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Simple and rapid method determination for metformin in human plasma using high performance liquid chromatography tandem mass spectrometry: Application to pharmacokinetic studies

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Abstract

A rapid and simple method for quantitation of metformin (MET) in human plasma by HPLC–MS/MS was developed and validated. The sample preparation consists of plasma deproteinization using acetonitrile. The mobile phase consisted of water–acetonitrile and formic acid (55/45/0.048, v/v/%) and the run time was 3 min. A pursuit C_{18} (100 mm × 2.0 mm i.d., 3 μm) column connected to a guard column MS-pursuit (0.20 mm × 0.20 mm i.d., 5 μm) was used. The range of the calibration curve was from 20 to 5000 ng/mL, the limit of quantitation being 20 ng/mL. The detection was performed on a mass spectrometer (ESI^+), using metoprolol as internal standard. The calibration curves have r^2 values of 0.995 (CV = 0.24%, n = 10). The accuracy and precision were between 90.74 and 106.7% and coefficients of variations (CV) of 1.10 and 4.35%, respectively. The method was applied to determine the pharmacokinetic parameters: C_{max} (1667.25 ng/mL) and T_{max} (3.89 h).
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1. Introduction

Metformin (Metformin HCl, *N*,*N*-dimethylimidodicarbonimidic diamide hydrochloride, MET, Fig. 1) is prescribed as a drug that contains antihyperglycemic properties. This drug was approved by the FDA in December 1994 and has been the only clinically available drug that can significantly improve insulin sensitivity in patients that suffer from Diabetes type II (non-insulin dependent). Typically Metformin reduces basal and postprandial hyperglycemia by about 25% in more than 90% of the patients. The usual dosage is 500 mg per tablet. Measuring the plasma concentration of MET is important for studying the pharmacokinetics of this drug to determine diabetic patient adherence with prescribed therapy and for general drug monitoring.

The metabolism of MET has also been documented by researchers and linked to specific areas of clinical research. Gastrointestinal absorption of MET is incomplete with an absolute bioavailability of 40–60% (under fasting conditions) [1,2] in combination with rapid elimination and 20–30% of an oral dose is recovered in feces [3,4]. Gastrointestinal absorption decreases as the MET dose increases, suggesting some form of saturable absorption or permeability/transit time-limited absorption [2] and the negligible hepatic metabolism of MET as happens in humans [4]. Side effects and the need for administration two to three times a day for larger doses can also reduce patient compliance and hinder successful therapy. Administration of a sustained-release, once-a-day dosage form could reduce the dosing frequency and improve patient compliance.

Various methods for determination and quantitation of MET in biological samples have been published [5–16]. However, most of them have a complex sample preparation. Nevertheless, with the advent of more specific and sensitive technique such as quadrupole MS/MS and ion trap $MS^{\wedge n}$, the use of more simple

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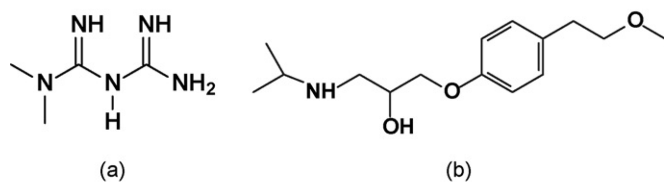

Fig. 1. Chemical structures of (a) MET and (b) metoprolol.

extraction and purification procedures such as protein plasma precipitation and liquid–liquid extraction (LLE) have become an alternative approach.

Although solid phase extraction (SPE) yields a more pure extract, the procedure is time consuming and depends of the kind of sorbent, choice of suitable solvent and pH conditions. The usual methods are precipitation [5–11], liquid–liquid extraction [12] or solid phase extraction [13–15]. Therefore, the actual trends have an approach that follows these kinds of procedure. However, liquid–liquid extraction generally involves several steps, including evaporation, which is time consuming and has an important effect on the yield. On the other hand, the precipitation procedures are simple and fast and achieve the same sensitivity level as standard LLE and significantly improve laboratory productivity and accuracy.

It is important to point out that precipitation could yield a final extract that causes ionic suppression as observed by Zhong and co-workers [11] and Gu et al. [7]. These authors described the use of APCI and a washing step after the denaturizing plasma step to solve this problem, in spite of this; they have used a high selective detection technique such as HPLC–MS/MS.

Therefore, the main purpose of this study was to develop and validate a rapid, simple and effective method for the extraction of MET from human plasma. To do this, we used mass spectrometry on an ESI source without decreasing the signal intensity or the ion suppression effect from endogenous components, as observed by Zhong and co-workers [11], and demonstrated good linearity (20–5000 ng/mL).

2. Experimental

2.1. Chemicals and reagents

Metformin hydrochloride reference standard (99.99%) was obtained from European Pharmacopeia (order code M0605000). Metoprolol (99.99%) was kindly donated by the INCQS (National Institute of Quality Control in Health) (Rio de Janeiro, Brazil) (Fig. 1). All HPLC solvents and reagents were HPLC grade and were purchased from Tedia Company, Inc. (Fairfield, OH, USA). The water was obtained by double-distillation, and purified additionally with a Milli-Q system®. Formic acid was purchased from Merck (Darmstadt, Germany).

2.2. Standards solutions

Stock solutions of 1 mg/mL MET and 1 mg/mL metoprolol were prepared as free base in Milli-Q water–methanol (50/50, v/v) and stored at −22 °C. Standard solutions of MET were obtained from stock solutions by serial dilutions with Milli-Q water–methanol (50/50, v/v). All solutions were prepared in glass volumetric flasks. They were used to spike the blank plasma samples prior to extraction.

2.3. Blank plasma

A pool of blank plasma samples from healthy volunteers was used for the validation of the method. They were kindly donated by HEMORIO (RJ, Brazil).

2.4. Sample extraction

50 μL of working solution MET (analyte) (appropriate concentration) and 50 μL of working solution Metoprolol (100 ng/mL) were added to 200 μL of blank plasma sample. The sample mixture was vortexed (Phoenix, SP, Brazil) for 10 s. Then, 40 μL of 0.5 M sodium hydroxide was added to achieve pH 12, and vortex-mixed for an additional 10 s. The 600 μL of acetonitrile was added to precipitate protein and the tube was capped and vigorously vortexed for 1 min and centrifuged at 19,975 × *g* for 5 min. The liquid phase was transferred to another tube (1.5 mL). Then, 100 μL of methanol–water (50/50, v/v) and 120 μL of 0.1 M HCl was added to the supernatant and the mixture was votexed for 30 s. The pH of this mixture was 2.0. To the final extract, we added 10 μL of 1 M ammonium hydroxide obtaining a pH 9. The mixture was again vortexed for 10 s. Finally, 20 μL of each sample was injected into the HPLC–MS/MS system.

2.5. Apparatus and chromatographic conditions

The analyses were performed on a Varian® Prostar HPLC system model 210, connected to a Varian® Prostar Autosampler Model 410, and coupled to a Varian® quadrupole MS/MS 1200 L Mass Spectrometer with an ESI source. The HPLC system was operated using an isocratic conditions on a Varian® pursuit C_{18} (100 mm × 2.0 mm i.d., 3 μm) column connected to a Varian® guard column MS-pursuit (0.20 mm × 0 20 mm i.d., 5 μm). The mobile phase consisted of a mixture of water–acetonitrile and formic acid (55/45/0.048, v/v/%) at a flow rate of 0.3 mL/min. The autosampler temperature was 25 °C and the column temperature was 30 °C.

Mass spectrometer operating conditions were: ionization positive mode, ESI; scan mode, centroid; detector, 2000 V; needle, 3000.0 V; shield, 600 V; capillary collision-induced dissociation (CID) for MET (m/z 130.1 → m/z 71.0) and metoprolol (m/z 268.2 → m/z 116.0) were −18.0 and 14.0 V, respectively. Manifold temperature, 42 °C; API house temperature, 45 °C and drying gas, 350 °C. Nitrogen pressures: main gas, 67.8 psi; drying gas, 24.2 psi; nebulizing gas, 52.7 psi. For CID, argon was used as the collision gas at a back-pressure of approximately 2.59 mTorr. Data acquisition was performed with MS Workstation (upgrade) SP1 Version 6.5 software (Varian®).

2.6. Ion suppression–matrix effect

One of the best tools for observing matrix effects is the post column infusion technique [17,18] (Fig. 2). The analyte of interest (100 μg/mL) was infused at a steady rate (10 μL/min) into the HPLC eluant stream which was the same as that used in the routine analysis of MET, i.e. water–acetonitrile and formic acid (55/45/0.048, v/v/%) at 0.3 mL/min that was directed into the MS system. At the same time, a control sample blank extract (matrix) was injected and the LC–MS assay was conducted while the standard mixture was infused (Fig. 2). The same experiment was done using a mobile phase instead of blank extract. By comparing the two results, it was possible to assess the effect of ion suppression on MS/MS. In this system, any ion suppression would be observed as a depression of the MS signal (Fig. 3).

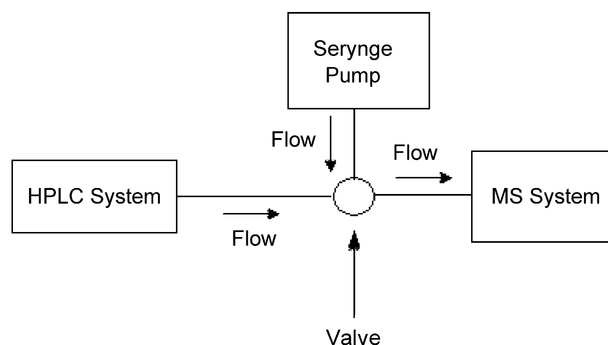

Fig. 2. The procedure set for conducting the post-column infusion experiment to detect matrix effects in an HPLC–MS–MS assay.

Fig. 3. Chromatograms of MET in plasma: (a) limit of quantitation of MET, (b) metoprolol, internal standard, (c) blank for MET and (d) blank for metoprolol, internal standard.

2.7. Validation procedure

The method was submitted to validation procedures, parameters and acceptance criteria based on guidance for bioanalytical method validation for human studies [19], and recommendations of Shah et al. [20], Causon [21] and Wieling et al. [22].

2.7.1. Calibration curve and linearity

A standard calibration curve consisted of nine concentration points of 20, 30, 50, 100, 200, 500, 1000, 2000, 4000 and 5000 ng/mL of MET, which were prepared on each day of the 5-day validation. Each concentration point was performed in duplicate. The calibration curve was prepared by fortifying a drug-free poll of plasma aliquots with a pre-determined MET concentration and metoprolol in a single concentration at 100 ng/mL. The calibration curve was constructed by plotting peak area ratio (y) of MET to the internal standard versus MET concentrations (x). Slope, intercept and correlation coefficient were calculated as regression parameters by a weighted linear regression model ($W = X^{-1}$).

2.7.2. Intra- and inter-day accuracy and precision

Assay precision and accuracy were evaluated intra- and inter-day by determining MET concentrations in eight replicates of quality control samples (QCs) at four different concentrations (20, 30, 2000 and 4000 ng/mL) daily for three separate days. These four concentrations cover the LOQ, low, medium and high range of the standard curve. Each run consisted of calibration standards, blank plasma samples with and without internal standard in duplicate and QC samples in eight replicates.

The accuracy of the method was determined as relative error in percentage ((difference between the mean calculated and added concentration)/added concentration) × 100, while precision was evaluated by intra- and inter-day coefficients of variations (CV, %). A precision (CV, %) and accuracy (RE, %) $\leq$15% for all QCs tested with exception of that related to the LOQ ($\leq$20%) are acceptable [18–21].

2.7.3. Limit of quantitation

The limit of quantitation (LOQ) was evaluated by spiking plasma with MET at a single concentration of 20 ng/mL ($n = 8$) and metoprolol at 100 ng/mL. The LOQ was determined in three different days (inter-day assay).

2.7.4. Recovery

The recovery of MET was determined by comparison of peak areas from blank human plasma fortified prior to the extraction procedure (addition of extraction solvent) and fortified after the extraction procedure, which represents 100% recovery. With this procedure we could evaluate, quantitatively the percentage of analytes extracted from plasma matrix. Recovery of MET was determined from plasma samples spiked with three different concentrations (30, 2000 and 4000 ng/mL ($n = 5$ at each concentration)). The recovery of the internal standard (metoprolol) was determined at the concentration of 100 ng/mL processed according to the described method.

2.7.5. Specificity

The specificity of this method in relation to endogenous plasma components was evaluated by analysis of a series of randomly selected blank plasma samples, such as hemolyzed ($n = 2$), lipemic ($n = 2$) and normal ($n = 4$). The contribution of endogenous matrix compounds and/or matrix effect was also investigated comparing the signal for analyte and IS in these blank samples with the signal obtained from analysis of hemolized, lipemic and normal plasma spiked with a concentration of analyte at limit of quantitation (20 ng/mL) and IS at the working concentration (100 ng/mL).

2.7.6. Stability studies

All of the stability studies performed were carried out using control human plasma samples spiked at 30, 2000 and 4000 ng/mL of MET. Each determination was performed in five replicates. The mean (±S.D.) and CV (%) values of the ratios between the five measurements were determined. The degradation of MET was evaluated by comparison of the results obtained in each study of stability with those observed from the analysis of fresh spiked samples at the same concentration. The results were expressed as mean variation.

The stability of MET in plasma was studied at room temperature and at −22 °C. The short-term stability in plasma was assessed at room temperature over the period needed to process a batch of study samples (6 h). The long-term stability of MET in human plasma stored at −22 °C was determined at 0, 01, 02, 04, 08, 14 and 77 days. Samples were analyzed immediately after preparation (reference values) and after storage. Prior to their analyses, samples were brought to room temperature and vortex-mixed well.

The three freeze-thaw stability was also determined. Spiked plasma was analyzed immediately after preparation and after repeated freeze (−22 °C) thaw (25 °C) cycles on three consecutive days. The stability of MET in extracted samples on an autosampler (25 °C) was also determined over the period needed to inject a batch of study samples (24 h).

2.8. Pharmacokinetic study

The study was conducted according to a single-dose, randomized, two-way crossover design and the washout period was 1 week between the treatments of the study. After a single oral administration of 850 mg of MET Hydrochloride® tablet reference drug (Glifage, Merck, Brazil) and test drug according the protocol assay, blood samples (10 mL each) were collected in vacutainers tubes. Following centrifugation at 19,975 × g for 15 min, the plasma was pipetted into polypropylene tubes and stored at −22 °C until analysis.

3. Results and discussion

3.1. Chromatography and specificity

HPLC analysis of hydrophilic substances, such as MET, in human plasma requires a very sensitive method, since the limit of quantitation is 20 ng/mL. On the other hand, for pharmacoki-

Table 1
Reported HPLC methods for the determination of MET in human plasma

Reference	Column	Mobile phase	Analyte and internal standard (Rt, min)	Flow (mL/min)	Detection (linearity)	Extraction	Volume injected (μL)	Plasma volume (mL)	LOQ (ng/mL)
[5]	Silica	25% ACN, 75% pH 7, 0.03 M $(NH4)_2HPO_4$	MET (6,8) Atenolol (7,8)	1,0	UV (240 nm) 10–2000 ng/mL	Protein precipitation (1.5 mL ACN/1 M HCl), washing with 1.5 mL of CH_2Cl_2	–	0,5	–
[6]	C18 Ion pair	Methanol, 0.05 mol/L $(NH_4)H_2PO_4$ and sodium octanesulfonate, 0.01 mol/L (35/65, v/v)	–	1,0	UV (233 nm)	Protein precipitation (ACN), washing CH_2Cl_2	20,0	–	–
[7]	C_{18} (Nucleosil)	AcetonitrileL:Methanol:10 mM ammonium acetate (20:20:60, v/v/v)	MET (1,20) Phenformin (2,04)	0,65	MS (APCI)	Protein precipitation (ACN), washing CH_2Cl_2	20,0	0,1	1,0
[8]	Ciano	Potassium dihydrogen orthophosphate and acetonitrile (60/40, v/v)	None	1,0	UV (234 nm)	Protein precipitation (HClO4)	50,0	0,25	62,5
[9]	μBondapak C_{18}	Sodium dodecyl sulphate 0.01 M, sodium dihydrogen phosphate 0.01 M, acetonitrile 40%	MET (3,4) Phenformin (4,5)	1,5	UV, 235 nm (100–4000 ng/mL)	Protein precipitation (300 μL of CAN)	100,0	0,2	200,0
[10]	Cation exchange column (nucleosil)	Tetramethylammonium phosphate, buffer pH 3.7 and acetonitrile (80/20, v/v)	MET (9,0) Buformin (15,0)	1,0	UV, 236 nm (20–4000 ng/mL)	Protein plasma precipitation (ACN)	50,0	0,2	20,0
[11]	C8	Acetonitrile:water:formic acid (70:30:1, v/v)	MET (2,63) Diphenhydramine (3,10)	0,5	MS (APCI) *m/z* 130 → 60 (2–2000 ng/mL)	Protein plasma precipitation (400 μL ACN)	20,0	0,250	2,0
[12]	Silica	Acetonitrile, 40 mM aqueous sodium dihydrogen phosphate (25:75, v/v), pH 6	MET (5,796) Ranitidine (7,677)	1,0	UV (234 nm) 15,6–2000	1-Butanol/*n*-hexane (50:50, v/v) in alkaline condition LLQ, back-extraction CH3COOH 1%	50,0	0,1	15,6 (s/n = 3)
[13]	C_{18}	Sodium dodecyl sulphate 0.002 M, potassium dihydrogen phosphate and acetonitrile 37.5%	MET (4,85) Phenformin hydrochloride (13,2)	1,0	UV (50–2000 ng/mL)	Ion pair solid phase extraction	–	1,0	5,0
[14]	Cation exchange column	Acetonitrile/methanol/water and ammonium acetate	[D6] MET	1,0	MS (ESI) (10–1000 ng/mL)	Cation exchange solid phase extraction	50,0	0,2	10,0
[15]	–	20 mM NH_4OAc and 5% HAc in ACN as the running buffer, pH 5.1	MET (2,48 min) Phenformin (3,47 min)	–	CEUV, 240 nm (200–3500 ng/mL)	Solid phase extraction (C_{18}) elution with methanol + 3% acetic acid	–	0,5	200,0
[16]	Cation exchange column	0.4 mol/L ammonium acetate	–	2,0	UV (232 nm)	Ultrafiltration (30 min)	50,0	0,3	50,0 (s/n = 10)

netics and bioequivalence studies, the high number of samples to be analyzed makes a rapid method necessary. Therefore, the HPLC conditions have to be optimized to reach both of those aims.

As described before, MET is very hydrophilic which makes its analysis using HPLC difficult. To solve this problem, previous studies have used normal column or ion exchange column (Table 1). The disadvantages are the long run time (>5 min) and/or the derivatization step before analysis and, in some cases, the need for a column-switching system.

Recently, Zhong and co-workers [11] reported that MET was chromatographed on a C_8 column with a retention time of 2.63. The mobile phase consisted of acetonitrile–water–formic acid (70:30:1, v/v) at a flow-rate of 0.5 mL/min. Gu et al. [7] showed that MET could be fast eluted (1.2 min) using a more elaborate mobile phase consisting of acetonitrile:methanol:10 mM ammonium acetate pH 7.0 (adjusted with triethylamine) (20:20:60, v/v/v) delivered at 0.65 mL/min. Both studies employed HPLC/MS/MS using atmospheric pressure chemical ionization (APCI) in the positive ion mode to decrease ion suppression effects observed when using ESI^+ mode.

In the present study, the system was operated using isocratic conditions on a pursuit C_{18} (100 mm × 2.0 mm i.d., 3 μm) using a very simple mobile phase consisted of milli-Q water–acetonitrile and formic acid (55/45/0.048, v/v/%) at a flow rate of 0.3 mL/min. The representative chromatograms of HPLC/MS–MS (ESI^+) analysis of the samples plasma spiked with MET and internal standard and of the blank plasma are shown in Fig. 3. There are no chromatographic peaks interfering with either the MET or the internal standard. With the sample processing and chromatographic conditions described, MET and the internal standard peaks are very symmetric, with retention times of 1.08 and 1.27, respectively.

Fig. 4 shows the fragmentation pattern mass spectra of MET obtained by HPLC–MS/MS–MS (ESI^+ mode). The fragmentation of MET had two very abundant ions *m/z* 60.0 and 71.0 (Fig. 5), which could be assigned to the β-cleavage at the N–R bond with rearrangement of one or, especially, two hydrogen atoms (*m/z* = 60) as shown in Fig. 5. The fragment *m/z* 71.0 has a more symmetric shape and the less line background effect. Therefore, the ion 71.0 was chosen to MET identification and quantitation.

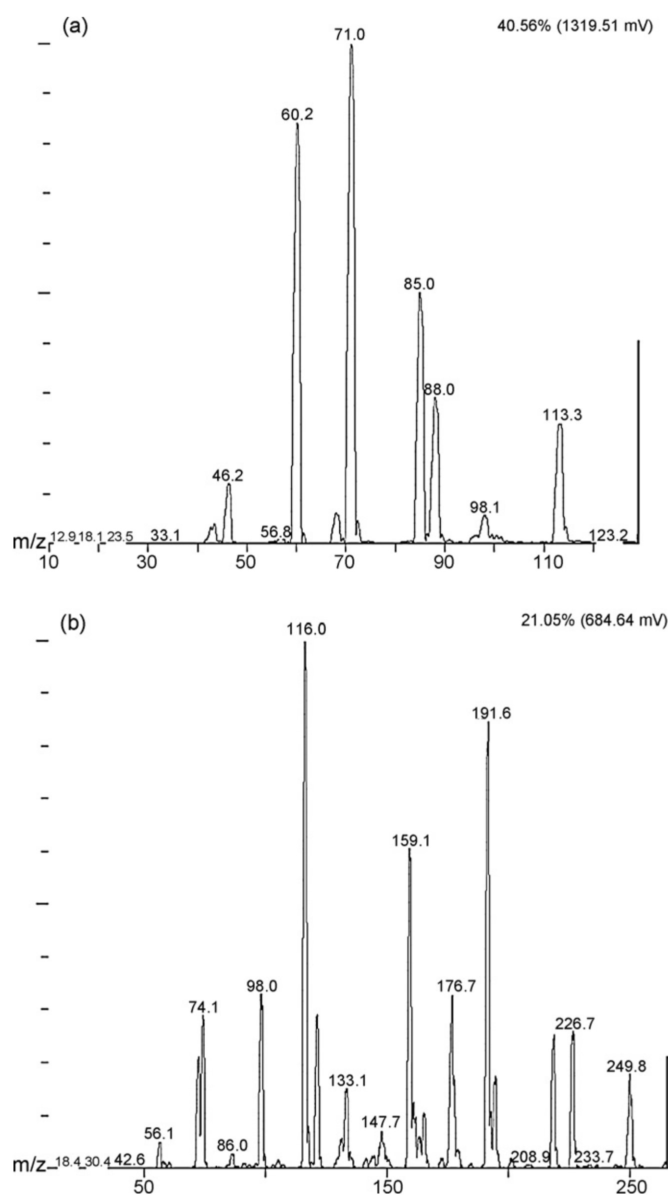

Fig. 4. Absolute breakdowns of (a) MET and (b) metoprolol.

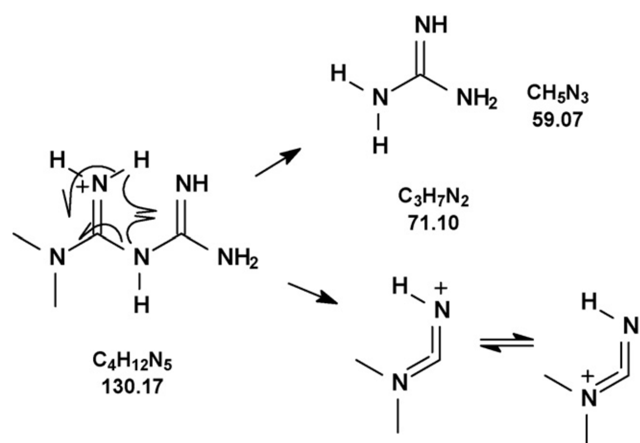

Fig. 5. Mechanism proposed to formation of monitored MET ion product *m/z* = 71.0.

3.2. Sample extraction procedure

Table 1 listed some data from the methods described in the current literature applied to MET determination and quantitation. The major researchers agree that the main problem of MET analysis from biological samples is due to its high polarity (octanol/water partition coefficient 0.01), which makes it difficult to extract the drug directly from biological fluids by organic solvent even in a very strong sodium hydroxide solution (1 M). To solve this problem Song et al. [23] developed a method based on the extraction of MET with Bromothymol Blue (as an ion pair) followed by a back extraction in the aqueous phase. However, they observed that for the ion-pair extraction using a medium polar organic solvent such as chloroform, the final

extract was not pure due the co-extraction of salts and some endogenous ionic substances.

Lai and Feng [15] have described a method using ion pair solid phase extraction (IPSPE) (Table 1), which, in spite of the high recovery, has not shown to be satisfactory when applied to patient samples as part of a medication adherence study. Firstly, the authors pointed out many reasons for these undesired results. Although the procedure for sample preparation and solid phase extraction were simple in comparison to many published methodologies, it was still not convenient enough for routine clinical use. Additionally, the requirement of solid phase extraction can be associated with high cost when used for routine clinical assessment.

Considering that the MET is very hydrophilic, liquid–liquid or solid phase extraction have some disadvantages. Our method use protein precipitation with acetonitrile.

Matrix ion suppression (reduced response) or ion enhancement (increased response) (also known as 'matrix effects') makes it a problem on LC–MS assays and depends of the extraction and/or clean-up procedure used to obtain the final extract. Therefore, during this method development, we adopted some approaches not only to check matrix effect but to avoid it, with the aim to minimize any change in the MS response of the analyte and IS that could be a result of the specific matrix of the sample being assayed (final extract).

The first approach adopted in present study was to select acetonitrile (100%) as a protein precipitant as described by Chen et al. [11]. However, the results showed that the analyte was not efficiently extracted from plasma. Another disadvantage was the matrix ion suppression observed. Wang et al. [7] reported a more complex procedure to extract MET, which consists of protein plasma precipitation using high amounts of acetonitrile at low pH followed by washing with 2 mL of dichloromethane. The authors have to use APCI in positive mode to decrease ion suppression as adopted by Zhong and co-workers [11].

Therefore, in this study it was decided to optimize the precipitation procedure without the washing step. For that, the precipitation step was performed using a higher ratio of ACN (dipolar–aprotic solvent) to plasma volume (3:1) at a pH near the pK_a of MET, which is enough to discard the majority of the protein present in plasma samples. The addition of hydrochloric acid decreases the pH, which was sufficient to protonate MET. The addition of NH_4OH solution maintained the pH near 9 ($<pK_a$ 12.4), with the aim to preserve a balance between the MS response and liquid chromatographic behavior. At this pH, the concentration of the charged analyte in the solution was enough to improve the MS performance and the chromatographic behavior. No peak tailing interactions with the HPLC system was observed, providing the best peak shape. With the improved sample clean-up procedure, the analytical column exhibited excellent stability. After more than 2600 injections the column keeps on very good condition.

3.3. Ion suppression–matrix effects

The ratio of acetonitrile/plasma (3:1) at alkaline pH allowed us to analyze MET by ESI^+ mode without employing APCI (Fig. 6). In spite of the fast run chromatography (less than 3 min), was not necessary to improve the HPLC conditions to select the region in chromatogram without matrix effect as could be seem in the background of chromatograms illustrated on Fig. 3.

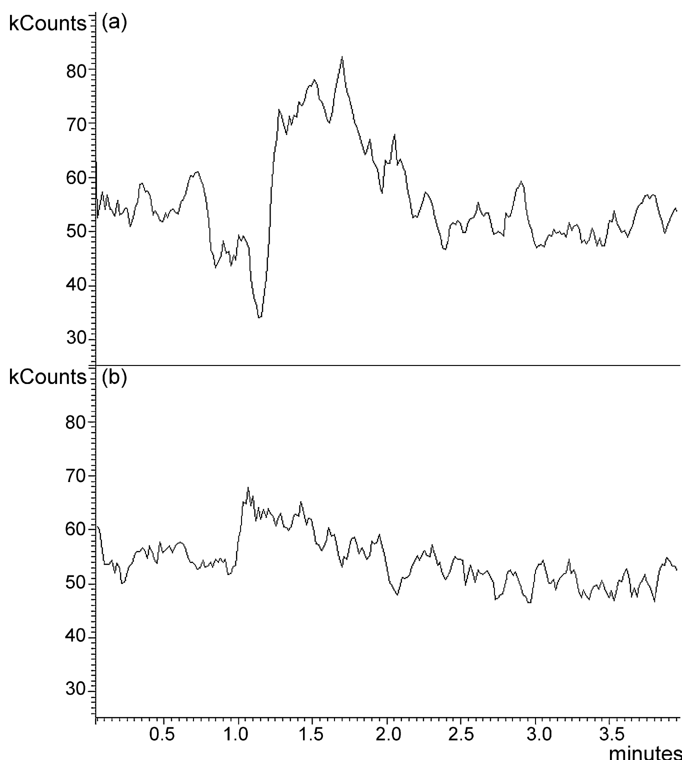

Fig. 6. Chromatograms of plasma blank and blank solvent (mobile phase) while infusing analyte through the syringe pump.

3.4. Linearity

Phenytoin, nadolol and metoprolol were evaluated as internal standards. The obtained results (not shown) showed that metoprolol gave better results.

The calibration curves were prepared over the concentration range of 20–5000 ng/mL of MET in human plasma. Regression analysis of the correlation between the chromatographic peak area ratios of MET/internal standard (metoprolol) versus known concentrations of MET yielded linear correlation over the concentration range analyzed. The corresponding mean (±S.D.), coefficients of correlation (r^2) for the curves prepared on the same day ($n = 2$) were $0.9942 \pm 4.89 \times 10^{-3}$ (CV = 0.49%), and curves prepared on different days ($n = 10$) were $0.9969 \pm 2.25 \times 10^{-3}$ (CV = 0.24%) used to validate the method. The regression parameters (coefficients from the curve estimation) to intra- and inter-day were $y = 0.0127x - 0.0796$ and $y = 0.013x + 0.0645$, respectively. The accuracy of fit was evaluated by means of analysis of variance (F_{test}, $\alpha = 0.05$) [20], the F_{table} were higher than $F_{calculated}$ for all calibration curves.

For concentration of calibration standards ranging from 20 to 5000 ng/mL, the precision around the mean values did not exceed 15% coefficient of variation.

Table 2
Intra and inter day precision and accuracy of MET in human plasma

Quality control ($n = 24$)	Concentration (ng/mL)	Inter day assay			Intra day assay		
		Mean	Precision (CV, %)	Accuracy (%)	Mean	Precision (CV, %)	Accuracy (%)
LLOQ	20	18.23	1.10	91.16	18.38	12.02	91.91
QC-Low	30	27.22	4.35	90.74	27.10	7.68	90.32
QC-Middle	2000	2133.92	4.08	106.70	2038.96	7.52	111.96
QC-High	4000	4136.68	3.65	103.42	3967.32	5.67	99.18

Table 3
Absolute and relative recovery of the analytical methods

Quality control ($n = 5$)	Absolute recovery (MET)		Relative recovery (MET)		Absolute recovery (MTP)	
	Recovery (%)	Precision (%)	Recovery (%)	Precision (%)	Recovery (%)	Precision (%)
QC-Low (30 ng/mL)	96.69	15.24	93.75	1.88	103.38	17.07
QC-Middle (2000 ng/mL)	88.80	6.35	92.00	5.38	96.98	11.65
QC-High (4000 ng/mL)	77.37	11.07	88.95	1.66	87.11	12.35

3.5. Precision and accuracy

Intra- and inter-day precision and accuracy of the method, assessed by analyzing quality control samples (20, 30, 2000 and 4000 ng/mL), are given in Table 2. The following validation criteria for precision and accuracy were used to assess the suitability of the method: the precision determined at each concentration level should not exceed 15% coefficient of variation except at the limit of quantitation where it should not exceed 20%; accuracy should be within 85–115% except at the limit of quantitation where it should be within 80–120%. As shown in Table 2, the intra-day precision was between 5.67 and 12.02% over 20, 30, 2000 and 4000 ng/mL concentration range of MET, and the corresponding accuracy varied from 90.32 to 111.16%. The inter-day precision was between 1.10 and 4.35% over the 20–4000 ng/mL quality controls concentration of MET, and the corresponding accuracy varied from 90.74 to 106.7%. The results demonstrate that the method has good reproducibility and accuracy. The precision and accuracy at the evaluated concentrations (20, 30, 2000 and 4000 ng/mL) were acceptable in view of international recommendations [18–21].

3.6. Recovery

The mean of absolute and relative recoveries of MET in plasma samples after extraction procedure are showed in Table 3.

3.7. Limit of quantitation

The criteria for the determination of the limit of quantitation of MET in plasma was based on a signal-to-noise ratio at least five times greater than any interference in blanks at the retention time of the analyte. The limit of quantitation was 20 ng/mL. At this level, the mean concentration found was 18 ± 2 ng/mL (CV was 12.02% and accuracy was 91.91%) (Table 2). These parameters were well within the acceptance criteria of the accuracy of 80–120% and precision of 20% for the limit of quantitation at a signal-to-noise ratio (s/n) of 756.

3.8. Stability studies

Stock solutions of MET (1 mg/mL) and internal standard (1 mg/mL) were stable at −20° C for at least 6 months.

Stability of MET in human plasma was studied under different conditions at three concentrations (30, 2000 and 4000 ng/mL) and compared with data obtained from freshly prepared samples. Results of stability assays of MET in human plasma are showed in Table 4.

3.9. Application to pharmacokinetic study

The present method was applied to pharmacokinetic study in twenty-six healthy volunteers who orally received 850 mg

Table 4
Results of long-term stability tests and stability in freeze-thaw cycles

Analyte	Stability in freeze-thaw cycle		Experimental concentration in plasma sample (ng/mL)						Variation of mean (%)
	Amount	Conc. (ng/mL)	Fresh prepared (0 h)			Prepared after 1848 h			
			Mean	S.D.	Precision (CV, %)	Mean	S.D.	Precision (CV, %)	
Metformin ($n = 5$)	QC-Low	30	30.18	2.75	9.1	26.8	0.74	2.78	11.2
	QC-Middle	2000	1983.72	85.67	4.32	1881.8	101.7	5.4	5.14
	QC-High	4000	4052.37	167.8	4.14	4234.8	118.4	2.79	−4.5

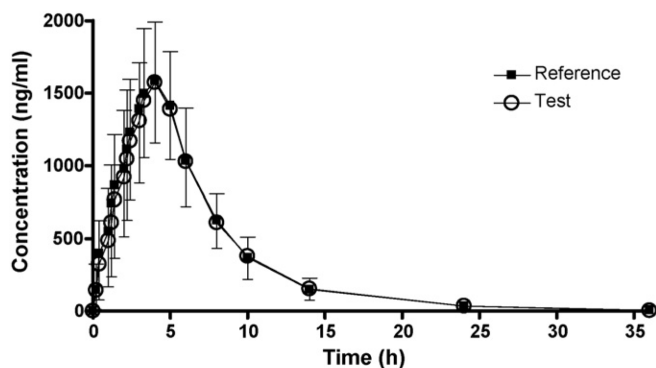

Fig. 7. Mean plasma concentration–time profile of MET after a single oral dose of a tablet of MET hydrochloride (850 mg) to healthy volunteers. Data are mean ± S.D. ($n = 26$) of the reference medicine and the test over the time.

of MET. A representative plasma concentration–time curve is shown in Fig. 7. The method was clearly adequate for monitoring plasma concentration profiles of MET during the 24 h sampling period.

4. Conclusion

The developed method proved to be useful and reliable for the determination of MET in human plasma. The results demonstrated that the pre-treatment procedure is simple, rapid and specific, avoiding degradation of the drug and matrix effects. This method was validated for a concentration ranging from 20 to 5000 ng/mL and has a good reproducibility and accuracy and is useful for clinical therapeutic drug monitoring and for bioequivalence studies.

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