The Thai Journal of Pharmaceutical Sciences

Volume 46
Issue 1 *2022*

Article 12

1-1-2022

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Original Article

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Received: Mar 04, 2021
Accepted: May 17, 2021
Published: Feb 07, 2022

ABSTRACT

Background: New specific and sensitive spectrofluorimetric fluorotag method has been developed and validated for the quantification of metformin hydrochloride. **Materials and Methods:** The derivatization reaction was carried out using 1,2-naphthoquinone-4-sulfonic sulfonate as (NQS) fluorotag in the presence of strong alkaline medium (potassium hydroxide) to form the highly fluorescent product. The fluorescence was observed at emission wavelength 453 nm after excitation at 359 nm. The different experimental conditions affecting the reaction product formation and stability were carefully studied and optimized as 2% w/v of potassium hydroxide solution, 0.5% w/v NQS reagent. **Results:** At optimum reaction conditions, good linear relationship was found and Beer's law range was found to between 5 and 30 µg/mL, (R^2 = 0.999). As per International Conference on Harmonization guidelines, the validation parameters were satisfied (%relative standard deviation <2.0) for accuracy and precision studies. Limit of detection and limit of quantification for proposed method were found to be 0.54 µg/mL and 1.6 µg/mL, respectively. **Conclusion:** The contemplated method was successfully employed for quantification of metformin in pharmaceutical dosage form, spiked human plasma and urine samples.

Keywords: 1,2-Naphthoquinone-4-sulfonic sulfonate, metformin hydrochloride, spectrofluorimetry, validation

INTRODUCTION

Metformin is 3-(diaminomethylidene)-1.1-dimethylguanidine, monohydrochloride and used as an oral antihyperglycemic drug for the management of Type II diabetes mellitus. It is invariably recommended either as monotherapy or as an adjunct to diet or with a sulfonylurea (combination) to reduce blood-glucose levels.[1]

Spectrofluorimetry has assumed a special status in drug analysis because of its greater sensitivity and specificity than absorption spectrophotometry. In spectrofluorimetry two wavelengths, excitation and emission are used. In conventional fluorescence, an emission spectrum is obtained by scanning the emission monochromator at various emission wave lengths (λ_{em}), at a particular excitation wavelength (λ_{ex}).[2,3]

Metformin is an antidiabetic drug and is frequently used in the formulations alone and with other antidiabetic drugs. The metformin is not having any aromatic ring and not useful for developing spectrophotometric method with approximate range of λ_{max}: 232 nm. Aliphatic structure is not useful for the analysis, as excipients and solvents interfere with the analysis. Though several reports are available in ultraviolet (UV) Spectrophotometric methods, the region of 200–230 nm is objectionable, because of solvent cut off region. Hence, advanced methods such as high-performance liquid chromatography (HPLC) are utilized wherein the sensitivity (specificity) is higher an account of separation in HPLC column, but chromatographic methods are complex, require expensive equipments and skilled operators.[4-9] In the present work, an equally sensitive, specific and simple spectrofluorimetric method is developed. Metformin is not having a fluorophore. Hence, metformin alone is not useful for the method development. However, metformin has functional groups that can participate in the chemical reactions. Therefore, a derivative method is developed for quantification of metformin in pharmaceutical preparations and biological samples. 1,2-naphthoquinone-4-sulfonic sulfonate (NQS),

potassium hydroxide solution are used for the derivatization of metformin in spectrofluorimetric method.[10-15] Many times, derivatization is a specific reaction; the specificity of the fluorimetric method will further enhanced.

MATERIALS AND METHODS

Instrumentation and Chemicals

The Shimadzu (Japan) RF-5301 PC Spectrofluoro photometer, which is equipped with 150 watt Xenon arc lamp, 1 cm non-fluorescent quartz cell, connected to RFPC software used for the measurement of fluorescence intensity of selected drug at selected wavelengths. The instrument was operated both at low and high sensitivity with excitation and emission slit width set at 5 nm. Analytical balance (Shimadzu AUX 220, Japan), pH meter (Elico, India), Ultra sonicator (Sonica, Italy), Cyclo mixer (Remi, India), Centrifuge (Remi, India), and UV-Chamber (Bio-technics, India) were used for the study.

All reagents which were used in the experimental work were of analytical grade. Analytical grade sodium hydroxide, potassium hydroxide and 1,2- Napthaquinone- 4- sulfonic acid sodium were purchased from SD Fine Chem. Ltd., Mumbai, India. Metformin was supplied by Dr. Reddys laboratories, Hyderabad as gift sample and tablet dosage form were procured from local pharmacy.

Preparation of Solutions

Preparation of standard stock solution

Metformin (25 mg) was weighed and transferred into 25 mL volumetric flask and dissolved in water. The flask was shaken and volume was made up to the mark with water. From this, 10 mL solution was diluted to 100 mL with distilled water to obtain standard solution of (100 µg/mL).

Procedure of the calibration graph for metformin

The standard solution of metformin (100 µg/mL) was used to prepare set of diluted standard solutions as follows. The concentrations (5.0–30 µg/mL) were prepared by pipetting appropriate volumes (0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 mL) of metformin standard solution into 10 mL volumetric flasks, 1.0 mL 2% w/v potassium hydroxide solution, and 0.5% w/v NQS reagent was added, and the volume was made up to mark with distilled water. The samples were scanned.

Analysis of metformin from tablet dosage form

Tablet powdered (Glycomet) was weighed equivalent to 25 mg of standard metformin in 100 mL volumetric flask. About 50 ml of distilled water was added and sonicated for 30 min. The solution was completed to the volume with distilled water, filtered and the first portion of filtrate was rejected. From that filtrate 10 ml of solution was transferred into the 100 mL volumetric flask to obtain the final metformin solution of concentration 25 µg/mL and subjected to proposed analytical method.

Analysis of metformin from spiked human plasma

Drug free human blood sample was collected from healthy volunteers into the heparinized tube and centrifuged at 4000 rpm for 30 min. The liquid component (plasma) transferred into a clean polypropylene tube and kept at 2–8°C. From this, collect 1 mL of plasma spiked with 1 mL of metformin (100 µg/mL) stock, mix well and subjected to further analysis as per proposed method. A blank experiment was carried out to the plasma sample without metformin.

Analysis of metformin from spiked urine sample

Drug free urine sample was collected from healthy volunteer (5 mL) in the early morning and added to methanol (5 mL). The content was mixed well and centrifuged at 4000 rpm for 30 min. The clear supernatant layer was collected and 1 ml of that spiked with 1 ml of metformin (100 ug/ml) stock solution. The samples were subjected to further analysis as per proposed method. A blank experiment was carried out to the plasma sample without metformin.

Selection of Analytical Wavelengths for Metformin Hydrochloride (MET)

Metformin solution (10 µg/mL) was prepared by adding 1.0 mL of potassium hydroxide solution and 0.5 mL of NQS reagent and analyzed using spectrofluorimeter. Initially, the emission wavelength and excitation wavelength of metformin are identified. Excitation wavelength (λ_{ex}) for metformin was found to be 360 nm and the emission wavelength (λ_{em}) was found at 453 nm.

Optimization of Concentration Potassium Hydroxide

The different concentration (1–10% w/v) of potassium hydroxide solutions was selected and fluorescence intensities of metformin were measured. Stock solution of metformin (100 µg/mL) 1 ml was added to 0.5 mL of 0.5% w/v NQS reagent, 1 ml of potassium hydroxide solution, and volume was made up to 10 mL in volumetric flask with distilled water. The solutions were scanned.

Optimization of Concentrations NQS Reagent

The different concentration (0.5–2.5% w/v) of NQS reagent was used for the derivatization of metformin. Stock solution of MET 1 ml (100 µg/mL) was added to 1 ml of 2.0% w/v potassium hydroxide solution, 1 ml of NQS reagent, and volume was made up to 10 mL in volumetric flask with distilled water. The solutions were scanned.

Optimization of Time for Reaction

Various concentrations of metformin solutions (5.0–30 µg/mL) were prepared by pipetting appropriate volumes (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mL) of metformin standard solution into 10 mL volumetric flasks, 1.0 mL 2% w/v potassium hydroxide solution and 1 ml 0.5% w/v NQS reagent were added. The volume was made up to mark with distilled water. The samples were scanned with interval of 15 min, to carry out the evaluation of the optimum reaction time by monitoring the color development and derivatization reaction of solution at room temperature.

Method Validation

The method was validated for linearity, precision, accuracy, selectivity, limit of detection (LOD), and limit of quantification (LOQ) by the following procedures.[16]

Linearity

The standard dilutions of metformin (5–30 µg/mL) were quantified by proposed spectrofluorimetric technique and fluorescence intensities were recorded. The calibration curve was constructed by plotting the analyte intensities against the drug concentrations. The linearity was evaluated by linear regression equation method.

Precision

The intra- and inter-day precision was evaluated for the proposed spectrofluorimetric method using responses, 3 times on the same day and three different days, respectively, for three different concentrations of metformin (5, 15, and 30 µg/mL). The results are reported in terms of relative standard deviation (% RSD).

Accuracy

The accuracy of the method was determined by calculating recoveries of metformin by the method of standard additions. The level of concentrations of metformin 80, 100, and 120% were spiked to the pre quantified sample of metformin and then subjected to the proposed analytical method. The percentage recovery and percentage % RSD were calculated for each concentration.

Sensitivity

LOD and LOQ parameters are used. The LOD and LOQ were separately determined based on standard calibration curve.

RESULTS AND DISCUSSION

Wavelength corresponding maximum absorbance was found at 232 nm for metformin [Figure 1]. This λ_{max} cannot be used for the analysis, because several excipients present in the formulation (tablet or suspension) may interfere. The solvent cut off wavelength is nearer (~220 nm). The perusal of structure of metformin indicated the absence of aromatic ring. The aliphatic chain cannot be considered for analysis for the reasons mentioned above. Keeping these points into consideration, we attempted this work with objective to develop simple, specific and sensitive spectrofluorimetric method with support of fluorotag for quantification of metformin in pharmaceutical and biological preparations.

In this method, primary aliphatic amines of metformin exhibit nucleophillic character in alkali medium to the presence of the lone pair of electrons on the nitrogen atom and reacts with electron deficient center of sulfonic group in NQS reagent, produced orange colored chromogen complex, which exhibit highest fluorescence intensity at 453 nm after excitation at 359 nm [Figures 2 and 3]. These conditions are used for further analysis.

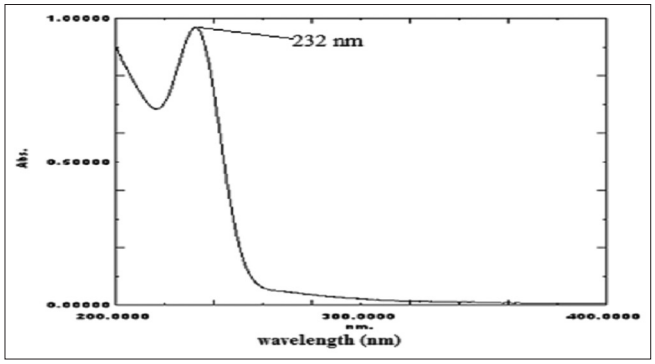

Figure 1: Ultraviolet-spectrophotometer scan of metformin solution in water

Effect of Medium on Fluorescence Intensity

Metformin reaction is evaluated for the highest fluorescence intensity. For this purpose, potassium hydroxide and sodium hydroxide solutions were tested. Fluorescence intensity was higher in potassium hydroxide solution than the sodium hydroxide solution [Figure 4]. Hence, potassium hydroxide solution was selected as medium for quantification.

Optimization of Potassium Hydroxide Solution for Fluorescence Reaction

The optimization of potassium hydroxide solution was carried out for metformin using different concentrations of potassium hydroxide solutions and samples were scanned. The potassium hydroxide concentrations-intensity profile is recorded in Figure 5. The Figure 5 indicated that the intensity was decreased above 2% w/v, suggesting that the reaction was not stable at higher concentrations. Thus, 2% w/v of potassium hydroxide was finalized and used for further analysis.

Optimization of NQS Reagent

The optimization of NQS reagent was carried out for metformin by preparing different concentrations of NQS solutions and samples were scanned. The maximum intensity was found with the 0.5 %w/v NQS reagent. The concentration intensity is given in Figure 6.

Optimization of Time for Reaction

The stability of the derivatization reaction of metformin with of NQS reagent in presence of potassium hydroxide solution was carried out by scanning the samples with interval of 15 min. The stability time profiles were recorded. It was observed that the reaction is stable within 10 min after preparation of samples. The developed color and derivatized reaction remained stable up to 1 h at room temperature.

Preparation of Calibration Curve

The fluorescent intensities of different concentrations of metformin were obtained. The graded fluorescence profiles were observed for gradual increase in concentration of metformin. The calibration curve is given in Figure 7, revealed that the metformin showed linear relationship between concentration (µg/mL) and fluorescence intensity in the range of 5–30 µg/mL. The r^2 value is 0.9993. The same method was used for further studies.

Method Validation

The responses for metformin at excitation wavelength of 359 nm and emission wavelength of 453 nm were found to

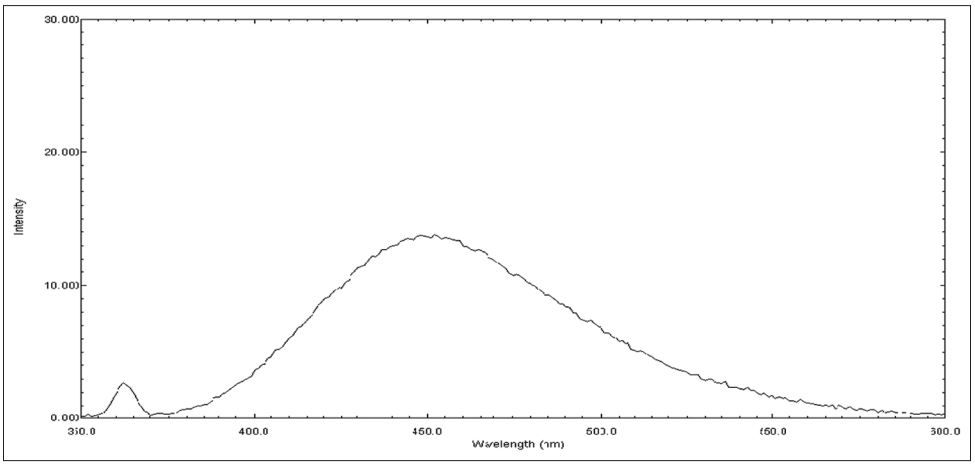

Figure 2: Fluorescent spectra of metformin (10 µg/mL) with 1,2-naphthoquinone-4-sulfonic sulfonate reagent and potassium hydroxide solution at excitation wavelength of 359 nm and emission wavelength of 453 nm

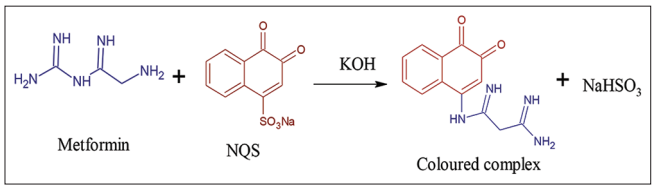

Figure 3: Probable chemical reaction between metformin and 1,2-naphthoquinone-4-sulfonic sulfonate

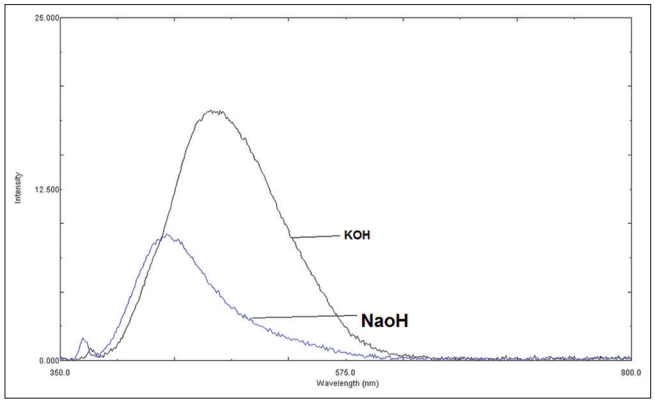

Figure 4: Fluorescent spectra for metformin with potassium hydroxide and sodium hydroxide solution at excitation wavelength 359 nm and emission wavelength at 453

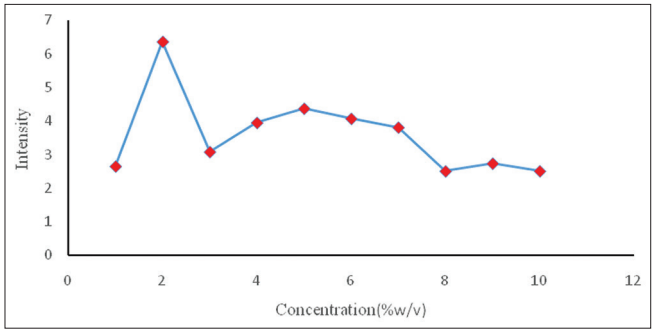

Figure 5: Effect of different concentration of potassium hydroxide metformin fluorescence (λ_{ex} at 359 nm and λ_{em} at 453 nm

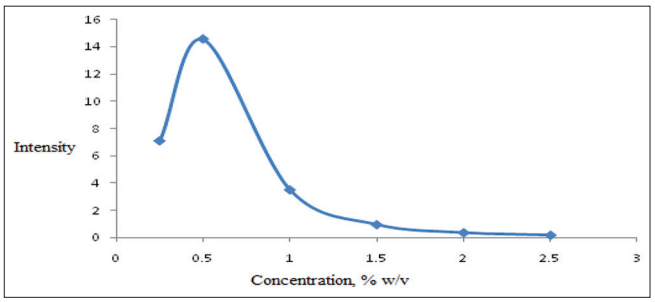

Figure 6: Effect of different concentration of 1,2-naphthoquinone-4-sulfonic sulfonate on concentration (µg/mL) metformin fluorescence (λ_{ex} at 359 nm and λ_{em} at 453 nm)

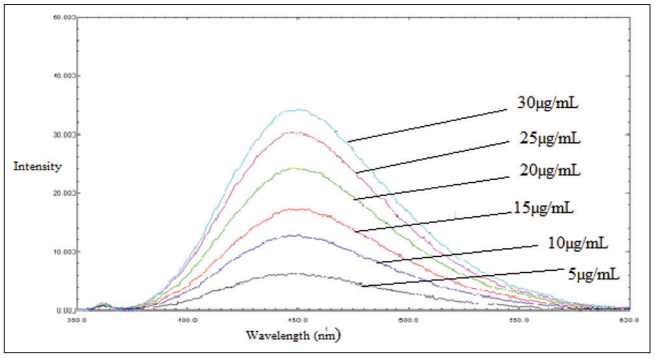

Figure 7: Linearity spectra of metformin solution by derivative method with (5–30 µg/mL) at l_{Ex} 359 nm and l_{Em} 453 nm

be linear in the concentration range of 5–30 µg/mL, with a correlation coefficient (r^2) value of 0.9993 [Figure 8]. The precision data were reported in Table 1. The % RSD of repeatability and inter-day analysis was less than 2.0. These statistical data are indicative of good precision. The mean of percentage recoveries and % RSD values were calculated and reported in Table 2. The % recovery of metformin for 500 mg was found to be satisfactory and metformin for 250 mg was also gave satisfactory results. Hence, the present proposed method is accurate. LOD and LOQ of the method were reported in Table 3, which indicates the sensitivity of the method.

Table 1: Precision for the metformin for spectrofluorimetric method

Sl.No.	Theoretical concentration (µg/mL)	Intra-day precision			Inter-day precision		
		Conc. found (µg/mL) AM±SD (*n*=3)	% Recovery AM±SD (*n*=3)	% RSD	Conc. found (µg/mL) AM±SD (*n*=3)	% Recovery AM±SD (*n*=3)	% RSD
1	5	4.97±0.097	99.50±1.74	1.54	4.984±0.11	99.68±1.85	1.74
2	15	14.54±0.35	96.93±0.94	1.19	14.80±0.11	98.71±1.85	1.55
3	30	29.31±0.32	99.39±0.93	0.89	30.23±0.49	100.76±1.21	1.35

Table 2: Accuracy of the fluorimetric method of metformin (Glycomet 500 mg and 250 mg) (Recovery studies)

Formulation	Recovery level (%)	Formulation content (µg/mL)	Amount of drug added-standard drug (µg/mL)	Amount found (mg) (AM±SD) (*n*=3)	Percent recovery (AM±SD) (*n*=3)	Percent RSD
GLYCOMET-500 mg	80	10	8	18.03±0.41	100.71±0.65	1.93
	100	10	10	20.35±0.14	101.78±0.14	0.62
	120	10	12	22.00±0.54	100.03±0.98	1.98
GLYCOMET-250 mg	80	10	8	17.50±0.41	97.27±0.57	1.99
	100	10	10	20.75±0.15	103.75±0.23	0.60
	120	10	12	22.33±0.80	101.52±0.81	2.0

Table 3: Optimized spectrofluorimetric characteristics of metformin

Parameters	Value
Excitation Wavelength (nm)	359
Emission Wavelength (nm)	453
Beers law range (µg/mL)	5-30
Regression equation	Y=1.1517X+0.3483
Correlation coefficient (r2)	0.9993
Limit of Detection (µg/ml)	0.54
Limit of Quantification (µg/ml)	1.6

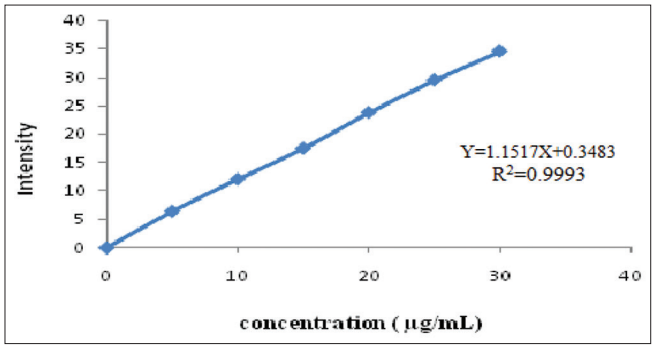

Figure 8: Standard plot for metformin estimation using derivative method at λ_{Ex} 359 nm and λ_{Em} 453 nm

Assay of Metformin

The proposed method was applied to the assay of commercially available tablets (Glycomet) containing metformin (500 mg and 250 mg). The results obtained for metformin were compared with the corresponding labeled amounts. The % assay in the commercial formulations was found to be in the range (95–105%) to by the proposed method. The % RSD for the formulations (Glycomet 500 mg and 250 mg) was <2, which indicates that the proposed method was accurate.

Analysis of Metformin from Spiked Human Plasma and Urine Sample

The proposed method was further extended to quantification of metformin from spiked human plasma and urine sample. The sample concentration was computed by linear regression equation method. The % recovery of metformin from spiked human plasma and urine sample were found to be 98 and 102%, respectively, and %RSD values were <2. These results were revealed that the proposed method having capability to quantify the drug molecule from spiked human plasma and urine sample without interference [Supplementary Table 1].

CONCLUSION

Spectrofluorimetric method has been developed and validated for quantification of metformin using NQS as fluorotag to improve the simplicity, selectivity, economic and its independence on expensive equipments, which makes the developed method, is of superior in normal quality control analysis. The results of contemplated spectrofluorimetric method validation and % recovery of metformin from pharmaceutical, spiked plasma and urine preparations were prime face evidence to employ fruitfully for quantification of metformin in different samples without any interference.

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SUPPLEMENTARY DOCUMENT

Supplementary Table 1 : Quantification of metformin from spiked human plasma and urine samples

Amount of drug spiked (μg/mL)	Plasma		Amount of drug spiked (μg/mL)	Urine	
	% Recovery (A.M±SD)*	% RSD		% Recovery (A.M±SD)*	% RSD
10	98.5±0.25	0.253	10	101.5±0.13	0.128