

## DEVELOPMENT OF PAPER-BASED ANALYTICAL DEVICE FOR DETECTING DIAZEPAM IN URINE

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### ABSTRACT

**Objective:** The aim of this study was to develop Paper-based Analytical Devices (PADs) with colorimetric method as a presumptive test for detecting diazepam in urine

**Methods:** Colorimetric method was used as a principle of this study. PADs were fabricated with wax-printing methods. Chosen colorimetric reagent was tested for selectivity with hydromorphone and codeine; and sensitivity by measuring the absorbance with UV-Vis spectrophotometer. PADs were tested for its sensitivity and stability. The intensity of color developed on PADs are measured with ImageJ. The ability of PADs to detect diazepam in urine was simulated with testing spiked urine sample to the PADs

**Results:** Zimmermann gave the most obvious prominent color change from colorless to purple-red color out of the four reagents. PAD is selective to diazepam when tested with hydromorphone and codeine. PAD is sensitive with a cut-off concentration at 100 ppm. PAD can detect diazepam in urine with the highest recovery percent at 92.8%±4,6

**Conclusion:** It can be concluded that PAD is quite selective and sensitive to detect diazepam in urine and can be done easily and fast for onsite analysis

**Keywords:** Paper-based analytical devices, Diazepam, Colorimetric, Detection method

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### INTRODUCTION

Drug abuse is an act of drug consumption that can harm the user and the environment around them [1] and is often linked with addiction [2]. One of the drugs that have the potential to cause addiction is diazepam. Diazepam (7-chloro-1-methyl-5-phenyl-3H-1,4-benzodiazepin-2-one), also known as valium, is a class IV psychotropic drug used as an antianxiety, anti-insomnia, anticonvulsant, sedative, hypnotic, and tranquilizer [3]. Diazepam works by increasing gamma-aminobutyric acid (GABA) inhibitory activity by binding to the GABA receptor in the limbic system and hypothalamus, causing increased frequency of chloride channel opening, therefore causing neuron excitation [4, 5].

Investigations are needed to control drug abuse. These investigations are usually divided into two steps: a presumptive test followed by a confirmation test [6]. While a confirmation test is usually more accurate, expensive, takes more time and involves instruments, a presumptive test is less expensive, more straightforward, preferably can be done rapidly and on site by handler with less skill needed than in a confirmation test. Several methods that earlier have been employed for diazepam determination are spectrophotometric [7, 8], Raman spectroscopy [9], thin layer chromatography (TLC) [10], high performance liquid chromatography (HPLC) [11, 12], and GC-MS [13, 14]. These methods are selective and sensitive to determine diazepam, but have some drawbacks. Most of them need sample preparation, expensive instrumentation and skilled operators. Because some of these methods are time-consuming, they cannot be used for on-site analysis. Therefore a simpler, cheaper, faster, on-site test that can detect diazepam is needed. Paper-based analytical devices (PADs) with colorimetric detection can be the solution to this problem.

PADs were first found in 2007 [15] for bioassays and now have been used in various applications such as environment monitoring, biomedical and pharmaceutical analysis, clinical diagnosis, and forensic investigation [16]. Some of the advantages of PADs are: inexpensive; require minimum instrumentation; require only a small amount of sample and reagent; simple fabrication and operation; easier visualization than a traditional microfluidic; does not require any power; portable; and disposable [17, 18].

One of the detection methods that are commonly used in PADs is the colorimetric method [6, 15, 19]; because of efficient detection, it can

easily be observed visually by color change, economical price, easy to operate, equipment-free, and stable [20, 21]. The aim of this study was to fabricate PADs as the development of a diazepam detection device using the colorimetric method. Zimmermann, hydrochloric acid, Marquis, and Vitalin-Morin [22, 23] are colorimetric reagents used in this research. This PAD is applicable for diazepam detection device that is faster, simpler, with a good performance.

### MATERIALS AND METHODS

#### Materials

All chemicals were analysis grade unless stated otherwise. 1,3-dinitrobenzene and ACS grade formaldehyde were purchased from Sigma Aldrich. Hydrochloric acid, nitric acid, sulphuric acid, ethanol, potassium hydroxide were purchased from Ensure. Acetone was purchased from Mallinckrodt Chemicals. HPLC grade methanol was purchased from J. T. Baker. Diazepam was purchased from Merck. Hydromorphone tablet was produced by Dilaudid. Whatman chromatography paper no. 1 was purchased from GE Healthcare. The measurement of absorbance was recorded by UV-Vis spectrophotometer Analytik Jena SPECORD 200 with 1 cm quartz cuvette. Wax was printed with Xerox ColorQube 8870 wax printer. For the measurement of color intensity, PADs were scanned by Printer Canon G3000. A standard solution of diazepam was made by dissolving 100 mg of diazepam in 100 ml ethanol.

#### Colorimetric reagent test

Colorimetric reagents used were Zimmermann, hydrochloric acid, Marquis and Vitalin-Morin. Reagents were tested against diazepam standard solution. Reagent preparation and test were carried out with the following procedures.

#### a. Zimmermann test

One gram of 1,3-dinitrobenzene dissolved in 100 ml ethanol (Zimmermann A). Fifteen grams of potassium hydroxide dissolved in 100 ml water (Zimmermann B) [22]. One ml of diazepam standard solution was transferred into a test tube. Five drops each of Zimmermann A and Zimmermann B then added to the test tube and the color change was observed.

### b. Hydrochloric acid test

Hydrochloric acid was diluted with water to get a concentration of 2 N. One ml of diazepam standard solution was transferred into a test tube. Ten drops of hydrochloric acid were then added to the test tube and the color change was observed.

### c. Marquis test

Five ml of formaldehyde was added to 45 ml of sulphuric acid [24]. One ml of diazepam standard solution was transferred into a test tube. Ten drops of Marquis reagent were then added to the test tube and the color change was observed.

### d. Vitalin-morin test

To make potassium hydroxide solution, 0.56 g of potassium hydroxide was dissolved in 100 ml ethanol. Two ml of diazepam standard solution were transferred into a test tube. Ten drops of nitric acid were then added to the test tube and the test tube was heated in the water bath to dry. Five ml of acetone and 1 ml of potassium hydroxide solution were then added to the test tube and the color change was observed [22].

### Specific reagent performance test against standard diazepam

Specific reagents chosen were tested for their selectivity and sensitivity. The tests were carried out with the following procedures.

#### a. Selectivity test

For the selectivity test, the diazepam standard solution was diluted to the concentration of 200 ppm. Hydromorphone and codeine were extracted from the tablet and also made into a 200 ppm solution. Specific reagents obtained from the previous test are tested against the three drugs. The reagent is considered selective if the color change occurs with diazepam alone.

#### b. Sensitivity test

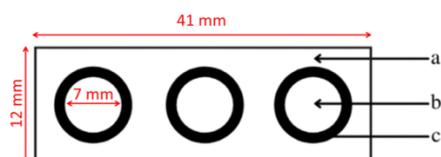
Diazepam standard solution was diluted to five different concentrations (1, 2, 3, 4, and 5 ppm). Three ml of each concentration was transferred into 5 test tubes. Two ml of selective reagent added to the test tube. Some of the solution then transferred to a micro plate for better color change observation. The absorbance of each solution was measured with a UV-Vis spectrophotometer.

#### Reagent optimization on whatman chromatography paper no. 1

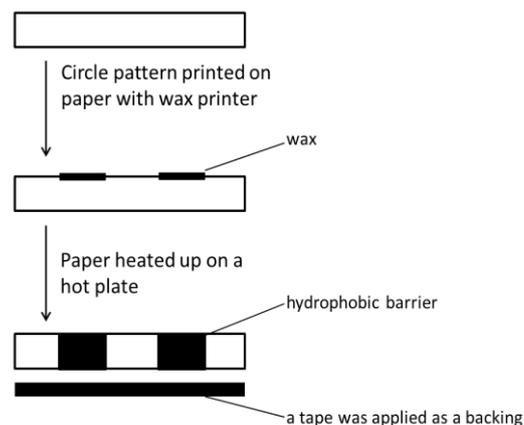
Whatman chromatography paper No. 1 was cut into 0.5 × 0.5 cm pieces. Two µl of specific reagent were then added to the paper with two different treatments. Papers with treatment A were tested with diazepam solution after the reagent was dried first, and papers with treatment B were tested with diazepam solution right after the reagent placement. The time taken for reagent to produce color was recorded.

#### Paper-based analytical device (PADs) design for diazepam analysis

The Paper-based Analytical Device (PADs) was designed as shown in fig. 1. Circle pattern was printed on Whatman chromatography paper No. 1 using Xerox ColorQube 8870 wax printer. One PAD consists of three circles for three tests to make sure that the results are indeed positive or negative. The printed paper then heated on a hot plate at 150 °C for 2 min to create a hydrophobic barrier in the paper. A tape was used as the hydrophobic barrier on the back of the paper. The fabrication scheme is shown in fig. 2. The performance of PADs was observed by sensitivity test and stability test. After each test, the paper was scanned with Printer Canon G3000 and the intensity of developed color was measured by ImageJ.



**Fig. 1: Design of the PAD (a) Whatman chromatography paper no. 1; (b) Hydrophilic area for reagent and sample; (c) Hydrophobic barrier to contain reagent and sample**



**Fig. 2: Fabrication scheme of PAD**

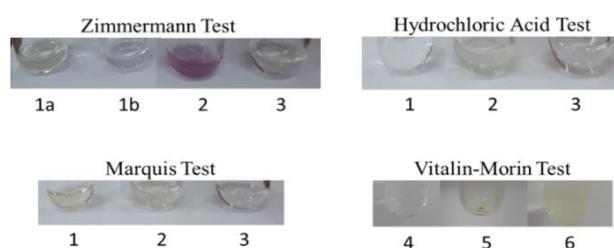
#### Application of PADs for detecting diazepam in urine

Calibration curve of diazepam concentration against color intensity was made by using standard diazepam with various concentrations (200 ppm, 400 ppm, 800 ppm, 1600 ppm, 2000 ppm, and 4000 ppm). Onto the PADs that have been made, 2 µl of reagent and 2 µl of diazepam solution were added. The color intensity was measured by ImageJ and plotted to a curve.

To demonstrate the application of PADs for detecting diazepam in biological samples, 2 ml of urine sample was spiked with 10 mg and 20 mg of diazepam to get 1000 ppm and 2000 ppm of diazepam in urine. Ethanol was added to each solution to 10 ml. Each of the spiked samples was then added to the PADs that contained the reagent. PADs were scanned and the color intensity was measured by ImageJ and the equation from the calibration curve was used to get the percent recovery.

#### RESULTS

The specificity of colorimetric reagents were tested against diazepam standard solution. The reagents tested were Zimmermann, hydrochloric acid, Marquis, and Vitalin-Morin. All tests were carried out in a test tube. Fig. 3 shows the color produced by these reagents when reacted with diazepam.



**Fig. 3: Colorimetric reagent test results. (1) Colorimetric reagents; (1a) Zimmermann A; (1b) Zimmermann B; (2) Diazepam standard solution reacted with reagents; (3) Ethanol as blank; (4) Diazepam standard solution; (5) Diazepam after heated with nitric acid; (6) Diazepam after acetone and potassium hydroxide were added**

Specific reagents chosen were tested for their selectivity. The selectivity test was carried out against diazepam, hydromorphone and codeine as they are in narcotic and psychotropic drug classes that are often found in a drug abuse case.

Selective reagent chosen was tested for sensitivity against diazepam with various concentrations. After reacting diazepam with Zimmermann, the absorbance was measured and recorded with UV-Vis Spectrophotometer.

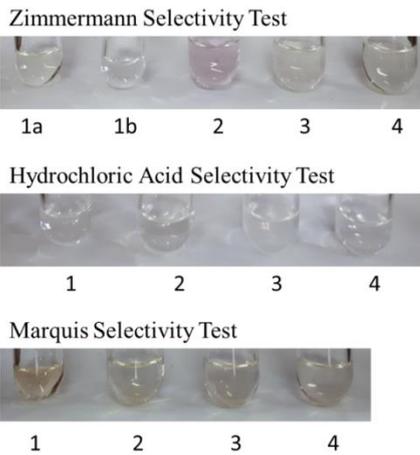


Fig. 4: Specific reagents selectivity test results. (1) Specific reagent; (1a) Zimmermann A; (1b) Zimmermann B; (2) Diazepam reacted with reagents; (3) Hydromorphone reacted with reagents; (4) Codeine reacted with reagents

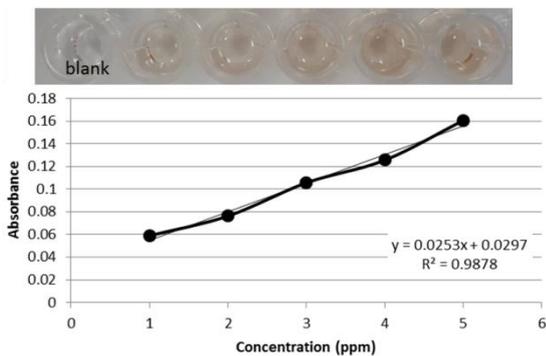


Fig. 5: Calibration curve of diazepam with Zimmermann reagent

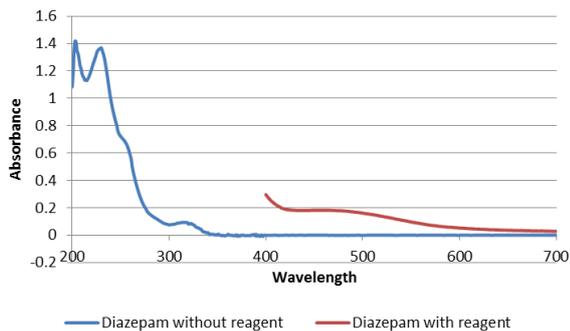


Fig. 6: Overlay spectrum of diazepam without reagent and diazepam with Zimmermann

Before applying reagent on PADs, an optimization of the reagent was carried out. This optimization was meant to test which method works the best on whatman chromatography paper no. 1 for diazepam detection.



Fig. 7: Color change on whatman chromatography paper no. 1 (a) Treatment A; (b) Treatment B. Diazepam solution concentration variation: (1) 200 ppm; (2) 400 ppm; (3) 800 ppm; (4) 1600 ppm; (5) 2000 ppm

PAD was tested for its sensitivity and selectivity against standard diazepam. The sensitivity of PAD was tested with various diazepam concentrations. To PADs that contained reagent, diazepam solutions were added. PADs then scanned and ImageJ measured the color intensity. Table 1 shows the color intensity measurement of PADs and fig. 8 shows the sensitivity curve with images of scanned PADs.

Table 1: Sensitivity of PAD

Concentration of diazepam (ppm)	Intensity
100	207896
200	234755
400	246804
800	282130
1600	316396
2000	328954
4000	449621

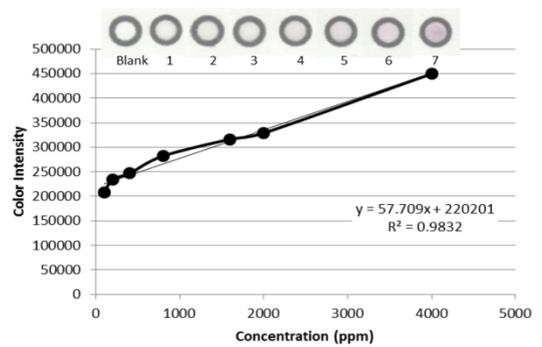


Fig. 8: Sensitivity curve of PAD

The stability of PAD was tested with diazepam solution with a concentration of 2000 ppm. PADs that contained reagent were left for 1 to 7 d and then diazepam solution was added. PADs then scanned and the color intensity was measured by ImageJ.

Table 2: Stability of PAD

Day	Color intensity
1	377024
2	206216
3	195913
4	156990
5	166736
6	154430
7	145043

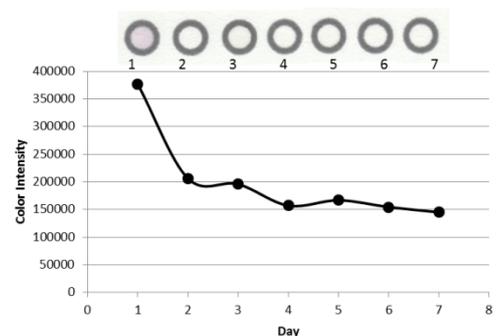


Fig. 9: Stability curve of PAD

The ability of PADs to determine diazepam in urine was tested with spiked urine samples. First, a calibration curve was obtained to calculate diazepam amount in urine.

Table 3: Color intensity measurement for calibration curve

Concentration (ppm)	Color intensity
400	271431
800	323573
1600	416974
2000	474475
4000	651165

Table 4: Application of PADs for detecting diazepam in urine

Concentration of diazepam in spiked urine (ppm)	Color intensity	Cx (ppm)	% Recovery	% Recovery average	Deviation standard
1000	339570	921.7	92.2	86.9	7.1
	337027	897.5	89.8		
	325534	788.0	78.8		
2000	443314	1910.4	95.5	92.8	4.6
	443069	1908.1	95.4		
	426640	1751.5	87.6		

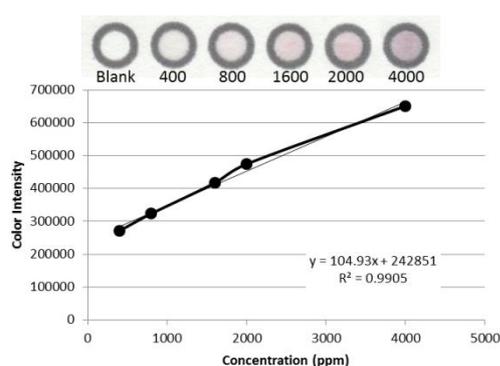


Fig. 10: Calibration curve of diazepam concentration versus color intensity for determination of diazepam in spiked urine

## DISCUSSION

The aim of this study was to develop a PAD with colorimetric method as a presumptive test for the determination of diazepam in urine. The best colorimetric reagent was chosen with testing all reagent candidates with standard diazepam. As shown in fig. 3, all of the reagents gave a specific color change that is in accordance with literatures when reacted with diazepam. Zimmermann produced purple color, hydrochloric acid produced yellow color, Marquis produced pale-orange color, and Vitalin-Morin produced yellow-orange color [22, 23]. 1,3-dinitrobenzene in the Zimmermann reagent forms Meisenheimer Complex when reacted with diazepam in alkaline condition. The yellow color that hydrochloric acid produces when reacted with diazepam is because of the benzophenone structure diazepam has reacted with hydrochloric acid. Vitalin-Morin also reacts with benzophenone derivative structure in diazepam and produce yellow-orange color [25], while Marquis reagent may form a colored complex with diazepam, but the reaction is unknown. However, out of all four reagents, only Zimmermann, hydrochloric acid and Marquis were chosen to get to the next step. Vitalin-Morin cannot get to the next step because it requires a gradual process that is not suitable for the design of PADs.

Reagents chosen were then tested for their performances. Fig. 4 shows that Zimmermann gives a positive result to diazepam only. Hydromorphone and codeine turn yellowish when reacted with Zimmermann because of the yellowish color of Zimmermann A reagent. Zimmermann only responds with a particular group that is activated methylene group in C3 at diazepam structure. It also requires C2 that binds with carbonyl and N1 that binds with an alkyl. Without these specific groups, Meisenheimer Complex, that causes the purple-red color, cannot be formed [25]. Hydrochloric acid turns diazepam solution to a yellowish color, but not with hydromorphone or codeine. From this finding, it can be said that hydrochloric acid is a selective reagent. However, the color produced by hydrochloric

acid with diazepam was barely visible. It can be seen that all three drugs turn yellowish when reacted with Marquis. Out of three reagents tested, only Zimmermann that is selective with diazepam and give a visible color change.

The sensitivity of Zimmermann was tested using a UV-Vis spectrophotometer. Fig. 5 shows that the greater the concentration, the more prominent the color and the higher the absorbance. Visually, it can be seen that the lowest concentration of diazepam tested gives a color change that is distinguishable from the blank, which is ethanol as the solvent. The calibration curve was obtained with equation  $y = 0.0253x + 0.0297$  and  $R^2$  value 0.9878. The absorbance measurement was also carried out on diazepam without the reagent. Fig. 6 shows the spectrum pattern difference between diazepam that have been reacted with Zimmermann and diazepam standard without any reagent added. Diazepam without reagent shows the maximum absorbance at 230 nm in the ultraviolet wavelength range, while diazepam with Zimmermann shows the maximum absorbance at 500 nm, in visible wavelength range.

Before applying reagent to the PAD, an optimization was carried out first to determine the best condition of reagent placement. From fig. 7, it can be seen that Whatman Paper with treatment B (b) shows a more prominent color than Whatman Paper with treatment A (a). Papers with treatment A were tested with diazepam solution after the reagent was dried first, and papers with treatment B were tested with diazepam solution right after the reagent placement. Both treatments start showing color after 1 minute of reaction between the reagent and diazepam. Because of these findings, treatment B was chosen to be applied to the PADs.

To ensure PADs performance, PADs were tested for its sensitivity and stability. Table 1 and fig. 8 show that the color intensity gets higher as the concentration gets higher. A calibration curve of diazepam concentration versus color intensity was obtained with equation  $y = 57.709x + 220201$  and  $R^2$  value 0.9832. Visually, the color difference between the lowest concentration tested and the blank is distinguishable. ImageJ can also measure the color intensity up to the lowest concentration. With these findings, it can be said that PADs are sensitive with a cut-off concentration at 100 ppm.

As shown in table 2 and fig. 9, the color intensity measured by ImageJ was decreasing from day to day until it gets stable on the fourth day onwards. But visually, on the scanned PADs, the color change only happened at day one. Although ImageJ reads intensity on day 2 to day 7, the color change is not visible to the naked eye. The insignificant color change after the first day may happen because the reaction between Zimmermann and diazepam can occur only in alkaline condition. When PADs that contain reagent were left for a few days, the alkali from Zimmermann may have evaporated and the paper was neutral again. Alkali is required to activate the methylene group in C-3 in the benzodiazepine structure. As previously explained, for dinitrobenzene in Zimmermann to react with diazepam, the methylene group must be activated first. From this result, it can be said that PADs were stable at day one. Sample

containing diazepam needs to be tested right after the Zimmermann reagent was placed.

To demonstrate PADs ability to detect diazepam in urine, PADs were tested against urine samples that had been spiked with diazepam. This test was meant to see if PADs can detect diazepam in urine and if the matrix found in urine affects the detection. Before determining diazepam in urine sample, a calibration curve was made using standard diazepam with various concentrations (400–4000 ppm). For determination of diazepam in urine, urine sample was spiked with diazepam to get 1000 ppm and 2000 ppm concentrations of diazepam. In the process of this, ethanol was needed to dissolve diazepam because of diazepam's solubility. The ratio of urine: ethanol used was 1:4. The test was done three times. The equation from the calibration curve (fig. 10) obtained was used to get percent recovery of diazepam in the urine.

As shown in table 4, the best percent recovery obtained was 92.2% for 1000 ppm and 95.5% for 2000 ppm. The low percent recovery indicates a matrix effect from urine to the color intensity. From two concentrations tested, only two percent recoveries qualified the requirement of diazepam determination (95–100%) [26]. However, these values are good enough for the development of a method.

## CONCLUSION

In summary, we have successfully developed a paper-based analytical device that was made from Whatman Chromatography Paper No. 1, with Zimmermann reagent for the presumptive test of diazepam. The device was considered rapid, economic, and sensitive with a cut-off concentration at 100 ppm. The color change produced by the device was visible within 1 minute. PADs can also detect diazepam spiked in urine samples with a percent recovery of  $92.8\% \pm 4.6$  at 2000 ppm of diazepam.

## ABBREVIATION

PAD: Paper-based Analytical Device, GABA: Gamma Amino Butyric Acid, TLC: Thin Layer Chromatography, HPLC: High Performance Liquid Chromatography, GC-MS: Gas Chromatography–Mass Spectrometry.

## FUNDING

Nil

## AUTHORS CONTRIBUTIONS

All the authors contributed equally.

## CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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