8.1 LITERATURE SURVEY FOR SELECTION OF FABRICATION TECHNIQUE FOR DEVELOPMENT OF MICROFLUIDIC DEVICE

Microfluidic is actually the science and technology of systems that process or manipulate small amounts of fluids, using channels with dimensions of tens to hundreds of micrometers. The generous amalgamation of biological, chemical as well as engineering science principles are required for development of microfluidic devices. The concepts of flow of fluids as well as diffusion of fluids from engineering sciences, the specific chemical reaction for the analyte of interest from chemical sciences and the correlation of the analyte with the biological systems from biological sciences helps in development of a successful diagnostic microfluidic device. Enormous advancements have been done in last decade in fabrication of microfluidic devices. One of the widely used materials for fabrication of microfluidic devices is PDMS (Polydimethylsiloxane) polymer. [1] Various techniques like wet and dry etching, thermoforming, polymer ablation, polymer casting etc have been used for PDMS based fabrication. [1, 2] These techniques require highly sophisticated arrangements for development of microfluidic device.

A new horizon for development of microfluidic devices have evolved which is paper based microfluidic devices for various applications. [4] From applications in medical diagnosis to forensic testing and up to pharmaceutical testing, paper microfluidics is everywhere. Widely used methods for its fabrication includes 3D printing, photolithography, jet printing, laser printing [5] wax printing etc. [6]. Also electrochemical biosensing by printed circuit technology is done by fabrication of paper microfluidic devices. [7] Most widely used principle for development of paper microfluidics devices is colorimetric reactions based on principle of ELISA. For detection of many biological agents for diagnostic and testing purpose, paper microfluidics has been developed. For detection of glucose [8], horse radish peroxide [9], C-reactive protein [10], alpha fetoprotein [11] etc paper microfluidics based on principle of ELISA has been developed. Recently, enzyme based paper microfluidic device encasing colorimetry assay for urea was developed [12] However, studies on colorimetric based paper microfluidics relying simply on chemical reactions between the selected reagents also has been reported. The studies for detection of toxic ions based on colorimetric assay were reported. [13] Also, literature for detection of certain analytes in biological fluid was reported where the paper was impregnated with index matching fluid [14]. For analytical applications also many paper microfluidics have been developed. For acid base titrations, a paper microfluidic device was developed. [15] Similarly for analysis of pharmaceuticals also devices have been developed. [16] A colorimetric based paper microfluidic device for identification of counterfeit malarial drugs was reported. The study was purely based on chemical reaction of analyte of interest with the reagent having specific reaction with it being impregnated on to the paper device. The fabrication techniques for paper microfluidics have also evolved and techniques of origami and wax printing in fusion have been used. Also the use of laboratory parafilm has been reported for fabrication of paper microfluidic devices. [17]

8.2 HIGHLIGHTS OF PAPER MICROFLUIDIC CHIP DEVELOPED AT OUR LABORATORY.

The kit developed at our laboratory is having novel fabrication procedure utilizing

paper as a main constituent. The fabrication of paper chip developed at our premises is very easy and can be accomplished in less than 10 min. The designing materials used for fabrication were also very economic and easily available at any analytical laboratory premises. Unlike traditional microfluidics which require complex attachments and pumping systems, paper microfluidic chip developed by us does not require any of this as capillary wicking through paper does all the needful circumventing the other necessities. The proposed microfluidic chip is highly cost effective and affordable. The paper chip thus developed also has advantage of being portable and thus would be ideal for resource limit settings and remote areas where facilities of sophisticated laboratories are limited. For onsite quality checking of herbal bulk goods by drug inspectors and quality regulators, the chip would aid as a first pass check and even for home diagnosis of MMA for critically anaemic patients it would be ideal.

8.3 SECTION –A

Estimation of Tadalafil as adulterant in herbal formulation by paper microfluidic device

The Spectrophotometric colorimetric method was developed for estimation for Tadalafil which can also be used for routine analysis of Tadalafil in laboratory premises. The assay thus developed was extrapolated for development of microfluidic chip.

8.3.1 Experimental

8.3.1.1 Brief outline for selection for herbal formulation prone to be adulterated and for the synthetic analogue prone to be counterfeited

In recent years the emergence of counterfeiting of phytopharmaceuticals has increased tremendously due to upsurge in their use in lieu of natural products. These natural products take long intervals for curbing the ailment, thus an illegal practice of adding synthetic analogues to these natural products have occurred. Erectile dysfunction has affected about 50% of men between the ages of 40-70 years in last 2 decades due to lifestyle changes leading to multiplication in usage of aphrodisiac medicaments [18]. One of the main constituent of aphrodisiac herbal medicines is Ashwagandha. Its botanical name is Withania Somnifera. Widely used synthetic aphrodisiac medicaments include Sildenafil, Tadalafil and verdenafil. Tadalafil has longer half-life and longer duration of action compared to other two drugs. [18] Unlike sildenafil, Tadalafil does not have likely affinity for phosphodiesterase-6 which is major cause for visual disturbances (one of major side effect of PDE5Is). [19] Therefore, Tadalafil is one of the widely used synthetic aphrodisiac medicines. Tadalafil is a carboline derivative and phosphodiesterase 5 inhibitor that is used primarily to treat erectile dysfunction, benign prostatic hyperplasia and primary pulmonary hypertension. [20] Other common side effects of PDE5I's are headache, flushing and visual disturbance. [19] Tadalafil is contraindicated in patients having history of cardiac arrest and overdose of Tadalafil can lead to penile haemorrhage. If it is added as an adulterant in herbal medicines without any ethical forte, patients unknowingly may suffer from severe consequences.

Owing to cease the untoward consequences which can occur due to adulteration of Ashwagandha formulations with Tadalafil we have a developed a very cost-effective, easy and rapid paper based microfluidic chip for qualitative as well as quantitative analysis of Tadalafil if counterfeited in Withania sominifera formulations.

8.3.1.2 Literature review

For analysis of tadalafil various methods are available in the literature. Quantitative analysis of Tadalafil by HPLC-UV in various fake drugs have been reported [21]Various methods have been reported for estimation of synthetic phosphodiesterase inhibitors in pharmaceutical preparation using techniques like HPLC [22], LCMSMS [23, 24], XRF [25], etc. Also Raman spectroscopy along with H^1 NMR and DOSY H^1 NMR has been used for used for checking analysis of illegal manufactured tadalafil. [26] Simple HPLC method for estimation of Tadalafil for bioanalytical and pharmaceutical analysis employing green analytical chemistry was also reported. [27]Similarly HPLC method for analysis of Tadalafil in dissolution studies also has been reported. [28] A UPLS-MS-MS method also has been reported for pharmacokinetic study of Tadalafil. Derivative spectrophotometry based UV method also have been reported for analysis of Tadalafil in bulk as well as dosage form.

8.3.1.3 Drug profile

Drug profile of Tadalafil is already mentioned in section 7.2.

8.3.1.4 Chemicals and materials

Reference standard of Tadalafil was obtained as a gift sample from Ami life sciences Pvt. Ltd. (Vadodara, India). The marketed formulation of TAD tablets with brand name ‘Tazzle 20’ tablets manufactured by Dr. Reddy’s Laboratories Ltd were procured from local pharmacy. The marketed formulations of Withania somnifera powder with brand name “Ashwagandha churna” manufactured by Shree Narayan Ayurvedic pharmacy and marketed by Lion were procured from local Ayurvedic pharmacy. Potassium permanganate was procured from Fischer Scientific Pvt Ltd. (India) and Sodium hydroxide, potassium hydroxide, sodium carbonate, sodium nitroprusside and potassium ferricyanides were purchased from Loba chem. chemicals Pvt Ltd (Mumbai, India). Isopropyl alcohol, n-hexane was procured from Rankem. The excipients used for preparation of placebo samples i.e. Lactose monohydrate, microcrystalline cellulose, talc, titanium dioxide, magnesium stearate, starch, cross povidone were procured from SD Fine Chemicals. Parafilm M was procured from Bermis NA. Filter paper no.1 was procured from Whatman plc. Aluminium foil was procured from Hindalco. Hotplate used was of Remi. Precision analytical balance (A X 120, by Shimadzu Corporation analytical and measuring Instruments division, Kyoto, Japan) for weighing of samples. Kangaroo paper punching machine was used. Paraffin wax was procured from ACS chemicals. Polycaprolactone manufactured by Mallooom%C2%AE and marketed by Amazon.in. Single distilled water required for study was prepared at laboratory premises. Other miscellaneous materials used were Black permanent marker, Paper clips, fevicol etc. Spectrophotometric analysis was done using UV 1700 instrument having UV probe software. Paper microfluidic chip

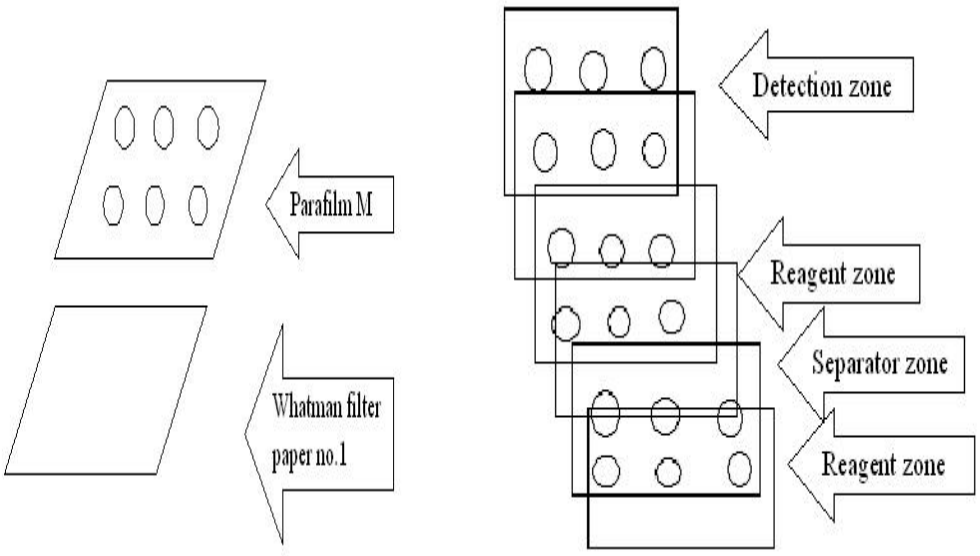
analysis were done using Redmi 5 Smartphone of model no.MDI1 with android version 7.1.2 N2G47H having photometrix app from play store.

8.3.1.5 Fabrication of paper microfluidic device

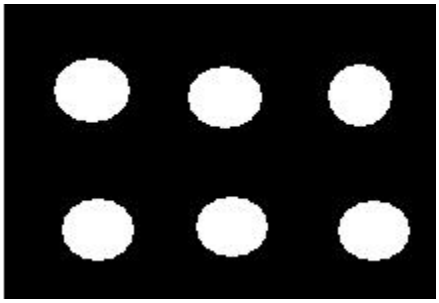
Fabrication procedure for paper microfluidic device was achieved using very minimal components and without any elaborates or complicated procedure. Three pieces of parafilm M of 2X2 cm size were cut and 6 equidistant holes were punched in it. Further Three pieces of Whatman filter paper no.1 of 2X2 cm size were cut.(a) Assembling was done using 1 layer of Parafilm M above 1 layer of Whatman filter paper no.1.(b) After assembling paper clips were tightened by firmly holding the sequenced assembly. The assembly was then sandwiched between two pieces of aluminum foil and heated on a hotplate for 2 minutes. (c) The MFD device was thus ready to use. Further 3X18 pieces of 6 mm diameter of Whatman filter paper no.1 were punched and impregnated 1X6 pieces of Whatman filter paper no.1 using 0.05 % $KMnO_4$ in SDW and 1X6 pieces of Whatman filter paper no.1 using 1 M Na_2CO_3 in SDW. The impregnated pieces were then allowed to air dry for 30 min. The 6 mm diameter impregnated pieces of Whatman filter paper no.1 were inserted inside the holes created by punching machine. 1X6 pieces were kept as a separator for the impregnated reagents. The 3 layers were then assembled in order of top layer $KMnO_4$, then separator and then last layer of Na_2CO_3 and glued using fevicol. Eliminating the punched area viz., testing zone other area of chip was colored using black marker pen for getting contrast images. The brief for fabrication for paper microfluidic device is shown in Figure 8.1. The paper microfluidic device thus fabricated needs to store in airtight container in cool and dry place and should be used within 7 days for best results.

Figure 8.1 Schematic diagram of paper microfluidic device during fabrication a) Orthogonal view of the layers of paper microfluidic device b) Isometric view of all layers of paper microfluidic device c) Pictorial presentation of final fabricated paper microfluidic chip

a) b)



c)



8.3.1.6 Reagents and sample preparation

8.3.1.6.1 Reagents preparation for colorimetric method

For preparation of 0.05% $KMnO_4$, 50 mg of $KMnO_4$ was weighed accurately using weighing balance and diluted up to 100 ml with SDW to get 0.05% $KMnO_4$. The solution of potassium permanganate thus prepared was filtered using 0.45 µ filter paper prior to use. For preparation of 1 M Na_2CO_3, 10.20 gm of Na_2CO_3 was weighed accurately using weighing balance and diluted up to 100 ml with SDW to get 1M Na_2CO_3.

8.3.1.6.2 Sample preparation

8.3.1.6.2.1 Sample preparation for Spectrophotometric colorimetric method

10 mg of TAD was weighed carefully using weighing balance and dissolved in SDW and made up to 10 ml to prepare 1000 µg/ml stock solutions. Aliquots of 0.1, 0.2, 0.3, 0.4, 0.5, 0.6 ml were withdrawn and diluted up to 10 ml to produce 10, 20, 30, 40, 50, 60 µg/ml respectively. Final dilution was done after addition of colorimetric reagents to the samples thus giving final solutions in concentration range of 5 – 30 µg/ml.

8.3.1.6.2.2 Sample preparation for paper microfluidic chip method

250mg of Tadalafil was dissolved in SDW and made up to 25 ml to prepare 10000 µg/ml stock solutions. Aliquots of 125, 250, 500, 750, 1000, 1250 µl were withdrawn and diluted up to 5 ml to produce 250, 500, 1000, 1500, 2000, 2500 µg/ml respectively for microfluidic chip method. Final dilution was done by addition of colorimetric reagents to the samples thus prepared giving final solutions in concentration range of 125 – 1250 µg/ml for microfluidic chip method.

8.3.1.6.3 Placebo sample preparation

The placebo was prepared by mixing lactose: talc: titanium dioxide: magnesium stearate: starch: cross povidone in % ratio of 77: 1: 1: 20: 1 making total of 10 gm of sample.

8.3.2 Experimental

The colorimetric method for tadalafil was first developed using a spectrophotometer. The method was validated using (ICH), Q2 (R1) guideline for precision, accuracy, linearity, range, LOD, LOQ, Specificity and Selectivity. [28] The developed method was then extrapolated for development of microfluidic chip by using the similar

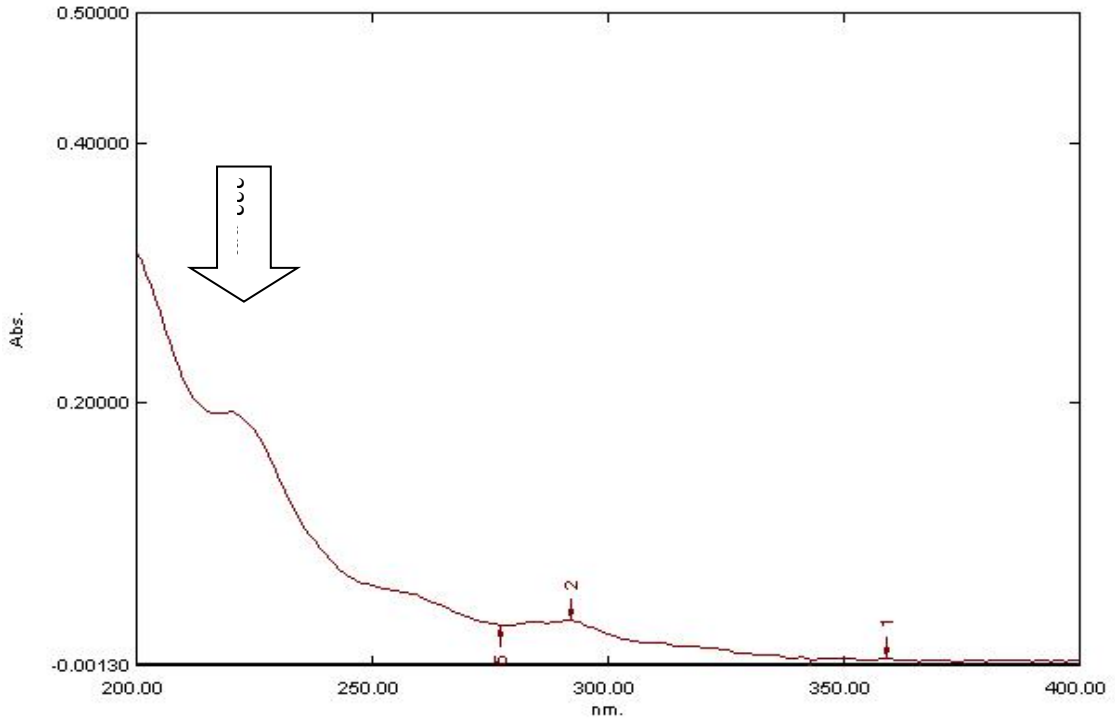
concentration of reagents but scaling down the volumes as test needed to be accomplished with microlitre/nanolitre of reagents. For more accurate results, photometrix app available in android Smartphone was utilized by which quantitative analysis was attainable.

8.3.2.1 Development of colorimetric method for Tadalafil

8.3.2.1.1 Prelimilinary trials for development of Colorimetric method for estimation of Tadalafil

By scanning Tadalafil solution of 10 µg/ml in single distilled water gave λmax of 222 nm which falls in UV (Ultraviolet) range. (Figure 8.2)

Figure 8.2 Spectra of Tadalafil for UV estimation



But for development of colorimetric based microfluidic chip, color needed to be developed, which fits in the visible range of 400-800 nm. For development of colorimetric method various trials were taken. Reactions based on various colorimetric principles were tried. Considering the fact that its structure which has a secondary amino group, trials using color reactions specific to secondary amine were carried out. Initially a specific color reaction for secondary amine using 5% sodium nitroprusside and 5% potassium ferricyanide along with 1 drop of 2N sodium carbonate was tried out. But the procedure required warming the test solution up to 90°C for 15 seconds and then cooling in air to get a strong purple-red color. The test was based on principle of oxidation of secondary amine to acetaldehyde. [29] As the procedure was cumbersome and it was not possible to extrapolate on paper based microfluidic chip, an alternate method was needed. Another method tried also

required heating at 80°C for 90 min as well as extraction into n-hexane which as previously stated was not possible to extrapolate on paper chip. [30]

8.3.2.1.2 Principle of developed Colorimetric method for estimation of Tadalafil

It was decided to utilize the oxidizing property of potassium permanganate to try some colorimetric chemical reaction. [31-34, 35] The ($MnO4^-$) ion in $KMnO_4$ is in the +7 oxidation state, thus acts as a strong oxidizing agent (10). The characteristic purple color of $KmnO_4$ is due to this ion. In acidic solution, ($MnO4^-$) ion is reduced to +2 oxidation state of (Mn2+) ion which is colorless.

$8\ H^+ + MnO_4^- + 5\ e^- \rightarrow Mn^{2+} + 4\ H_2O$ (in acidic solution)

Although in basic solution (MnO_4^-) ion is reduced to +6 oxidation state of ($MnO4^{2-}$) which gives a green colored ion.

$MnO_4^- + e^- \rightarrow MnO_4^{2-}$ (in alkaline solution)

And in neutral medium, ($MnO4^-$) gets reduced to the +4 oxidation state of (MnO_2) which is a brown colored product.

$2\ H_2O + MnO_4 - + 3\ e- \rightarrow MnO_2 + 4\ OH^-$ (in neutral medium)

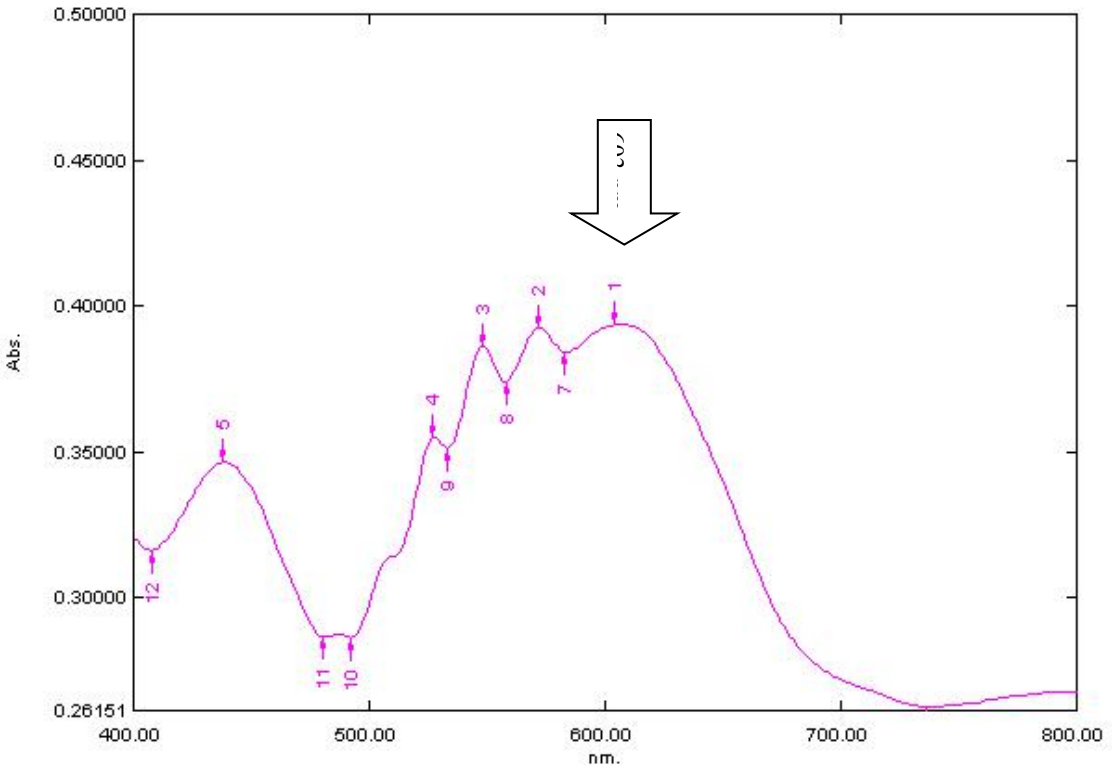
Taking oxidizing property of potassium permanganate in alkaline medium as basis, it was decided to design a colorimetric method for estimation of Tadalafil. Now selection of appropriate base for the method needed to be decided. Trials were taken using potassium hydroxide (KOH) and Sodium hydroxide (NaOH) and Sodium carbonate (Na_2CO_3). But no prominent colorimetric reaction was observed using KOH, thus it was decided to move forward with NaOH as alkaline medium for the study. Tadalafil in presence of $KMnO_4$ is oxidized and (MnO_4^-) ion in presence of is reduced to +6 oxidation state of ($MnO4^{2-}$) which gives a dark green colored ion. But the reaction is not specific for Tadalafil. Similar colorimetric reaction takes place with Sildenafil as well as Verdenafil. Thus, further colorimetric reaction was tried using Na_2CO_3. Using Na_2CO_3 as basifying reagent, a prominent orange colour is obtained which is specific for Tadalafil (Figure 8.4) Now, the reagents to be incorporated for the study were decided but their concentration for getting maximum and precise absorbance still needed to be optimized.

8.3.2.2 Method optimization and development

By using OFAT (One factor at a time) approach, keeping concentration of Na_2CO_3 constant as 0.5 N, different concentrations of $KMnO_4$ were taken. The different concentrations of $KMnO_4$ taken were 0.01%, 0.05%, 0.5% and 1%. 0.01% turned out to be very dilute whereas 1% turned out to be much intense. Thereby trials were continued using 0.05% and 0.5%. On reaction of 0.05% $KMnO_4$ with 0.5 N Na_2CO_3, visible orange color was observed whereas on reaction of 0.5% $KMnO_4$ with 0.5 N Na_2CO_3, orange color was superseded with purple color of $KMnO_4$ and thus reaction gave purple color instead on orange. Thereby, it was decided to move forward with 0.05% $KMnO_4$. But still the orange color developed was not prominent which can be easily visible by naked eyes on microfluidic chip. Thus, it was decided to increase the concentration of Na_2CO_3 to 1 N. Analysis of sample using reagents 1N Na_2CO_3 with 0.05% $KMnO_4$ gave the best results. After optimization of reagents being utilized for study, volumes of reagents and sequence in which they should be added for optimum color development needed to be determined. Also the optimum range of drug perceivable on paper chip needed to be determined. For quantification of drug by

optimized colorimetric assay method, it was needed to plot calibration curve for the drug. For development of spectrophotometric colorimetric method using optimized reagents in ratio of 1:1, spectra of 10 µg/ml solution was primarily taken on UV 1700 instrument having UV probe software for data manipulation and analysis. The spectra gave maximum absorbance at λ of 603 nm, thus λmax selected was 603nm, and scanning speed was fast. (Figure 8.3)

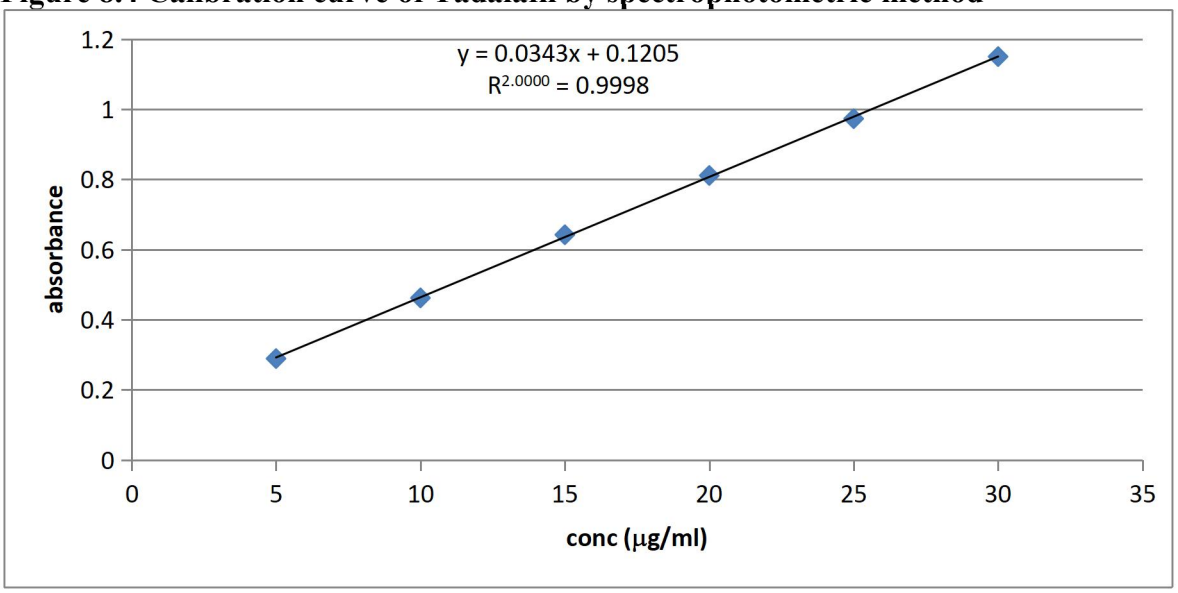
Figure 8.3 Spectra of Tadalafil for colorimetric estimation



After trying for a vast range of solutions, 5 - 30 µg/ml was selected as optimum range for assay of Tadalafil for Spectrophotometric method and 125 – 1250 µg/ml for microfluidic chip method. For visibility of colour on paper microfluidic chip, higher range was used for it. For spectrophotometric method this much high range was creating much noise and thus different range was selected for both methods. After sample preparation for range of 10 – 60 µg/ml as described in section 8.3.1.6.2.1, to 5 ml of calibration sample, 2.5 ml of 1N Na_2CO_3 was added followed by 2.5 ml of 0.05% $KMnO_4$ giving 5 – 30 µg/ml as final dilution. Figure 8.4 represents the calibration curve for Tadalafil by spectrophotometric method. Formation of orange colored solution was achieved by chemical reaction of optimized reagents by Spectrophotometric method. For optimum orange color development it took around 2 min and the color was stable for up to 30 min which was sufficient for Spectrophotometric analysis. For paper microfluidic chip method, as described in section 8.3.1.6.2.2, samples were prepared in range of 250- 2500 µg/ml. Then to 5 ml of calibration sample, 2.5 ml of 1N Na_2CO_3 was added followed by 2.5 ml of 0.05% $KMnO_4$ giving 125 – 1250 µg/ml as final dilution. Formation of orange colored solution was achieved by chemical reaction of optimized reagents by paper microfluidic chip method. For optimum orange color development it took around 2

min and the color was stable for up to 30 min which was sufficient for paper microfluidic chip analysis.

Figure 8.4 Calibration curve of Tadalafil by spectrophotometric method



8.3.2.3 Application of developed method in fabricated paper microfluidic device

Fabrication of microfluidic chip which is economic, easy and rapid to produce was our objective. Various trials were taken initially using different hydrophobic and hydrophilic materials for creating microfluidic channels. Primary trial included use of paraffin wax and laboratory filter paper. Micro channels were first made using stencil and pencil and then paraffin wax was deposited in to the micro channels. Then the assembly was heated on a laboratory hotplate for development of microfluidic channels. But on application of thus developed chip, liquid leaked out of the micro channels thus eliminating it as worthy method for our study. Another trial was taken for development of polymer based microfluidic device. Polycaprolactone moldable thermoplastic pellets were utilized for development of microfluidic device. A micro mould in desired pattern of microfluidic device was used for creating micro channels in to the microfluidic device. Dunfield has reported the successful use of parafilm M for development of MFD. [19] Using Parafilm M as hydrophobic material, various hydrophilic materials were tried viz., Kimwipes, Whatman filter paper grade 42, Whatman filter paper no. 1, Whatman filter paper grade 41, out of which Whatman filter paper no. 1 as hydrophilic layer along with Parafilm M as hydrophobic layer gave the best results. The fabrication of paper microfluidic chip was done as described in section 8.3.1.5. Now scaling down of developed colorimetric method was required as test needed to work in micro/nano liters of volumes for its working in paper microfluidic chip. But as discussed earlier in section 8.3.2.2, during development of assay itself caution was taken in selection of range suitable for visibility of color on paper chip, thus range

(125-1250 µg/ml) was used here for development of assay using paper microfluidic chip. Keeping the concentration of reagents similar as used in spectrophotometric colorimetric, volume was reduced to 5µl of reagent solutions and well as sample solution. The reagents like Na_2CO_3 and $KMnO_4$ were impregnated on to 1X6 pieces of Whatman filter paper no.1 and air dried for 30 minutes as described in section 8.3.1.5. The Sample solution was deposited using micropipette on the testing area and the color change was detected. For Qualitative analysis, appearance of orange color confirms presence of Tadalafil in the sample whereas for Quantitative analysis, the image was captured in the 1X1 cm area in the photometrix app (Figure 8.5) and then calibration curve was plotted. (Figure 8.6) Real time color processing in pixels was achieved by using the photometrix app. The photometrix app can be freely downloaded from the android playstore. After installation of the application, the application needs to be opened. Then Univariate analysis option is to be selected. First calibration curve needs to be plotted. On left top corner a circle with star icon appears for defining the settings for our method. The settings page is divided into 4 sections. 1) Sampling- here the number of samples needs to be defined for calibration curve, we have adjusted 6 samples in each calibration curve, also region of interest for sample to be defined, we have selected 64X64 m^2 area for it. 2) Camera specifications to be defined. We have kept flash mode auto and resolution for images 640X480 pixels for our study. Data can be sent directly by email, using a 3rd option, but we saved data on our android phone itself. The other minor specifications like chart items sizes were included in 4th section, which were set as default by the application. A digital image of the chip was taken and analyzed by measuring the average RGB (Red, Green, and Blue) intensity of the color developed on the paper circle. A plot of the Tadalafil concentration versus the average RGB intensity was generated. Results show that the intensity of the orange color developed on the paper test was consistent and proportional to the amount of Tadalafil present in the sample. With Tadalafil concentrations ranging from 125, 250, 500, 750, 1000, 1250 µg/ml, a linear calibration plot was obtained with a detection limit of 7.25 µg/ml.

8.3.2.4 Application of developed methods

8.3.2.4.1 Application of developed methods for marketed formulation of Tadalafil

Success of any analytical method is based on its application for routine analysis. Firstly both developed methods were applied for analysis of Tadalafil marketed formulation. For it 20 tablets were weighed and finely powdered. An accurately weighed portion of the powder equivalent to 5 mg of Tadalafil was transferred to volumetric flask and made up to 100 ml with SDW and sonicated for 10 min to prepare 50 µg/ml of sample. The solution was filtered through Whatman filter paper (No. 42). The above prepared solution was then pretreated for development of color by taking 5 ml of above prepared sample,

adding 2.5 ml of 1N Na_2CO_3 and 2.5 ml 0.05% $KMnO_4$ making it 25 µg/ml and analyzed by Spectrophotometer at 603 nm for the content of Tadalafil using the calibration curve for estimation of assay by spectrophotometric colorimetric method. The result of assay is described in Table 8.1. The result of assay was average of six replicates. For application by Paper microfluidic chip method, sample preparation was same as for spectrophotometric colorimetric method. For it accurately weighed portion equivalent to 25 mg TAD was dissolved in SDW into 100 ml volumetric flask giving 250 µg/ml of TAD. Then using a micropipette 5 µl of sample was deposited on the testing area of Paper microfluidic chip and the image of color developed was captured using the sampling icon in univariate analysis in photometrix app by android Smartphone camera. The concentration of sample obtained by Paper microfluidic chip method on photometrix app was 267.16 giving % assay to be 106.86. The assay was repeated 6 times. The result of assays in six replicates gave very minor standard deviation of ±0.69. The calibration curve along with sampling data is represented in Figure 8.7. The result of assay represented in graph is of only 1 sample as the mobile application automatically calculates and mentions it in the graph.

8.3.2.4.2 Application of developed methods for marketed formulation of Withania somnifera formulation, Placebo and deliberately prepared counterfeit sample of Withania somnifera formulation by adulteration with Tadalafil

The ultimate aim of the study was to detect the counterfeiting of Tadalafil if added as adulterant in Withania somnifera herbal formulation. For checking counterfeiting, sample preparation of standard Ashwagandha powder formulation needed to be done. For that 2.5 gm Ashwagandha powder was taken in 25 ml methanol. It was then refluxed for 5 hours at 80°C, sonicated and centrifuged for 15 min each respectively. The supernatant thus formed was collected. 5 ml of sample thus formed was taken in a 10 ml volumetric flask; to it 2.5 ml of 1N Na_2CO_3 and 2.5 ml 0.05% $KMnO_4$ was added. The resulting solution was analyzed by Spectrophotometer at 603 nm for the content of Tadalafil using the calibration curve by spectrophotometric colorimetric method. No orange color was developed thus validating the method to be selective for only estimation of Tadalafil and also showing that the marketed formulation of Ashwagandha powder had no trace of adulteration with synthetic analogue Tadalafil for enhancement aphrodisiac activity. This test was repeated 3 times for checking precision of method. Also 5 µl aliquot of alcoholic extract was deposited on the paper microfluidic chip where also no orange color was visible signifying the integrity of marketed formulation of Ashwagandha powder. This test was repeated 3 times for checking precision of method.
Also specificity of test was checked by application of both methods (Spectrophotometric and paper micro fluidic chip method) on placebo samples. The placebo sample was prepared as discussed in section 8.3.1.6.2. 100 mg of placebo sample was dissolved in 100 ml of SDW and the resulting solution was filtered through 0.45 µ filter paper. 5 ml of this filtered solution was taken in 10 ml volumetric flask; to it 2.5 ml of 1N Na_2CO_3 and 2.5 ml 0.05% $KMnO_4$ was added. The resulting solution was analyzed by Spectrophotometer at 603 nm for the content of Tadalafil using the calibration curve by spectrophotometric colorimetric method. No

orange color was developed thus validating the method to be specific for only estimation of Tadalafil. This test was repeated 3 times for checking precision of method. Also 5 µl aliquot of placebo sample was deposited on the paper microfluidic chip where also no orange color was visible signifying the specificity of method. This test was repeated 3 times for checking precision of method.
Then to 5 ml sample of Ashwagandha alcohol extract deliberately 5 ml of 40 µg/ml Tadalafil solution was added to prepare 20 µg/ml deliberately prepared counterfeited sample. Now taking 5 ml of this deliberately counterfeited sample, 2.5 ml of 1N Na_2CO_3 and 2.5 ml 0.05% $KMnO_4$ was added making 10 µg/ml of sample. Again due to presence of Tadalafil, orange color was observed. This solution was then analyzed by Spectrophotometer at 603 nm for the content of Tadalafil using the calibration curve by spectrophotometric colorimetric method. Assay results are represented in Table 8.1. The results are average of 6 runs. For microfluidic chip method, to 5 ml sample of Ashwagandha alcohol extract deliberately 5 ml of 500 µg/ml of TAD was added giving 250 µg/ml of sample deliberately prepared counterfeited sample. Then taking 5 µl of the counterfeited sample, aliquot was deposited on paper microfluidic chip, due to presence of Tadalafil, orange color was developed and the image of color developed was captured using the sampling icon in univariate analysis in photometrix app by android Smartphone camera. Assay results are represented in Table 8.1. The results are replicate of 6 runs.

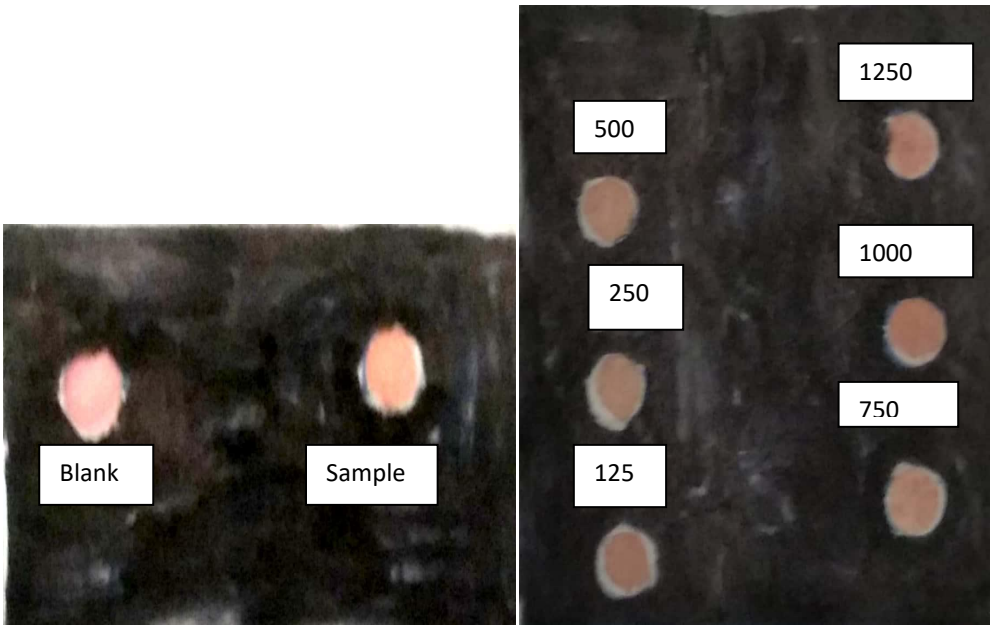
8.3.2.5 Method validation

The spectrophotometric colorimetric method as well as the Paper microfluidic chip method were validated using (ICH), Q2 (R1) guideline for precision, accuracy, linearity, range, LOD, LOQ, Specificity and Selectivity. [28] Precision study was undertaken as intraday as well as intraday. For spectrophotometric colorimetric method as well as for Paper microfluidic chip method using procedure described above in section 8.3.2.2 and 8.3.2.3, 3 replicates of Tadalafil with concentrations, 125 (LQC), 500 (MQC), 1000 (HQC) µg/ml were prepared 3 times a day as well on 3 different days and %RSD was calculated for paper microfluidic chip method and 3 replicates of Tadalafil with concentrations, 5 (LQC), 15 (MQC), 25 (HQC) µg/ml were prepared 3 times a day as well on 3 different days and %RSD was calculated for spectrophotometric method %RSD below 2% signifies the method to be precise. LOD and LOQ were calculated from Standard deviation and slope values of 3 replicates of calibration curve. Accuracy calculation by spectrophotometric colorimetric method was undertaken using recovery method at 80, 100, and 120 % levels whereas for Paper microfluidic chip method it was only done for 100% level in 3 replicates. The summary of validation parameters are represented in Table 8.1.

Table 8.1 Summary of validation parameters

Parameters	**Tadalafil**	
	Spectrophotometric colorimetric method	**Paper microfluidic chip method**
Analytics	By Analytical wavelength 603 nm	By Visibility of naked eyes and RGB value on android phone camera
Linearity range (µg/ml)	5 – 30	125-1250
Regression equation	y = 0.0343x + 0.1205	y = 0.0001x + 0.206
Correlation coefficient (R^2)	0.9998	0.8680
Intraday precision (%RSD)	0.12	0.35
Inter day precision (%RSD)	0.58	0.69
LOD (µg/ml)	0.49	7.25
LOQ (µg/ml)	1.46	21.74
Accuracy (% Mean Recovery)	98- 102	90- 110
Assay %(Marketed formulation)	101.09± 0.25	106.86± 0.69
Assay (Deliberately adulterated with TAD)	100.46±0.49	105.33±1.89
Specificity (Placebo)	Specific	Specific
STD ASH powder	Selective	Selective

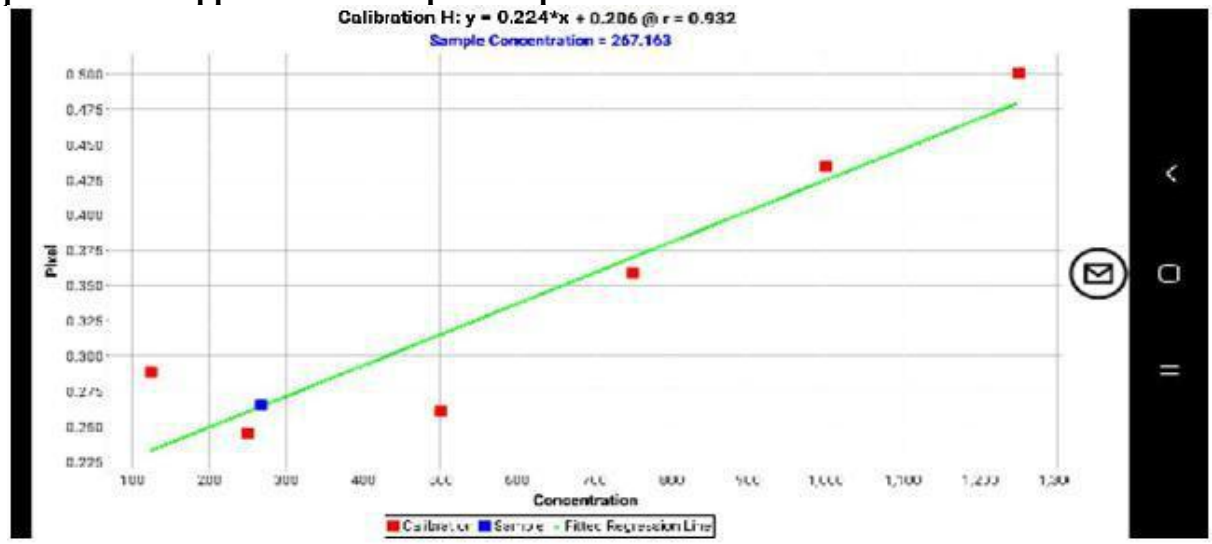
Figure 8.5 Qualitative and Quantitative tests of Tadalafil on MFD device



a) **b)**

*Conc in µg/ml

Figure 8.6 Calibration curve of Tadalafil by Smartphone android based photometrix app for the developed samples



8.3.2.6 Comparison of both developed methods by statistical analysis

Statistical analysis was accomplished utilizing t-test test for comparing the two developed methods. The t-value obtained was -7.34847 and p value obtained was 0.0000012. From the results it can be said that there is significant difference between the two results at $p< 0.05$ significance level.

8.4 SECTION –B

Estimation of methyl malonic acid as early biomarker for pernicious anaemia using paper microfluidic device

The Spectrophotometric colorimetric method was developed for estimation for MMA which can also be used for routine analysis of MMA in laboratory premises. The assay thus developed was extrapolated for development of microfluidic chip.

8.4.1 Introduction

8.4.1.1 Underlying cause for pernicious anaemia

Pernicious anaemia may be caused due to 2 reasons. Lack of intrinsic factor for uptake of Vitamin B_{12} or scarce intake of food containing Vitamin B_{12}. If the underlying cause for pernicious anaemia is due to intrinsic factor, medical treatment is required. However if its due to lack of food containing Vitamin B_{12}, intervention can be done by giving adequate food containing Vitamin B_{12}. According to one of the study undertaken in 2014, 375 million people in whole world are vegetarians, out of which maximum percentage share of 38% is from India mainly due to religious reasons. [37] Cyanocobalamine is essential water-soluble micronutrient. [36] For maintaining nerve integrity as well for meiosis, Cyanocobalamine is much essential vitamin in humans. [38] Cyanocobalamine acts a coenzyme for conversion of methyl

malonyl CoA to succinyl CoA in Kreb's cycle. As it is synthesized only in animals by their gut flora, it is solely found in animal based food products like milk, eggs, meat, pork, fish etc. Thus, for vegan diet followers, its deficiency stands out to be a serious matter of concern. [39, 40] Schilling test is done for finding serum Vitamin B_{12} deficiency, but the test is tedious and costly. [25] It was tried to develop a simple and inexpensive diagnostic MFD which can give rapid results.

8.4.1.2 Selection of selective biomarker for pernicious anaemia

Many studies have suggested, Homocysteine as well as Methyl malonic acid as potential indicators of Vitamin B_{12} deficiency and folate deficiency in various situations like in pregnancy, in cancer patients as well as in anaemic patients. [42,43] Nevertheless as per research, elevated levels of Methymalonic Acid (MMA) and homocysteine have been identified as better indicator of B_{12} and folate deficiency than checking the actual serum B_{12} level itself, because normal or high serum levels of Vitamin B_{12} are also found in Vitamin B_{12} deficient state in some studies. [44, 45] However, homocysteine is better indicator of folate (Vitamin B_9) deficiency than for Vitamin B_{12} deficiency. [46, 47] Therefore, MMA was selected a selective biomarker to be checked in urine for occurrence of pernicious anaemia for our study.

8.4.1.3 Literature review

It was tried to develop a simple and inexpensive diagnostic MFD which can give rapid results.A GC-MS method has been developed for MMA estimation which requires 24 hours collection of urine. In it derivatization to dicyclohexyl ester of MMA is done for GCMS analysis. [48] Also a HPLC method along with precolumn derivatization with diazo salt is reported. [49] A colorimetric method for determination of MMA in urine was also developed by A.J Giorgio et al using ion exchange resin for extraction of MMA and diazotized with p-nitroaniline for color development. [50] Another method for determination of MMA includes a TLC method for elution followed by detection using 2, 7-dichlorofluorescein solution. [51] Similarly, a TLC method for semiquantitative analysis of MMA using Fast Blue B as detecting reagent was reported. [52] Also an ELISA kit is available for MMA detection. [53, 54] We have developed a colorimetry based paper microfluidic device for estimation of methyl malonic acid being released in urine as biomarker for Vitamin B_{12} deficiency for patients suffering from pernicious anaemia.

8.4.1.4 Biomarker profile

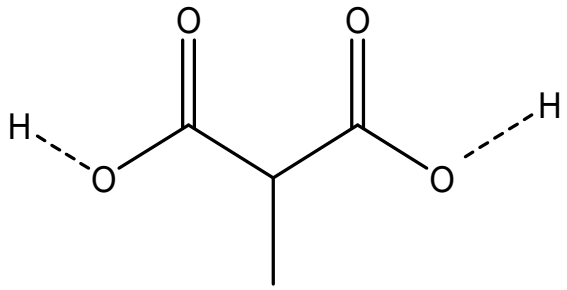
Chemical name: Methyl malonic acid

IUPAC Name: 2-Methylpropanedioic acid

Molecular Formula: $C_4H_6O_4$

Molecular Weight: 118.09 gm/mol

Figure 8.7 Chemical Structure of MMA



Appearance: White crystalline powder

Melting point: 134 °C

pKa: 3.07, 5.76

Solubility: Freely soluble in water

Category: MMA is a vital intermediate in the metabolism of fat and protein and thus its appearance in urine acts as marker for Vitamin B_{12} deficiency which is main cause for pernicious anemia.

8.4.1.5 Chemicals and materials

Reference standard of MMA was procured from TCI chemicals, India. Fast blue B salt was procured from Dimer chemicals Pvt Ltd. Vadodara, Gujarat. Glacial acetic acid and ammonia were purchased from Fisher scientific, India and Merck, India respectively. Ethanol was procured from Rankem. Parafilm M was procured from Bermis NA. Filter paper no.1 was procured from Whatman plc. Aluminium foil was procured from Hindalco. Hotplate used was of Remi. Precision analytical balance (A X 120, by Shimadzu Corporation analytical and measuring Instruments division, Kyoto, Japan) for weighing of samples. Kangaroo paper punching machine was used. Paraffin wax was procured from ACS chemicals. Polycaprolactone manufactured by mallooom%C2%AE and marketed by Amazon.in. Single distilled water required for study was prepared at laboratory premises. Other miscellaneous materials used were Black permanent marker, Paper clips, fevicol etc. Spectrophotometric analysis was done using UV 1700 instrument having UV probe software. Paper microfluidic chip analysis were done using Redmi 5 Smartphone of model no.MDI1 with android version 7.1.2 N2G47H having photometrix app from play store.

8.4.1.6 Paper chip fabrication

Fabrication procedure for paper microfluidic device was already discussed in section 8.3.2.3. Only the impregnated reagents were different for developing a MFD for detection of MMA as a biomarker for pernicious anaemia. The 3 layers were then assembled in order of top layer FBB, then separator and then last layer of Ammonia and glued using fevicol. Eliminating the punched area viz., testing zone other area of chip was colored using black marker pen for getting contrast images. The paper microfluidic device thus fabricated needs to be stored in airtight container in cool and dry place and should be used within 7 days for best results.

8.4.1.7 Reagents and sample preparation

8.4.1.7.1 Reagents preparation

For preparation of 0.5% FBB, 0.25 gm of FBB was weighed accurately using weighing balance and transferred to 100 ml glass beaker. To it 45 ml of ethanol was added followed by 3 ml water and 2 ml GAA. The volume of resulting solution was 50 ml. Magnetic bead was immersed to the resulting solution. Then it was kept for stirring on magnetic stirrer at 300 rpm for 30 min. On completion of 30 min the magnetic stirrer was switched off and the FBB reagent was ready to use. Also ammonia solution was required for the colorimetric analysis but it was used in concentrated form for colour development.

8.4.1.7.2 Sample preparation for Spectrophotometric colorimetric method

100mg of MMA was weighed carefully using weighing balance and dissolved in SDW and made up to 100 ml to prepare 1000 µg/ml stock solutions. Aliquots of 0.2, 0.4, 0.6, 0.8, 1.0, 1.2 ml were withdrawn and diluted up to 10 ml to produce 20, 40, 60, 80, 100, 120 µg/ml respectively. Final dilution will be done by addition of colorimetric reagents to the samples thus giving final solutions in concentration range of 10 – 60 µg/ml.

8.4.1.7.3 Sample preparation for paper microfluidic based colorimetric method

Sample preparation for paper microfluidic device needed to be done by serial dilution method. The range which gave positive results by this method encases a huge paradigm of 50 – 10000 µg/ml. The procedure followed was, 1 gm of MMA was weighed carefully using weighing balance and dissolved in SDW making volume up to 10 ml in a volumetric flask which gave 100000 µg/ml of Stock solution 1. Then from this Stock solution 1, 1 ml aliquot was withdrawn and transferred to another 10 ml volumetric flask making up volume and giving a 10000 µg/ml of Stock solution 1a. Again, from Stock solution 1, 0.5 ml aliquot was withdrawn and transferred to 10 ml volumetric flask and volume was made up giving 5000 µg/ml of Stock solution 1b. From Stock solution 1a, 1 ml aliquot was withdrawn and diluted 10 times giving Stock solution 2a of 1000 µg/ml. And further from Stock solution 2a, 1 ml aliquot was diluted 10 times giving Stock solution 3a of 100 µg/ml. Similarly from Stock solution 1b, 1 ml aliquot was withdrawn and diluted up to 10 times giving Stock solution 2b of 500 µg/ml and further on diluting Stock solution 2b to 10 times gave 50 µg/ml Stock solution 3b. Thus, concentrations covering whole range of 50-10000 µg/ml were prepared consisting of 50, 100, 500, 1000, 5000 and 10000 µg/ml solutions.

8.4.1.7.4 Artificial urine preparation for application of both methods

100 ml artificial urine was prepared in SDW at laboratory premises. For its preparation, salts; potassium chloride 20 mg and sodium chloride 800 mg were weighed and transferred in 100 ml volumetric flask. To it buffers; disodium hydrogen phosphate 114 mg and potassium dihydrogen phosphate 20 mg were added. 20 µl

Queen yellow food colour was added to it for characteristic yellow colour of urine. The volume was made up to 100 ml with SDW and mixed uniformly in the volumetric flask. The final pH of the urine was set in between 7.5 to 8 using HCl or NaOH. [37]

8.4.2 Experimental

Primarily Spectrophotometric colorimetric analytical method was developed for estimation of MMA. The method was validated using (ICH), Q2 (R1) guideline for precision, accuracy, linearity, range, LOD, LOQ, Specificity and Selectivity. [28] The developed method was then extrapolated for development of microfluidic chip by using the similar concentration of reagents but scaling down the volumes as test needed to be accomplished with microlitre/nanolitre of reagents. For more accurate results, photometrix app available in android Smartphone was utilized by which quantitative analysis was attainable.

8.4.2.1 Development of colorimetric method for Methyl malonic acid

As it is apparent from structure (Figure 8.8), no chromophoric group is present in methyl malonic acid; coupling with an appropriate reagent was the only resort for development of colorimetric method for it. Various trials were taken for development of color, which fits in the visible range of 400-800 nm for colorimetric based paper microfluidic chip method. Colorimetric reactions for acids can be proceeded with diazonium salt formation using agents like sulphanilic acid [55], paranitroaniline [56], fast blue B [57] etc. As per the literature survey [58], coupling of extracted methyl malonic acid with diazotized p-nitroaniline was tried initially. Its procedure included heating sample up to 95°C for 3 min and cooling in ice-bath. Although chromogen was observed by application of above method, this was not possible to deploy in to paper microfluidic device. [59] Also MMA, in presence of tetrazotized o-dianisidine, gave color reaction on the TLC plate. [60] Considering all this in mind, it was decided to follow to diazonium salt formation principle, and only changing the salt for diazonium formation at laboratory premises. From literature survey [61], it was investigated that β-napthol leads to formation of diazonium dye on coupling with fast blue B salt giving purple colored azodye. Also alkylresorcinol in presence of fast blue B leads to formation of azo derivatives for colorimetric determination. [62] Therefore, it was decided to utilize fast blue B as coupling reagent for methyl malonic acid.

8.4.2.2 Sample preparation

The range 10 – 60 µg/ml selected for Spectrophotometric colorimetric method did not give clear distinction of violet color of azodye colored complex formed in-between various concentrations on paper microfluidic device because the volumes of sample and reagents are largely scaled down in it. Thus range which gave positive results by paper microfluidic based colorimetric method encases a huge concentration paradigm of 50 – 10000 µg/ml. The range thus selected gave clear distinction between the palate of violet color formed for serial concentrations. But the selected range for paper microfluidic devices cannot be used for Spectrophotometric method as we already know the absorbance scale for spectrophotometric analysis runs from scale 0 to 1 only and absorbance of 100 µg/ml also runs out of the absorbance scale.

8.4.2.3 Method optimization and development

As discussed in section 8.4.2.1, it was decided to utilize FBB as coupling agent for forming a diazonium salt with MMA. The procedure for preparation of FBB by which it would give the optimum chemical reaction still needed to be optimized. The solvent for FBB reagent preparation as well as the acidifying medium needed to be selected. As per literature study [62], solvents tried for development of chromogen were methanol, propanol and ethanol. FBB was freely soluble in all solvents though residues of FBB salt remained in all of them, but needed stirring for minimum 30 minutes on magnetic stirrer at room temperature. 0.5% FBB concentration was considered in every solvent. Also acidifying agent needed to be decided. As per literature study [56], Glacial acetic acid was reported to give better results as acidifying agent while preparing FBB thus it was selected as optimal acidifying agent for our method. Now by using OFAT (One factor at a time) approach, keeping Glacial acetic acid 1% as constant, methanol, propanol and ethanol were tried as solvents for dilution. For chromogen formation with MMA, basifying agent also needed to be decided. 10% NaOH, 10% KOH, 10% Na_2Co_3, 10% Ammonia solution were tried as basifying agent. Using OFAT (One factor at a time) approach, 4 ml of 50 µg FBB prepared in methanol with 1%GAA was made to react with 5 ml 100 µg/ml of MMA and mixed. Then to this sample, 1 ml of 10% NaOH was added. Similar procedure was followed for other basifying agents also. Better results with stable chromogen formation were achieved by using 50 µg FBB prepared in ethanol with 1% GAA as solvent and 10% ammonia as basifying agent. But still the chromogen was not intense enough to be visible on paper microfluidic device. Thereby, concentration of ammonia was increased to 50% which was still not enough thus finally it was decided to use ammonia as concentrated solution itself. Various trials were taken for optimization of volumes of reagents to be added. Finally it was decided to keep 90% ethanol, 4% GAA, 6% water for preparation of 0.5% FBB reagent by mixing all the above stated reagents and stirring for 30 minutes on magnetic stirrer. For 50 ml, the exact volumes and weight of reagents and sample taken are presented in section 8.4.1.7.1. The chromogen formed by chemical reaction of 0.5% FBB, concentrated ammonia with MMA is violet coloured having λmax of 526 nm. After impregnation of reagents 0.5% FBB and ammonia on paper microfluidic device it remains stable for 7 days for chromogenic reaction with MMA as a biomarker in urine for pernicious anaemia. Figure 8.8 represents the overlain spectra and Figure 8.9 represents calibration curve of MMA by spectrophotometric colorimetric for the developed samples.

Figure 8.8 Overlay spectra of MMA with range of concentration 10- 60µg/ml

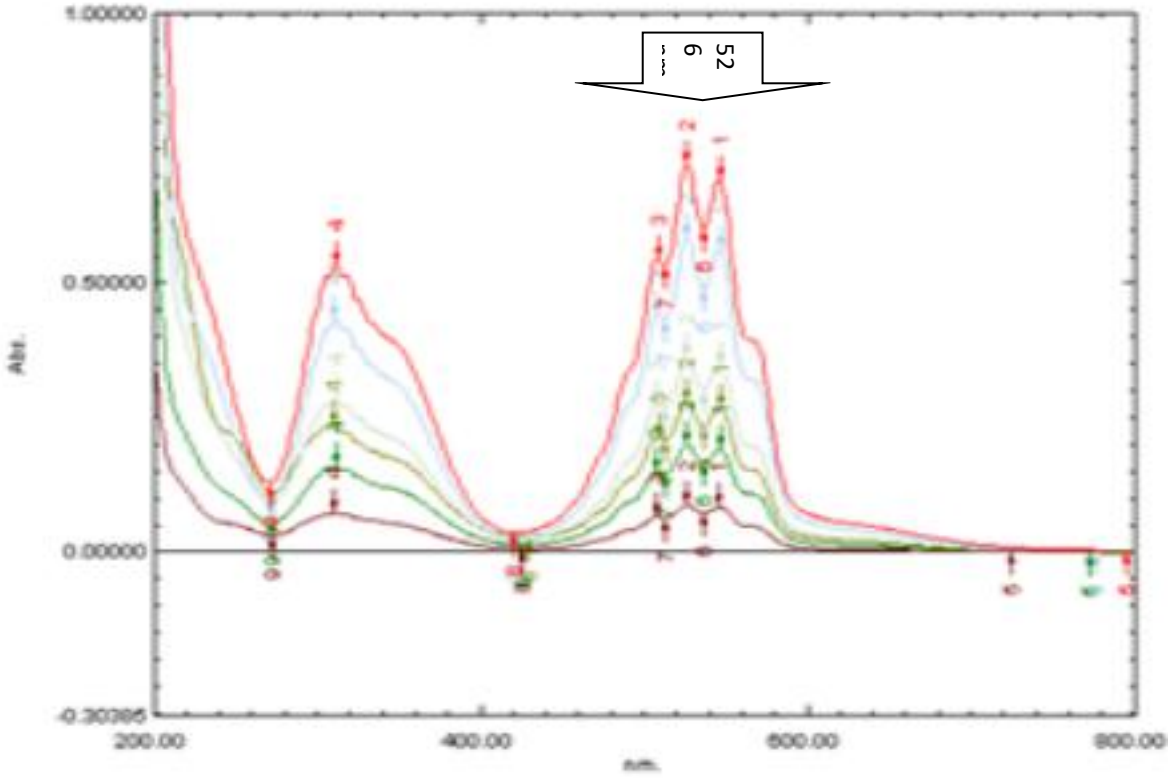
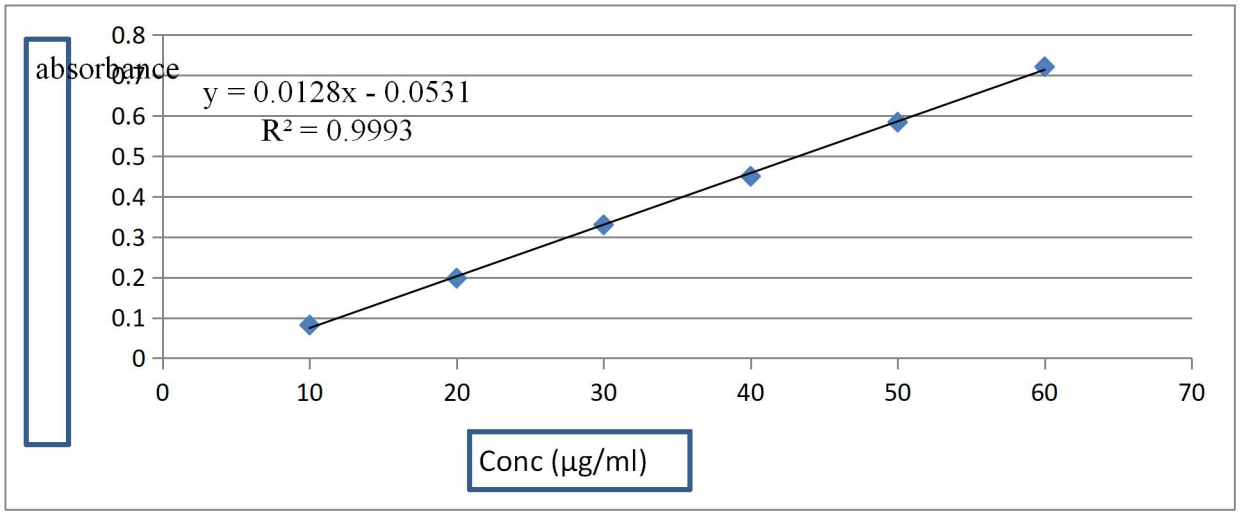


Figure 8.9 Calibration curve for spectrophotometric colorimetric method for estimation of MMA with range of concentration 10-60 µg/ml



8.4.2.4 Application of developed method in fabricated paper microfluidic device

Fabrication of microfluidic chip for this study was decided as discussed in section 8.3.2.3. The fabrication of paper microfluidic chip was done as described in section 8.4.1.6 and Figure 8.2. Now scaling down of developed colorimetric method was required as test needed to work in micro/nano liters of volumes for its working in paper microfluidic chip. As discussed earlier in section 8.4.2.2, 50 – 10000 µg/ml was

selected as optimum range of MMA sample for development of paper microfluidic devices whereas volume was reduced to 5µl of sample solutions. The reagents Ammonia and FBB were impregnated on to 1X6 pieces of Whatman filter paper no.1 respectively and air dried for 30 minutes as described in section 8.4.1.6. The sample solution was deposited using micropipette on the testing area and the color change was detected. For qualitative analysis, appearance of violet color confirms presence of MMA in the sample whereas for quantitative analysis, the image was captured in the 1X1 cm area in the photometrix app (Figure 8.10) and then calibration curve was plotted. (Figure 8.11) Real time color processing in pixels was achieved by using the photometrix app. The photometrix app is to be used as discussed in section 8.3.2.3. A plot of the MMA concentration versus the average RGB intensity was generated. Results show that the intensity of the Violet color developed on the paper test was consistent and proportional to the amount of MMA present in the sample. With MMA concentrations ranging from 50, 100, 500, 1000, 5000, 10000 µg/ml, a linear calibration plot was obtained.

8.4.2.5 Application of developed methods

8.4.2.5.1 Application of developed methods on artificial urine prepared at laboratory premises spiked with MMA

Success of any analytical method is based on its application for routine analysis. Both developed methods were applied for analysis of MMA in artificial urine spiked with known concentration of MMA. Artificial urine was prepared as mentioned in section 8.4.1.7.4. 1gm of MMA was transferred to volumetric flask and made up to 10 ml with SDW and sonicated for 5 min to prepare 100000 µg/ml of Stock solution. The solution was filtered through Whatman filter paper (No. 42). The stock solution was diluted 10 times giving 10000 µg/ml of solution. From the above prepared solution, 0.5 ml aliquot was withdrawn and volume was made up to 50 ml with artificial urine giving 100 µg/ml of concentration in sample. The above prepared solution was filtered through a 0.2 µm Nylon 6, 6 membrane filter, Ultipor® N66® from Pall Life Sciences, USA; prior to use and then pretreated for development of color by taking 5 ml of above prepared sample, adding 4 ml of 0.5% FBB reagent and 1 ml ammonia solution making it 50 µg/ml and analyzed by Spectrophotometer at 526 nm for the content of MMA using the calibration curve for estimation of MMA by spectrophotometric colorimetric method. The result of assay is described in Table 8.2. The result of assay was average of six replicates. For application by Paper microfluidic chip method, sample preparation was same as for spectrophotometric colorimetric method. From the 100000 µg/ml stock solution prepared previously for spectrophotometric colorimetric method, 2 ml aliquot was withdrawn and volume was made up to 50 ml with artificial urine giving 2000 µg/ml of sample. This solution was filtered through a 0.2 µm Nylon 6, 6 membrane filter, Ultipor® N66® from Pall Life Sciences, USA; prior to use. Then using a micropipette 5 µl of sample was deposited on the testing area of Paper microfluidic chip and the image of color developed was captured using the sampling icon in univariate analysis in photometrix app by android Smartphone camera. The concentration of sample obtained by Paper microfluidic chip method on photometrix app was 1940.795 giving % assay to be 97.039. The assay was repeated 6 times. The result of assays in six replicates gave very minor standard deviation of ± 0.893. The calibration curve along with sampling data is represented in Figure 8.12. The result of

assay represented in graph is of only 1 replicate as the application automatically calculates and mentions it in the graph.

8.4.2.6 Method validation using ICH Q2 (R1) guideline

The spectrophotometric colorimetric method as well as the Paper microfluidic chip method were validated using (ICH), Q2 (R1) guideline for precision, accuracy, linearity, range, LOD, LOQ. [15] Precision study was undertaken as intraday as well as intraday. For spectrophotometric colorimetric method as well as for Paper microfluidic chip method using procedure described above in section 8.4.2.3 and 8.4.2.4, 3 replicates of MMA with concentrations, 10 (LQC), 30 (MQC), 50 (HQC) µg/ml for spectrophotometric colorimetric method and 50 (LQC), 500 (MQC), 5000 (HQC) for paper microfluidic method were prepared 3 times a day as well on 3 different days and %RSD was calculated. %RSD below 2% signifies the method to be precise. LOD and LOQ were calculated from Standard deviation and slope values of 3 replicates of calibration curve. Accuracy calculation by spectrophotometric colorimetric method was undertaken using recovery method at 80, 100, and 120 % levels whereas for Paper microfluidic chip method it was only done for 100% level in 3 replicates. The summary of validation parameters are represented in Table 8.2.

Table 8.2 Summary of validation parameters

Parameters	**MMA**	
	Spectrophotometric colorimetric method	**Paper microfluidic chip method**
Analytics	By analytical wavelength 526 nm	By visibility of naked eyes and RGB value on android phone camera
Linearity range (µg/ml)	10-60	50-10000
Regression equation	y = 0.0128x - 0.0531	y=0.008x+152.380
Correlation coefficient (R^2)	0.9996	0.986
Intraday precision (%RSD)	0.57	0.49
Inter day precision (%RSD)	0.62	0.79
LOD (µg/ml)	0.78	2.05
LOQ (µg/ml)	2.35	6.15
Accuracy (% Mean Recovery)	98- 102	95- 105
Assay %(Spiked In artificial urine)	99.67±0.05	97.04± 0.89

Figure 8.10 Colorimetric test of MMA on MFD device

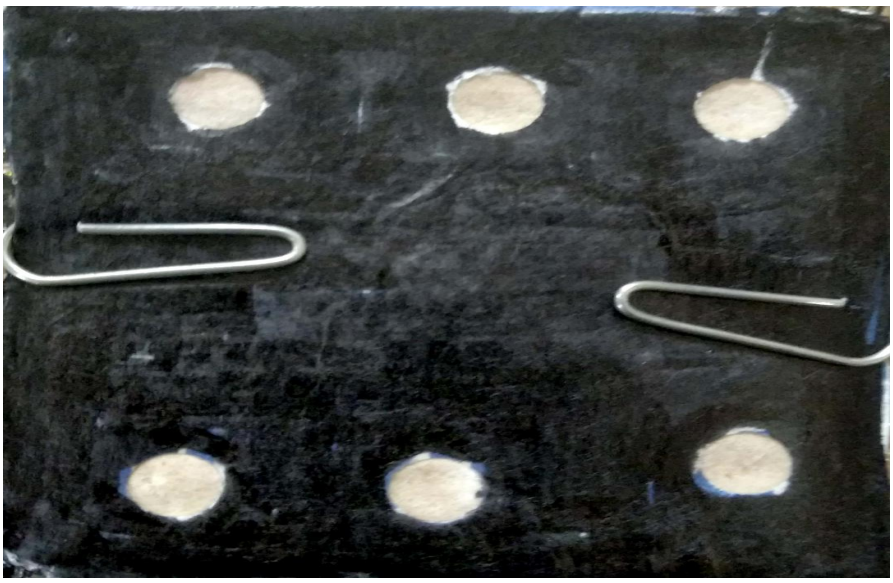
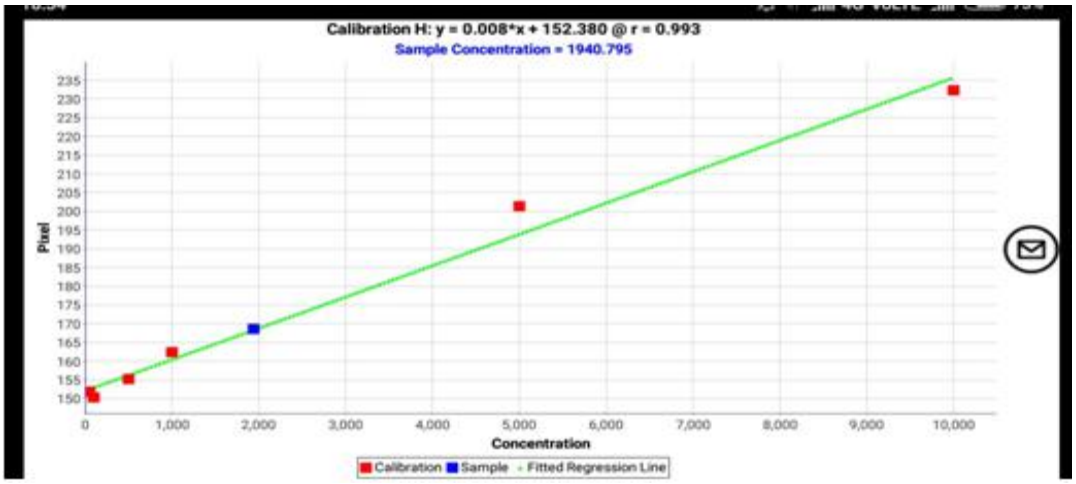


Figure 8.11 Calibration curve of MMA by Smartphone android based photometrix app for the developed samples



8.4.2.7 Comparison of both developed methods by statistical analysis

Statistical analysis was accomplished utilizing t-test test for comparing the two developed methods. The t-value obtained was 3.4821 and p value obtained was 0.00295. From the results it can be said that there is significant difference between the two results at $p < 0.05$ significance level.

8.5 CONCLUSION

A rapid, easy and very innovative method for Tadalafil testing along with a novel testing device was developed. A novel paper microfluidic device was fabricated by making hydrophobic channels in to Whatman filter paper no.1 using parafilm M. Also a new colorimetric assay method was developed for spectrophotometric estimation of

Tadalafil which can be extrapolated for application to paper microfluidic device. The developed method was very much suitable for qualitative as well as quantitative analysis of Tadalafil and also for checking its adulteration in herbal Ashwagandha formulations. For more precise analysis, real time color processing in pixels was achieved by using photometrix app available in android Smartphone. Thereby, allowing onsite analysis of Tadalafil without any laboratory preparations. Similarly, a rapid, easy and very innovative method for MMA testing along with a novel testing device was also developed. The colorimetric assay method developed for spectrophotometric estimation of MMA was also extrapolated for application to paper microfluidic device. The developed method was very much suitable for qualitative as well as quantitative analysis of MMA and also for diagnosis of MMA at home for critically anemic patients. For MMA also, more precise real time color processing in pixels was achieved by using photometrix app available in android Smartphone. Future scope of the work is to develop a quantitative method which gives assay value having no statistically significant difference with the standard spectrophotometric method used in analytical laboratories.

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