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Development of a Novel Method for the Analysis of the Drug in Low Concentration Using an Antidiabetic Drug 'Voglibose' for the Study

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Authors' Contributions

This work was carried out in collaboration between all authors. Author SK designed the study, performed the experiments and done the analysis, and wrote the draft of the manuscript. Author DKA contributed in designing the experiments and also modified the draft of the manuscript. Author AP contributed towards modification of manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aim: To develop a novel method for the analysis of the drug in low concentration using an anti diabetic drug 'voglibose' for the study.

Study Design: Analysis of the drug plays an important role in its development. Assay and content uniformity are the two tests required for establishing quality of a pharmaceutical product. Drug dissolution testing is routinely used to provide critical in vitro drug release profiles. Normally, all these tests are done using HPLC to obtain and analyze the chromatograms but, this procedure becomes challenging as the concentration of the drug reduces. In such conditions, alternative methods have to be established or different instruments have to be used. Voglibose is one such antidiabetic drug which is used in very low concentrations (0.2 mg to 0.3 mg per tablet) in

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pharmaceutical industries for the treatment of postprandial hyperglycemia. The existing methods of analysis for assay and content uniformity have a number of challenges. In dissolution studies the drug gets diluted to further lower levels and hence the conventional methods do not produce any results. Currently no method is available for dissolution studies for this drug. Thus the paper aims at establishing a suitable method for the analysis of the drug.

Methodology: The main aim of this work is to develop a common method suitable for the analysis of the drug. The paper mentions of applying the concepts of drug enzyme interaction as a method of transduction for the analysis of the drug. Silicon wafer surfaces were used as a sensor surface to study the drug- enzyme interactions which caused fluorescence and florescent images were obtained by the fluorescent microscope and further processed using image processing techniques to quantify the concentration of the drug.

Results: It was observed that fluorescence was obtained only due to drug enzyme interaction. The corresponding fluorescent images were captured and their intensity values were analyzed using image processing techniques and the concentrations of different samples were quantified. The method is also used to quantify the concentrations as used in dissolution method. There were other two samples prepared which mentions that fluorescence is not observed by single coating either alpha amylase or voglibose on the silicon surfaces.

Conclusion: This work was successfully carried out towards developing a common method suitable for the analysis of the drug-"Voglibose" used in low concentration; for all the parameters including assay, content uniformity and dissolution. This work also contributes for dissolution studies for the drug which had no methods available till date for quantifying the concentration of the drug corresponding to 0.3mg in 900ml of buffer. Thus the work establishes a novel method that can be used by the pharmaceutical industry for the analysis of drug in low concentrations.

Keywords: Biosensing; voglibos; su-8; immobilization; fluorescent images processing.

1. INTRODUCTION

This paper mentions of the application of biosensing to the area of analysis of the drug. The pharmaceutical industry invests a great amount of time and resources for drug discovery and development. The drugs are subjected to a variety of laws and regulations that govern patenting, testing, safety, efficacy and marketing of the drugs. Thus analysis of drug plays an important role in the pharmaceutical industry during its development phase.

During the analysis of the drug, assay and content uniformity (CU) are the two tests generally required for establishing quality of pharmaceutical product. Assay testing is done by pooling the content of multiple units (tablets/capsules) together while in case of CU, evaluation of the units is done individually. The evaluation procedure usually remains the same for both the parameters (assay and content uniformity) which involves grinding the product, dissolving/extracting the drug using an appropriate solvent and then assaying it using an analytical technique such as chromatographic/ spectroscopic [1]. Another important parameter during the analysis of the drug is drug dissolution testing which is routinely used to provide critical in vitro drug release information for quality control purposes [2]. Dissolution study is conducted to assess batch-to-batch consistency of solid oral dosage forms such as tablets and to predict release profiles inside the body. The dissolution test is also evaluated using chromatographic/spectroscopic techniques. The procedures of assay, content uniformity and dissolution are routinely done for all the drugs. The analysis of the drug becomes challenging when the concentration of the drug reduces as the techniques involved for the analysis like spectroscopy/ chromatography do not produce results with the regular procedures. Hence either the procedures have to be modified or expensive instrumentation has to be used to obtain the results.

This paper focuses on establishing a common method for the analysis of the drug in low concentration. One such drug is voglibose1, an antidiabetic drug used in very low concentrations of about 0.2 mg to 0.3 mg per tablet which is supposed to be the drug with lowest concentration used in pharmaceutical industry. Being a highly reactive antidiabetic drug with the lowest concentration, it is the best choice for the study [3]. Voglibose is a research product of Japan based company Takeda Pharma. It is an alpha glucosidase inhibitor (AGI) used for lowering blood glucose levels in patients with

diabetes mellitus that can be taken orally for longer period [4]. This plays an important role in controlling the post prandial hyperglycemia (PPHG) by inhibiting the function of alpha glucosidase helping in the digestion of complex carbohydrates and cleaving oligosaccharides into monosaccharides [5]. Alpha glucosidase inhibitor behaves as a classic competitive inhibitor by competing with the oligosaccharides for the binding site.

The mechanism of action of different AGIs are similar but not identical. Fig. 1. shows the chemical structure of a voglibose drug molecule.

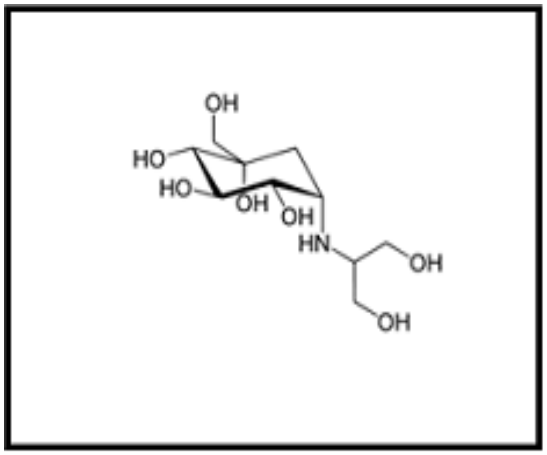

Fig. 1. Structure of a voglibose drug molecule

Most commonly known AGIs are acarbose, miglitol and voglibose which help in attenuating the rate of absorption of sucrose by acting as competitive inhibitors on the luminal enzymes. The literature reveals that miglitol and voglibose have equal efficacy in reducing PPHG as compared to acarbose. The clinical benefit of voglibose is its better safety profile as compared to miglitol and acarbose [6]. Looking at the efficacy and safety profile amongst the available AGIs, voglibose has a preferential choice in the management of postprandial hyperglycemia in the treatment of type-2 diabetes mellitus.

Every drug should have a suitable and validated method of analysis. If a suitable method is not available then it becomes essential to develop and validate a simple, sensitive, accurate, precise, reproducible method for the estimation of drug samples. Voglibose absorbs UV in low wavelength region and hence cannot be detected with high sensitivity with normal processes. Various spectroscopic methods have been developed so far for the estimation of voglibose in bulk and formulations. “Japanese Pharmacopeia” (JP) describes the post derivatization methods with fluorescent detectors for the estimation of voglibose at an excitation wavelength of 350 nm and an emission wavelength of 430 nm [1]. Mallikarjuna Rao *et al*. explains of UV- Spectroscopic method for estimation of voglibose at 282 nm in bulk and tablets [7]. Sai Kishore et al. developed and validated RP-HPLC (reverse phase- high performance liquid chromatography) method for quantitative analysis of voglibose in pure and pharmaceutical formulations and was detected at 282 nm [8]. Shubhangi C et al. used two different methods (LC_FD and LC_MS) for the analysis and detection of voglibose [9]. Woo J. S and Ryu J. K mention of quantitative determination of voglibose in tablet using HPLC fluorescence detection with post column derivatization and mass spectroscopic detection at a wavelength of 272 nm [10]. The above mentioned methods for the analysis and detection of voglibose, suffer with many challenges and limitations in the industry such as low response, overlapping peaks, requirements for expensive instrumentation and highly skilled manpower for this kind of study and achieving precise results.

Dissolution is a test parameter used to evaluate the drug absorption rate in the body. This is done by diluting the drug with 900 ml of buffer and obtaining the chromatograms. In case of voglibose, the normal concentration of the drug is about 0.3 mg per tablet and in dissolution studies, 0.3 mg gets diluted in 900 ml of buffer reducing the concentration of the drug to a greater extent. Currently voglibose dissolution test is not possible as the low concentration drug gets further diluted and no peaks are observed in the chromatogram [11]. Because of the above mentioned challenges, there is a requirement for a novel method for the quantification of this drug. In this paper, a method is established for the quantification of drug based on measuring the signal generated because of the drug enzyme interaction on a sensor surface [12].

In this work, we have used the immobilization approach in order to get a strong drug enzyme interaction [13]. Immobilization is a crucial process in biosensors field where enzymes are fixed to or within solid supports and interacts with its target [14]. This helps in production of a stable layer with an attached biomolecule with sufficient binding sites capable of maintaining its structure and activity to recognize the target analyate [15]. The first step towards this sensing was to identify

the analyate to which the drug had specific binding. Our recently published work on docking and chemical analysis reveals that voglibose has a very strong binding with alpha amylase thus inhibiting the latter action [16]. So, alpha amylase was chosen as an enzyme to interact with voglibose. In order to facilitate immobilization process, alpha amylase was first immobilized on a polymer coated silicon surface by drop casting method. The polymer used was SU-8, a negative photoresist commonly used in microfabrication processes [17]. This was followed by flushing of the drug (voglibose) on the immobilized surface. The interaction between the drug and the enzyme was confirmed by capturing the images under the fluorescent microscope [15]. The captured fluorescent images were processed using image processing techniques to quantify their intensities with respect to the concentration of the drug under study [18].

2. MATERIALS AND METHODS

Silicon wafers (P-type, <100>, single side polished) were purchased from Siegert Wafer GmbH, Germany. The SU-8 (2000.2) polymer was received from Microchem. The drug voglibose was obtained from Tirupati Medicare Pvt Ltd, HP, India as a raw product. Alpha Amylase (fungal diastase) was obtained from Anthem Cellutions, Bangalore, India. The buffer used was acetate buffer prepared using acetic acid and sodium acetate with pH of 5.2.

2.1 Experimental Methods

2.1.1 Methods of detection of voglibose

The regular methods of detection of voglibose is by the use of HPLC with other instrumentation. In spite of this, the existing methods can perform the test for assay and content uniformity only and no method is available for dissolution studies. But the method mentioned in the paper discuss a common method to test assay, content uniformity and dissolution which is based on biosensing. Prior to this, a silicon surface is used as the substrate. The enzyme alpha amylase is immobilized over the surface and the drug is made to interact with it. This generates fluorescence and the fluorescent images are obtained. These fluorescent images are then quantified for their intensity values which is proportional to the concentration of the drug.

2.1.2 Surface modification of silicon wafer surface

In order to enable a successful immobilization, sensor surface has to be modified [19]. This was achieved by first cleaning the silicon wafer by RCA method (Radio Corporation of America) to remove the organic and metallic contaminants [15]. RCA is a standard wafer cleaning procedure used in microelectronics industry to remove the metal contamination and organic impurities. It was followed by coating on the cleaned silicon wafer using a negative photoresist SU-8 of 1 micron thickness commonly used in microfabrication. For this, SU-8 2000.2 was spin coated and baked (pre and post baked) for optimized timings using a slow ramp up and ramp down at room temperature. Fig. 2. shows the steps involved in the surface modification of the silicon wafer surface prior to immobilization.

2.1.3 Sample preparation

The above mentioned modified SU-8 sensor surface was cut into small pieces to perform the experiments. Enzyme alpha amylase and voglibose were both prepared in the acetate buffer of pH 5.2 favorable for the enzyme activity. Stock solution of alpha amylase was prepared as 5 mg/ml in acetate buffer solution. Voglibose of different concentrations corresponding to 0.3 mg/ml, 0.6 mg/ml and 1 mg/ml along with 0.3 mg of voglibose in 900 ml of buffer to mimic dissolution studies were prepared.

2.1.4 Immobilization of drug on modified sensor surface

Alpha amylase of 5 mg/ml concentration was immobilized on the sensor surface by drop casting method using a fine micropipette. These surfaces were incubated for an hour at 40 degree Celsius, washed using buffer solution and DI water and then dried. Then different concentrations of voglibose prepared as mentioned above were used to flush over the surface and incubated for an hour at 40 degree Celsius, followed by washing in acetate buffer and distilled water before drying. Amongst the voglibose solutions prepared, one sample corresponds to the concentration of 0.3 mg in 900 ml of buffer solution mimicking the concentrations of dissolution studies. The other concentration corresponding to 0.3 mg/ml is the concentration of the drug used in the tablet form. One of the sensor surfaces was coated with only

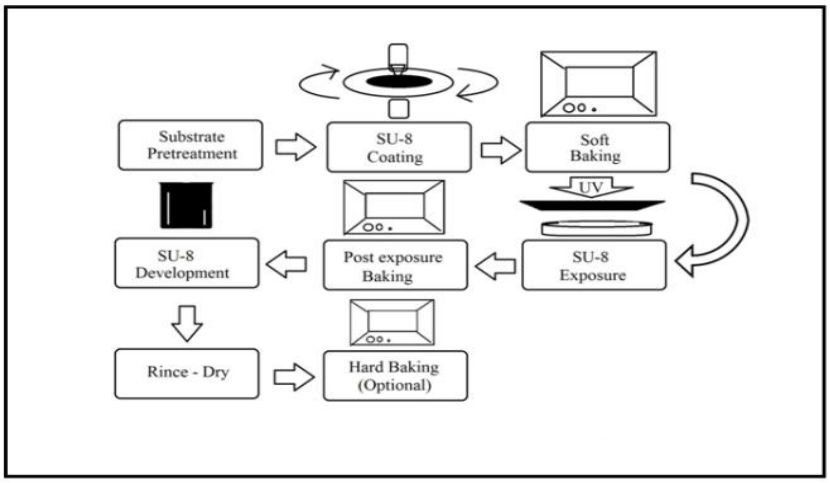

Fig. 2. Steps involved in the surface modification of silicon surface prior to immobilization.

alpha-amylase of 5 mg/ml concentration by drop casting method while another sample was prepared without the immobilization of alpha amylase but only voglibose of 0.3 mg/ml coated on it as negative controls. Then all the samples were observed under the fluorescence microscope and fluorescent images were captured. These images were processed using image processing techniques to quantify the concentration of the drug as mentioned earlier.

2.1.5 Drug-enzyme interaction

The interaction between alpha amylase and voglibose resulted in fluorescent images which were captured and observed under the Carl Zeiss fluorescence microscope (Model No: AXIOIMAZER Z1). Earlier, the drug-enzyme interaction was confirmed by using molecular docking and chemical analysis as described in introduction section. Fig. 3. depicts the full process flow for the drug-enzyme interaction.

3. RESULTS AND DISCUSSION

The results of the work done can be classified into three parts. Firstly, it was observed that fluorescence was obtained only due to drug enzyme interaction. The corresponding fluorescent images were captured and their intensity values were analyzed using image processing techniques. It was observed that the intensity value of the images increased as the concentration of the drug increased with maintaining the enzyme concentration constant. The drug was used in three different concentrations (0.3 mg/ml, 0.6 mg/ml and 1 mg/ml) with the enzyme concentration being constant at 5 mg/ml, as mentioned earlier. Fig. 4. shows different fluorescent images captured (A-C) and their corresponding histograms (D-F) due to the drug-enzyme interaction. In the images (A-C), the observed fluorescence is represented by "f" against the sensor surface background ("b").

As it can be seen from the figure that the average intensity values of the fluorescent images increases as the concentration of the drug increases. Table 1 shows different average intensities for different concentrations of voglibose.

Table 1.Tabulation of increasing concentration of voglibose and corresponding intensity values

S.No	Concentration of Voglibose	Average Intensity Value
1.	0.3 mg/ml	141
2.	0.6 mg/ml	164
3.	1 mg/ml	170

The plot in Fig. 5 also depicts the linear relationship between the voglibose concentrations with respect to their average intensity values.

As mentioned earlier that dissolution studies for this drug has not been possible till date because of drug getting more diluted resulting in getting no peak in the chromatograms. So far, no method is available for dissolution studies for this drug. In this work, experiments were conducted even for such dilutions and it was observed that

even for such low concentrations (0.3 mg/ 900 ml), fluorescence was observed because of drug enzyme interaction. The average intensity value of the captured images was found to be 119 which is comparable. Fig. 6. (A &B) shows the captured fluorescent image of the sample and its histogram corresponding to dissolution concentration of the drug with the enzyme concentration beings constant.

From the literature studies, it is known that the pharmaceutical concentration of voglibose is 0.3 mg per tablet. Hence, further the experiments were narrowed down to the concentrations of drug corresponding to 0.3 mg/ml (concentration in tablet form) and 0.3 mg in 900 ml of buffer (depicting concentrations of the dissolution studies). Repetitions were done to confirm the coincidence of the intensity values in both the cases and the experiments were found reproducible with minimum deviation in the observed values. The Table 2 shows the intensity values corresponding to the two different concentrations of the drug.

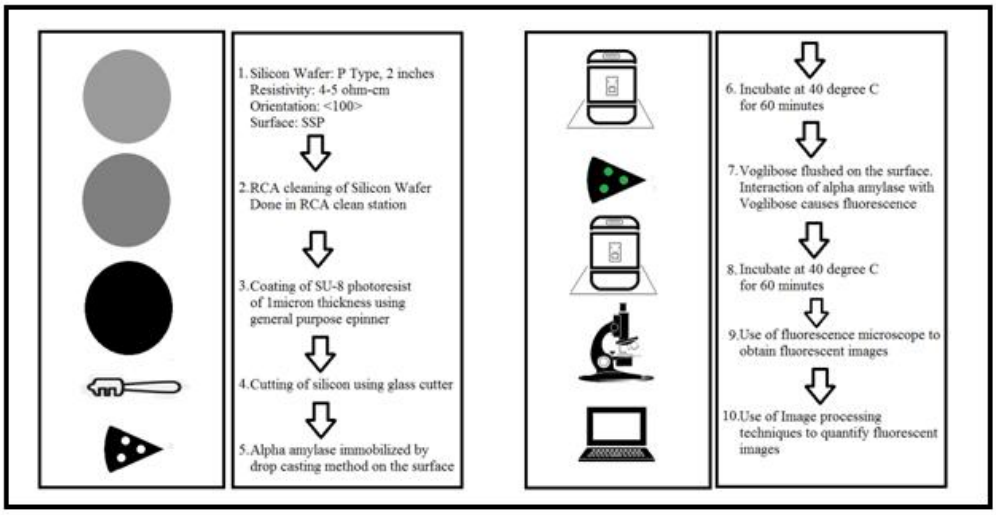

Fig. 3. Process flow for drug-enzyme interaction on the immobilized silicon surface

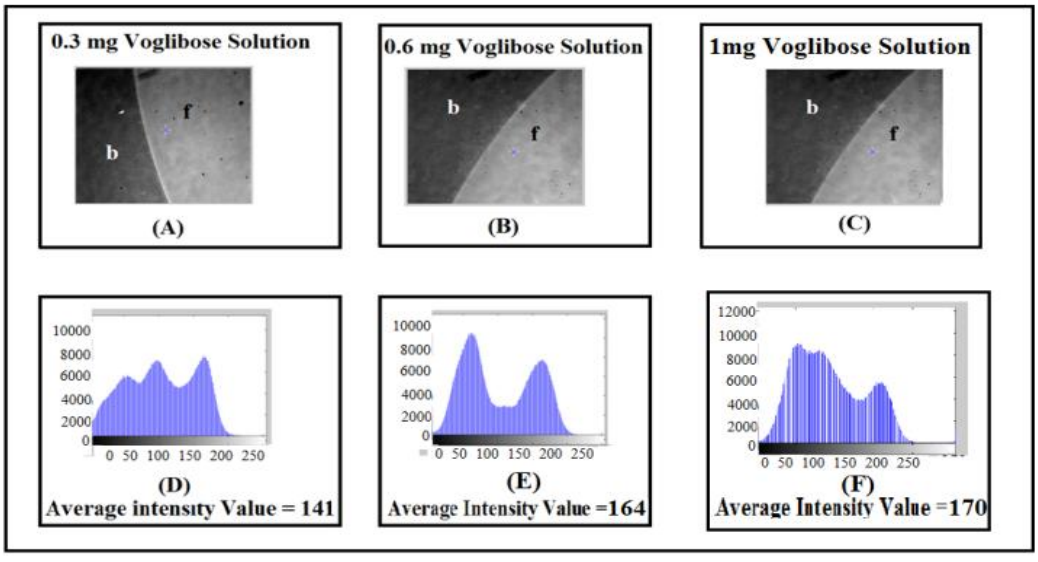

Fig. 4. Fluorescent images of sensor surface and their corresponding histograms with various concentrations of voglibose

Two different samples were also prepared simultaneously as negative controls containing only either alpha amylase or only voglibose coated on the silicon surfaces. It can be noted from the Fig. 7 (A&B) that fluorescence was not observed in these samples thus confirming the observed fluorescence attributed to drug enzyme interaction.

Hence it can be stated that the average intensity values of the drug corresponding to dissolution concentration is in the range of 110 to 120 and the average intensity value of the drug corresponding to 0.3 mg/ml which depicts the concentration of the drug in tablet form is in the range of 140 to 150 (Table 2). It can also be observed that there is distinct difference in the

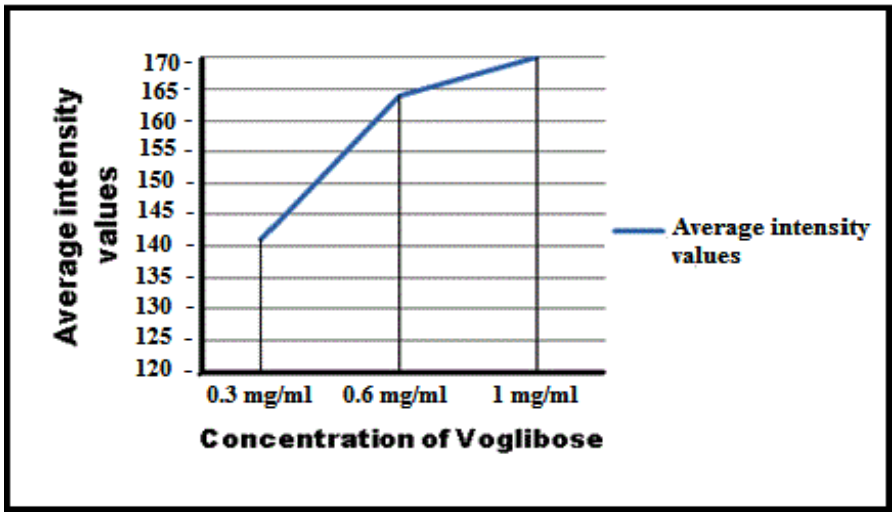

Fig. 5. Plot between the voglibose concentrations and their average intensity values

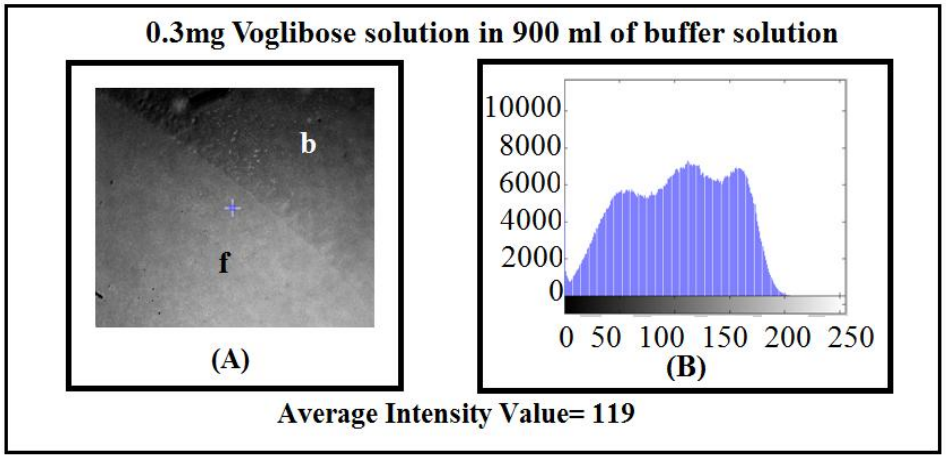

Fig. 6. Fluorescent images of dissolution study for voglibose

Table 2. Average intensity values for voglibose in two different experiments

S. No	Concentration of the volgibose	Average intensity values of experiment 1	Average intensity values of experiment 2
1	0.3 mg in 900 ml buffer solution	119	117

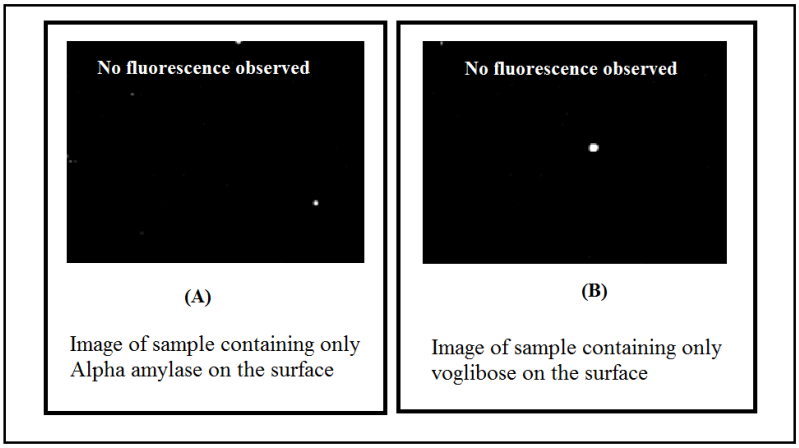

Fig. 7. Samples containing only alpha amylase or voglibose (negative controls)

intensity values obtained for different concentrations.

4. CONCLUSION

This work was successfully carried out towards developing a common method suitable for the analysis of the drug-"Voglibose" used in low concentration; for all the parameters including assay, content uniformity and dissolution. The method has been established by applying the concepts of drug enzyme interaction as a method of transduction for the analysis of the drug thus quantifying the concentrations of the drug as low as 0.3 mg/ml which is equivalent to the Voglibose concentration in tablet form. This work also contributes for dissolution studies for the drug which had no methods available till date for quantifying the concentration of the drug corresponding to 0.3 mg in 900 ml of buffer. Thus the work establishes a novel method that can be used by the pharmaceutical industry for the analysis of drug in low concentrations. The same concept can be extended on microcantilevers to generate an electrical readout of the system to quantify the concentration of the drug. The method also can be used to devise a dedicated system for image acquisition and image analysis to give an automated readout of the concentration of the drug.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

ACKNOWLEDGEMENTS

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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