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Research Article

Cost effective paper-based colorimetric devices for a simple assay of dopamine in pharmaceutical formulations using 3,3',5,5'-tetramethylbenzidine-silver nitrate as a chromogenic reagent

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ABSTRACT

Paper-based devices using smartphone-based detection have been widely used for colorimetric analysis due to their simplicity, ease of operation, and handheld capability. Herein, we demonstrate simple colorimetric measurement of dopamine on paper-based devices, which were fabricated in the 96-well plate shape using wax printing technique. The detection reaction was based on the 3,3',5,5'-tetramethylbenzidine (TMB)-silver nitrate redox reaction. The oxidized TMB possessed the blue color which could be observed by naked eyes. In the presence of dopamine, the blue color of the oxidized TMB decreased according to the reduction of the oxidized TMB. In optimal condition, the responses were fit to the logarithmic curve with r^2 of 0.9932. The method showed good precision (%RSD<6.6%), and recoveries (92.8 - 106.2%) with the detection limit of <0.6 mM. Moreover, the results were well agreeing with those obtained from the conventional UV-Vis spectrophotometry, which were not statistically difference (P > 0.05). The developed method did not require any complex or time-consuming preparation and was successfully employed for the determination of dopamine injections.

1. INTRODUCTION

Dopamine belongs to a group of catecholamine neurotransmitters which plays an important role in mammalian cardiovascular, endocrine and central nervous system.[1] Several disorders of the nervous system are associated with the disturbances of dopamine transmission including Parkinson's disease, depression, and schizophrenia.[2,3] Therefore, determination of dopamine in biological fluids has a great importance for clinical purposes. Moreover, dopamine hydrochloride intravenous injection has been prescribed for the treatment of the shock syndrome due to various serious conditions such as low blood pressure, myocardial infarction, trauma and renal failure.[4, 5] Hence, efficient analytical methods for quality control of dopamine dosage forms is mandatory. The United States and British Pharmacopeias recommend non-aqueous titration for the assay of dopamine in raw material and high-performance liquid chromatography (HPLC) for the assay of dopamine injections.[6, 7]

In addition, several analytical methods have been investigated for determination of dopamine in biological fluids and pharmaceutical products e.g. spectrophotometry,[8] fluorescence,[9] electrochemistry,[10] chemiluminescence,[11] electro-luminescence,[12] capillary electrophoresis-electrochemical detection (ECD),[13] and HPLC-ECD/fluorescence detection.[14] These methods are time-consuming, expensive and require skilled operators and complicated maintenance. Thus, a simple, rapid and low-cost method for determination of dopamine becomes essential.

Microfluidic paper-based analytical devices were firstly introduced by Whiteside group,[15] which have gained attention due to their simplicity, low-cost, low reagent and sample consumption, portability and disposability.[16-18] Several detection techniques have been implemented to paper-based devices i.e. colorimetry, fluorescence, electrochemistry, electrochemiluminescence, and chemilumi-nescence.[19-23] Among these detection techniques, the colorimetric detection is the most commonly used because it is easy to perform and requires simple equipment.[18] The color products can be visually observed or quantitated using a smartphone or a scanner to take the images of the colored area and analyzing by image processing software.[24] ImageJ is an image analysis freeware which was developed by National Institutes of Health (NIH). This software can be used to convert Red, green and blue (RGB) to grayscale images. Then, the mean grayscale intensities are plotted against the analyte concentration.

A simple colorimetric method for the determination of dopamine has been reported, which was based on the 3,3',5,5'-tetramethylbenzidine (TMB)-silver nitrate ($AgNO_3$) redox reaction (Figure 1).[25] Subsequently, the blue color solution was generated from the oxidized TMB (oxTMB). The presence of dopamine could cause the fading of the blue color that can be quantitatively measured by UV-Vis spectrophotometer.[25] Herein, we demonstrate a simple and low-cost paper-based device using the colorimetric reaction of $AgNO_3$-TMB for the measurement of dopamine in pharmaceutical formulations. Then, a smartphone photograph of colored areas was taken and analyzed by ImageJ. Up to the present, colorimetric measurement of dopamine on paper-based devices based on the TMB - $AgNO_3$ redox reaction has not been reported.

2. MATERIALS AND METHODS

2.1. Instruments and analytical conditions

2.1. Chemicals

TMB, 3-hydroxytyramine hydrochloride (dopamine hydrochloride) and dimethyl sulfoxide (DMSO) were purchased from Tokyo Chemical Industry (Tokyo, Japan). $AgNO_3$ was obtained from POCH (Gliwice, Poland). Acetic acid was purchased from RCI Lab scan (Bangkok, Thailand). Sodium acetate was obtained from Ajax Finechem (New South Wales, Australia). All chemicals were of analytical grade. Sterile water was purchased from Thai Nakorn Pattana (Nonthaburi, Thailand). Whatman® filter paper No.1 was supplied by GE Healthcare Life Sciences (Chicago, USA) and laminating film with the thickness of 125 µm was purchased from HIC (Osaka, Japan).

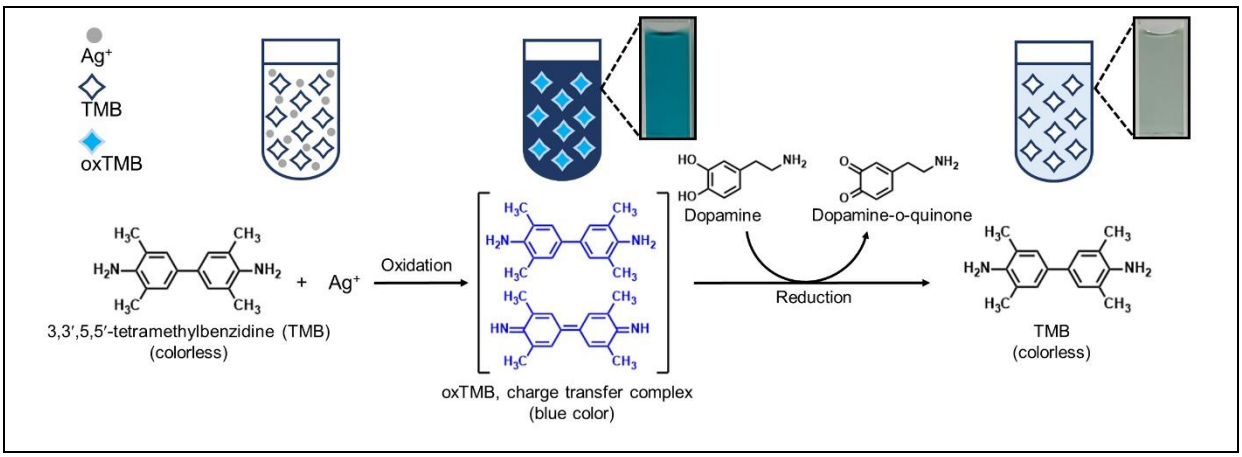

Figure 1. Mechanism of the TMB - $AgNO_3$ redox reaction for colorimetric measurement of dopamine.

Stock solutions of 40 mM TMB and 40 mM $AgNO_3$ was separately prepared by dissolving 0.0481 g TMB and 0.0339 g $AgNO_3$ in 5 mL DMSO and sterile water, respectively. The stock standard solution of dopamine was prepared by dissolving 0.0189 g dopamine in 10 mL sterile water (10 mM). Working standard solutions were diluted from the stock solution with water to obtain 1 - 8 mM dopamine. The stock solutions were protected from light and stored at room temperature, while the stock solution of dopamine was kept at 4°C.

2.2. Fabrication of paper-based devices

Fabrication of paper-based devices relies on patterning of hydrophilic - hydrophobic regions on a Whatman® paper in order to create capillary channels or reservoirs on the paper.[18] The patterns of paper-based devices were drawn using Microsoft® PowerPoint software. Whatman® qualitative filter paper sheet grade 1 with pore size of 11 µm and thickness of 180 µm was cut into the A4 size (21.0 x 29.7 cm). The designed paper featuring a number of circular shapes with a diameter of 6 mm and line thickness of 0.8 mm (2.25 pt) was printed on the filter paper (ca 300 circular shapes/A4 paper size) using Fuji Xerox® Colorqube8870 wax printer (Bangkok, Thailand). Subsequently, the patterned paper was heated on a hot plate for 5 min to allow the wax to penetrate through the paper. Finally, the paper-based devices were laminated underneath with a clear laminating film.

2.3. Determination of dopamine on paper-based devices

Firstly, 100 µL of 40 mM TMB and 100 µL of 40 mM $AgNO_3$ were mixed in 300 µL of 0.2 mM sodium acetate buffer (pH 5.0) and vortexed for 1 min. Then, 5 µL of the mixture was added to the reservoir on the paper-based devices using micropipette and allowed to dry for 3 min using a hair dryer. Next, 3 µL of dopamine solution was added and dried for 3 min. After 12 min (total reaction time of 15 min), a color change on the paper-based devices was visually observed and photos of the colored areas were taken in a light box using smartphone camera (Samsung Galaxy A7 (2015) model (Seoul, the Republic of Korea)). The setup for taking photo including three desk lamps, the paper-based devices and smartphone was fixed in order to control the light intensity for reproducible measurement. Then, the images were converted to grayscale and the color intensity was analyzed using the freeware ImageJ (version 1.51g).

Various parameters affecting the dopamine determination on paper-based devices were investigated including the concentrations of TMB (5 - 10 mM), reaction times (3 - 23 min), and stoichiometric ratios of TMB and $AgNO_3$ (1:3, 1:2, 3:4, 1:1, 4:3 and 2:1). The optimized condition was evaluated from the color intensity and the stability of the color product.

2.4. Method validation

The optimized condition for the analysis of dopamine on the paper-based devices was validated in terms of calibration curve (logarithmic model), accuracy, precision, limit of detection (LOD) and limit of quantitation (LOQ). A calibration curve fitted to a logarithmic model was established by plotting the gray intensity against five different concentrations of dopamine (1 - 8 mM). Triplicate analyses were done for each concentration. Method precision was determined from intra-day (n = 3) and inter-day (n = 3) experiments, calculated from the relative standard deviations (RSDs) of the gray intensity. Recovery of the method was estimated by spiking different concentrations (80, 100 and 120 percent of nominal concentration, 1 mM dopamine) into the sample solutions (n = 3). The LOD and LOQ were based on the gray intensity of blank and SD of blank, which were calculated from eq. 1 and eq. 2.[26]

$$LOD = 3.3SD_{blank} + \text{gray intensity of blank} \quad (1)$$

$$LOQ = 10SD_{blank} + \text{gray intensity of blank} \quad (2)$$

2.5. Application

The validated method was applied for the assay of two strengths of dopamine injections (10 and 25 mg/mL). Sample solutions were freshly prepared by pipetting 303 µL and 153 µL of 10 and 25 mg/mL dopamine injections, respectively into a 5-mL volumetric flask and adjusted to volume with water. Then, 3 µL of sample solution was added on the paper based-device. The determination of dopamine in the injections was also carried out using UV-Vis

spectrophotometry and the results obtained from two methods were statistically tested by t-test.

3. RESULTS AND DISCUSSION

3.1. Determination of dopamine on paper-based devices

The determination of dopamine based on the TMB - $AgNO_3$ redox reaction using UV-Vis spectrophotometry has been demonstrated by Zhu et al.[25] Firstly, the oxidation of TMB by silver ions yielded the blue charge-transfer complex of diamine in TMB and the diimine in the oxidation product (oxTMB),[27] which can be observed by naked eyes and can be quantitatively measured using spectrophotometry at the wavelength of 655 nm. Subsequently, the color of the reaction mixture from colorless to blue was observed. Then, addition of dopamine resulted in the fading of blue color because it acted as the reducing agent to oxTMB (Figure 1). In the presence of dopamine, the absorbance gradually decreased with an increasing concentration of dopamine. Zhu et al. demonstrated the dopamine determination using UV-Vis spectrophotometry. The reaction mixture contained of 100 µL of 1 mM TMB, 50 µL of 1.5 mM $AgNO_3$, 340 µL of 0.2 M sodium acetate buffer and 10 µL of dopamine. However, transferring the UV-Vis spectrophotometric condition to the paper-based devices could not be achieved because of the lower capacity of the reservoirs on the paper. The mass of reagents (TMB and $AgNO_3$) and dopamine on the paper reservoirs were 5 - 25 times fewer than those in the spectrophotometry, which were insufficient to produce the blue colored product on the paper. Therefore, factors affecting the colorimetric reaction on the paper-based devices were further investigated.

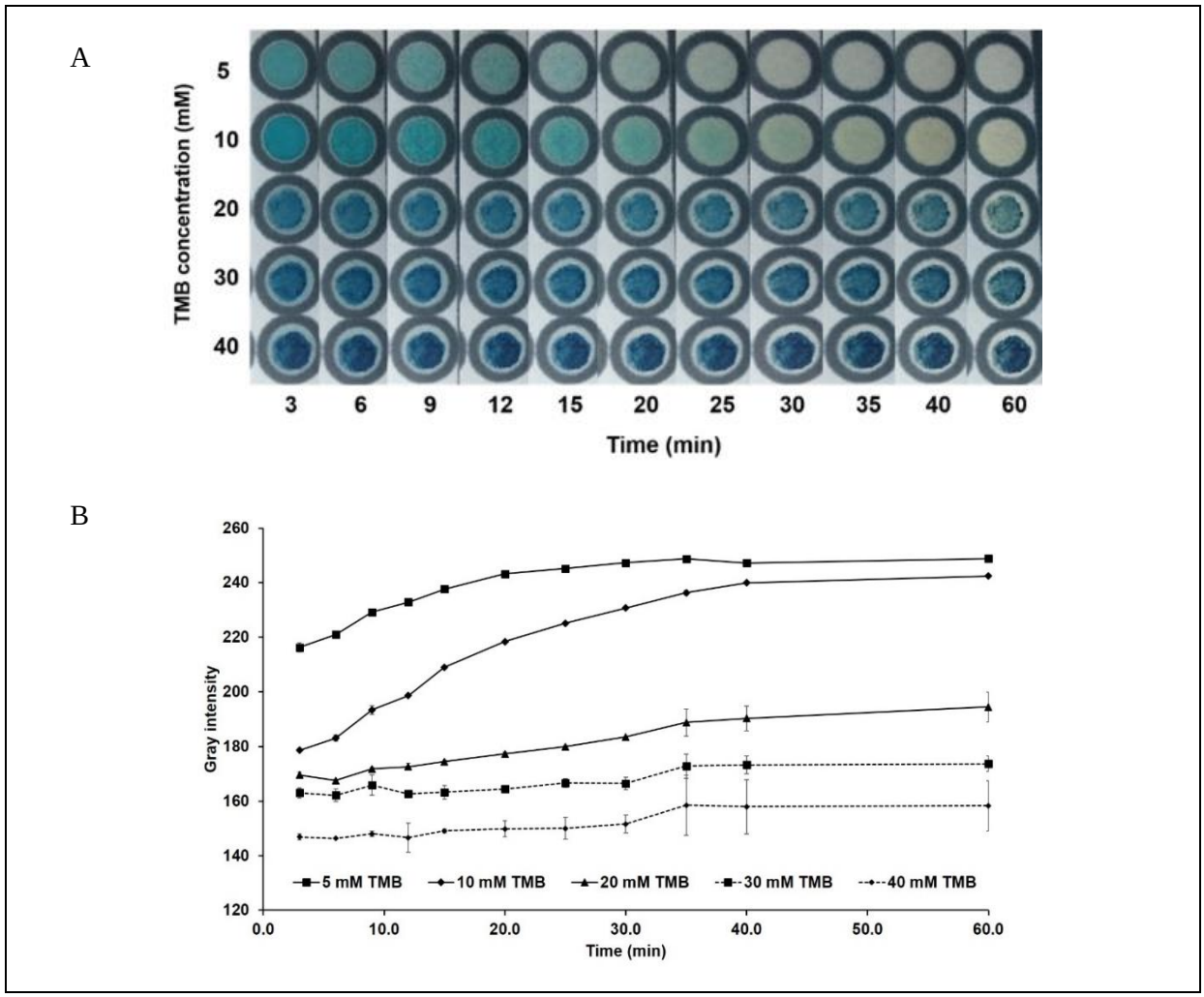

Figure 2. Effects of TMB concentrations on the blue color of colorimetric probe and their color stability after exposed to the ambient atmosphere for 3-60 min: a) a photograph of the blue charge-transfer complex on the paper-based device, b) the gray intensity of the blue charge-transfer complex.

3.1.1. Effects of TMB concentrations

Initially, 1 mM TMB and 0.75 mM $AgNO_3$ (stoichiometric ratio of 4:3) was utilized in the reaction mixture in order to produce the blue charge-transfer complex. The blue color intensity on the paper-based devices was directly proportional to the amounts of oxTMB. However, the blue color was not observed when the concentrations of TMB were less than 1 mM. Therefore, varying TMB concentrations to 5 - 40 mM with 3.75 - 30 mM $AgNO_3$ using the constant stoichiometric ratio of 4:3 was investigated. After the addition of 5 µL of TMB and $AgNO_3$ mixture, the reaction zone was dried with the hair dryer for 3 min. The images of the colored areas were taken at 3 to 60 min in order to investigate the color stability since TMB is light and air sensitive.[28] The photos were taken by the smartphone camera and converted from red, green and blue (RGB) color to grayscale mode by the ImageJ software, which has the intensity in the range of 0 - 255. The display intensity of zero represents that the image is totally dark, while the completely bright image possesses the intensity of 255. Figure 2 shows the effects of TMB concentrations on the blue color complex, it was found that the gray intensity of blue charge-transfer complex obtained from the low concentrations of TMB (5 - 10 mM) significantly decreased over time from 216.31±1.73 to 248.78±0.45 for 5 mM TMB and from 178.63±0.69 to 242.46±0.41 for 10 mM TMB. The decreasing rates were 0.4%/min and 0.9%/min for 5 and 10 mM TMB, respectively. Whereas the reaction mixture containing TMB concentrations in the range of 20 - 40 mM offered the stable color product for approximately 30 min with the remaining color intensity of 91.8 - 97.9%. Then, the intensity slightly decreased about 6.7 - 14.7% during 30 - 60 min with the rate of 0.1 - 0.3%/min. TMB at the concentration of 40 mM was selected as the optimal concentration for further optimization since it provided the highest blue intensity.

3.1.2. Effects of reaction times

Effects of reaction times on the colorimetric measurement of dopamine using TMB - $AgNO_3$ redox reaction was investigated. The mixture of 40 mM TMB and 30 mM $AgNO_3$ (5 µL) was added into the reservoirs and the photos were taken after the addition of 1 mM dopamine (3 µL) from 3 to 23 min. The gray intensity showed a gradual increase from 3 - 11 min and the maximum color intensity was obtained during 15 - 23 min. Therefore, 15 min was selected as the optimized reaction time (Figure 3).

3.1.3. Stoichiometric ratios of TMB and $AgNO_3$

TMB is a chromogenic substrate that can be readily oxidized by $AgNO_3$, to form the blue charge-transfer complex between the neutral amine (TMB) as a donor and diiminium structure (dication, oxTMB) as an acceptor via the $\pi-\pi$ interaction (Figure 1).[25] The proposed mechanism indicated that two moles of TMB

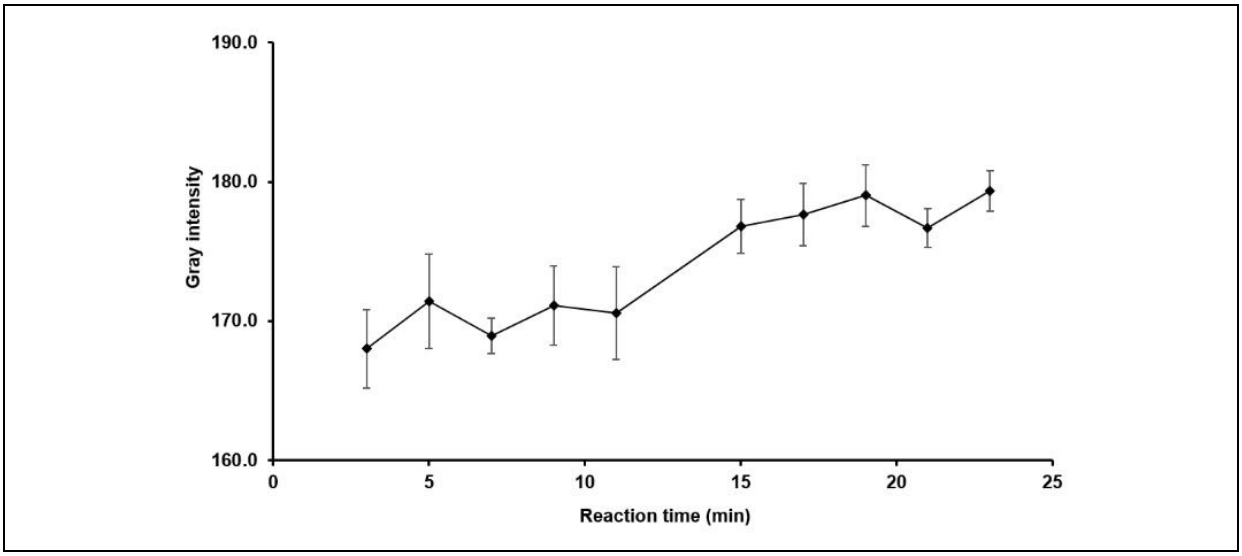

Figure 3. Effects of the reaction times.

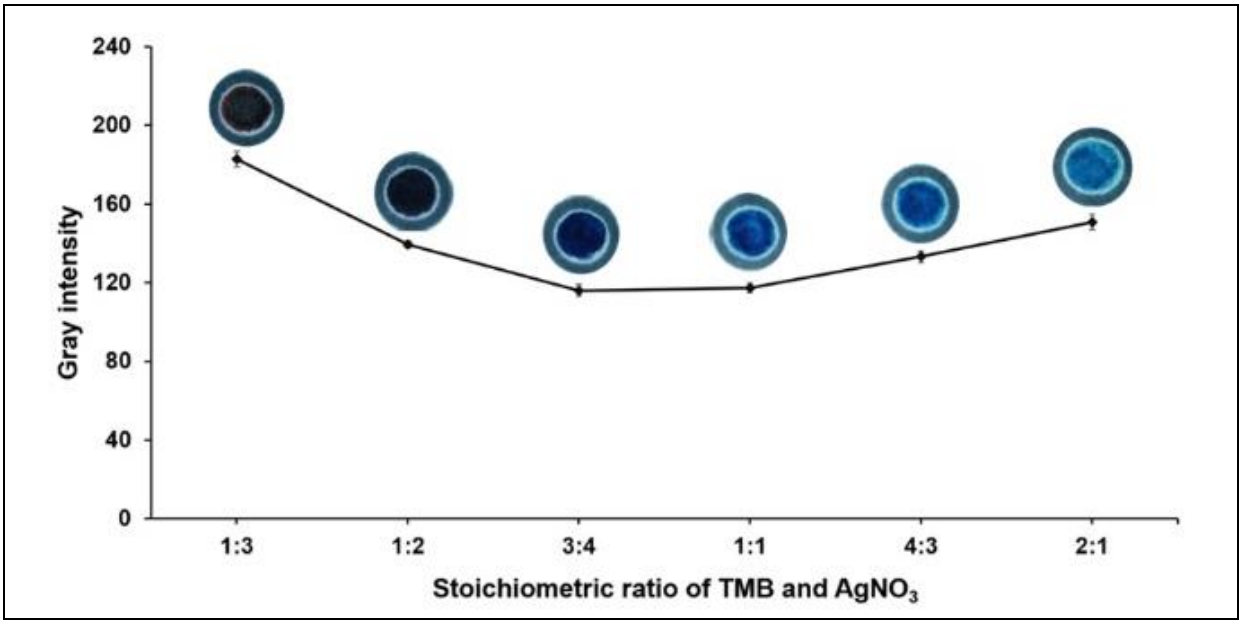

Figure 4. Effects of the stoichiometric ratios of TMB and $AgNO_3$.

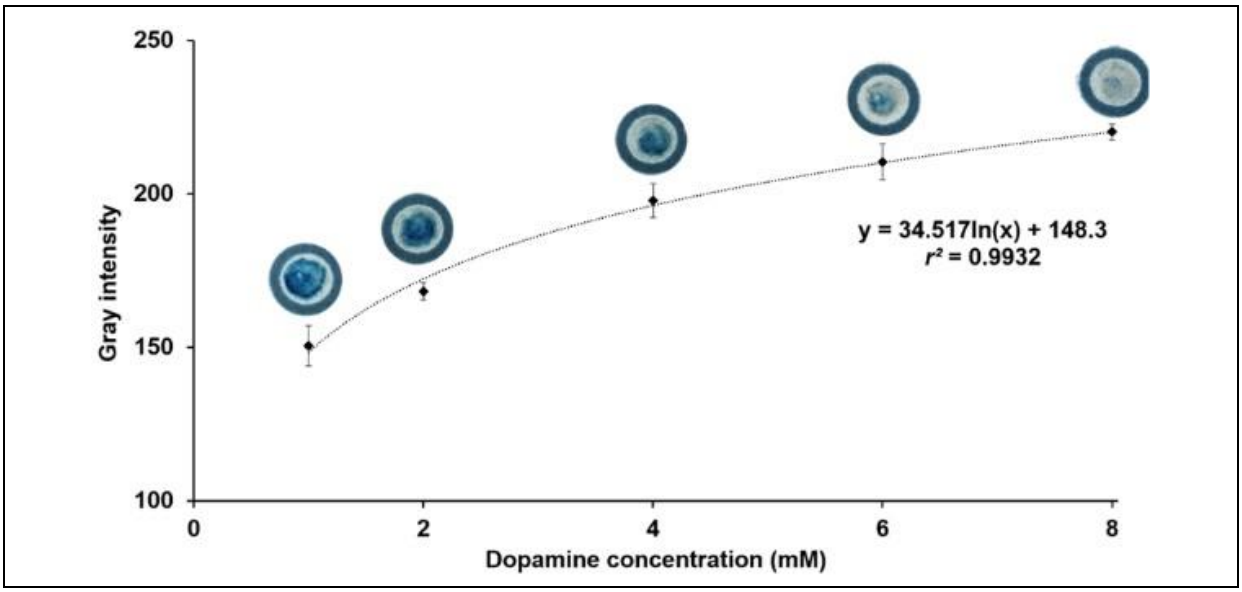

Figure 5. A calibration curve of dopamine.

Table 1. Validation data

Precision (%RSD)			LOD (mM)	LOQ (mM, n = 9)	%Recovery (n = 3)			
Concentration (mM)	Intra-day (n = 3)	Inter-day (n = 9)			%Added			Average (%RSD)
					80	100	120	
1	1.8	4.4						
4	2.7	2.8	0.57	1.0 (4.4)	110.8	106.2	92.8	99.9 (6.8)
8	1.1	1.2						

requires one mole of $AgNO_3$ for the complete reaction. However, excessive amounts of $AgNO_3$ could be generated side products such as silver nanoparticle, which may interfere the blue color formation.[29] Stoichiometric ratios of TMB and $AgNO_3$ at 1:2, 3:4, 1:1, 4:3 and 2:1 were examined. The TMB: $AgNO_3$ of 2:1 provided the lower blue color product with the gray intensity of 150.8±3.91 comparing with the ratio of 1:1 with the gray intensity of 117.3±2.53 (Figure 4). The blue intensity rapidly increased when the ratio of TMB and $AgNO_3$ changed from 2:1 to

1:1 and remain stable at ratio of 3:4. When the stoichiometric ratio of TMB and $AgNO_3$ was 3:4, the color change from blue to purple. With the excess amount of $AgNO_3$ may cause the conversion from the silver ions to the neutral silver. Subsequently, the silver aggregates into nanostructure possessed the purple color and could interfere the colorimetric assay.[29] Therefore, the stoichiometric ratio of 1:1 was selected as the optimal stoichiometric ratio. The optimal condition for the measurement of dopamine on paper-based devices was in 40 mM TMB and 40 mM $AgNO_3$ using the reaction time of 15 min after the introduction of dopamine on the paper-based device.

3.2. Method validation

A calibration curve was established by plotting the gray intensity against the dopamine concentration in the range of 1 - 8 mM. The responses fitted the logarithmic model with r^2 = 0.9932 (Figure 5). Precision, recovery, LOD, and LOQ of the developed method were evaluated (Table 1). The intra-day (n = 3) and inter-day (n = 9) precision showed RSDs of < 3.5% and < 4.4%, respectively. The LOD and LOQ calculated from eq.1 and 2 were 0.57 and 1.01 mM (RSDs of < 4.4%), respectively. The mean recoveries of dopamine (n = 3) was 99.9 with RSDs of < 6.8%. The recovery data indicates that there was no sample matrix effect on the quantitation of dopamine in the samples. The validation data shows that the method was appropriate for the analysis of dopamine in pharmaceutical formulations.

3.3. Applications

Applications of the developed method was illustrated in the assay of dopamine in pharmaceutical formulations. The method was applied to determine dopamine in injections with strengths of 10 and 25 mg/mL. Percent label amounts of the samples (n = 3) were within 100.9 - 102.0 (RSDs < 2.8%), which was in the USP limit of 95.0 - 105.0%. Importantly, the quantitation of dopamine in the injections was also performed independently by conventional spectrophotometry (percent label amounts of 98.9 - 102.2 with RSDs < 1.5%). The results obtained from two methods were well agreeing, which were not statistically significant difference (P > 0.05) tested by t-test.

4. CONCLUSIONS

Colorimetric assay on the paper-based devices for the detemination of dopamine in pharmaceutical formulations using smartphone-based optical detection offers reliability, simplicity, low-cost, portability and environmentally friendliness. The cost of paper-based devices with the reagents for assay of dopamine in triplicate was approximately 0.04 USD. This method illustrates the TMB - $AgNO_3$ redox reaction on the paper-based devices that could be applied for the determination of various analytes e.g. thiols, epinephrine and norepinephrine. Also, the reaction product could be alternatively detected by an electrochemical detector, which is an altenative for paper-based devices. Furthermore, the method shows the potential for equipment-free detection because the chemical reaction on the paper is visible to naked eyes. The analytical platform is made from cellulosic material, which is compatible with many chemicals, easily functionalized and easy to store. Therefore, the devices can be used for other chemical reactions involving in the analytical methods. From practical aspect, the simple and reliable method using less equipment is desirable for the quality control of pharmaceutical products and also other analytical purposes in the resource-limited setting.

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Conflict of interest

The authors have no conflict of interest.

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