substances **(**6-(3,4-diaminophenyl)-5-methyl-4,5-dihydropyridazine-3(2*H*)-one and *p*-anisaldehyde was purchased from Sigma Aldrich.

Column Selection and Mobile Phase Optimization

For adequate retention times of **(**6-(3,4-diaminophenyl)-5-methyl-4,5-dihydropyridazine-3(2*H*)-one and *p*-anisaldehyde on different columns like Zodiac C18, Kromasil C18 and Zorbax C18 of different dimensions were evaluated. On Zorbax C18 and Kromasil C18 early elution, blank interference and inadequate separation of analytes was observed. However, on Zodiac C18 column the separation and the responses for GTI-A and GTI-B were found to be suitable.

Different composition of mobile phase using ammonium acetate buffer and methanol were studied. Good separation and responses were observed using gradient composition of ammonium acetate

buffer and methanol was optimized with column oven temperature of 25 ˚C at a flow rate of 1.0 mL per minute. Under these conditions the retention times of GTI-A and GTI-B were observed to be about 1.29 and4.29 minutes.

Instrumentation

Chromatography

The Liquid Chromatography system used was Shimadzu LC-2010 series. The analytical column was a Zodiac C18 (125 × 4.6 mm) 5µm. The mobile phase consisted of mobile phase-A (Ammonium acetate buffer) and mobile phase-B (Methanol). The flow rate was 1.0 mL/min and the run time was 15 minutes. Column oven temperature was maintained at 25 ˚C. Injection volume was 10µL. The gradient optimized (**Table.1**) was as follows.

Table: 1: Gradient Program

Time	Mobile phase-A	Mobile phase-B
0.01	80.0	20.0
5.00	40.0	60.0
8.00	25.0	75.0
10.0	25.0	75.0
15.00	80.0	20.0

Standard and Sample Preparation

Standard Preparation

Preparation of GTI-A Stock solution:

Weighed accurately about 100.0 mg of GTI-A standard in to a 100 mL volumetric flask, dissolved and made up to volume with methanol.

Preparation of GTI-B Stock solution:

Weighed accurately about 100.0 mg of GTI-B into a 100 mL volumetric flask, dissolved and made up to volume with methanol.

Preparation of GTI-A 1.0% solution (With respect to test concentration):

Transferred 1.0 of GTI-A stock solution in to a 100 mL volumetric flask, dissolved and diluted to volume with methanol.

Preparation of GTI-B 1.0% solution (With respect to test concentration):

Transferred 1.0 of GTI-B stock solution in to a 100 mL volumetric flask, dissolved and diluted to volume with methanol.

Sample Preparation

Weighed accurately and transferred about 50.0 mg of the test sample in to a 50 mL volumetric flask, dissolved and diluted in to the mark with methanol.

RESULTS AND DISCUSSION

The developed RP-HPLC method for the determination of GTI-A and GTI-B in pimobendan sample was validated. The linearity was assessed by preparing and analyzing six calibrators of GTI-A in the concentration range of 0.25-1.5 µg/mL and GTI-B in the concentration range of 0.25-1.5 µg/mL (Tables 2 & 3). The linearity was established by plotting the peak area counts of analytes versus concentration of analytes in the concentration range of 0.25µg/mL to 1.5µg/mL. The slope, intercept and regression coefficient were determined by the least squares linear regression analysis.

Table 2: Linearity of GTI-A at different level.

S.No	Conc. [%]	Area Response achieved in Linearity
1	0.0061	2393
2	0.025	8052
3	0.050	18335
4	0.100	34360
5	0.125	42048
6	0.150	51398

Table 3: Linearity of GTI-B at different level.

S.No	Conc. [%]	Area Response achieved in Linearity
1	0.0021	2451
2	0.025	18622
3	0.050	39737
4	0.100	79382
5	0.125	97483
6	0.150	118323

The precision and accuracy were evaluated by spiking GTI-A and GTI-B and determining the % RSD < 5%. Data is summarized in Tables 2 & 3. System precision was done by six replicated injections of the standard preparation. The slope, intercept and regression coefficient were determined by the least squares linear regression analysis. Linearity correlations of the peak area counts and concentration of the analytes was achieved $r^2 = 0.999$. This is represented graphically in Figure 2 & 3.

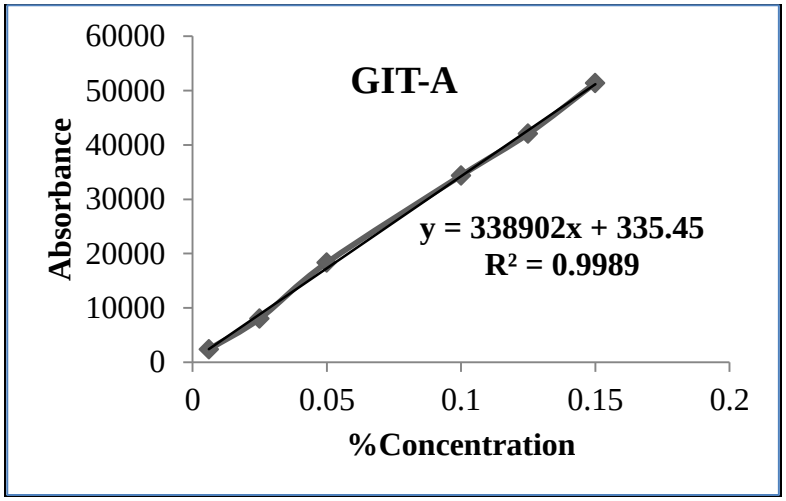


Figure: 2 Linearity graph of GTI-A

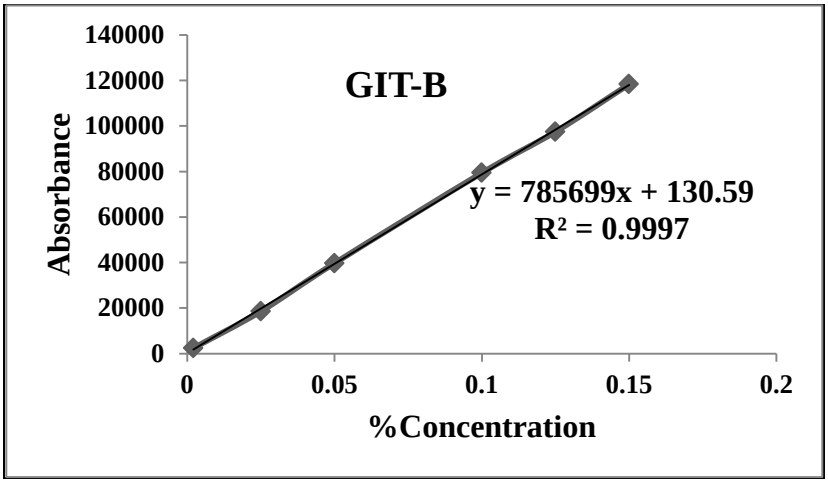


Figure: 3 Linearity graph of GTI-B

The LOQ was calculated on the basis of the lowest concentration of analytes that gives Signal to Noise ratio not less than 10 and for LOD not less than 3. LOD was found 0.02µg/mL and 0.006µg/mL. LOQ was found 0.06µg/mL and 0.02µg/mL. GTI-A and GTI-B was not found in the pimobendan sample accuracy experiments were performed by spiking GTI-A and GTI-B and determining the % RSD. Recoveries of the spiked amounts of analytes were calculated (Figure 4). Data is summarized in Table 4 & 5.

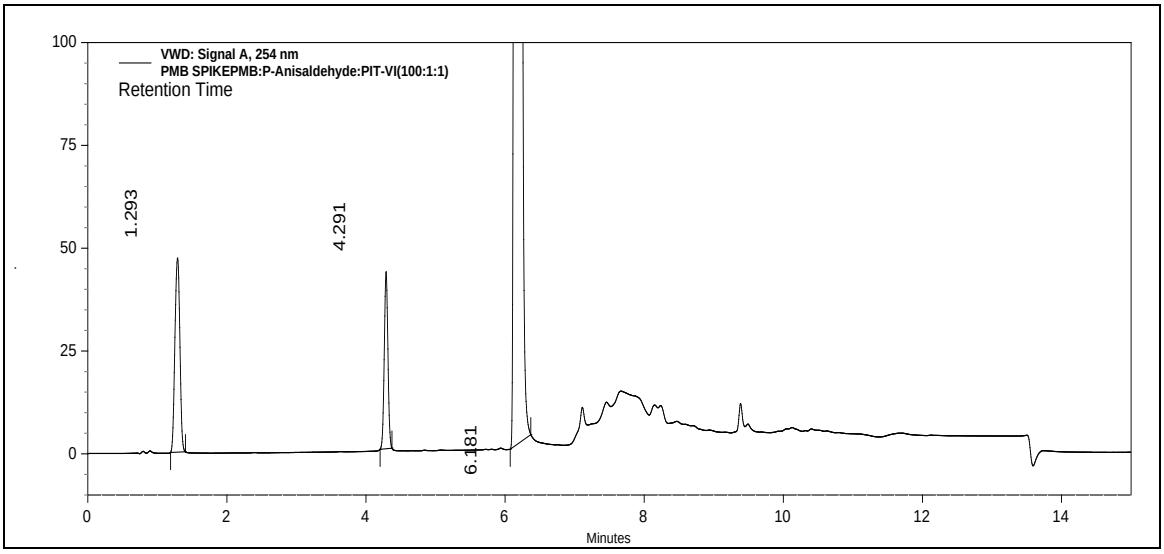


Figure 4: Accuracy of GTI-A and GTI-B in Pimobendan sample at Working Level.

Table 4: Accuracy of spiking GTI-A at different level.

Level	Amount of GTI-A present in Analysed sample	Amount Added with respect to sample µg/	Amount found with respect to sample µg/	% Recovery
50% Recovery 1	NIL	0.51	0.52	101.96
50% Recovery 2	NIL	0.49	0.50	102.04
50% Recovery 3	NIL	0.50	0.51	102.00
100% Recovery 1	NIL	1.10	1.18	107.27
100% Recovery 2	NIL	1.10	1.16	105.45
100% Recovery 3	NIL	1.00	1.07	107.00
150% Recovery 1	NIL	1.50	1.51	100.67
150% Recovery 2	NIL	1.52	1.57	103.29
150% Recovery 3	NIL	1.51	1.58	104.64
Mean				**103.81**
% RSD				**2.30**

Table 5: Accuracy of spiking GTI-B at different level.

Level	Amount of GTI-B present in Analysed sample	Amount Added with respect to sample µg/mL	Amount found with respect to sample µg/mL	% Recovery
50% Recovery 1	NIL	0.50	0.51	102.00
50% Recovery 2	NIL	0.50	0.51	102.00
50% Recovery 3	NIL	0.51	0.51	100.00
100% Recovery 1	NIL	1.11	1.12	100.90
100% Recovery 2	NIL	1.09	1.10	100.92
100% Recovery 3	NIL	1.05	1.06	100.95
150% Recovery 1	NIL	1.50	1.51	100.67
150% Recovery 2	NIL	1.51	1.52	100.66
150% Recovery 3	NIL	1.51	1.52	100.66
Mean				**100.97**
% RSD				**0.64**

CONCLUSION

A method on HPLC was developed for screening and quantification of (6-(3,4-diaminophenyl)-5-methyl-4,5-dihydropyridazine-3(2*H*)-one and *p*-anisaldehyde in the pimobendan samples. This HPLC method was cost effective, sensitive and specific for the detection of (6-(3,4-diaminophenyl)-5-methyl-4,5-dihydropyridazine-3(2H)-one and *p*-anisaldehyde in the pimobendan samples. The high levels of (6-(3,4-diaminophenyl)-5-methyl-4,5-dihydropyridazine-3(2*H*)-one and *p*-anisaldehyde in the formulation might be dangerous if this product is not properly tested by drug quality control laboratories. The described method presents a highly reliable technique for rapid detection of genotoxic impurities in these active pharmaceutical ingredients with accuracy and precision.

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