Advanced Chemicobiology Research
https://ojs.wiserpub.com/index.php/ACBR/

UNIVERSAL WISER PUBLISHER

Research Article

A Novel, Simple Method for Detection of Caffeine in Urine as a Predictive Marker for Liver Diseases

Sarah Ali Qutb, Mariam Ahmed Moustafa, Mirna Fawzy Nazmi, Ahmed Hany Abed Mahmoud, George Safwat Mounir Abdel Masih, Safa Gamal Abdel Fattah Suleiman, Mary Karam Arian Amen, Mohamed Omar Ewis Khamis, Ayman Saber Mohamed*

Zoology Department, Faculty of Science, Cairo University, Giza, Egypt
E-mail: ayman81125@cu.edu.eg

Received: 17 June 2022; **Revised:** 17 August 2022; **Accepted:** 25 August 2022

Abstract: Drugs, chemicals, and endogenous substrates are all metabolized by hepatic cytochrome P450 (CYP) which plays a role in the aetiology of a variety of liver illnesses. Caffeine is completely metabolized by CYPs. Caffeine excretion in the urine is a sign of liver damage. The study aimed to develop and validate an inexpensive, simple, and rapid method for determining urinary caffeine as a diagnostic marker for liver diseases. Caffeine in urine sample underwent hydrolysis. Strip containing phosphomolybdic acid (PMA) was used to detect the hydrolysis product of caffeine in urine. The interaction of caffeine with PMA produced a blue color on the strip. The current method helps analyze caffeine in urine, providing a simple, rapid, and economic routine analysis.

***Keywords*:** liver function markers, diagnosis, caffeine, urine, phosphomolybdic acid

1. Introduction

The liver is in charge of a number of functions, including primary detoxification of different metabolites, protein synthesis, and the manufacture of digestive enzymes. Furthermore, metabolism, red blood cell management, glucose synthesis, and storage are all controlled by the liver [1]. It can be challenging to diagnose liver disorders since their symptoms are frequently ambiguous and easily mistaken for those of other medical conditions. Specific markers, on the other hand, can assist physicians in diagnosing and monitoring liver illnesses. Numerous end products and enzymes in the metabolic pathway of the liver are subjected to alterations and may be used as biochemical markers for liver disease [2].

Markers of hepatic necrosis, hepatic obstruction, liver biosynthetic capacity, hepatic steatosis, hepatic fibrosis, and hepatic tumor can all be classified as markers of liver illness [3]. Liver function tests are helpful in determining the severity of a patient's liver disease [4]. However, these tests have some drawbacks, such as a lack of sensitivity, which can be shown in various liver diseases like cirrhosis, noncirrhotic portal fibrosis, congenital hepatic fibrosis, and others [5].

Caffeine is the world's most widely used psychoactive substance [6]. Caffeine is completely metabolized in the liver via the cytochrome P450 (CYP) oxidase enzyme system. Paraxanthine, theobromine, and theophylline are the most common metabolites of caffeine. CYP enzymes are heme proteins that catalyze the metabolism of a wide range

DOI: https://doi.org/10.37256/acbr.2120231639

of endogenous and exogenous substrates [7]. CYPs enzyme expression is mostly downregulated in the hepatic tissue in response to inflammation or illness, resulting in the alterations of drug activity and toxin release [8]. As a result, CYP inhibition could be used as a biomarker for liver health. By measuring caffeine in urine over time after a single dose of caffeine, the activity of human CYPs, which completely catabolizes the caffeine, can be examined [9]. Thereby, the goal of this study was to develop and validate an inexpensive, simple, and rapid method for determining urinary caffeine as a diagnostic marker for liver diseases.

2. Materials and methods

2.1 *Chemicals*

The phosphomolybdic acid (PMA) solution, chromatography paper strips, citric acid, sodium hydroxide (NaOH), and glacial acetic acid were purchased from Sigma-Aldrich (St. Louis, MO, United States).

2.2 *Ethical consideration*

The National Institute for Diabetes and Endocrinology (NIDE) approved experimental protocols and procedures in this study with the Helsinki Declaration of 1975 (Ethical approval No. IDE00242).

2.3 *Inclusion criteria*

The individuals were selected from healthy males and females (5 for each gender) aged between 20 and 30 years.

2.4 *Exclusion criteria*

Individuals with any chronic systemic diseases, smokers, patients under any medication, and individuals with a history of any illness for the past six months. Also, individuals who drank caffeine in the week before the experiment were excluded.

2.5 *Study area*

The experiments were performed at Cairo University between January and March 2022.

2.6 *Strip preparation*

The strip was developed in our laboratory by immersing the chromatography paper strip in a 10% PMA solution and then drying it.

2.7 *Sample preparation*

In the following procedures (Figure 1), the urine sample from a healthy individual was used. The material was divided into two cups (each containing 50 ml). Only one cup was given 0.5 g of caffeine.

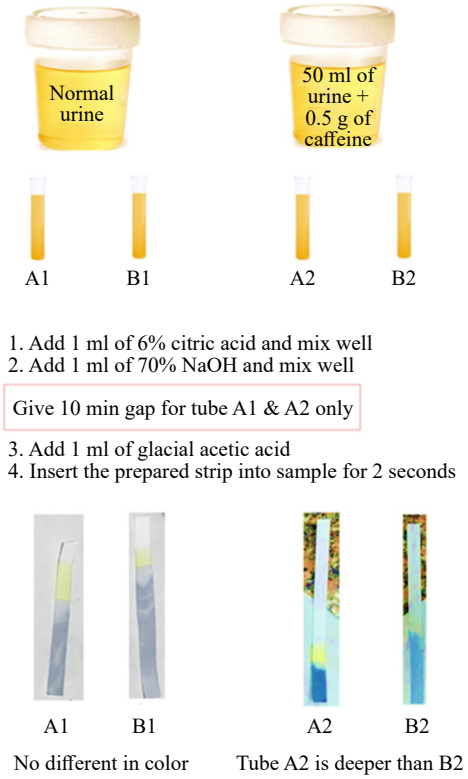

Figure 1. Experimental procedures

2.8 *Procedures*

1. From each cup, 0.5 ml of urine was transferred into four tubes (A1, B1 & A2, B2).
2. For all tubes, 1 ml of 6% citric acid was added and mixed well to increase caffeine solubility.
3. Next, 1 ml of 70% NaOH was added and mixed well to start the alkaline hydrolysis of caffeine.
4. For tubes A1 & A2, a 10 minutes gap was given to complete the hydrolysis of caffeine. In contrast, the following steps were conducted and worked directly on tubes B1 & B2 without a time gap.
5. An amount of 1 ml of glacial acetic acid was added to neutralize the excess NaOH.
6 Finally, the prepared strips were inserted into all tubes for 2 seconds and the color change from yellow to blue was observed.

2.9 *Validation of the method*

2.9.1 *Limit of detection (LOD)*

LOD of the proposed method was investigated experimentally by using different concentrations of caffeine samples versus the caffeine-free sample (control). The test was run until there was no longer a visual difference between the caffeine samples and caffeine-free sample in the colored reaction product. Analysis was performed on five replicates for each concentration.

2.9.2 *Precision and accuracy*

The test strips were used to detect the caffeine in samples with concentrations of 2.5, 5.0, 10.0 and 20.0 mg/ml. The caffeine standards described above were analyzed 10 times for the intraday assay (n = 10) and daily for 10 successive days for the interday assay (n = 10).

2.9.3 *Statistical analysis*

The results obtained from the caffeine strip tests were estimated by naked eyes and recorded by taking pictures. LOD was determined by probit regression using Statistical Package for Social Sciences (SPSS) for Windows (version 20). The reported LOD values are the lowest amount of caffeine at which the probit model predicts 95% positive detection.

3. Results

A blue color was created in tubes A1 & B1 (no caffeine). Tube A2 produced a darker blue tint in the caffeine sample compared to tube B2 (Figure 1).

3.1 *LOD*

The LOD for quantitative detection of caffeine was 17.34 mg/ml by using the strip reader (Figures 2 and 3).

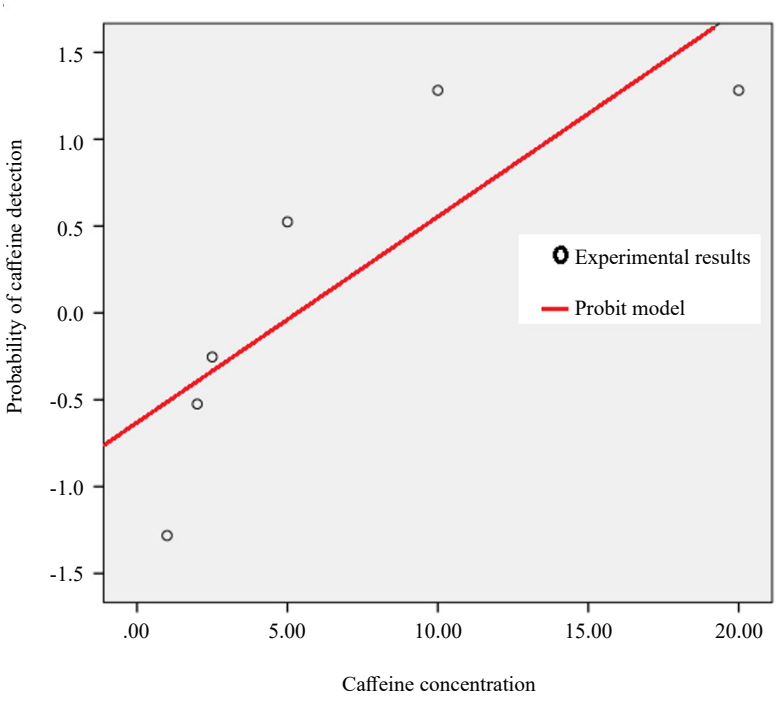

Figure 2. LOD of caffeine is 17.34 mg/ml at 95% confidence interval

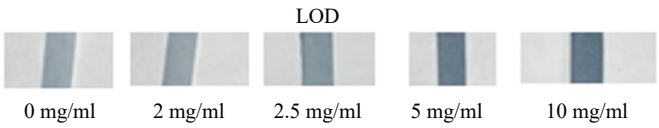

Figure 3. LOD of the caffeine strips

3.2 *Precision and accuracy*

The current study revealed that the new caffeine strip is precise and accurate, especially above the concentration of 2.5 mg/ml of caffeine (Table 1).

Table 1. Intraday and interday variability of the caffeine strip

Caffeine concentration (mg/ml)	Intraday		Interday	
	No. of the positive result	No. of the negative result	No. of the positive result	No. of the negative result
2.5	8	2	7	3
5.0	9	0	8	2
10.0	10	0	10	0
20.0	10	0	10	0

4. Discussion

The formation of molybdenum (Mo) blue using chemical reduction has many applications in analytical chemistry [10]. The principle of the current method depends on the reduction of PMA by caffeine to form Mo blue (Figure 4).

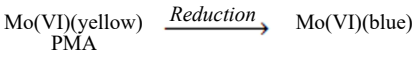

Figure 4. The reduction of PMA converts its color from yellow to blue

This reaction cannot be occurring due to the weak basicity of caffeine. Therefore, caffeine first undergoes alkaline hydrolysis using NaOH, producing caffeidine (N,3-dimethyl-5-(methylamino) imidazole-4-carboxamide) [11] (Figure 5). To optimize the hydrolysis time, we found that it takes 10 minutes for the complete hydrolysis of caffeine. In the second step, glacial acetic acid was added to create the acidic medium required for the reduction of PMA by caffeidine [12].

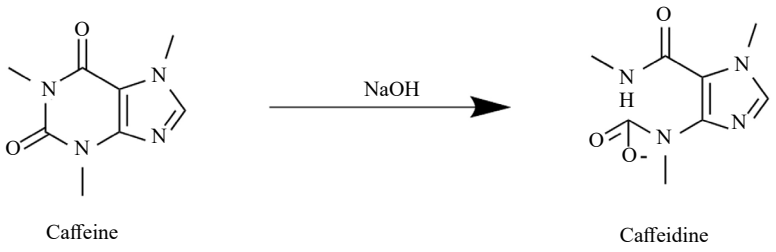

Figure 5. The alkaline hydrolysis of caffeine

The biggest problem we faced in this study is the low specificity of this method where a wide range of reductants present in urine could produce blue color with PMA. To overcome this problem and increase the specificity of this method, we tested the sample 2 times. In the first test, we allow 10 minutes for the alkaline hydrolysis to complete. While in the second test, we added the glacial acetic acid directly after NaOH addition without the 10 minutes gap. As caffeine needs to be hydrolyzed to react with PMA, it cannot interfere with the core of the first test. While in the second

test, it reacted with PMA and increase the intensity of the blue color (Figure 6). Thus, the sample containing caffeine produced different degrees of blue color on the two strips.

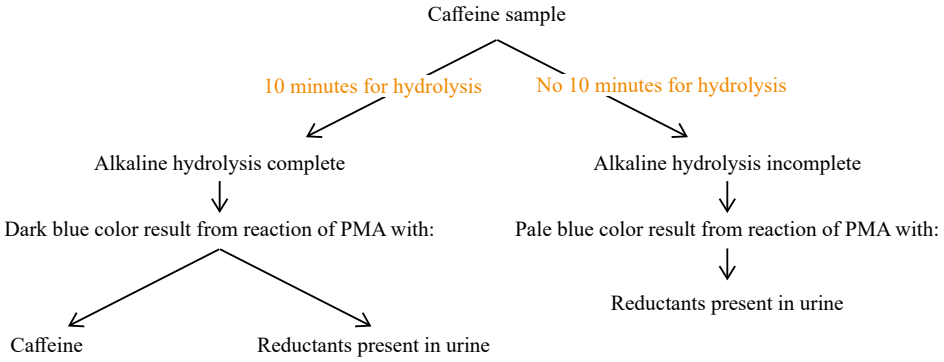

Figure 6. Two tests were performed on the caffeine sample producing a different degree of blue color

On the other hand, if the sample doesn't contain caffeine, it will produce the same color on the two strips (Figure 7).

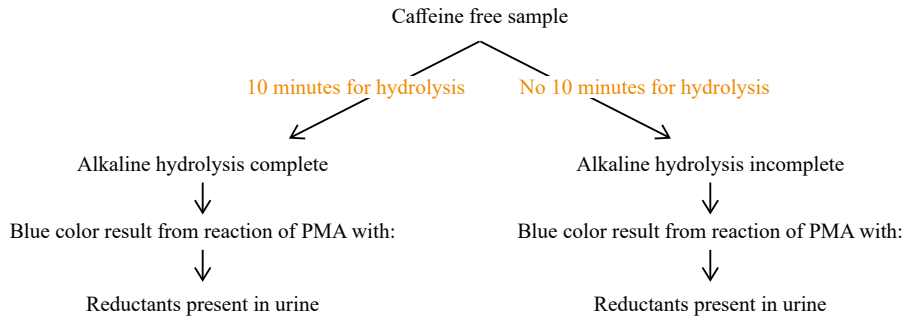

Figure 7. Two tests were performed on the caffeine-free sample producing the same blue color

5. Conclusion

The current method uses PMA strips to analyze caffeine in urine and provides a simple, quick, and cost-effective routine examination.

Conflict of interest

There is no conflict of interest for this study.

References

[1] Lala V, Goyal A, Minter DA. *Liver function tests*. Treasure Island, Florida: StatPearls; 2021. https://www.ncbi.nlm.nih.gov/books/NBK482489/

[2] Giannini EG, Testa R, Savarino V. Liver enzyme alteration: a guide for clinicians. *Canadian Medical Association Journal.* 2005; 172(3): 367-379. https://doi.org/10.1503/cmaj.1040752

[3] Derosa G, Maffioli P. Traditional markers in liver disease. In: Patel VB, Preedy VR. (eds.) *Biomarkers in liver*

disease. Berlin: Springer; 2017. p.3-22.
[4] Thapa BR, Walia A. Liver function tests and their interpretation. *The Indian Journal of Pediatrics*. 2007; 74(7): 663-671. https://doi.org/10.1007/s12098-007-0118-7
[5] Yap CYF, Aw TC. Liver function tests (LFTs). *Proceedings of Singapore Healthcare*. 2010; 19(1): 80-82. https://doi.org/10.1177/201010581001900113
[6] Graham TE. Caffeine and exercise: metabolism, endurance and performance. *Sports Medicine*. 2001; 31(11): 785-807. https://doi.org/10.2165/00007256-200131110-00002
[7] Lu D, Ma Y, Zhang W, Bao D, Dong W, Lian H, et al. Knockdown of cytochrome P450 2E1 inhibits oxidative stress and apoptosis in the $cTnT^{R141W}$ dilated cardiomyopathy transgenic mice. *Hypertension.* 2012; 60(1): 81-89. https://doi.org/10.1161/HYPERTENSIONAHA.112.191478
[8] Bezirtzoglou EEV. Intestinal cytochromes P450 regulating the intestinal microbiota and its probiotic profile. *Microbial Ecology in Health and Disease.* 2012; 23: 18370. https://doi.org/10.3402/mehd.v23i0.18370
[9] Furge LL, Fletke KJ. HPLC determination of caffeine and paraxanthine in urine: An assay for cytochrome P450 1A2 activity. *Biochemistry and Molecular Biology Education*. 2007; 35(2): 138-144. https://doi.org/10.1002/bmb.28
[10] Crouch SR, Malmstadt HV. Mechanistic investigation of molybdenum blue method for determination of phosphate. *Analytical Chemistry*. 1967; 39(10): 1084-1089. https://doi.org/10.1021/ac60254a027
[11] Ivankovic S, Seibel J, Komitowski D, Spiegelhalder B, Preussmann R, Siddiqi M. Caffeine-derived N-nitroso compounds. V. Carcinogenicity of mononitrosocaffeidine and dinitrosocaffeidine in bd-ix rats. *Carcinogenesis.* 1998; 19(5): 933-937. https://doi.org/10.1093/carcin/19.5.933
[12] Nalumansi I, Birungi G, Moodley B, Tebandeke E. Preparation and identification of reduced phosphomolybdate via molybdenum blue reaction. *Oriental Journal of Chemistry*. 2020; 36(4): 607-612. http://dx.doi.org/10.13005/ojc/360403