A new RP-HPLC method for metformin determination in human serum

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ABSTRACT

Background: Anticoagulant in the sample bottles could form complex with metformin thereby affecting its concentration. This necessitates the need to develop HPLC method for determination of metformin in serum

Objectives: This study was aimed at developing a simple, less tedious RP-HPLC method for the determination of metformin in human serum.

Methods: Blank serum (2 mL) was spiked into solution containing 2 mL metformin (2-65 µg mL-1) and 1 mL caffeine (5.0 µg mL-1) as internal standard (IS) centrifuged at 3000 rpm for 5 minutes. Portion (0.5 mL) of the resultant solution was injected into the HPLC auto sample machine (Agilent 1260 infinity). The optimized conditions included a mobile phase of methanol-water (80:20 v/v) containing 0.1 % orthophosphoric acid, isocratic elusion mode, an injection volume of 10 µL, flow rate of 1.2 mL min-1, at 35 °C and detection wavelength of 255 nm. Calibration curve (0.40 to 12.2 µg mL-1) was constructed by plotting the peak area ratios against their corresponding concentrations. The method was validated according to ICH guidelines.

Results: Metformin and caffeine eluted at 1.29 and 1.42 minutes respectively. The method was precise (4.77 % RSD), accurate (% Er of 0.22 and % recovery of 99.78 %) with a linear calibration curve (r = 0.988). LOD and LOQ of the developed method are 1.93 and 5.87 µg/mL respectively. All the parameters were within the acceptable limits.

Conclusion: The developed method was found to be simple, precise, accurate and rapid for the routine therapeutic metformin monitoring in human serum.

Keywords: Therapeutic metformin monitoring, RP-HPLC, serum, isocratic elution.

Une nouvelle méthode RP-HPLC pour la détermination de la metformine dans le sérum humain

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RÉSUMÉ

Contexte : L'anticoagulant présent dans les flacons d'échantillons pourrait former un complexe avec la metformine, ce qui en modifierait la concentration. D'où la nécessité de mettre au point une méthode CLHP pour le dosage de la metformine dans le sérum.

Objectifs : Cette étude visait à développer une méthode RP-HPLC simple et moins fastidieuse pour la détermination de la metformine dans le sérum humain.

Méthodes : Du sérum vierge (2 mL) a été dopé dans une solution contenant 2 mL de metformine (2-65 µg mL-1) et 1 mL de caféine (5,0 µg mL-1) comme standard interne (SI), centrifugé à 3000 rpm pendant 5 minutes. Une portion (0,5 mL) de la solution résultante a été injectée dans l'automate d'échantillonnage HPLC (Agilent 1260 infinity). Les conditions optimisées comprenaient une phase mobile de méthanol-eau (80:20 v/v) contenant 0,1 % d'acide orthophosphorique, un mode d'élusion isocratique, un volume d'injection de 10 µL, un débit de 1,2 mL min-1, à 35 °C et une longueur d'onde de détection de 255 nm. Une courbe d'étalonnage (0,40 à 12,2 µg mL-1) a été construite en traçant les rapports de surface des pics en fonction des concentrations correspondantes. La méthode a été validée conformément aux directives de l'ICH.

Résultats : La metformine et la caféine ont été éluées respectivement en 1,29 et 1,42 minutes. La méthode était précise (? 4,77 % RSD), exacte (% Er de 0,22 et % de récupération de 99,78 %) avec une courbe d'étalonnage linéaire (r = 0,988). La LOD et la LOQ de la méthode développée sont respectivement de 1,93 et 5,87 µg/mL. Tous les paramètres sont dans les limites acceptables.

Conclusion : La méthode développée s'est avérée simple, précise, exacte et rapide pour le suivi thérapeutique de routine de la metformine dans le sérum humain.

Mots-clés : Suivi thérapeutique de la metformine, RP-HPLC, sérum, élution isocratique.

INTRODUCTION

Metformin (Figure 1) is chemically[1], 1-dimethylbiguanide hydrochloride, a biguanide oral antidiabetic given to supplement treatment by diet modification when such modification has not proven effective on its own. The biguanides are generally preferred in obese patients because they are not associated with weight gain.[1] Metformin is a first-line drug, recommended by the World Health Organization (WHO) in the management of type 2 diabetes.[2] It regulates blood glucose without causing weight gain or hypoglycemia.[2] Major disadvantages of metformin therapy are interindividual variations in its pharmacokinetic profiles and to some extent, lactic acidosis that results due to high metformin concentration.[3,4] For optimal glycemic control, metformin concentration should be within the range of 0.75 to 5.00 µg/mL.[5]

Several bioanalytical methods for the analysis of metformin in plasma have been reported.[6,7,8,9] However, the effect of anticoagulants in the sample bottles needs to be studied as they may affect its concentration of metformin as seen with gentamicin in heparinized bottles.[10] This can be achieved by developing a new method that addresses the drawbacks reported by the existing HPLC methods for the determination of metformin in plasma (human and animals) such as tediousness and several extraction steps among others.[11,12] The aim of this study was to develop a simple HPLC-UV method for the determination of metformin in human serum.

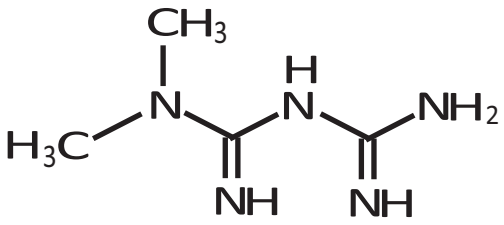

Figure 1: Chemical structure of metformin

MATERIALS AND METHODS

Equipment and reagents

Standard metformin powder, caffeine standard powder, HPLC grade methanol, HPLC grade water were all obtained from Sigma Aldrich (Germany). HPLC column: Eclipse Plus C18 (100 mm×4.6 mm i.d., 3.5µ particle size), Shimadzu D439300179 digital analytical weighing balance, Thermo Electron Corporation Centra CL2 centrifuge, HPLC sample bottles 1.5 mL, HPLC machine used was Agilent technologies (Model 1260 Infinity Series). FTIR machine (Agilent technologies model 1200 Infinity Series).

Preparation of solutions

Preparation of suitable dissolution solvent for metformin standard powder

Although metformin is highly soluble in water, it was observed that the solvent that gives better resolution both for the drug and internal standard (caffeine) is methanol:water (M:W) in a ratio 60:40 v/v. This solvent was used in dissolving the metformin and IS throughout the analysis.

Preparation of standard solution

Ten milligrams (10 mg) each of metformin and caffeine standard powders were separately weighed and dissolved into two labeled volumetric flasks containing M:W (10 mL) to obtain 1000 µg/mL stock solutions. The caffeine stock solution was further diluted to obtain 5 µg/mL. Working solutions of metformin (2-64 µg/mL) each containing caffeine (5 µg/mL) spiked with serum were prepared using serial dilution.

Optimization of chromatographic conditions

Chromatographic conditions were optimized by analysing the solutions of metformin and caffeine separately and then in combination. The solutions (metformin and caffeine) were further analysed after spiking with serum and adjustments to the chromatographic conditions were made (Table 1). Mobile phase was degassed through an online degasser and the column was conditioned with the mobile phase. Also, samples were injected after allowing the chromatographic base line to equilibrate for 10 minutes.

Method validation

The developed method was validated according to International Conference on Harmonization (ICH) guidelines.[13]

Linearity, LOD and LOQ

Various metformin solutions (2-64 µg/mL) containing caffeine (5 µg/mL) spiked with serum were vortex mixed and centrifuged at 3000 rpm for 5 min. A quantity (0.5 mL) of each solution was injected into the HPLC machine operated at the optimized chromatographic conditions. Six-point calibration curve was constructed by plotting the peak area ratios (metformin/caffeine) against their corresponding concentrations. Linear regression equation, coefficient of correlation (r) and standard

deviation at intercept on y-axis were computed using LINEST function in Microsoft Office Excel 2007. Thereafter, limit of detection (LOD) and limit of quantification (LOQ) were calculated.

The limit of detection (LOD) was determined by studying the calibration curve using samples containing the drug in the range of LOD. The standard deviation of y-intercepts of the regression lines was used as standard deviation. LOD is expressed as:

$$\text{LOD} = \frac{3.3a}{S},$$

The limit of quantitation (LOQ) was determined using the expression:

$$\text{LOQ} = \frac{10a}{S}$$

Where *a* in each case is the standard deviation of y-intercepts of the regression lines determined through LINEST function in Microsoft Office Excel® 2007.

Accuracy and recovery

The accuracy of this method was checked by standard addition method, where 80, 100 and 120 % of a preanalysed 16 µg/mL solution of metformin containing IS and serum were added to same (16 µg/mL solution) to obtain 28.8, 32 and 35.2 µg/mL solutions of metformin. The mixtures were centrifuged as described under preparation of calibration curve before finally injecting into the HPLC machine. After obtaining the chromatograms, the metformin content was determined by subtracting the peak area ratio of metformin/caffeine (IS) of the preanalysed unspiked solution (16 µg/mL) from that found in each of the spiked solutions (28.8, 32 and 35.2 µg/mL) and interpolating the final concentrations from the calibration curve. Accuracy was expressed as percentage relative error (% Er) and percentage recovery.

Intraday and interday precision

Metformin solution (16 µg/mL) containing caffeine and serum was analyzed six times within a day at one hour intervals and three times for three consecutive days to determine the intra-day (repeatability) and inter-day (intermediate) precisions respectively. Precisions were expressed as percent relative standard deviation (% RSD) in both cases.

RESULTS

Chromatograms of metformin and caffeine alone are presented in Figures 2 and 3 respectively, while the obtained chromatogram of metformin and caffeine spiked with human serum is shown in Figure 4. Optimized chromatographic conditions are listed in Table 1. The constructed calibration curve of metformin in human serum is presented in Figure 5. Validation and calibration curve parameters of the developed method are listed in Table2.

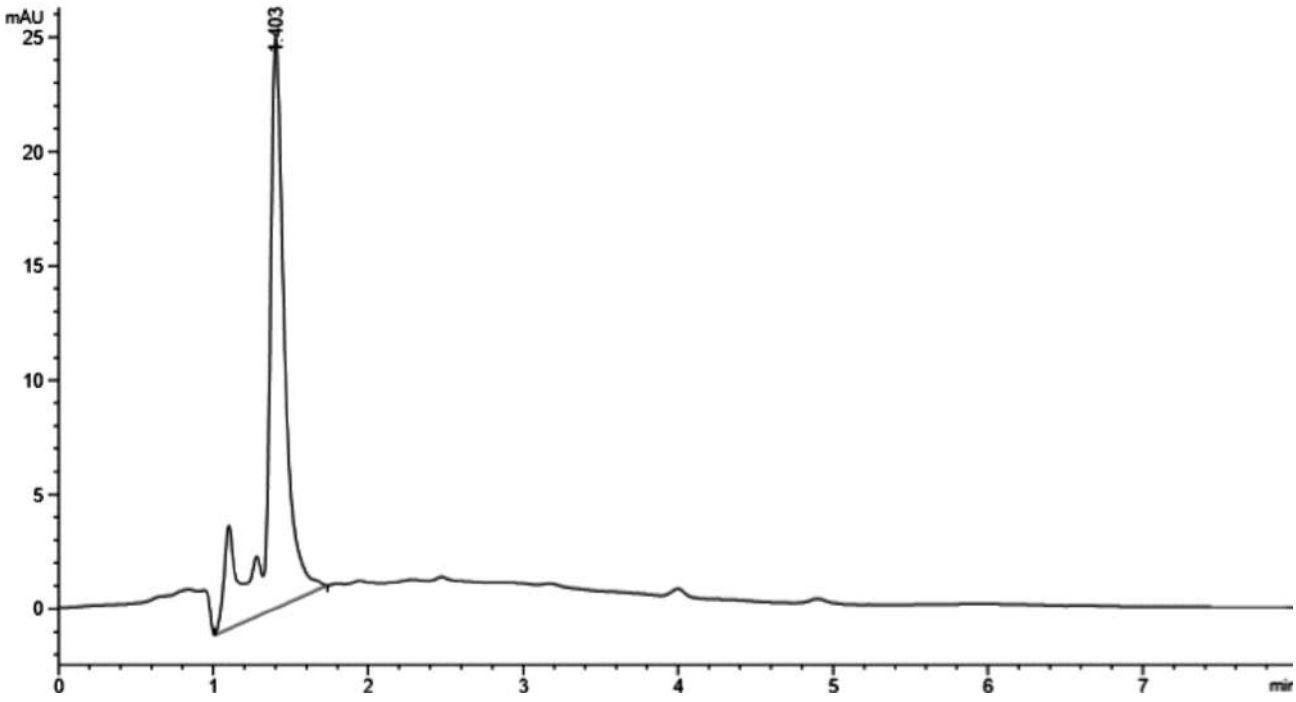

Figure 2: HPLC chromatogram of Metformin alone

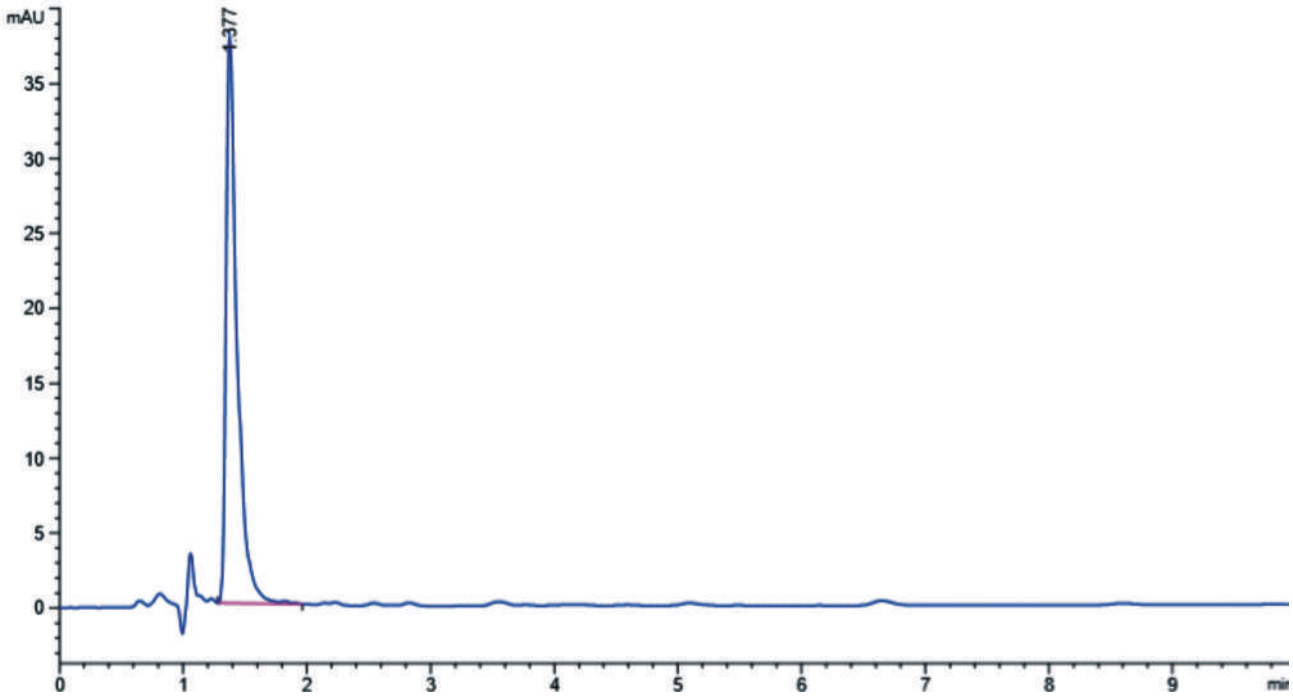

Figure 3: HPLC chromatogram of caffeine alone

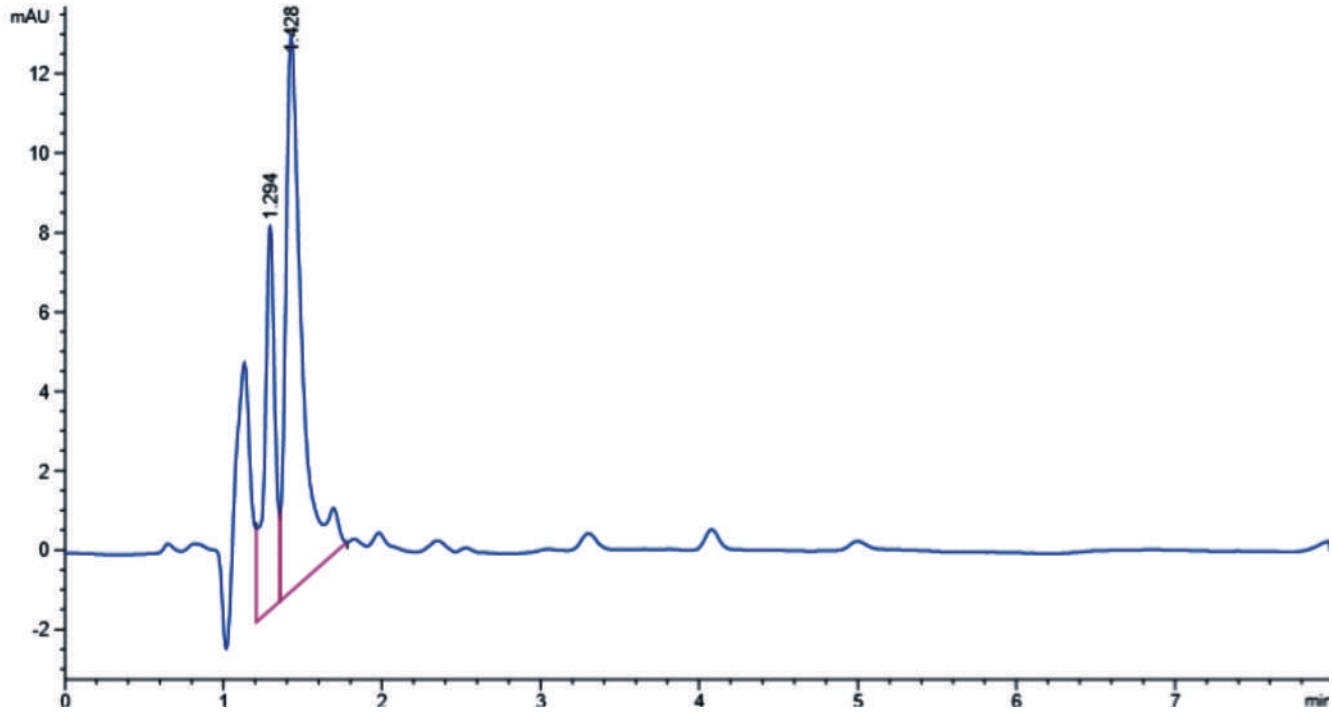

Figure 4: HPLC chromatogram of metformin and caffeine spiked with human serum

Table 1: Optimized chromatographic conditions

S/No.	Parameters	Descriptions
1	Mobile phase	Methanol: Water (80:20)
2	Column	Eclipse Plus C18
3	Column size	C18 4.6 x 100, 3.5 µm
4	Wavelength	255 nm
5	Column temperature	35 °C
6	Flow rate	1.2 mL/min
7	Injection volume	10 µl
8	Runtime	7 minutes

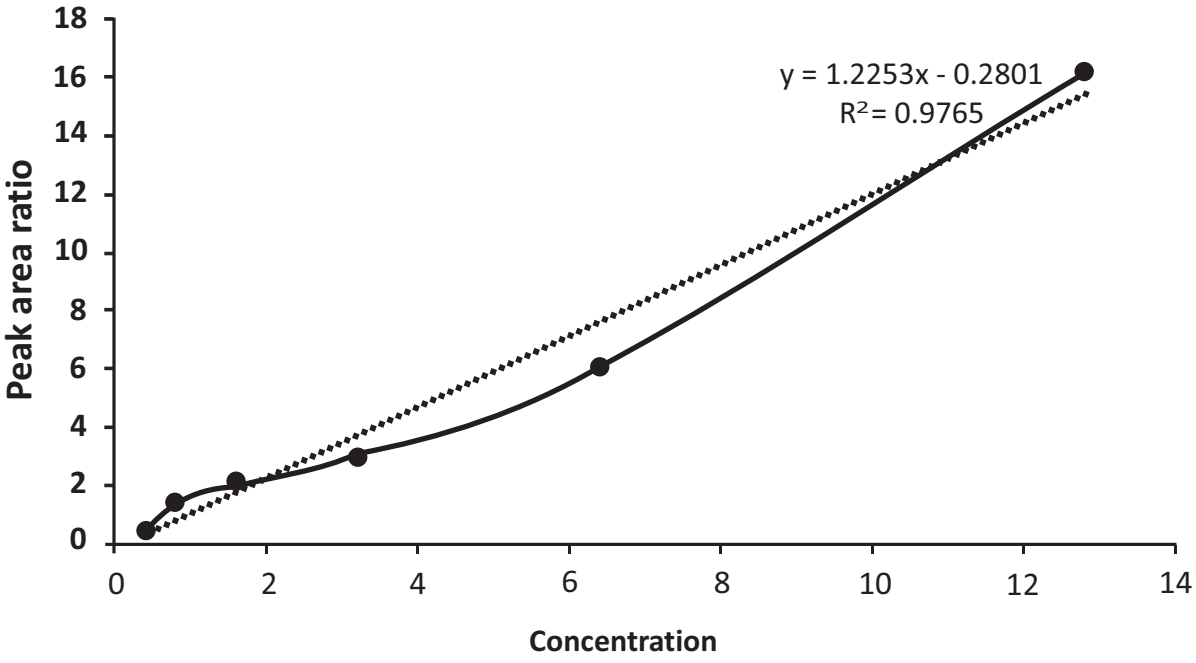

Figure 5: Calibration curve of metformin in serum using caffeine as internal standard

Table 2: Validation and calibration parameters of the developed method

S/No.	Parameters	Values
1.	Precision	
	Intraday	(69.97, 72.07, 71.28,74.16 74.91)
	Mean	72.48
	RSD ± SD	2.82 ± 2.04
	Interday	(69.97,74.91,76.79)
	Mean	73.89
	RSD ± SD	4.77 ± 4.76
2.	Accuracy	
	% Er (Mean ± SD)	0.22 ± 1.86
	% Recovery (Mean ± SD)	99.78 ± 1.86
3.	Calibration curve range	0.4-12.2 µg/mL
4.	Coefficient of correlation (r)	0.9882
5.	LOD	1.93 µg/mL
6.	LOQ	5.87 µg/mL

DISCUSSION

Several columns were tried for this method to obtain the one that best fits the stated objective. Techspher 50DS 25 cm x 4.6 mm was found to elute metformin at a longer time and did not adequately resolve metformin from the internal standard. However, Eclipse Plus C18 (4.6 x 100, 3.5 m) was found to elute the analyte in less than two minutes and resolved metformin from the internal standard (Figures 2-4) under the optimized chromatographic conditions (Table 1). Eclipse Plus column was adopted throughout the analysis because it saves time as analysis can be conducted in a short time which is an advantage over other columns that take much longer time before metformin is eluted.

Different ratios of the mobile phase methanol-water which is the polar part of the chromatographic system and its resultant effect on the separation of polar drugs were used to observe the resolution between metformin and caffeine (IS). The best resolution was achieved with a solvent system of methanol and water (80:20 v/v). This mobile phase was found to be simple when compared with 34 % acetonitrile and 66 % aqueous phase (10 mM KH_2PO_4 and 10 mM sodium lauryl sulfate) at pH 5.2 used and reported by Chhetri.[9] The mobile phase was also found to be cheaper when compared with that of Murthy.[14] A shorter retention time (1.29 min.) was recorded compared to 9.26 and 2.15 minutes that was reported.[9,14]

Metformin and caffeine (IS) absorbed well at a wavelength of 255 nm when scanned in various compositions of the mobile phase, thus it was selected as optimum wavelength. To ensure uniformity of the system operation throughout the analysis, equilibrating the column with mobile phase prior to injection of the sample into the chromatographic system was ensured. Peak parameters such as height, asymmetry and tailing were considered while maintaining flow rate and baseline drift. Other factors like resolution, repeatability, tailing factor and theoretical plate number where also checked prior to starting analysis every time and were in conformity with the set guidelines.[13]

There was strong relationship (within the range of 0.4-12.2 µg/mL) between metformin/caffeine peak area ratios against their corresponding concentrations ratio as indicated by coefficient of determination (r^2) which is tending to unity (Figure 5). This clearly shows the linearity of the method. The method was found to be highly sensitive as it can accurately detect metformin at a very low concentration of up to 1.93 µg/mL and can quantify 5.87 µg/mL of the drug in analytical samples (Table 2). This method can therefore be used in therapeutic monitoring of metformin in diabetic patients that require such intervention.

With good technique and reliable methodology, the precision of an analytical method should be < 15 % CV.[15] The developed method was found to be highly precise as both the intraday and interday precisions were less than five percent (Table 2). A % CV of 11.42 and 14.5 was reported in HPLC-UV method for simultaneous determination of metformin and other drugs in human plasma.[16] Similarly a % CV of 4.54 and 6.97 was also reported.[9] The accuracy (0.22) of the method expressed as the measure of percentage relative error are within the range (1 - 5 %) for moderately accurately procedure.[15] This shows the high precision of the developed method. The method was also found to recover almost all the analyte as indicated by a close 100 % recovery with almost zero percent error (Table 2). This shows that the method recovery is excellent especially when compared with the 98.88 % reported in a RP-HPLC method for analysis of metformin.[17]

CONCLUSION

The developed method was found to be simple, precise, accurate and rapid for the routine therapeutic metformin monitoring in human serum.

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