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Research Article

RP-HPLC Method Developed for the Determination of Metformin in Human Saliva

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ABSTRACT

Several extraction steps involved while analyzing metformin in plasma necessitates the need to develop a simple less tedious RP-HPLC method for metformin analysis in human saliva.Blank saliva (2 mL) was spiked with 2 mL solution (12.5 μg mL^{-1}) of metformin and 1 mL solution (0.5 μg mL^{-1}) of caffeine as internal standard (IS). The mixture was vortex mixed and centrifuged at 3000 rpm for 10 minutes. A portion (0.5 mL) of the resultant solution was injected into the HPLC 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 mLmin^{-1}, at 35°C and detection wavelength of 232nm. Calibration curve(1.25to25.0 μg mL^{-1}) was prepared by plotting the peak height ratios of metformin Vs IS against their corresponding concentrations. The method was validated according to ICH guidelines. Metformin and caffeine eluted at 1.6 and 2.6 minutes respectively. The method was precise (<1 % RSD), accurate (% Er of 1.00 and % recovery of 99.98 %) with linear calibration curve (r= 0.9987). The developed method can be used for determination of metformin in human saliva.

Keywords: Metformin; Saliva; Isocratic elution; RP-HPLC

INTRODUCTION

Metformin (Figure 1) is chemically 1,1-dimethylbiguanide hydrochloride, a biguanide antidiabetic appearing as white or almost white crystals with melting point of 222 – 226 °C and content range 98.5 – 101.0 % dried substance, freely soluble in aqueous medium, slightly soluble in alcohol, and insoluble in acetone and in methylene chloride solvents.[1] Physicochemical features of metformin showed that the drug has pKa values of 2.8 and 11.5, appeared very largely as water loving cationic species at physiological pH values. The metformin pKa values renders metformin a stronger basic in nature than most other basic drugs with less than 0.01% of the drug as unionized form in blood. The lipid solubility of the unionized species of the drug is very little as shown by its low LogP value (Log10 of the partition coefficient of the unionized form between octanol and water) of -1.43. These parameters indicate low lipophilicity and, consequently, rapid passive movement of metformin through cell membranes is much unlikely. The LogP of metformin is less than that of the other biguanide phenformin (-0.84) because the di-methyl substituents on metformin makes it less lipophilic than the bulky phenyl ethyl side chain in phenformin. More derivatives of metformin are presently being investigated with the aim of producing prodrugs that are more lipophilic with better oral absorption.[2,3]

Some developed methods for determination of metformin in biological fluids have been reported in the literature. An HPLC method for determination of metformin in brain sections and plasma of rats treated with polysaccharides was reported to be developed and validated. They used ranitidine HCl as internal standard and pentafluorophenylpropyl column (HS F5, 150 × 4.6

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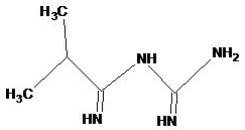

Fig. 1: Structure of metformin

mm id), a mobile phase consisting of ammonium acetate-acetonitrile in ratio (47.8:52.2), rate of flow 1.3 mL/min, injection volume 20 μL and detection λ_{max} 233 nm. The method successfully resolved metformin from IS with retention time (9.56 and 4.91 mins) and (12 and 6 mins) for metformin and internal standard in plasma and brain samples respectively.[4] Metformin was also reported to be quantified in HPL-MS/MS method for comparative pharmacokinetic study among 3 metformin formulations in healthy Mexican volunteers in a single-dose, randomized, open-label, 3-period crossover study. The method utilized C18 (4.6 × 100 mm id) column, a mobile phase consisting of acetonitrile-formic acid (40:60), column temperature of 21°C, flow rate 0.8 mL/min and retention time of metformin 1.8 min.[5] The concentrations of metformin in plasma were also determined using HPLC method in a pharmacokinetic interaction study between metformin and teneligliptin in healthy adults. The column used was (μBondapak Phenyl, 300 ×3.9 mm, 10 mm; Waters) isocratic elution. The employed mobile phase is ternary consisted of acetonitrile-6.7 mM potassium dihydrogen phosphate-triethylamine (500:1500:1) adjusted with phosphoric acid to pH 7.6. The analyte was detected using (SPD-6A; Shimadzu Corporation, Kyoto, Japan) spectrophotometer at λ_{max} of 234 nm.[6] A RP-HPLC method for estimation of metformin in plasma was reported that utilized propranolol as IS, C18 (150 × 4.6 mm id) column at 40°C, flow rate 1 mL/min, binary mobile phase consisted of phosphate buffer pH 7.0-acetonitrile in ratio (50:50) and retention time of 7.5 and 9.5 minutes for metformin and IS respectively. The method was applied only for bioequivalence studies using 2 metformin products and suggests that, it should be used for pharmacokinetic study of metformin.[7] A HPLC method for concurrent determination of metformin and three ACE inhibitors (lisinopril, captopril and enalapril) was also reported. The method utilized caffeine as IS and C18 (250 × 4.6 mm id) column at temperature 25°C, pH adjusted to 3.0 with phosphoric acid and mobile phase composition of acetonitrile-water in ratio (50:50). The flow rate was 1 mL/min and detection λ 218 nm. The method LODs were 3.26 and 0.98 ng/mL and LOQs 9.26 and 2.22 ng mL^{-1} for lisinopril and metformin respectively. The retention time of the analysed drugs was not mentioned and the displayed chromatogram makes it difficult to ascertain that because the chromatogram is not well labelled. The method was reported to be applied in the determination of the drugs in their finished product and suggested its application for pharmacokinetic studies of the drugs.[8] To the best of our literature search, there is no report on analytical method for the analysis of metformin in human saliva. This research is aimed at developing and validating an RP-HPLC method for the analysis of metformin in human saliva which may be utilized for pharmacokinetic and bioequivalence studies involving the drug in saliva. The non-invasive procedure for sample collection in this method presents a more convenient way to patients and ease of compliance to study as compared to blood collection using invasive techniques that are painful and may be harmful to the subject unless strict safety measures are followed.

MATERIALS

Equipment and reagents

Standard metformin powder, caffeine standard powder, HPLC grade methanol, and HPLC grade water were obtained from Sigma Aldrich (Germany).HPLC column: Chemisil ODS C18 (200 mm×4.6 mm i.d., 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). A double scanning UV/Vis spectrophotometer (Model SP 3000) was also used.

METHODOLOGY

Preparation of suitable solvent (diluent) for dissolution of metformin

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 (80:20) (M:W). This solvent was used for dissolving the metformin and internal standard throughout the analysis.

Preparation of stock solution of metformin and internal standard (caffeine)

A stock solution of metformin was prepared by accurately weighing and dissolving 2 mg of pure metformin powder in 20 mL of M:W to obtain a concentration of 100 μg/mL. A stock solution of caffeine was prepared by accurately weighing and dissolving 2 mg of pure caffeine powder in 20 mL of M:W to obtain a concentration of 100 μg/mL. Further dilutions were appropriately made where necessary.

Optimization of Chromatographic conditions

Separation was achieved chromatographically using the column Chemisil ODS® C18 (200 mm × 4.6 id). The mobile phase consisted of methanol-water containing 0.1

% orthophosphoric acid as additive. Isocratic separation conditions were achieved through varying and optimizing the mobile phase ratios, injection volume, temperature, detection wavelength and the rate of flow after several trials. These stated conditions allowed the detection of metformin and caffeine (internal standard) with ample sensitivity.

Preparation of metformin-saliva sample

A diluted solution of metformin (12.5 μg/mL) was prepared from the stock solution and a portion (2 mL) was added to blank saliva (2 mL) followed by 1 mL of a solution (0.5 μg/mL) of the caffeine internal standard to obtain a mixture containing (5.0 μg/mL) of metformin. The mixture was vortex mixed and centrifuged at 3000 rpm for 10 min which gave a relatively clear solution. A quantity (0.5 mL) of the clear solution was injected into the HPLC auto sample machine and the chromatogram was obtained which resolved metformin from the internal standard.

Preparation of calibration curve of metformin

Various concentrations (3.125, 6.25, 12.5, 25.0, 50.0 and 62.5 μg/mL) were prepared from the stock solution and portions (2 mL each) were transferred into series of 5 mL sample bottles each containing blank saliva (2 mL) followed by 1 mL of a solution (0.5 μg/mL) of the caffeine internal standard to obtain mixtures containing 1.25, 2.5, 5.0, 10.0, 20.0 and 25.0 μg/mL respectively of metformin and 100 μg/mL of the internal standard. The mixtures were vortex mixed and centrifuged at 3000 rpm for 10 min resulting in clear solutions. A quantity (0.5 mL) of each clear solution was injected into the HPLC machine and the respective chromatograms were obtained. The peak height ratios of metformin/internal standard obtained were then plotted against the corresponding concentrations.

Validation of Method

This method was validated with respect to linearity, precision, accuracy, percentage recovery, limit of detection and limit of quantitation.[9]

Accuracy and recovery

Accuracy of this method was checked by standard addition methods, where 80, 100 and 120 % of a 10 μg/mL solution of metformin were added to same and treated with saliva and the IS as described in the methodology to obtain final concentrations 18, 20 and 22 μg/mL 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 height ratio of metformin/internal standard of the unspiked solution (10 μg/mL) from that found in each of the spiked solutions and interpolating the final concentrations from the calibration curve. Accuracy was expressed as percentage relative error (%Er).

$$\%\,\mathrm{Er} = \frac{x-\mu}{\mu} \times 100$$

Where x is the mean and μ is the expected value. While the percentage recoveries were computed using formula.

$$\%\ \text{Recovery} = \frac{\text{measured concentration}}{\text{added concentration}} \times 100$$

Linearity, Limit of detection and limit of q uantification

Various metformin solutions (1.25-25 μg/mL) containing caffeine (0.5 μg/mL) spiked with saliva were vortex mixed and centrifuged at 3000 rpm for 10 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:

$$\mathrm{LOD} = \frac{3.3\sigma}{\mathrm{S}}$$

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

$$\mathrm{LOD} = \frac{10\sigma}{\mathrm{S}}$$

Where σ is standard deviation of y-intercepts of the regression lines determined through LINEST function in Microsoft Office Excel® 2016.

Intraday and interday precision

Metformin solution (20.0 μg/mL) containing caffeine and saliva 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.

$$\%\mathrm{RSD} = \frac{\mathrm{s}}{\mathrm{x}} \times 100$$

Where *s* is the standard deviation and *x* is the mean.

RESULTS AND DISCUSSION

The optimized chromatographic conditions of the developed RP-HPLC method are shown in Table 1 while the chromatograms obtained are presented in Figures 2 and 3 and Figure 4. Calibration parameters for RP-HPLC method are shown in table 2. Calibration curve of metformin in saliva is shown in Figure 5. The results of the validation parameters for the method are shown in Table 3.

Table 1: Optimized chromatographic conditions of the method

Parameters	Descriptions
Mobile phase	Methanol:Water (80:20)
Column	Chemisil ODS® C18
Column size	200 mm×4.6 mm i.d., 5μ particle size
Additive	0.1% orthophosphuric acid
Detection wavelengthλ	232 nm
Column temperature	35 °C
Flow rate	10 μL
Injection volume	1 mL/min
Runtime	6 min
Retention time	1.6 min

Table 2: Calibration curve parameters of the developed method

Parameter	Value obtained
Linearity range (μg/mL)	1.25 – 25.0
Correlation coefficient (r)	0.9987
Regressionequation	A = Cy + x
Slope (y)	1.7997
Intercept (x)	0.5297

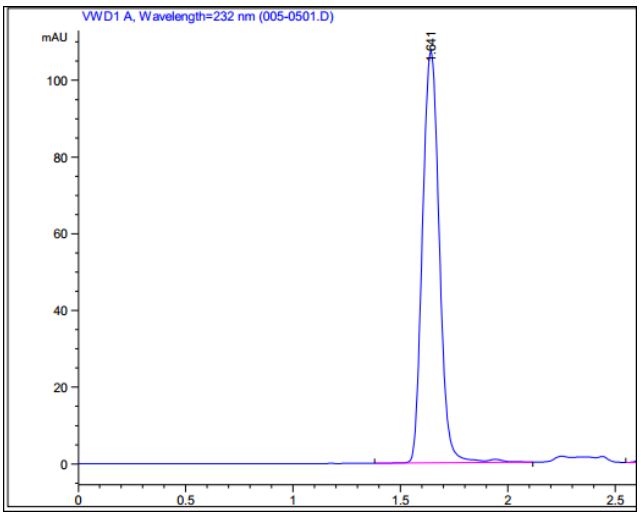

Fig. 2: RP-HPLCchromatogram of metformin alone at 232 nm

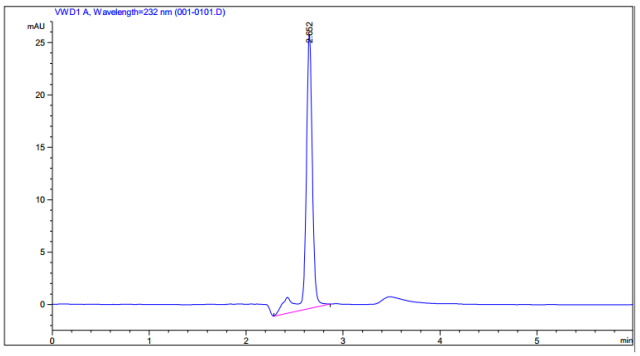

Fig. 3: RP-HPLC chromatogram of internal standard alone at232 nm

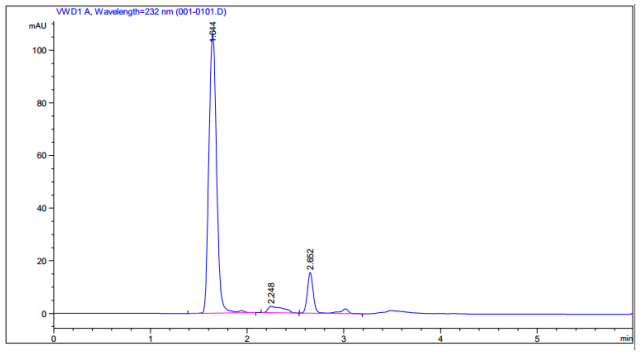

Fig. 4: RP-HPLCchromatogram of metformin and internal standard spiked with saliva

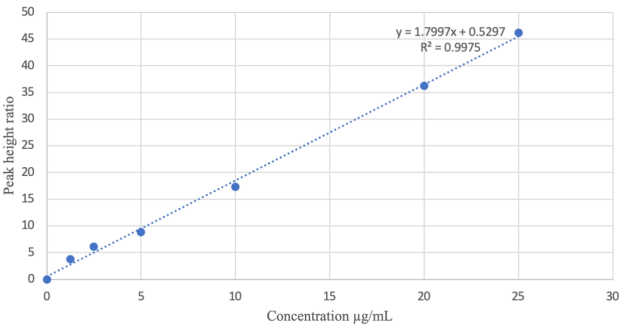

Fig. 5: RP-HPLC calibration curve of developed method for metformin

Table 3: Validation parameters of the developed method

Parameter	Value obtained
Precision Intraday (% RSD) ± SD	0.5 ± 0.12
Interday (% RSD) ± SD	0.7 ± 0.11
Accuracy ± SD (% Er)	1.00 ± 0.12
Recovery ± SD (%)	99.98 ± 0.10
Limit of detection (ng/mL)	0.22
Limit of quantification (ng/mL)	0.67

The peak height ratios of metformin and internal standard against their corresponding concentrations were determined. The linear relationship between the peak height signals (A) and their corresponding concentrations (C in ng/mL) is given by the regression equation of the type A = Cy + x (Table 2). The coefficient of correlation (r)is approaching unity(0.9987). This shows a direct proportionality relationship between peak height signals and concentrations which give the good correlation. The solvent methanol-water (80:20) appears to be the best as it serves as solvent for both metformin and caffeine as well as utilized solvent.

For the optimization of chromatographic conditions, various shackles were systematically countered to get to optimum conditions. Mobile phase being the polar part of reverse phase HPLC system always has a profound effect on the separation of drug molecules which are mostly polar in nature. Due to this, the selection of mobile phase was critically considered. Different ratios of methanol-water were used to observe the separation between metformin and IS (caffeine). We achieved the best separation with the mobile phase composition methanol and water in the ratio 80:20 (v/v) without buffers. It was observed that detection wavelength for both metformin and IS responded well at a λmax of 232 nm when scanned in various mobile phases, thus it was selected as optimum wavelength. The peak parameters such as height, asymmetry and tailing were considered while maintaining flow rate, baseline drift etc. This analytical procedure involved the use of internal standard as there was extraction in of the drug after addition of the internal standard in human saliva to account for loses if any. The uniformity of the system operation throughout the analysis was checked by initially equilibrating the column with mobile phase prior to injection of the sample to be analysed into the chromatographic system. Theoretical plates, tailing factor, resolution and repeatability were checked prior to starting analytical work every time. All the factors were found satisfactory and according to the set guidelines.[9]

The low % RSD ($\leq$ 0.70) computed shows the precision of the method. With good technique and reliable methodology, the precision should be < 15 % CV.[10] There was a reported % CV of 9.8 and 8.11 in HPLC method for determination of metformin in brain sections and plasma of rats[4]. This shows that the precision of each of the developed methods is satisfactory. The accuracy ($\leq$ 1.00) of this method expressed as the measure of percentage relative error are within the range (1 – 5 %) for moderately accurately procedure.[10] The average percentage recovery for the method was found to be 99.98 % showing that the method has good recovery especially when compared with the 98.20 % reported by Valentina P, et al.[7] in an HPLC method for the analysis of metformin in human plasma. The LOD ($\leq$ 0.22 ng/mL) and LOQ ($\leq$ 0.67 ng/mL) for the developed method are satisfactory. This shows that the method is sensitive for the analysis of the drug inhuman saliva.

CONCLUSION AND RECOMMENDATION

From the results obtained, it can be concluded that RP-HPLC method for the analysis of metformin in human saliva was developed and validated. All the validation parameters fall within the acceptable limit for analytical method; hence the proposed method can successfully be applied for quantitative estimation of metformin in pharmacokinetic, interaction and bioequivalence studies involving metformin.

CONFLICT OF INTEREST

The authors declare no conflicts of interest

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