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LETTERS

Development and validation of Hand-held device for the rapid detection of Metformin in biological samples: A forensic application

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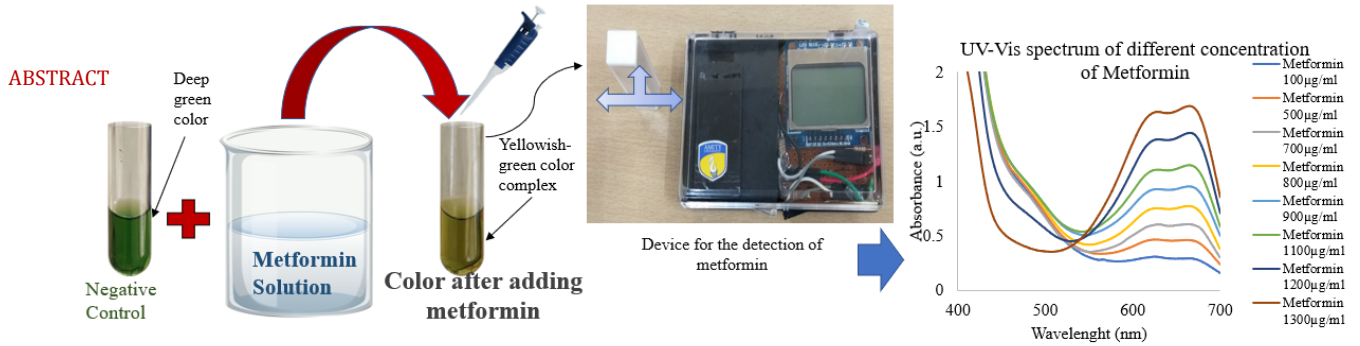
Research Article



Metformin is a widely used anti-diabetic drug that enhances glycaemic control by advancing the insulin sensitivity abatement of intestinal glucose absorption. Due to its versatility, it is also emerging as an abused drug leading to toxicity and fatal conditions. Detection of metformin with the existing qualitative and quantitative approaches necessitate complex and intricate instrumentation, fully facilitated laboratories, and trained scientist and technicians. Henceforth, in the current study, we have developed a hand-held electronic device-based detection system for metformin using the basic principles of spectrophotometry. It is based on the formation of a yellowish-green color complex (wavelength 680nm) which involves the oxidative coupling of 3-methyl 2-benzothiozoline hydrazone (MBTH) catalyzed by iron. The method developed has been optimized at different pH, reaction temperature, and reaction time and found to be specific, sensitive, and linear in the range of concentration 100-1000 µg/ml with the limit of detection (LOD) of 120.5 µg/ml.

Keywords: Metformin, hand-held device, UV-Visible spectrophotometry, MBTH, rapid detection

INTRODUCTION

Metformin, scientifically named as, 'N, N-dimethylimidodicarbonimidic diamide', comes under the class of biguanides, is an anti-diabetic hypoglycemic drug that is orally administered and largely used in the treatment of Type 2 Diabetes/non-insulin-dependent diabetes mellitus.[1-5] Metformin improves glycaemic control by promoting the sensitivity of the insulin which employs the obstruction in the process of hepatic gluconeogenesis and reduction in intestinal glucose absorption.[6-9] Apart from being an anti-diabetic drug, metformin is also used as an antineoplastic.[10,11] International Diabetes Federation's recent data states that as of 2021, 537 million adults are suffering from diabetes mellitus, and approximately 629 million adults by 2045.[12-14] With widespread diabetes mellitus, the considerable amount of antidiabetic drug users has increased. As the usage of metformin drugs raised by 97%, drug abuse cases of metformin are also elevating.[15,16] Additional therapeutic uses of this drug can also be the potential factor leading to metformin abuse and toxicity. Medical conditions like lactic acidosis and hypoglycemia correspond to metformin toxicity.[17-19] With the expansion of epidemics of diabetes mellitus, drug abuse and toxicity cases are also increasing which demands the development of an on-the-spot detection system for metformin.

Numerous analytical methods are available for metformin detection in surface water, pharmaceutical preparations, and human plasma which employ Thin Layer Chromatography (TLC),[20] Liquid Chromatography,[21-23] Capillary Electrophoresis,[24] Cation Exchange Chromatography, Liquid chromatographic techniques coupled with Ultra-Violet Spectroscopy,[25-27] Tandem Mass Spectrometry.[28] Nevertheless, such protocols are usually convoluted, tedious, time-consuming,

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complicated, and expensive, and generally require a specific laboratory environment, specialized trained personnel, and auxiliary equipment to operate instruments. So, modern detection techniques and methods should be developed which are convenient to use, cost-effective, handy, can be used in the field, and give on-the-spot results.[29,30]

This study intends to develop a sensitive and handy device for the on-the-spot detection of metformin in pharmaceutical preparations and biological matrices (after pre-treatments). The extraction procedure was investigated for the isolation of metformin from biological samples including blood and urine. In the current study, a convenient-to-use and cost-effective hand-held device have developed based on the colorimetric assay. Colorimetric detection is an easy and better way to evaluate the results because change can be easily noticed with the naked eye. Therefore, the colorimetric method was adapted to develop a device as it also offers other advantages of being expeditious and integral. We have used a dye-based assay employing 3-methyl 2-benzothiozoline hydrazone (MBTH) reagent.[31] This colorimetric method has collaborated with an electronic device with an inbuilt chargeable battery. The device consists of a sample cuvette, switch, photodiode detector, laser, battery, wires, and some plastic assets. A colorimetry-based handheld electronic device has been developed for the detection of metformin.

MATERIAL AND METHODS

Chemicals and reagents

Metformin Hydrochloride (N, N-dimethylimidodicarbonimidic diamide hydrochloride) and 3-methyl 2-benzothiozoline hydrazone [MBTH] reagent were of analytical grade and were supplied from Sisco Research Laboratories Pvt. Ltd. Other chemicals and reagents were of analytical grade.

Preparation of standard and working solutions

Metformin Hydrochloride standard stock solution

1.0 mg/ml standard stock solution was prepared by adding 100 mg of metformin hydrochloride in 100 ml of deionized water. The solution was stored at 4°C in air-tight amber-colored bottles. The working solution of metformin was prepared in the concentration range of 100-1300 µg/ml by serial dilutions.

MBTH standard stock solution

0.2% (w/v) MBTH was prepared by adding 0.2 g of MBTH in 100 ml of deionized water. The solution was stored in air-tight amber-colored bottles.

Ethanolic Ferric chloride [eth. $FeCl_3$] standard stock solution

0.5% (w/v) $FeCl_3$ was prepared by adding 0.5 g of $FeCl_3$ in 100 ml of ethanol. The solution was stored in air-tight amber-colored bottles.

Instrumentation and UV-Visible Spectrophotometer parameters

Research analysis has been executed on UV-Visible spectrophotometer, executed on UV visible spectrophotometer (model no. UV-2600 UV-Visible spectrophotometer, Shimadzu, Tokyo, Japan) with the spectral range from 400nm to 700 nm. It was used to record the spectra range from 400nm to 700 nm. Distilled water was used as the blank for the baseline correction. λ_{max} for the resulting colored complex was observed at 680nm. Spectra obtained were re-plotted using MS Excel for the data interpretation.

Selection of wavelength and reaction mechanism

The wavelength at maximum absorption (λ_{max}) of the colored complex was found to be stable with the different concentrations of metformin and MBTH. This spectrophotometric method is based on the reaction between the amino groups of metformin hydrochloride with MBTH in the presence of eth. $FeCl_3$ where λ_{max} was observed at 680nm**.**

Stability of the color complex

The stability of the colored product was assessed under different parameters i.e., variation in temperature, reaction time, and pH.

Optimization of reaction parameters for the colorimetric assay

Reaction temperature optimization

The reaction has been optimized at different temperatures ranging from 5 ^{0}C to 35 ^{0}C. The reaction has been performed under these temperature ranges and the absorbance and stability of the color complex have been studied.

pH optimization

The reaction has been optimized at different pH ranging from 2 to 8 using 0.1 N HCl and NaOH. The reaction has performed under these mentioned pH ranges and the absorbance and stability of the developed colored complex were noticed. The other reaction parameters remain constant.

Reaction time optimization

The reaction has been optimized at different reaction times ranging from 0.5 min to 4 min. The reaction has performed under these mentioned reaction time ranges and the absorbance and stability of the developed colored complex were noticed. The other reaction parameters remain constant.

Extraction procedure

300µl of the urine sample/300µg spiked blood tissue sample was taken in a centrifugation tube, and 5 ml of 1- butanol: hexane (50:50, v/v) was added under an alkaline medium (0.1 M NaOH). The mixture was vortexed for 5 minutes and then centrifuged for 7 minutes at 3000 rpm followed by back extraction into 5 ml acetic acid. The resulting supernatant was taken to evaporate to dryness.[32]

Testing protocol

1 ml of the extracted sample containing the drug was taken in a test tube followed by the addition of 1 ml 0.5% (w/v) ethanolic $FeCl_3$, then the mixture was incubated for 5min. 1 ml of 0.2% (w/v) MBTH was added to the resulting solution and again incubated for 3 mins and absorbance was recorded at 680 nm.

Interference study

The effect of other ingredients present in the pharmaceutical preparations was studied. Different compounds with similar structures, functional groups, and other categories of similar anti-diabetic drugs are also tested with the same method to check the specificity of the colorimetric assay. Different compounds with similar structures, functional groups (like aniline and cysteine), and other categories of similar anti-diabetic drugs (like Metco, Metform 500, and Galpride M1) have also experimented with the same method to check the specificity of the colorimetric assay.

Precision and accuracy

Precision and accuracy of the developed device were tested at two distinct domains i.e., 'between-batch' and 'within-batch' evaluation. The variant concentration of metformin was spiked in the biological sample for in-vitro analysis (200 µg/ml, 400 µg/ml, 600 µg/ml, 800 µg/ml, and 1000 µg/ml) and assessed on the same day and after the interval of 1 week. The process was replicated five times.

Fabrication of electronic hand-held device

Electronics

A cost-effective portable electronic device has been developed. It comprises of light source: green LED, sample cuvette (quartz) of 4 ml, photodiode detector, the display unit of 128×64 graphic LCD with a built-in controller (NT7108 or equivalent), Arduino Uno microcontroller board with a USB connection, and a power jack, lithium-ion battery as a power source, wires, and some plastic assets.

Working

This mini handheld device is based on the colorimetric assay for the detection of metformin. It is based on the mechanism which involves the oxidative coupling of MBTH catalyzed by iron. It includes the loss of two electrons by MBTH to form an active coupling electrophilic intermediate which results in the formation of the colored complex after electrophilic substitution (see Figure 1).

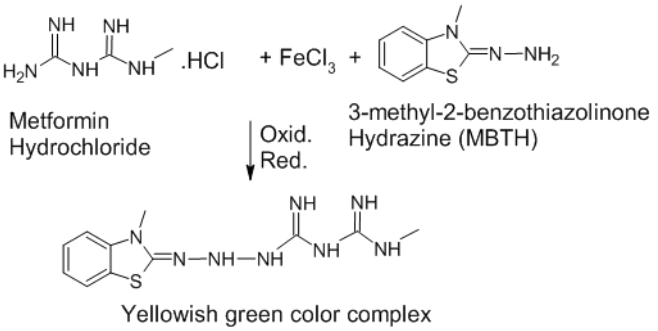

Figure 1. Reaction mechanism of the colorimetric assay

The sample was put in the cuvette (sample holder) and then placed in the chamber formulated in the device. LED as a light source having a monochromatic emission profile excites the atoms from the ground state to the excited state.[33,34] It emits the light or corresponding absorption wavelength of the questioned solution. The light source working is controlled by a microcontroller processing unit and light is permitted to pass through the sample chamber horizontally in the perpendicular plane. A fraction of light is absorbed and the rest is transmitted through it.[35] A photodiode detects the transmitted light and the resulting signal is then filtered and amplified. The filtered and amplified signal is then transmitted to the circuit unit for the analog-to-digital conversion.[36] The processing unit then processes the signal using standard calibrated values to answer the concentration level.[35,37] The final results are then displayed on the 128×64 graphic LCD unit (see Figure 2 and 3).

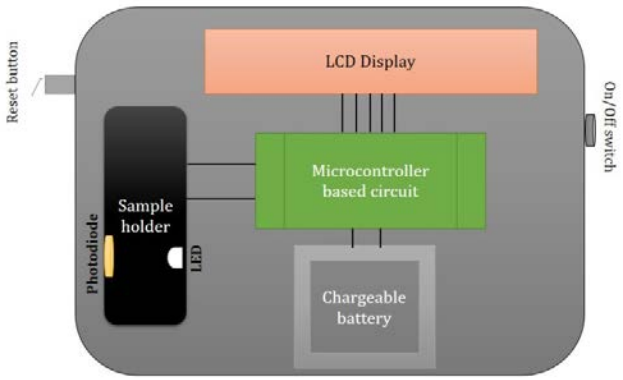

Figure 2. Schematic layout of the hand-held device

Figure 3. Real image of the hand-held device for the detection of metformin

Result and Discussion

Formation of color complex

The yellowish-green Color complex formed by the interaction

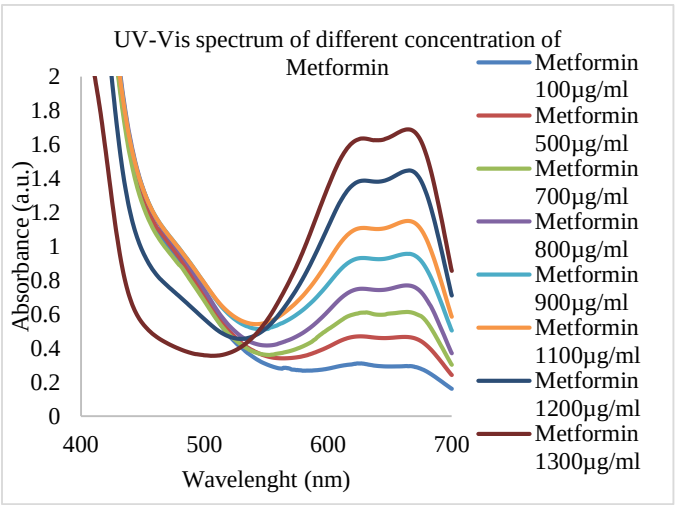

Figure 4. UV-Visible spectrophotometer spectrum at different concentrations of metformin showing the formation of colour complexes

of metformin and MBTH is characterized by a typical resonance band at 680nm. Two peaks were observed explaining the formation of one intermediate product at 630nm as a result of the reaction between metformin and ethanolic $FeCl_3$ and the final color complex of the intermediate product and MBTH at 680nm (see Figure 4).

Optimization of reaction parameters for the colorimetric assay

pH optimization

The reaction has been optimized at different pH ranges from 2 to 8. Experiments are performed under these mentioned pH ranges and the absorbance and stability of the color were observed. The other reaction parameters remain fixed. It was observed that the color complex was stable at neutral pH i.e. pH=7 (see Figure 5a). In the acidic medium, the color of the complex was changed whereas, in the alkaline medium, the mixture got aggregated and form a red-colored precipitate.

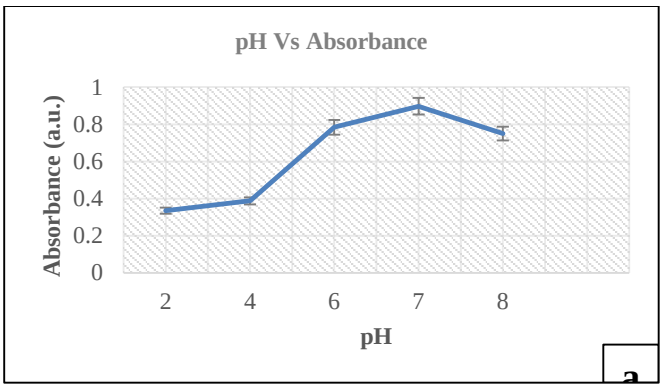

Figure 5a. Optimization of reaction parameters: pH versus absorbance graph

Reaction temperature optimization

The reaction has been optimized at different temperatures ranging from 5 ^{0}C to 35 ^{0}C. Experiments are performed under these temperature ranges and the absorbance is observed. It was observed that 20 ^{0}C – 35 ^{0}C range was found to be the optimum range wherein, 30 ^{0}C temp was found to be the most favorable temperature to carry out the experiment (see Figure 5b).

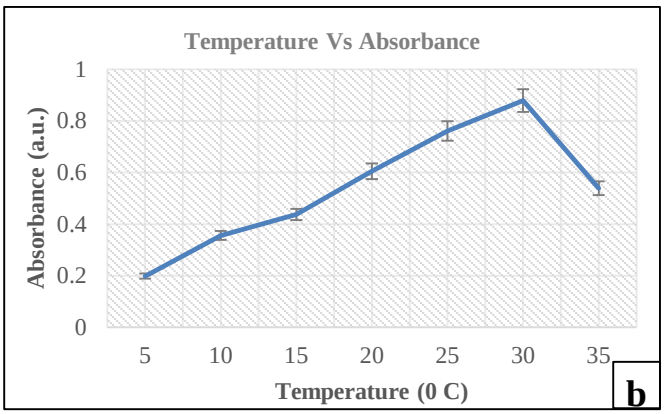

Figure 5b. Optimization of reaction parameters: Reaction temperature versus absorbance graph

Reaction time optimization

The reaction has been optimized at different reaction times ranging from 0.5 min to 4 min. Experiments were carried out under these mentioned reaction time ranges and the absorbance and stability of the developed colored complex were noticed. The other reaction parameters remain fixed. It was observed that 3 mins was the optimum reaction time (see Figure 5c).

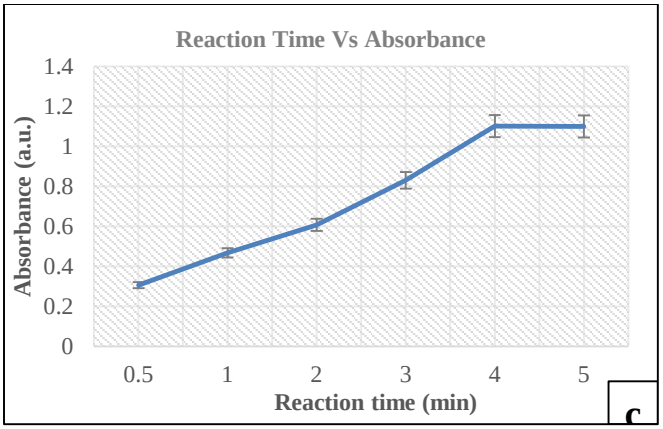

Figure 5c. Optimization of reaction parameters: Reaction time versus absorbance graph

Effect of metformin concentration

The concentration of metformin was varied to observe the interaction of metformin with MBTH in the range of 100 µg/ml to 1000 µg/ml. it was observed that the absorption increases simultaneously with the increase in the concentration of metformin following linearity (See Figure 5d).

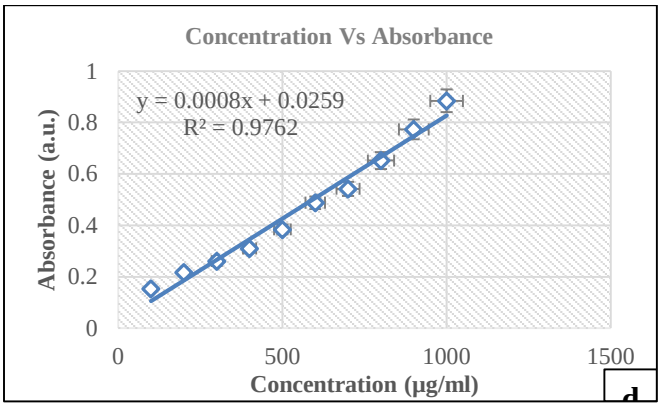

Figure 5d. Optimization of reaction parameters: Metformin concentration versus absorbance

Stability of the color complex

The yellowish-green colored complex was formed at optimum conditions in the presence of metformin on reaction with MBTH reagent. The color was found to be stable under optimized conditions.

Interference study

The effect of other ingredients present in the pharmaceutical preparations was studied. It was observed that none of the other compounds with a similar structure and functional group could

produce the same-colored complex employing this colorimetric assay. Metformin from different brands and companies was also tested for testing the interference (if occurred because of different ingredients, binding agents, etc). No interference from the ingredients and binding agents was observed in the formation of the color complex.

Linearity

The proposed method for the detection of metformin was found to be linear in the range of concentration 100 µg/ml to 1000 µg/ml with the value of R^2; Coefficient of determination at 0.9762 (See Figure 5d).

Precision and RSD (Relative Standard Deviation) %

Inferences for the precision/ RSD%, mean, the standard deviation for the developed method is compiled in Table 1. The values for the 'between-batch' and 'within-batch' precision (%) lie in the spectrum 0.179 to 0.526 and 0.129 to 0.385 respectively.

Table 1 Interday and Intraday precision results for the developed method

Concentration (µg/ml)	Within Batch (n=5)			Between Batch (n=5)		
	Mean (x)	X ± SD	Precision (RSD) (%)	Mean (x)	X ± SD	Precision (RSD) (%)
200	0.2168	0.2168 + 0.000837	0.385	0.2166	0.2166 + 0.00114	0.526
400	0.311	0.311 + 0.001	0.371	0.3118	0.3118 + 0.001304	0.418
600	0.4872	0.4872 + 0.001643	0.337	0.4868	0.4868 + 0.001924	0.395
800	0.652	0.652 + 0.001871	0.286	0.6512	0.6512 + 0.002387	0.366
1000	0.8824	0.8824 + 0.00114	0.129	0.882	0.882 + 0.001581	0.179

[n = No. of samples for each set of concentration]

Validation of handheld device via standard UV-Visible spectrophotometry and Gas Chromatography method

Validation of the developed method was done by comparing the data obtained by UV-Visible spectrophotometer, Gas Chromatography, and the data collected by the developed handheld device. Different concentrations of metformin were taken in the range of 100 µg/ml to 1000 µg/ml and analyzed with a UV-Visible spectrophotometer and Gas Chromatography. A similar concentration range was taken to record the readings through the developed device. Data obtained from the three methods were tabulated to validate the newly developed method (see Table 2).

Correlation study

To check the efficacy of the device for known methods, a correlation study was performed between the developed device with UV-VIS spectrophotometry; and the developed device with Gas Chromatography. Verification of device values was done by testing three unknown concentrations of metformin from all the stated three methods (Hand-held device, UV-Visible spectrophotometer, and Gas Chromatography), and it was observed that the data obtained from the respective methods were compatible and similar with the correlation coefficient (r) between hand-held device method and UV-Visible Spectrophotometer is found to be 0.9866 whereas 'r' between the hand-held device and Gas Chromatography is found to be 0.9853 (Table 3). It was observed that the developed method shows a promising substitute approach to detect and quantify the metformin in the biological samples (after extraction) (Table 4).

Application

The number of anti-diabetic drug users is rising with the increase of the epidemic of diabetes mellitus. Metformin; a biguanide, is the most common line treatment of Type 2 diabetes. It is also used in weight loss and works as an antineoplastic drug as well. Use of metformin raised by 97%. With this

Table 2 Validation of handheld device via standard UV-Visible spectrophotometry method and Gas Chromatography by correlating the data obtained by developed device, UV-Visible spectrophotometer, and Gas Chromatography and at different concentrations of metformin.

Comparative Analysis						
Concentration (in µg/ml)	UV-Visible Spectrophotometer		Gas Chromatography		Hand-held Device	
	Abs.	Unknown Conc. (µg/ml)	Peak Area	Unknown Conc.	Sensor Value	Unknown Conc.
Control	0.08		No Detection		932	
100	0.154		4008267		930	
200	0.217		4890000		921	
300	0.261		8132400		914	
400	0.31		9780000		902	
500	0.388		12067200		895	
600	0.488		12225000		886	
700	0.524		14078400		872	
800	0.652		15032640		865	
900	0.773		18100800		857	
1000	0.883		18790800		849	
Unknown sample 1	0.247	259	6843120	257	918	239
Unknown sample 2	0.354	537	12155810	556	892	564
Unknown sample 3	0.502	688	13587000	670	879	681

expanded metformin usage, drug abuse cases have also been increasing. Lactic acidosis and hypoglycemia are associated with metformin toxicity. The proposed method successfully developed the handheld device for the identification of metformin in the biological samples in the case of drug abuse. Laboratory instrumental techniques are usually convoluted, tedious, time-consuming, complicated, and expensive, and generally require a specific laboratory environment, specialized trained personnel, and auxiliary equipment to operate instruments. So, this modern detection device which is convenient to use, cost-effective, and handy, can be used in the field and give on-the-spot results.

Table 3 Correlation of the developed protocol against UV-VIS Spectrophotometry and Gas Chromatography

	Correlation coefficient		
	Device	*UV-VIS Spectrophotometry*	*Gas Chromatography*
Device	1		
UV-VIS Spectrophotometry	0.986665902	1	
Gas Chromatography	0.98537134	0.967376608	1

Table 4 Detection of metformin from biological samples via developed method and simultaneous comparison with standard methods involving UV-Vis Spectrophotometry and Gas Chromatography

S. No	Biological Sample	Extraction Method	UV-VIS (Absorbance)	Gas Chromatography (Peak area)	Device (value)	Conc. (µg/ml)
1.	Blood	Liquid-Liquid Extraction	0.218	4889961	919	200
			0.309	9779995	900	400
			0.486	12224200	884	600
			0.653	15032519	864	800
			0.885	18790756	846	1000
2.	Urine	Liquid-Liquid Extraction	0.216	4889981	920	200
			0.311	9779987	901	400
			0.489	12224655	884	600
			0.655	15032623	862	800
			0.890	18790800	848	1000

CONCLUSION

We have developed a handy portable electronic device for the detection of metformin in pharmaceutical preparations and biological matrices (after pre-treatments). It promises rapid, on-the-spot detection as it is based on the principle of colorimetry involving oxidative coupling of 3-methyl 2-benzothiozoline hydrazone (MBTH) with metformin. The developed method is specific, linear, and sensitive. The detection system was based on the colorimetric assay and electronic readout device which caters to effortless handling, cost-effectiveness, portability, and rapid detection. For detection, we have used photodiodes which are coupled with analog-to-digital converters and a digital readout screen. It works as a simple colorimeter with a chargeable battery. Interday and intraday precision analysis was observed in the respectable range. This method promises rapid monitoring of metformin with effortless handling, cost-effectiveness, and portability. The method is very helpful and used to detect metformin in biological samples.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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