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HPLC-UV determination of metformin in human plasma for application in pharmacokinetics and bioequivalence studies

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Abstract

In this study, a simple, rapid and sensitive HPLC method with UV detection is described for determination of metformin in plasma samples from bioequivalence assays. Sample preparation was accomplished through protein precipitation with acetonitrile and chromatographic separation was performed on a reversed-phase phenyl column at 40 °C. Mobile phase consisted of a mixture of phosphate buffer and acetonitrile at flow rate of 1.0 ml/min. Wavelength was set at 236 nm. The method was applied to a bioequivalence study of two drug products containing metformin, and allowed determination of metformin at low concentrations with a higher throughput than previously described methods.

Keywords: Metformin; Human plasma; Quantification; Reversed-phase chromatography; Pharmacokinetics; Bioequivalence

1. Introduction

Metformin is a disubstituted biguanide (*N*-1,1-dimethylbiguanide) indicated for the treatment of type 2 diabetes as an adjunct to diet and exercise, either as a single oral agent or in combination with sulfonylureas, alpha-glucosidade inhibitors, or insulin. Its adverse effects are generally tolerable and self-limiting. Although metformin's exact mechanism of action is not completely understood, its main blood glucose-lowering activity appears to be primarily through suppression of hepatic glucose output. Its therapeutic blood glucose-normalizing action is dependent on the presence of circulating insulin. Metformin has an absolute oral bioavailability of 40–60%. Gastrointestinal absorption is complete at 6 h with peak plasma concentrations reached after 2–3 h. Metformin pharmacokinetics can be described by a two-compartment open model, with a plasma elimination half-life of 2–6 h, which corresponds to rapid elimination from a central compartment (beta half-life), and a terminal elimination half-life of 8–20 h, corresponding to a slower elimination from a deep compartment (gamma half-life) [1,2].

Metformin quantification in plasma samples is required for pharmacokinetic studies [3], therapeutic drug monitoring [4], tests of new anti-diabetic drugs [5] and bioequivalence assays. Several HPLC methods have been used for this purpose. Techniques used are chromatography-tandem mass spectrometry (LC–MS–MS) [5–8], reversed-phase HPLC [9–18], ion-pair HPLC [3,4,19] and cation-exchange HPLC [20–23]. UV [3,4,9–12,14,15,17–23] including DAD [13] detection have been described. Sample pre-treatment methods include protein precipitation [3,5,11,12,14,20,21,23], solid phase extraction [4,9,15,18], metformin derivatization [13,19], ultrafiltration [22] and liquid–liquid extraction followed by back extraction [10,17].

Three criteria must be met for the plasma metformin assay to be functional within clinical laboratories: determination of metformin at low concentrations, simple, rapid and efficient sample pre-treatment and reasonable elution time [4]. These criteria are also valid for metformin quantification method for bioequivalence assays, since a very large number of samples are generated in this kind of study. Most of the above mentioned HPLC methods do not meet all three criteria. Those which allow determination of low concentrations of metformin present long elution times [13,20,21], while methods that provides adequate elution time lack sensitivity [3,11,14,15,22,23]. Some of them require complicated and time-consuming sample

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pre-treatment [4,10,13,15,17,19] and some do not use internal standards [13,14,18,22].

In this study, a simple, rapid and sensitive HPLC method with UV detection, adequate sensitivity and short elution time is described for determination of metformin in plasma samples from bioequivalence assays.

2. Experimental

2.1. Chemicals

Hydrochloric acid (analytical grade), methanol (HPLC grade), acetonitrile (HPLC grade) and phosphate buffer (analytical grade) were purchased from Merck (Brazil). Ultra-pure water was produced by distillation and additional purification with a MilliQ Academic (Millipore) system. Metformin was obtained from Shivam Chambers (India) and propranolol (internal standard, I.S.) was kindly supplied by FURP (Brazil).

2.2. Calibration standards and quality control plasma samples

Stock solutions (1000, 100, 10 and 1 μg/ml) of metformin in methanol and a stock solution (10 μg/ml) of propranolol (I.S.) in methanol were prepared and stored at −20 °C. Preparation of calibration standard plasma samples (30, 100, 250, 500, 1000, 2000 and 4000 ng/ml) was accomplished daily by introducing known amounts (15–200 μl) of metformin stock solutions (10 and 1 μg/ml) and 50 μl of I.S. stock solution in 10 ml glass tubes, evaporating it to dryness at 40 °C under a nitrogen stream and adding 500 μl of drug-free plasma. Quality control plasma samples (90, 1500 and 3500 ng/ml) were prepared in 50 ml and 100 ml volumetric balloons by spiking drug-free plasma with known amounts (90–175 μl) of metformin stock solutions (1000 and 100 μg/ml), aliquoted and stored at −20 °C.

2.3. Sample preparation

Fifty microliters of I.S. solution in methanol (10 μg/ml) were introduced in glass 10 ml tubes and evaporated to dryness at 40 °C under a nitrogen stream. Then, 500 μl of plasma and 50 μl of hydrochloric acid 0.05 M were added and vortex-mixed for 30 s. Protein precipitation was accomplished by adding 2 ml of acetonitrile. The mixture was centrifuged at 1900 × *g* for 10 min and filtered through a Durapore® membrane (13 mm 0.45 μm). The resulting filtrate was evaporated to dryness at 40 °C and under a nitrogen stream. The extracts were reconstituted with 300 μl of mobile phase and 25 μl were injected into the chromatographic system.

2.4. Chromatographic conditions

The chromatographic system consisted of a Merck Hitachi series L-7000 model including pump (L-7110), vacuum degasser (L-7612), auto injector with variable injection valve (L-7200), UV–vis detector (L-7400) and column oven (L-7300). The system was connected through a LD-7000 interface to HSM software in a computer system for data collection and processing.

Separation was performed on a 150 mm × 4.6 mm i.d., 4 μm particle size MetaSil-Phenyl column (MetaChem®). Mobile phase consisted of a mixture of phosphate buffer 0.02 M (pH 7.0) and acetonitrile (50:50, v/v); it was prepared daily and degassed before use. Flow rate and column temperature were 1.0 ml/min and 40 °C, respectively. Wavelength was set at 236 nm.

2.5. Assay performance

Assay performance was evaluated through determination of specificity, recovery, linearity, quantification limit, imprecision, accuracy and stability [24,25].

Specificity was investigated by analyzing six drug-free plasma samples (four normal plasma samples, one hemolised plasma sample and one lipemic plasma sample) for interference of endogenous compounds. Recoveries of metformin and I.S. were determined by comparing the response of pre-treated quality control plasma samples (90, 1500 and 3500 ng/ml) with the response of identical standards prepared in the mobile phase which did not undergo sample pre-treatment. The standard curve was obtained through analysis of calibration standard plasma samples and plot of peak area ratios of metformin and propranolol versus the corresponding metformin concentrations (30, 100, 250, 500, 1000, 2000 and 4000 ng/ml). Linearity of the standard curve was evaluated using least-squares linear regression analysis. Quantification limit was defined as the lowest metformin concentration that could be determined with mean value deviation and coefficient of variation less than 20%, using five plasma samples. Intra- and inter-day imprecision and accuracy were determined by repeated analysis of quality control plasma samples (90, 1500 and 3500 ng/ml) on the same day and on different days. Stability of metformin in plasma samples stored at −20 °C, in plasma samples after freeze–thaw cycles and in pre-treated plasma samples was evaluated.

2.6. Application

The method was used to determine metformin concentration in plasma samples from 24 healthy volunteers, after oral administration of a tablet containing 850 mg of metformin.

3. Results and discussion

3.1. Assay performance

The proposed method is suitable for metformin quantification in plasma samples. It showed specificity, since propranolol (I.S.) and metformin were well resolved and no interfering peaks from endogenous components of normal, hemolised and lipemic plasma were observed (Fig. 1). Retention times were 7.5 min for metformin and 9.5 min for internal standard. Average recovery was 93.7% for metformin and 112.2% for propranolol (Table 1). The method was linear over the range 30–4000 ng/ml and the calibration curve could be described by the equation $y = 0.0012\ (\pm 4.95\text{E-}5) \times -0.0667\ (\pm 0.0232)$ ($r^2 = 0.9994$). The

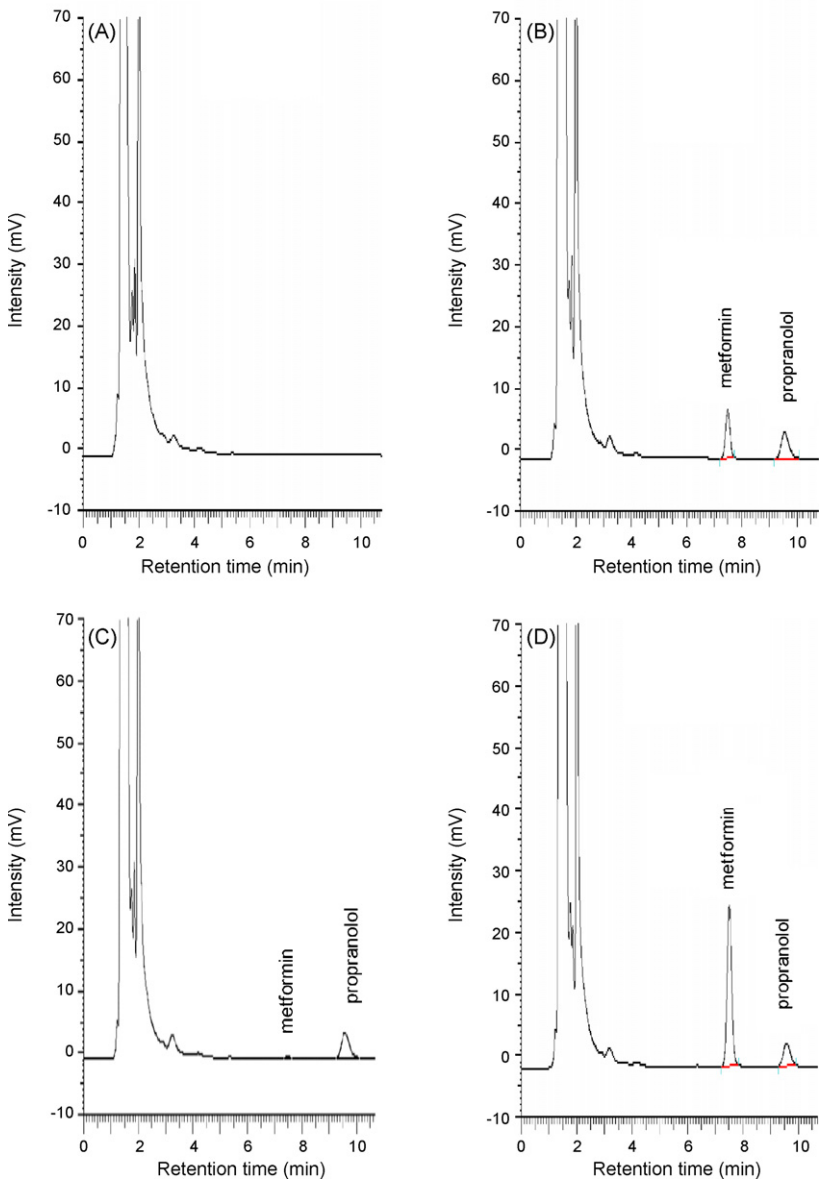

Fig. 1. HPLC-UV chromatograms obtained for blank plasma (A), blank plasma spiked with metformin 600 ng/ml + I.S. (B), blank plasma spiked with metformin 30 ng/ml (LOQ) + I.S. (C) and plasma from volunteer 2 h after oral administration of 850 mg of metformin (metformin concentration = 2258 ng/ml) + I.S. (D). Retention times are 7.5 min (metformin) and 9.5 min (propranolol, I.S.).

Table 1
Average recoveries of metformin and propranolol from plasma samples after protein precipitation with acetonitrile ($n = 6$ plasma samples at each concentration)

Concentration (ng/ml)	Recovery % (C.V.%)	
	Metformin	Propranolol
90	87.3 (2.32)	114.2 (1.14)
1500	95.7 (0.71)	112.6 (0.46)
3500	98.2 (1.52)	109.9 (2.54)
Average	93.7	112.2

C.V. = coefficient of variation.

lower quantification limit (LOQ) was 30 ng/ml, with imprecision of 8.43% (intra-day) and 11.28% (inter-day) and accuracy of 98.3% (intra-day) and 94.0% (inter-day) (Table 2). Imprecision and accuracy for quality control plasma samples ranged from 1.60 to 4.21% and from 87.0 to 103.2% (Table 2). The lower accuracy value reported for the 3500 ng/ml quality control plasma sample (Table 2) could indicate occurrence of bias, but this possibility was excluded by a residual analysis of the calibration curve. Stability of metformin in plasma samples was observed after three freeze–thaw cycles and for 236 days at −20 °C. Reconstituted organic extracts were stable for 48 h at room temperature.

Due to the high polarity of metformin, which makes it very difficult to extract it from biological fluids using organic solvents, protein precipitation is the most common method used

Table 2
Intra- and inter-day imprecision and accuracy of the determination of metformin concentration in plasma samples ($n = 5$ plasma samples at LOQ, $n = 6$ plasma samples at each quality control concentration for intra-day imprecision and accuracy and $n = 3$ plasma samples at each quality control concentration for inter-day imprecision and accuracy)

Concentration (ng/ml)	Imprecision (%)		Accuracy (%)	
	Intra-day	Inter-day	Intra-day	Inter-day
30 (LOQ)	8.43	11.28	98.3	94.0
90	1.83	4.21	93.4	103.2
1500	1.60	3.35	92.8	98.5
3500	2.85	2.99	87.0	89.3

for plasma sample preparation. It has been commented that the use of this simple and rapid procedure sacrifices sensitivity and is not effective in removing endogenous substances [4,10,22]. In order to overcome these problems, David et al. [19] and Tache et al. [13] describe methods based on derivatization reactions and Amini et al. [10] and Keal and Somogyi [17] describe liquid–liquid extraction procedures followed by back extraction, but these are too complicated and time-consuming for application in bioequivalence assays. In the present study, protein precipitation with acetonitrile was found to be highly effective in removing endogenous substances, since all of such components had retention times lower than 4 min and did not interfere with metformin or propranolol (I.S.) (Fig. 1). It also produced higher average recoveries for the analyte and higher sensitivity than previously described methods which used liquid–liquid extraction followed by back extraction [17], ultrafiltration [22], ion-pair solid phase extraction [9] or solid phase extraction [15] for sample preparation.

This method has an elution time of 10 min per plasma sample, that is shorter than those of others methods with similar sensitivity [13,20,21].

Short elution time, good separation between metformin, propranolol and endogenous substances and baselines with low background (Fig. 1) were accomplished by using a phenyl chromatographic column. C18 and C8 analytical columns were also tested in the development of the method but the results were not satisfactory, since metformin retention volume was very close to the column dead volume and no separation could be achieved. The phenyl column has an increased ability to interact with the polar metformin moiety, which allows higher resolution volumes and superior separation performance for metformin when compared to C18 and C8 columns [26]. This could be explained by different mechanisms of retention of C8, C18 and phenyl columns. Retention of solutes on the C8 and C18 stationary phases results from partitioning mechanisms, since these groups work as a highly hydrophobic organic phase in liquid–liquid equilibrium. The retention mechanism of the phenyl column is mainly based on partitioning when a methanol–water mobile phase is used, but also based on adsorption when an acetonitrile–water mobile-phase is used, as in the case of the proposed method [27]. Due to its high polarity, metformin does not interact with the hydrophobic phase in C8 and C18 chromatographic columns, but it is adsorbed by the phenyl column stationary phase. Van de Merbel et al. [18], however, have a different explanation for metformin retention in phenyl columns. These authors state that, as metformin is positively charged at pH 7.0, its retention is not caused by reversed-phase interactions, but by interactions with free silanol groups which are apparently more available in phenyl columns than in C18 columns.

The proposed method allows determination of metformin at lower concentrations than previously described methods, with a simple, rapid and efficient sample pre-treatment and has short elution time, thus fulfilling all required criteria for the plasma metformin assay to be considered functional in pharmacokinetic and bioequivalence studies in healthy volunteers.

3.2. Application

Mean plasma concentration–time curve of metformin, after oral administration of 850 mg to 24 healthy volunteers is shown in Fig. 2. Bioequivalence studies protocols generally recommend plasma sample collection for a time period corresponding to three to four times the drug plasma elimination half-life [28], which brings terminal concentrations values of about 6% of the peak concentration value; for metformin, mean peak plasma concentration of about 1700 ng/ml (Fig. 2) will produce plasma concentrations after a time period corresponding to four metformin half-lives of approximately 100 ng/ml. Since the method LOQ was 30 ng/ml, its sensitivity is adequate for bioavailability studies.

In the present study, plasma elimination half-life ranged from 1.6 to 9.8 h, with a mean value of 3.8 h. Most subjects had plasma elimination half-life below 4.0 h and had metformin plasmatic concentrations below the method LOQ 24 h after drug administration. Few subjects, which showed elimination half-life values greater than 4.0 h, had detectable metformin plasmatic concentrations 24 and 36 h after drug administration. Area under plasmatic concentration time curve from 0 to 36 h ($AUC_{0–36h}$) and from 0 h extrapolated to infinity ($AUC_{0–\infty}$) were calculated and the ratio $AUC_{0–36h}/AUC_{0–\infty}$ was higher than 85% for all subjects, as recommended by FDA guidelines [28].

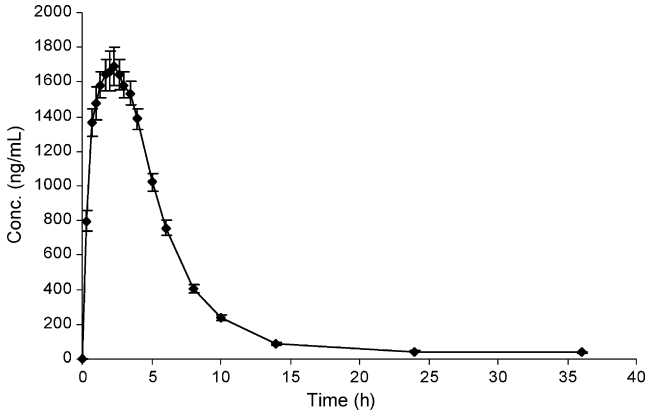

Fig. 2. Mean plasma concentration–time curve of metformin, after oral administration of 850 mg to 24 healthy volunteers. Vertical bars indicate mean standard error.

4. Conclusion

The method has proven to be simple, specific, sensitive, precise and accurate and is suitable for metformin quantification in plasma samples from bioequivalence, bioavailability and pharmacokinetic studies in healthy volunteers. It was successfully applied to a bioequivalence study of two drug products containing metformin.

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