



## Rapid Spectrophotometric Method using Mannich Reaction for Metformin Determination in Pharmaceutical Tablets and Human Urine.

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### ABSTRACT

Although there are many analytical methods available for estimation of metformin in biological samples and pharmaceutical preparations, to our knowledge there is no specific spectrophotometric method using its derivatization by the Mannich reaction. A simple spectrophotometric method was developed by studying the UV evolution of the complex obtained between metformin, formaldehyde and uranine using the Mannich reaction. Measurements of metformin were achieved at 437 nm using UV-visible spectroscopy. To assess the validity of the method in a clinical situation the extraction was adapted to extract metformin from human urine that had been tested by our method. The spectrometric correlation coefficient ( $R^2$ ) was found to be 0.98 over metformin concentrations of 5– 30  $\mu\text{g/mL}$ . This range includes the clinical levels reached during treatment. The method was validated for linearity, accuracy, and precision. The within-assay limit of detection and quantification (sensitivity) were found to be 0.001  $\mu\text{g/mL}$ . This method for quantification of metformin is simple, precise and accurate. It is suitable as an alternative to existing clinical methods and for the analysis of metformin in pharmacodynamic studies of urinary excretion as well as pharmaceutical formulations and *in vitro* dissolution studies.

**Keywords:** Metformin, Mannich reaction, spectrophotometric method, quantitative determination in urine.

### INTRODUCTION

Metformin hydrochloride (1, 1-dimethyl biguanide hydrochloride) is a widely used oral antidiabetic drug. The structural formula is shown in Fig. 1a. Originally, metformin was developed as a non-insulin treatment of hyperglycemia in patients with type 2 diabetes mellitus. It acts by improving peripheral sensitivity to insulin, reducing gastrointestinal glucose absorption and hepatic glucose production (Caspary 1971, Hundal et 2000, Borstet al 2001). Although the exact mechanism of metformin's action has not yet been fully determined, in recent years it has become common in the treatment of metabolic syndrome in human polycystic ovary syndrome (Nestleret) and has been found to improve vascular function (Katakam), prevent pancreatic cancer (Schneider), and reverse fatty liver diseases (Lin) in experimental animals. Thus, re-evaluations pharmacodynamics and its pharmacological activity is underway. This and issues related to clinical practice necessitates the accessibility of a simple and reliable, inexpensive methods of following metformin in biological fluids.

Several methods have been reported for the determination of metformin in human serum or plasma (Yuen). Many other methods have been used to determine metformin either in bulk drug- or tablet

formulations. These methods utilize capillary electrophoresis (Hamdan), spectrofluorometry (El-Bagary), potentiometry (Khaled), conductometry (Sartori 2009), voltammetry (Gholivand), UV-visible (Saxena, Chaturvedi), FTIR and Raman (Gunasekaran) spectrophotometry. Spectrophotometric methods have been used because of their rapidity, low cost, and reliability criteria. For example, Chaturvedi developed two spectrophotometric methods to determine three active ingredients in multicomponent tablets. Compared to conventional separation techniques these methods were shown to be accurate, simple and rapid. (Aryne) suggested a UV spectrophotometric method for the quantitation of metformin in both bulk drugs and pharmaceutical tablets. Using mathematical equations and five different wavelengths this method is highly accurate and easy for routine analysis. (Mubeen determined metformin in bulk drugs and tablet formulations using a spectrophotometric method based on chemical reaction. Their method was based on the assessment of the absorbance of the product of a reaction occurring between metformin and ninhydrin to give a violet color chromogen that can be monitored by UV-Vis spectrophotometry. However, these methods have not been shown to be useful for studies of biological fluids.



In humans, metformin is primarily cleared by glomerular filtration and tubular secretion, approximately 79 to 86% of an intravenous dose being recovered in urine (Tucker, Sirtori) Therefore, pharmacodynamic studies require analyses of metformin in human urine. But, previous attempts using HPLC have failed (Charles). Here we report a new simple, inexpensive, reliable and rapid spectrophotometric method for determination of metformin in chemical preparations and in human urine. In this method metformin is isolated using column chromatography and then complexed by the Mannich reaction.

## MATERIALS AND METHODS

### Reagents and materials

The working standard of metformin HCl (99.1% pure) and linagliptin were obtained from Benta Labs Ltd, Lebanon. The 1-butanol, hexane, acetic acid, formaldehyde, uranine (Fluorescein disodium salt), NaOH, acetonitrile, dibasic ammonium phosphate, were analytical reagent grade and purchased from Sigma-Aldrich. Deionized water was used for dilution. Determination of the pH was done using a Mettler Toledo (OH, USA) pH-meter.

### UV-visible Spectroscopy

The solutions were monitored by Cary UV spectrophotometer (Varian Inc., CA, USA). After the Mannich reaction the complexed metformin was followed by measuring the absorbance value at  $\lambda = 437$  nm. The metformin concentration was calculated against an internal metformin standard [22]. (Somogyi)

### Standard solutions

Stock aqueous solution of metformin was transferred into a volumetric flask to produce a solution with a concentration of 100 mg/L. The solution was shaken for 20–30 min at room temperature until complete dissolution of the metformin crystals. Samples for analysis were prepared by mixing 1 mL of aqueous uranine solution 10 mg/L, 1 mL of pure formaldehyde and different volumes of metformin stock solutions (0, 0.5, 1, 1.5, 2,– 2.5 and 3 mL). De-ionized water was transferred to each sample to reach a final volume of 10 mL. The final concentration of uranine was 10 mg/L and the metformin concentrations varied from 5–30mg/L. The composition of the prepared solutions is shown in Table 1.

### Extraction procedure

The extraction procedure described by Amini (Amini) was modified as follows. To alkalize the test medium 10 mL of 10 M NaOH to 10 mL of urine. Analytes were then extracted using a 10 mL mixture of 1-butanol: hexane (50:50 v/v). The tubes were vortex mixed for 30 s and centrifuged at 3000 g for 3 min. The supernatant (organic layer) was transferred into clean glass tubes and acidified with 1 mL of 0.2% acetic acid, vortex-mixed for 60 s and centrifuged at 3000 g for 3 min at room temperature. The

supernatant was removed by aspiration and the remaining aqueous layer was evaporated to dryness *in vacuo*. The residue was reconstituted with 5 mL deionized water.

To fortify the samples, pure metformin was added to the reconstituted aqueous medium. The composition of the samples and solutions used to analyze metformin in urine after extraction is summarized Table 2.

### Purification of the fraction by column chromatography

Column chromatography was performed on silica gel 60 (0.063-0.2 mm/70–230) mesh. The mobile phase was a mixture of acetonitrile-water with 4 mM dibasic ammonium phosphate pH = 7.8. Pre-coated plates of TLC silica gel 60 F<sub>254</sub> (Merck, Germany) were used for monitoring fractions and spots were detected with UV light (254 and 365 nm) and then sprayed with 3% H<sub>2</sub>SO<sub>4</sub> followed by heating to 110 °C. The first fractions were discarded after which the fractions containing metformin were collected and analyzed. In order to check the completeness of separation of a mixture containing more than one amine compound, a mixture of metformin and linagliptin (Boehringer Ingelheim, sigma), 2 ppm of each was poured into the chromatographic column. TLC was performed on the collected fractions and show that only one amine is collected and the two amines were shown to be completely separated. The first fraction collected containing the metformin and the second containing was the linagliptin. (Bidlingmeyer)

### Calibration curves and recovery

The calibration curve was prepared using distilled water dilutions of stock solution. Tests of linearity were performed on 5-30 mg/L metformin.

Recovery experiments were performed by adding metformin to samples. The percentage of recovery (%R) was calculated as follows:

$$\%R = [(C_r - C_f) / C_r]$$

$C_r$  = Real concentration of metformin in the samples;

$C_f$  = Concentration of metformin obtained by the internal standard addition curve.

## RESULTS AND DISCUSSION

### Identification of the metformin-formaldehyde-uranine complex

The Mannich reaction (Eq. 1) consists of an amino alkylation of an acidic proton placed next to a carbonyl functional group with formaldehyde and ammonia or any primary or secondary amine. The final product is a  $\beta$ -amino-carbonyl compound. Reactions between aldimines and  $\alpha$ -methylene carbonyls are also considered Mannich reactions because these imines form between amines and aldehydes [25] (Mannich 1912).

**Table 1:** The composition of samples used in metformin analysis.

Volume of metformin [100 mg/L] (mL)	Volume of uranine [100 mg/L] (mL)	Volume of formaldehyde (mL)	Volume of added water (mL)
0	1	1	8
0.5	1	1	7.5
1	1	1	7
1.5	1	1	6.5
2	1	1	6
2.5	1	1	5.5
3	1	1	5

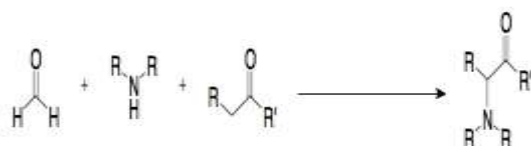
**Table 2:** The composition of different sample used for the determination of metformin in urine of an old diabetic man.

Volume of extracted sample from urine (mL)	Volume of added metformin (mL)	Volume of formaldehyde (mL)	Volume of added uranine (mL)	Volume of added water (mL)
1	0	1	1	7
1	0.5	1	1	6.5
1	1	1	1	6
1	1.5	1	1	5.5
1	2	1	1	5
1	2.5	1	1	4.5
1	3	1	1	4

**Table 3:** Validation parameters: standard linearity; intercept, slop, regression coefficient ( $R^2$ ), range, precision, recovery and limit of detection (LOD) of the spectrophotometric method used for metformin determination.

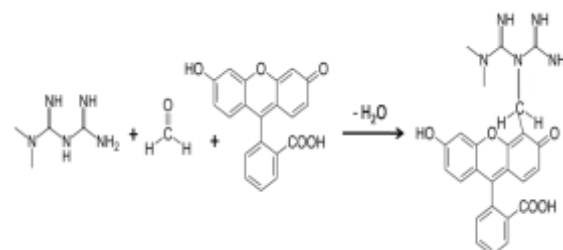
Method	Spectrophotometer
Standard linearity	
Intercept	9.79
slop	0.096
$R^2$	0.99
Range ( $\mu\text{g/mL}$ )	5 to 30
Precision (n = 5) (RSD %)	2.1
Mean recovery (%) $\pm$ standard deviation	97.9 $\pm$ 2.1
Detection limit	0.001 $\mu\text{g/mL}$

We hypothesized that the reaction between uranine, formaldehyde and metformin must be similar to the Mannich reaction involving a primary or a secondary amine with an aldehyde and an enolizable carbonyl compound in Eq. (1).



Eq. 1. Mannich reaction [25]

The overall reaction is given in Eq. (2).



Eq. 2. Mannich reaction using metformin as amine.

Metformin + formaldehyde + uranine  $\rightarrow$  Mannich derivative.

### **UV spectra of Metformin, and complexes obtained by mixing metformin, uranine and formaldehyde according to the Mannich reaction**

Metformin has a maximum absorbance at 233 nm, as shown in Fig. 1b, while Uranine presents a spectrum with maximum absorbance at 487 nm. After mixing metformin and formaldehyde the absorption spectrum show a blue shift and appears at 223 nm. After adding uranine a new spectrum with a maximum at 438 nm appears that is characteristic for the derivative obtained by the Mannich reaction. This confirms that the Mannich reaction has occurred forming a new compound that allows quantification of metformin in the human urine. Other biological fluids are being tested at the same spectrum. Figure 1c presents the absorption spectrum of the complex obtained when mixing metformin, formaldehyde and uranine. This spectrum presents maxima at 223, 274 and 437 nm, the band at 437 nm is characteristic for the derivative of the reaction and easy to use for quantification.

### **Evolution of the complex metformin-formaldehyde-uranine absorption spectrum with varying concentrations of metformin**

Figure 2 illustrates the change of the complex spectra obtained by the Mannich reaction at five different concentrations of metformin, ranging from 5 mg/L to 30 mg/L in the presence of constant concentrations of formaldehyde and uranine (10 mg/L). As shown in Fig. 2, the intensity of the UV absorption spectrum increases with the metformin concentration, while the wavelength is not changed.

### **Reliability criteria of the method**

A calibration curve was built to examine the linearity of the method. The least squares method was used to calculate the regression equation. A high linear correlation was obtained between the absorption evolution of the complex and the additional concentration of metformin. Correlation coefficients were higher than 0.99 in a concentration range of 5-30 mg/L. The precision of the method was evaluated with relative standard deviations (RSD) of metformin determination in five samples. The % RSD was 2 %. The detection limit of the method is 0.01 µg/mL as defined by the signal/noise ratio = 3 (MacDougall). Fig. 3 shows the regression curve of complex absorption as a function of metformin concentration.

### **Spectrophotometric method for quantification of metformin using the internal standard addition model**

A spectrophotometric method using the internal standard addition was examined to quantitatively determine metformin concentrations in samples. A calibration curve was described by the following equation:

$$A^* = aC + b$$

which is equivalent to

$$A^* = (A_0^*/C_0) \times C_{add} + A_0^*$$

Where  $A^* = (A/A_0)$  normalized absorbance intensity (arbitrary values) is equal to the ratio of the absorbance intensity after adding the internal standard (A) to the absorbance intensity before adding the internal standard ( $A_0$ ),  $C_0$ : solute concentration to be estimated.  $C_0$  is determined by the negative intercept of the curve with the abscissa [26] (Rima),

$A_0^*$ : normalized absorbance intensity of the starting solution, and  $C_{add}$  (known added concentrations).

The plot of  $A^*$  vs.  $C_{add}$  is shown in Figure 4.

The internal standard added = the added metformin ( $C_0$ ). Different concentrations ( $C_{add}$ ) were added to this initial solution.

The average recovery for five samples spiked with metformin as described above (Table 1) was calculated to be 97.9% ±2.1. Table 3 summarizes the validation parameters of this method.

### **Application of the method on the determination of metformin in human urine**

Twenty four hours after oral administration of one tablet of Glucophage (metformin hydrochloride, 1000 mg; benta, Lebanon) a sample of urine was collected from a 60 years old diabetic man. Ten ml urine was mixed with 10 ml of a basic 10 M NaOH solution and transferred to a centrifuge tube. Ten ml of 1-butanol and hexane (equal parts) was then added to the tube and stirred for 30 seconds and centrifuged for 3 minutes. The aqueous phase was aspirated and evaporated to dryness with a rotary evaporator. The residue was dissolved in 5 ml of distilled water for analysis. The obtained crude sample was treated through column chromatography to isolate the metformin from the urine. Afterward The Mannich reaction was performed on the isolated metformin sample as described above. The absorption spectrum of the urine residue solution was identical to the absorption spectrum of a pure metformin in aqueous solution, data not shown.

Metformin was added to the urine in order to develop the internal standard curve. Figure 5 shows the absorption spectra of metformin and the added metformin (Table 2) that were used to develop the internal standard curve, Figure 6.

The specimen's metformin concentration (calculated against the internal standard curve) was 73.28 mg/L × 10 (dilution) = 732.8 mg/L, since the metformin has been extracted from a volume of 10 ml of urine. The estimated concentration of metformin in urine is equal to half (366.4 mg/L). A 12 hour collection of urine following the administration of 850 mg metformin yielded 183.2 mg, 21.15% of the administered dose.

A 25 year old woman was administered 850 mg and a 25 year old man received 1000 mg metformin, and respectively collected 12 hours and 24 hours urine

samples. The samples were kept at room temperature during the collections.

The protocol of extraction and method of analysis were as described above. Figure 7 presents the man's metformin absorption spectrum and Figure 8, the metformin spectrum from the woman's urine. The recovered metformin concentration in the man's 24 hours urine and woman's 12 hours urine were 38% and 36.7%, respectively.

#### Reliability criteria of the method

The proposed method has been validated according to International Conference on Harmonization guidelines for validation of analytical procedures [27] which include limit of detection, limit of quantitation, linearity, repeatability and intermediate precision, recovery, specificity, and accuracy.

#### Limit of detection

The limit of detection (LOD) and limit of quantitation (LOQ) of the developed method were determined by analyzing progressively lower concentrations of the metformin working solution (5 µg/mL). The LOD is defined as the smallest concentration of the analyte that gives a signal to noise ratio of 3. The LOQ is the smallest concentration of the analyte that gives a signal to noise ratio of 10. The LOD and LOQ of metformin were found to be 0.05 and 0.01 µg/mL, respectively.

#### Accuracy (Linearity)

Over the selected concentration range (1, 10, 15, 20, 25 and 30 µg/mL metformin), a linear relationship was obtained between the absorption intensity and concentration (Fig. 3). All concentrations were tested in quintuplicate ( $n = 5$ ) (EMA 2006). Further testing has shown that this method is linear over the range of 5mg/L – 30 mg/L. The linear regression analysis demonstrates an excellent relationship between the absorption intensity and concentration of metformin added with correlation coefficients ( $R^2$ ) of 0.99, data not shown.

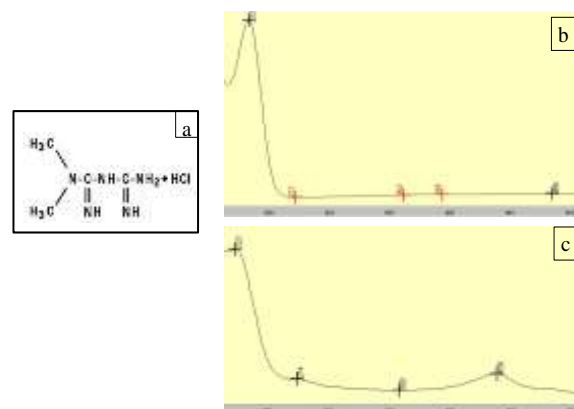
#### Precision

The precision of the method was determined by comparing results of multiple assays on the same time vs. assays of the same specimen done on four different days; repeatability (intra-day) and intermediate precision (inter-day). All assays were performed in quintuplicate ( $n=5$ ) using standard solutions of metformin (100 µg/mL). Samples were stored at room temperature. The mean values and standard deviations of absorption measurement are listed in Table 3.

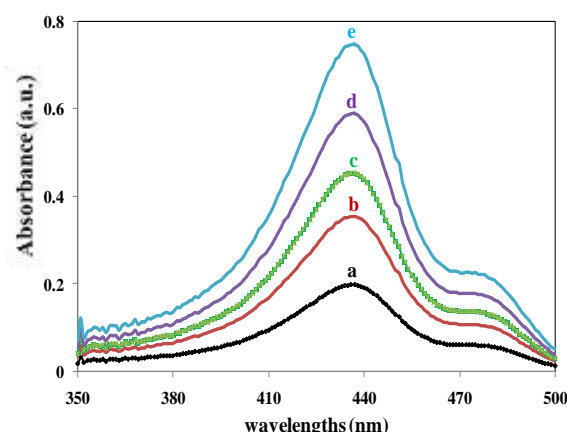
#### Recovery

The recovery of the method was determined by the standard addition method on selected samples. A known amount of metformin was added to a solution with an initial concentration  $C_0$  (10 µg/mL), at three levels 50%, 100%, and 150% ( $n = 5$ ). The percentage recovery was

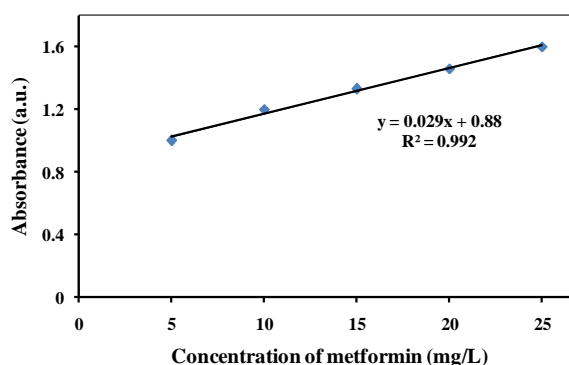
calculated according to (Rima) and data are presented in Table 3. The recovery was found to be 97.9%.



**Figure 1:** Chemical structure of metformin (a), and UV spectra of metformin in aqueous solution 10 mg/L (b), and of the product after mixing metformin (10 mg/L), formaldehyde (1 mL) and Uranine (10 mg/L) in aqueous solution (c).

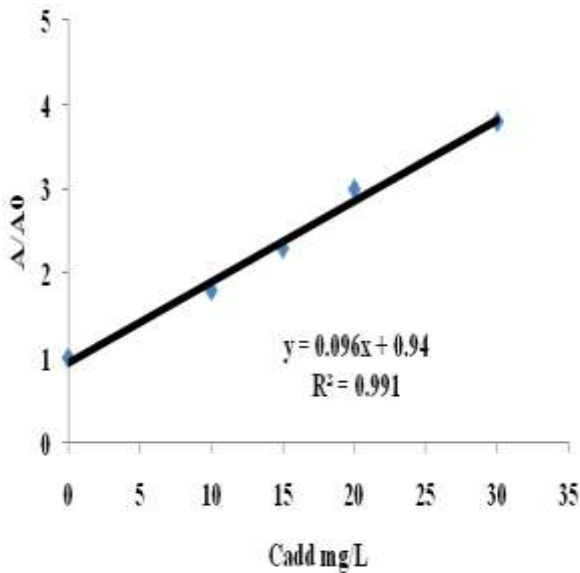


**Figure 2:** Evolution of the absorption spectra of the complex obtained by mixing metformin, formaldehyde and uranine in aqueous solutions. The concentration of the initial metformin is a: 10 mg/L. The added concentration of metformin to the initial solution is b: 10, c: 15, d: 20, and e: 25 mg/L.

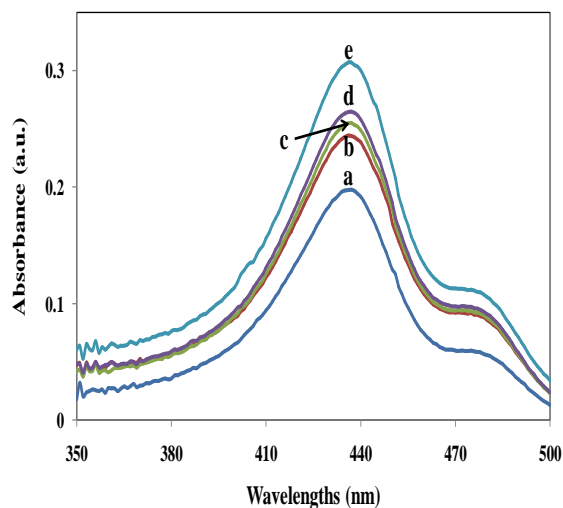


**Figure 3:** Regression curve of the colored product obtained after the Mannich reaction on metformin at different initial concentrations of metformin

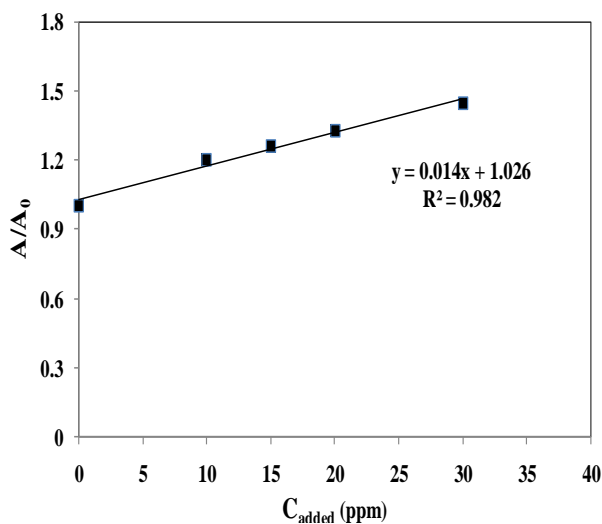




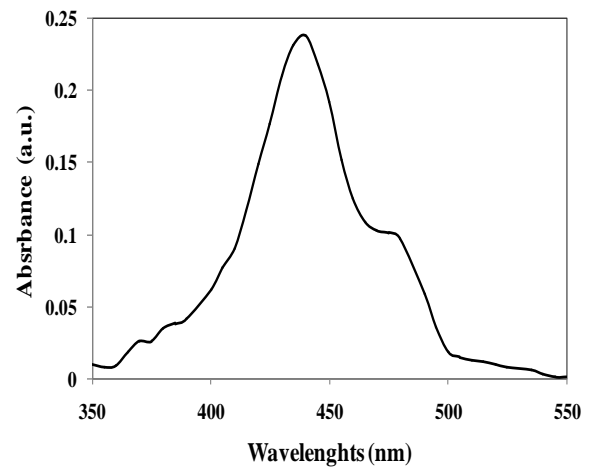
**Figure 4:** Internal standard method model for the quantitative determination of metformin.



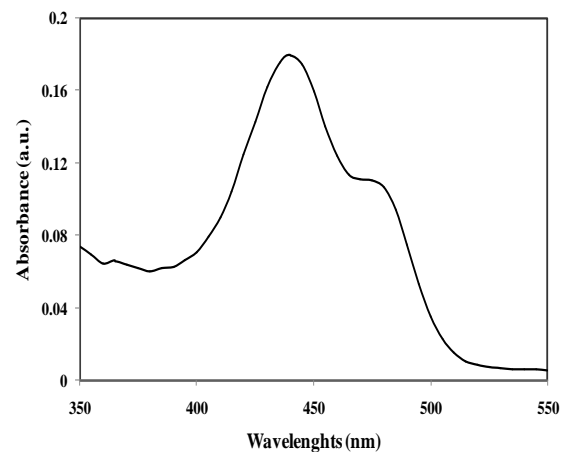
**Figure 5:** Absorption spectra of metformin in urine and fortified with a: 0, b: 10, c: 15, and d: 20 and e: 25 mg/L of pure synthetic metformin.



**Figure 6:** Internal standard addition curve to determine metformin in human urine.



**Figure 7:** Absorption spectrum of metformin extracted from the urine of young non diabetic man (25 years old) administrated by glucophage (1000 mg).



**Figure 8:** Absorption spectrum of metformin extracted from urine of young non diabetic women (25 years old) administrated by glucophage (850 mg).

**CONCLUSION**

This novel spectrophotometric Mannich reaction-based method is simple, reliable and specific for the quantitative determination of metformin content in tablets or human urine. Furthermore, this method could be applied to testing all pharmaceutical or non-pharmaceutical amine compounds in biological fluids. An important advantage of this method of metformin analysis is related to the spectroscopic selectivity by exciting the specific band of the complex obtained by the Mannich reaction.

The metformin recovered in the urine of normal young men and women, 38% and 36.7%, respectively are reasonable and compare well with the excretion by the diabetic subject. The apparent sex differences and the actual effect of the patient’s renal function remain to be resolved, but this is beyond the scope of this manuscript. However, these results lead us to question whether a modified dose form or chemically modified metformin might have different pharmacodynamics that could be salutary.

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