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Simultaneous assay of metformin and glibenclamide in human plasma based on extraction-less sample preparation procedure and LC/(APCI)MS

Cristina Georgita [a], Florin Albu [a], Victor David [b], Andrei Medvedovici [a,b,*]

[a] *S.C. Labormed Pharma S.A., Splaiul Independentei No. 319E, Bucharest 060044, Romania*
[b] *University of Bucharest, Faculty of Chemistry, Department of Analytical Chemistry, Sos. Panduri, No. 90, Bucharest 050663, Romania*

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Abstract

Separation of metformin and glibenclamide was achieved within a single chromatographic run on a Zorbax CN column, under isocratic conditions, using acetonitrile and aqueous component (0.01 moles/L ammonium acetate adjusted at pH 3.5 with acetic acid) in volumetric ratio 1/1. Plasma sample preparation is based on protein precipitation by means of organic solvent addition. 1,3,5-Triazine-2,4,6-triamine (IS1) was used as internal standard for metformin, while gliquidone (IS2) played the same role for glibenclamide. Detection was performed with an ion trap mass analyzer, using atmospheric pressure chemical ionization (APCI). A single MS stage was used for detection of metformin and IS1, by extracting ion chromatograms corresponding to molecular ions. MS/MS detection in the SRM mode was used for glibenclamide (*m/z* transition from 494 to 369 Da) and IS2 (*m/z* transition from 528 to 403 Da). The method produces linear responses up to 2000 ng/mL for metformin and 400 ng/mL for glibenclamide, respectively. Low limits of quantification were found in the 40 ng/mL range for metformin and at the 4 ng/mL level for glibenclamide. Precision was characterized by relative standard deviations (RSD%) below 9%. The analytical method was successfully applied to a single dose, open-label, randomized, two-period, two-sequence, crossover bioequivalence study of two commercially available anti-diabetic combinations containing 400 mg metformin and 2.5 mg of glibenclamide per coated tablet.

Keywords: Metformin; Glibenclamide; Anti-diabetic combination; Simultaneous assay; Human plasma; Extraction-less sample preparation; APCI; MS detection; Method validation; Bioequivalence; Pharmacokinetic parameters

1. Introduction

Metformin (1-(diaminomethylidene)-3,3-dimethyl-guanidine) is an anti-diabetic drug from the biguanide class [1]. Glibenclamide (5-chloro-*N*-[2-[4-(cyclohexylcarbamoyl-sulfamoyl) phenyl]ethyl]-2-methoxy-benzamide), also known as glyburide, is an anti-diabetic drug belonging to the sulfonylurea class, used in the treatment of type 2 diabetes [2]. Metformin combined with glibenclamide is a second-line drug designed for type 2 diabetes mellitus treatment when either drug alone does not improve glycaemic control [3].

Literature data report several individual methods for the determination of metformin and glibenclamide in biological samples. HPLC separations with UV or fluorescence detection are usually not suitable for the assay of glibenclamide in human plasma after administration of its therapeutic doses since these methods do not have enough sensitivity and/or specificity. Therefore, LC/MS methods using either electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI) modes are mainly used to assay glibenclamide in plasma samples [4–8].

For the assay of metformin in human plasma, different types of HPLC-UV methods (based on ion-exchange, ion-pair, or reversed-phase separation mechanisms) are used with different types of complex, time-consuming sample preparation procedures, such as chemical derivatisation, liquid–liquid extraction or solid phase extraction [9–11]. The analytical challenge is due to the high polarity of metformin which makes it difficult to extract from biological fluids and causes its rapid elution by

* Corresponding author at: University of Bucharest, Faculty of Chemistry, Department of Analytical Chemistry, Sos. Panduri, No. 90, Bucharest 050663, Romania. Tel.: +40 723 330 423; fax: +40 21 221 36 92.
E-mail addresses: avmedved@yahoo.com, qc@labormedpharma.ro (A. Medvedovici).

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reversed phase chromatography (poor separation against residual plasma endogenous matrix). Selectivity issue for metformin assay in plasma samples may also be solved through the use of mass spectrometric detection [12–14].

A single literature reference relates to the simultaneous determination of metformin and any of the sulfonylurea (i.e. glibenclamide) in biological fluids. This HPLC method is based on UV detection and uses an ion pair solid phase extraction technique [15].

The present study refers to the simultaneous assay of metformin and glibenclamide in human plasma samples, based on extraction-less sample preparation procedure followed by an LC separation on a nitrile-modified silicagel and MS detection. The method was fully validated and used for a bioequivalence study involving two commercially available coated tablet formulations containing metformin and glibenclamide.

2. Experimental

2.1. Materials

Metformin and glibenclamide, as standard reference substances were obtained from European Pharmacopoeia, Council of Europe, Strasbourg, France (glibenclamide, batch 1a, cat. no. EP G0325000 and metformin, batch a, cat. no. EP M0605000). Gliquidone (IS2) was obtained from LGC Promochem, Wesel, Germany (cat. no. BP 580). 1,3,5-Triazine-2,4,6-triamine (IS1), was obtained from MIKROMOL GmbH, Luckenwalde, Germany (cat. no. MM 0056.04). Ammonium acetate and acetic acid were pro analysis grade from Merck (Darmstadt, Germany). Acetonitrile-gradient grade was also produced by Merck (Darmstadt, Germany). Water for chromatography (resistivity minimum 18.2 MΩ and residual total organic carbon, TOC: maximum 30 ppb) was produced within the laboratory by means of a TKA Lab HP 6 UV/UF instrument and used during experiments.

2.2. Instrumentation

Experiments were performed on Agilent 1100 series LC/MSD (Agilent Technology, Waldbronn, Germany) system consisting of the following modules: degasser (G1379A), quaternary pump (G1311A), thermostated autosampler (G1329A), column thermostat (G1316A), APCI standard interface (G1947A), ion trap mass spectrometric detector SL series (G24450), and nitrogen generator (5183–2003). System control and data acquisition were made with the Agilent ChemStation for LC 3D software Version 10.02 incorporating the MSD Trap Control software Version 5.2 from Brucker Daltronics. The system was operationally qualified before and after the bioequivalence study (according to the producer OQ/PQ built-in procedures).

2.3. Chromatographic method

A single Zorbax CN column (Agilent Technologies, USA), 150 mm length, 4.6 mm internal diameter and 5 μm particle size, fitted with a Phenomenex C18 security guard cartridge (2 mm × 4 mm) was used during the validation stage and entire bioequivalence study. The column was validated before and after study completion, by computing the reduced plate height ($\bar{h}$) in case of the fluoranthene peak (a variation from 3.6 to 3.7 was noticed during the whole process, meaning around 1500 injected samples). The column was thermostated at 25 °C.

Isocratic elution was applied, using a mobile phase containing 50% aqueous 0.01 moles/L ammonium acetate solution, adjusted to pH 3.5 with acetic acid and 50% acetonitrile, at a flow rate of 1 mL/min. Injection volume was 50 μL.

2.4. MS detection

MS detector was operated in the positive ion mode. Detection for metformin and IS1 was achieved in a single MS stage. Data were obtained from extracted ion chromatograms corresponding to the protonated molecular ions (m/z = 130 Da for metformin and m/z = 127 Da for IS1). Glibenclamide and IS2 were detected in MS/MS mode using the following transitions: for glibenclamide: m/z transition from 494 to 369 Da; for IS2: m/z transition from 528 to 403 Da. Consequently, five acquisition data segments were created within a chromatogram: segment 1, 0–1.91 min, column effluent oriented to purge through the divert valve; segment 2, 1.91–2.68 min, column effluent to APCI, detection of IS1 (molecular ion m/z = 127 Da); segment 3, 2.68–4.10 min, column effluent to APCI, detection of metformin (molecular ion m/z = 130 Da); segment 4, 4.10–5.20 min, column effluent to APCI, glibenclamide detection (isolation of the precursor ion m/z = 494 Da, monitoring of the product ion m/z = 369 Da); segment 5, 5.20–7.00 min, effluent to APCI, IS2 detection (isolation of the precursor ion m/z = 528 Da, monitoring of the product ion m/z = 403 Da). Spectral isolation windows were of ±2 Da. Fragmentation voltages used for glibenclamide and IS2 were 1.2 and 1.6 V, respectively. The ion trap functioning was optimized according to the automated Tune Expert procedure for each analyte feed to the interface. Ion trap accumulation time varied from 250,000 μs for metformin and IS1 to 200,000 μs for glibenclamide and IS2. The corresponding ICC target was set at 30,000 for all compounds. Each data is the result of averaging of eight spectra. The multiplier voltage was 2085 V and the dynode voltage 7 kV. Extraction ion optic voltages were optimized for each of the target compounds (capillary exit from 70.5 to 127.9 V, octopole RF voltage from 50 to 300 V, skimmer from 15 to 38.7 V, octopole 1 dc from 4.95 to 14.4 V, octopole 2 dc from 0 to 2.9 V).

Structures of the analytes, their ionization patterns and MS spectra are given in Fig. 1.

2.5. Interface parameters

The parameters controlling APCI were the following: drying gas (N_2) temperature, 300 °C; vaporizer temperature, 350 °C; drying gas flow, 5 L/min; pressure of the nebulizer gas, 60 psi; capillary voltage, 2336 V (segment 2), 2434 V (segment 3), 4500 V (segment 4 and 5); high voltage end plate offset, −500 V;

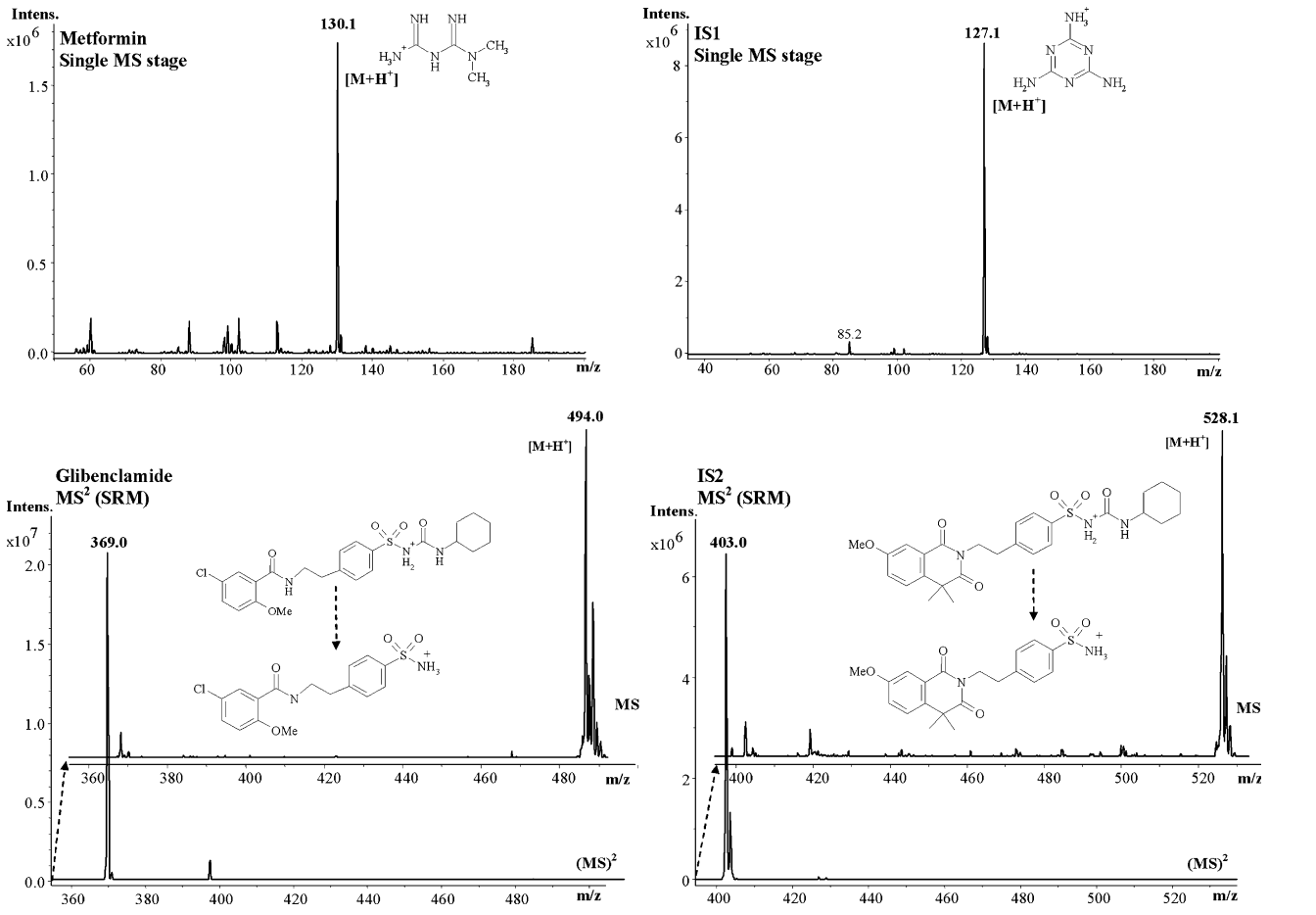

Fig. 1. Structures of analytes, their ionization patterns and corresponding mass spectra.

corona discharge, 4000 V (segment 2 and 3), 5000 V (segment 4 and 5).

2.6. Sample preparation

An aliquot of 0.4 mL from a solution in acetonitrile containing 0.5 μg/mL of IS1 and 0.5 μg/mL of IS2 was added to 0.2 mL plasma sample and vortexed for 5 min at 2000 rpm, followed by centrifugation at 25 °C with 800 × *g* for 5 min; the supernatant is quantitatively transferred and diluted with 0.3 mL of HPLC grade water. After a vortex period of 3 min at 2000 rpm, the sample is transferred in the injection vial and placed in the autosampler. A 50 μL volume was injected to column.

Protein precipitation by means of acetonitrile addition allows keeping glibenclamide and IS2 dissolved. Dilution of the supernatant with water is used to avoid solvent focusing effects on injection of higher volumes.

2.7. Methodology and pharmacokinetic application

The developed method was applied to an open-label, randomized, two-period, two-sequence, analytically blind, crossover study carried out on 24 healthy volunteers (male/female ratio = 14/10) with a mean age of 24.4 and a standard deviation $s = 4.4$ years; a mean bodyweight of 65.9 and a standard deviation $s = 7.7$ kg; a mean BMI of 21.8 and a standard deviation $s = 1.6$ kg/m^2 receiving one dose containing a combination of 400 mg metformin hydrochloride and 2.5 mg glibenclamide of the tested product (T) and one of the reference product (R) as coated tablets, in the sequence determined by randomization, with a 5 days wash-out period between consecutive administrations. Venous blood samples were collected pre-dose (0 h) and at the following post-dose intervals of time: 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 6, 7, 8, 10, 12, and 24 h. The study protocol was formally approved by the Romanian Drug Agency and by the National Ethics Committee. A written consent was priory obtained from volunteers. The whole study was conducted following all applicable guidelines for good clinical practices.

Medical examinations were performed in the screening and at the beginning of each study period (in-house day), in every single blood sampling days and at the end of each study period. The pharmacokinetic parameters considered for evaluation of the bioequivalence between tested and reference products were: C_{max}, observed maximum plasma concentration for each analyte; T_{max}, sampling time of the maximum plasma concentration; t_{half}, terminal elimination half life time; AUC_{last}, area under plasma concentration/time plot until the last quantifiable value; AUC_{total}, area under plasma concentration/time plot extrapolated to infinity. Pharmacokinetic parameters were determined by means of the KineticaTM software (Version 4.4.1.) from

Thermo Electron Corporation, USA. The analysis of variance (ANOVA) was performed on the pharmacokinetic parameters. Then, the 90% confidence intervals of the pharmacokinetic parameters characterizing the tested/reference products were determined. Details on the statistical data interpretation may be found in ref. [16].

3. Results and discussions

3.1. Method development

The difference in terms of the intrinsic polarity between target compounds is obvious. Metformin is characterized by a greater polarity ($\log K_{ow} = -2.64$) while glibenclamide is highly apolar ($\log K_{ow} = 4.79$). The use of a stationary phase based on nitrile chemically modified silicagel will enhance on retention of metformin (based on π–π interactions) and reduces hydrophobic interactions with glibenclamide, concomitantly conserving the reversed phase character of the separation.

The evident dissimilar hydrophobic character of target compounds leads to the choice of two appropriate internal standards. First one, namely 1,3,5-triazine-2,4,6-triamine or melamine (IS1) is close to metformin in terms of polarity ($\log K_{ow} = -0.38$). Gliquidone (IS2), (1-cyclohexyl-3-*p*-[2-(3,4-dihydro-7-methoxy-4,4-dimethyl-1,3-dioxo-2(1*H*)-isoquinolyl)ethyl], exhibits a hydrophobic character very similar to glibenclamide ($\log K_{ow} = 4.65$). Retention of glibenclamide and IS2 exponentially increases with the decrease of the organic solvent content in the mobile phase, while retention of metformin linearly decreases with the increase of the aqueous component (see Fig. 2) due to its water solubility. On the studied interval (±5%), retention of IS1 is practically unaffected by the mobile phase composition.

Variations with ±0.1 units of the pH in the aqueous component of the mobile phase do not affect the retention data or the detector response. However, the buffer concentration in the aqueous component of the mobile phase seems to have a more complex influence on retention characteristics and detector responses. This operational parameter readily influences the behaviour of metformin and IS1, while glibenclamide and IS2 remain unaffected. Metformin response is drastically enhanced with the increase of the concentration of ammonium acetate in the mobile phase within the interval 0.1–0.01 moles/L and remains unchanged to higher concentrations. The response of IS1 is halved on the same interval and became stable above 0.01 moles/L. The concentration of the buffer set at 0.01 moles/L seems a good compromise in terms of retention and detector response.

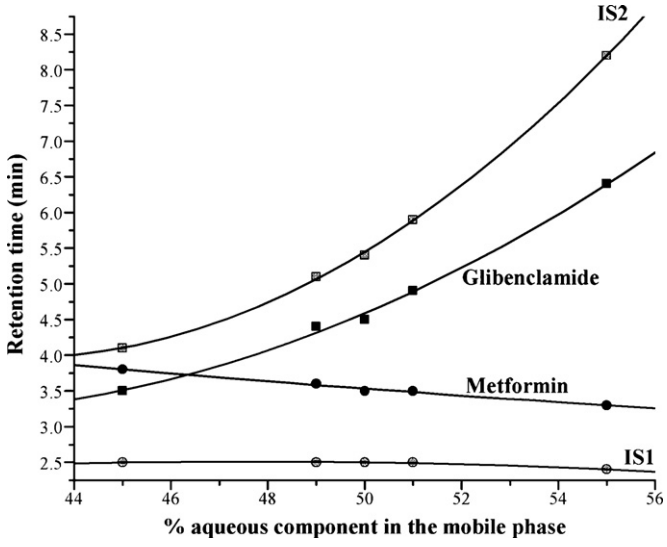

Fig. 2. Retention behaviour of target compounds with respect to changes in the mobile phase composition.

Influence of temperature on separation was studied over the 20–30 °C interval. Retention data were placed within the normal variation interval, illustrating that column temperature control does not represent a critical parameter.

Column batch to batch variations may induce sensible modifications in the retention of the target analytes. Retention behaviour acts in an opposite way for the pairs of analytes. On a stationary phase batch exhibiting less retention for metformin and IS1, an increased retention could be observed for glibenclamide and IS2. However, such variations are not affecting the overall method selectivity.

All analytes produce protonated molecular ions $[M+H]^+$ within the APCI module. Some fragmentation could already be observed at this stage for metformin, glibenclamide and IS2, without exceeding 15% from the intensity of molecular ions. Isolation of molecular ions for IS1 and metformin leads further to increased CID fragmentation (even at low fragmentation voltages) and formation of low *m/z* product ions. The poor ability of the ion trap mass analyzer to capture low *m/z* fragments results in reduced sensitivity and poor reproducibility. This is explaining our choice of using a single MS detection stage for IS1 and metformin. Glibenclamide and IS2 protonated molecular ions, under CID conditions, can readily eliminate cyclohexylisocyanate (125 Da), leading to product ions $[M+H-125]^+$.

3.2. Method validation

The method validation strategy fulfilled the guidelines mentioned in refs. [17,18] and responds to following criteria: selectivity; linearity (linearity domains and quantification limits), precision, accuracy, stability of analytes and samples.

3.2.1. Selectivity of the chromatographic method

As a reduced selectivity is produced through a single MS stage on IS1 and metformin detection, the major concern consists on the effect of the residual co-extracted plasma matrix on these compounds (their corresponding capacity factors are 1.5 and 2.4, respectively, below the recommended threshold of 3 often cited in literature). Six independent blank plasma samples have been tested, together to all pre-dose collected samples resulting from volunteers. No interference could be observed, residual peak areas in blank samples being situated below 12.3% from peak areas of target compounds at LLOQ level. The separation is illustrated in Fig. 3. Detail in the figure indicates spiked LLOQ levels of metformin and glibenclamide in

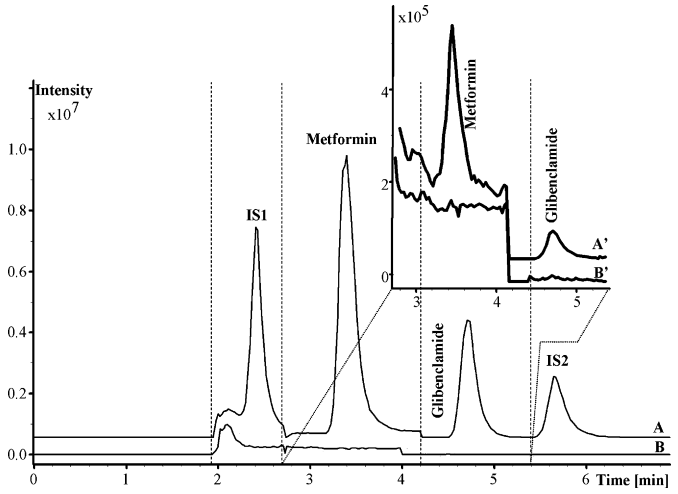

Fig. 3. Chromatogram of a plasma sample spiked with 1000 ng/mL metformin, 200 ng/mL glibenclamide and 1000 ng/mL from each of the internal standards (A) overlaid to a blank plasma sample (B). Zoomed window illustrates peaks of metformin and glibenclamide at LLOQ levels (A′) overlaid to a blank sample trace (B′).

plasma, overlaid to a blank pattern. Although co-medication was strictly forbidden during the bioequivalence study, method selectivity was also checked for possible interferences arising from acetylsalicylic acid, bromhexine, clorpheniramine, caffeine, codeine, paracetamol and ascorbic acid. No interference was observed.

3.2.2. Linearity, linearity domains and quantification limits

Linear relations between peak area ratio (target compound/corresponding IS) and the concentration values were obtained over the following intervals: 50–2000 ng/mL for metformin; 5–400 ng/mL for glibenclamide, respectively. Evaluation of the linearity domains was achieved at eight different concentration values and five replicates per level. Resulting correlation coefficients were 0.9989 for metformin and 0.9997 for glibenclamide. The linear regression function for metformin was characterized by a slope ($B \pm 2s$; where s is the standard deviation) of $2.1 \times 10^{-3} \pm 8.0 \times 10^{-5}$ and an intercept ($A \pm 2s$) of $(-7.2 \pm 7.0) \times 10^{-2}$. The corresponding parameters for glibenclamide were $B = 1.12 \times 10^{-2} \pm 2.0 \times 10^{-4}$ and $A = (1 \pm 3.8) \times 10^{-2}$. Over the linearity procedure, RSD% computed for absolute peak areas of IS1 and IS2 fall within 13.2 and 9.7%. Both internal standards were added to the samples at 1000 ng/mL level. Linear relations between the peak areas of IS1 and IS2 and their corresponding concentrations is valid over the interval 250–3000 ng/mL, being characterized by correlation coefficients of 0.9983 and 0.9986, respectively. During study completion, a calibration was performed for each analytical sequence containing samples from one volunteer. The normal variation interval (mean ± 2S.D.) of the slopes corresponding to the calibration functions ($n = 24$) was $(2.5 \pm 0.6) \times 10^{-3}$ for metformin and $(1.4 \pm 0.9) \times 10^{-2}$ for glibenclamide.

Evaluation of the quantification limit (LOQ) and subsequent calculation of the low limit of quantification (LLOQ) and the limit of detection (LOD) has been achieved in three different ways: (1) $\mathrm{LOQ} = [(10 \times s_A) - A]/B$, where B is the slope of the linear regression, A the intercept and s_A is the standard deviation calculated for A, LLOQ = LOQ/2; LOD = LOQ/3.33 [18]; (2) $\mathrm{LOQ} = [2 \times t \times (s_A + s_B \times C_{av})]/(B + 2 \times t \times s_B)$, where s_B is the standard deviation calculated for B, C_{av} the mean concentration value from the set used for the linear regression and t is the Student coefficient considered for $n - 2$ ($n = 8$) degrees of freedom and a confidence level of 95% ($t = 2.132$) [19,20]; (3) based on signal to noise ratios, indicating a ±20% relative bias from nominal and a precision better than 20% at LLOQ [17].

These results were compared with those based on evaluation of the mean residual peak areas in blank samples. Consequently,

Table 1
Precision of the method for simultaneous assay of metformin and glibenclamide in plasma samples

Analyte	Spiked concentration level (ng/mL)	Parameter	Intraday ($n = 10$)		Interday ($n = 6$)	
			Mean ± 2s	RSD%	Mean ± 2s	RSD%
Metfomin	150	Peak area (units × 10^7)	1.2 ± 0.17	7.3	1.07 ± 0.13	6.0
		Analyte/IS peak area ratio	0.22 ± 0.04	8.0	0.22 ± 0.04	7.1
		Experimental conc. (ng/mL)	140 ± 17	6.1	139 ± 15	5.4
	800	Peak area (units × 10^7)	8.2 ± 0.7	4.1	7.3 ± 1.0	6.7
		Analyte/IS peak area ratio	1.6 ± 0.2	6.8	1.6 ± 0.1	4.3
		Experimental conc. (ng/mL)	814 ± 105	6.5	791 ± 65	4.1
	1600	Peak area (units × 10^7)	13.3 ± 1.2	4.4	12.1 ± 1.4	5.9
		Analyte/IS peak area ratio	3.4 ± 0.2	3.1	3.2 ± 0.5	7.1
		Experimental conc. (ng/mL)	1658 ± 101	3.1	1551 ± 216	7.0
Gilbenclamide	15	Peak area (units × 10^7)	0.20 ± 0.03	7.1	0.18 ± 0.03	8.8
		Analyte/IS peak area ratio	0.16 ± 0.01	2.5	0.16 ± 0.02	5.2
		Experimental conc. (ng/ML)	13.5 ± 0.7	2.7	13.6 ± 1.5	5.5
	160	Peak area (units × 10^7)	2.1 ± 0.13	3.1	2.0 ± 0.1	3.4
		Analyte/IS peak area ratio	1.7 ± 0.2	5.7	1.7 ± 0.2	5.0
		Experimental conc. (ng/ML)	149 ± 17	5.8	153 ± 15	5.1
	320	Peak area (units × 10^7)	4.1 ± 0.4	4.6	4.0 ± 0.4	4.9
		Analyte/IS peak area ratio	3.3 ± 0.2	3.8	3.4 ± 0.3	4.4
		Experimental conc. (ng/ML)	293 ± 22	3.8	306 ± 27	4.4

Table 2
Intra-sequence precision calculated for QC plasma samples run out during the study ($n = 48$)

Analyte	QC sample concentration (ng/mL)	Calculated concentration (ng/mL) Mean ± 2s	RSD%
Metformin	150	150.6 ± 27	9.0
	800	794 ± 120	7.6
	1600	1672 ± 250	7.5
Glibenclamide	15	14.3 ± 2.6	9.4
	160	152 ± 23	7.6
	320	304 ± 52	8.5

LLOQ for metformin was found at 40 ng/mL level, and for glibenclamide at 4 ng/mL level.

3.2.3. Precision

Precision was evaluated for repeatability and intermediate reproducibility on spiked plasma samples, at three different concentration levels. Precision was assessed by means of RSD% values computed for absolute peak areas, peak area ratios and concentrations resulting from interpolation in the corresponding linear regressions. Repeatability study was achieved by injection of 10 replicates from a single prepared spiked plasma sample at three concentration levels within a single day experimental session. Intermediate reproducibility refers to six different samples and three concentration levels processed in different experimental sessions (days). Each sample within an experimental session was injected three times, the averaged value being considered for further computation. Data are presented in Table 1. None of the individual values obtained during the evaluation of precision was placed outside the allowed accuracy interval (%bias ±15%).

On analytical study completion, during each analytical sequence, a quality control (QC) set (three concentration levels, two replicates for each level) was run out. Intra-sequence precision was evaluated for all QC samples ($n = 48$), in terms of concentration (calculation was achieved by using the linear regression equation obtained for the calibration corresponding to the analytical sequence). Experimental data for both target compounds are shown in Table 2.

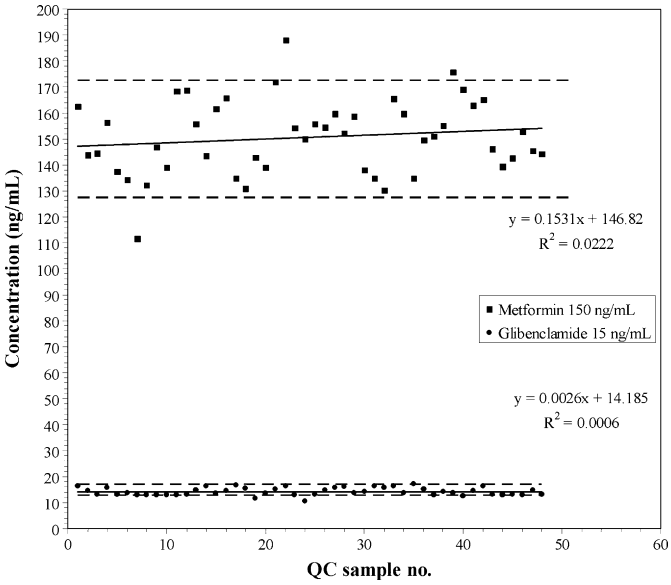

Fig. 4. Illustration of the method accuracy through QC sample data obtained on study completion at the low concentration level (150 ng/mL for metformin and 15 ng/mL for glibenclamide).

3.2.4. Accuracy

The bias (calculated as percentage) between the concentration values determined for the QC samples and the known values, should act as an accuracy indicator. In Fig. 4, the variation of %bias characterizing QC samples at the low concentration level (set around 3 × LLOQ) is shown for metformin and glibenclamide. This should be considered as the worst case, taking into account that concentrations are close to LOQ.

One can observe that only three individual values for metformin and two individual values for glibenclamide fall outside the accepted interval (±15%). In none of the cases, two values at the same concentration level within a QC set fall outside the allowed accuracy interval.

Table 3
Stability data for target compounds and samples

Analyte	Concentration (ng/mL)	Freeze and thaw ($n = 5$)		Long term ($n = 6$)		Short term ($n = 5$)		Post-preparative ($n = 7$)	
		Mean	RSD%	Mean	RSD%	Mean	RSD%	Mean	RSD%
Metformin	100	99	11.2	102	7.1	103	10.4	102	9.3
	500	466	7.1	480	5.4	471	4.0	454	5.2
	2000	1906	5.7	1996	3.9	1888	8.1	1879	5.8
Glibenclamide	10	9.1	6.3	10.1	11.3	9.3	6.1	9.2	5.7
	100	95	8.4	104	6.2	94	7.3	94	6.5
	400	388	4.9	389	7.5	389	5.9	400	5.8

Stock solution ($n = 6$)	Concentration (ng/mL)	Peak area	
		Mean	RSD%
IS1	1000	8.95×10^7	7.0
IS2	1000	1.75×10^7	13.1

Table 4
Pharmacokinetic parameters determined during bioequivalence assessment of two coated tablets containing a combination of metformin hydrochloride (400 mg) and glibenclamide (2.5 mg)

Analyte	Drug	Statistic	C_{max} (ng/mL)	T_{max} (h)	T_{half} (h)	AUC_{last} (ng/mL h)	AUC_{tot} (ng/mL h)
Metfomin	Tested (T)	Mean	1201.1	2.6	8.1	7989.2	8724.2
		RSD%	27.6	42.0	32.4	24.3	22.1
	Reference (R)	Mean	1258.0	2.7	7.9	8437.7	9181.9
		RSD%	24.1	42.3	30.8	17.9	16.2
90% Confidence interval for the ratio of the means T/R			86.1–104.4	–	–	88.0–100.8	87.2–100.9
Glibenclamide	Tested (T)	Mean	43.0	3.5	6.1	197.6	220.6
		RSD%	52.7	23.5	58.7	50.1	49.9
	Reference (R)	Mean	45.6	3.2	6.4	212.5	235.3
		RSD%	50.9	35.2	55.5	49.4	50.4
90% confidence interval for the ratio of the means T/R			84.6–104.9	–	–	82.2–104.3	81.6–102.7

Recoveries of the target compounds were determined at three concentration levels (100, 400 and 2000 ng/mL for metformin and 10, 40 and 400 ng/mL for glibenclamide). Recoveries of the internal standards were studied at a single concentration level (1000 ng/mL). Five replicates were made for each concentration level. Recoveries were evaluated with respect to the corresponding standard solutions resulting from spiking analytes to an aqueous media (water to acetonitrile 1:2, v/v) or to bulk precipitated blank plasma. The mean recoveries found for metformin and glibenclamide were 56 and 73.4%, respectively (relative standard deviations were 8.2 and 13.4%, for $n = 15$) when comparing to standard aqueous spiked samples. Experimental values were higher with respect to standard samples spiked in protein precipitated bulk blank plasma (96.5% for metformin and 100.7% for glibenclamide, with relative standard deviations of 7.5 and 9.2%, respectively). Recoveries of IS1 were 65% (with respect to standard spiked aqueous samples, RSD% = 7.6, $n = 15$) and 96.8% (with respect to standard spiked protein precipitated bulk plasma samples, RSD% = 5.3, $n = 15$). Recoveries of IS2 were 78.3% (with respect to standard spiked aqueous samples, RSD% = 7.5) and 101.2% (with respect to standard spiked protein precipitated bulk plasma samples, RSD% = 6.9). Lower recoveries obtained for metformin and IS1 may be explained by the signal suppression due to residual plasma matrix effects on ionization within the interface. Additional studies carried out to emphasize the effects of the anticoagulants used for blood sample collection (lithium heparin; ammonium heparin, potassium edetate, citrate) on signal suppression/enhancement effects reveals no significant differences.

3.2.5. Stability of analytes and samples

Stability studies for metformin and glibenclamide were made on spiked plasma samples having same concentration as the QC samples used on bioequivalence study completion. The stability of the stock solutions of the internal standards IS1 and IS2 (10 μg/mL) was checked over a 14 days period (6 samplings). Before each analysis, the IS stock solutions were spiked to blank plasma samples at 1000 ng/mL level; the sample was processed according to the procedure and injected in the chromatographic column. Freeze and thaw stability (over 5 consecutive cycles), long term stability at −40 °C (during 14 days, 6 samplings), short-term stability (during 24 h, 5 samplings) and post-preparative stability (during 24 h, 7 samplings) were considered at three concentration levels. Each individual sample was injected three times consecutively; data considered for further computation being based on averaged values. Stability was assessed considering criteria taken from precision (relative stan-

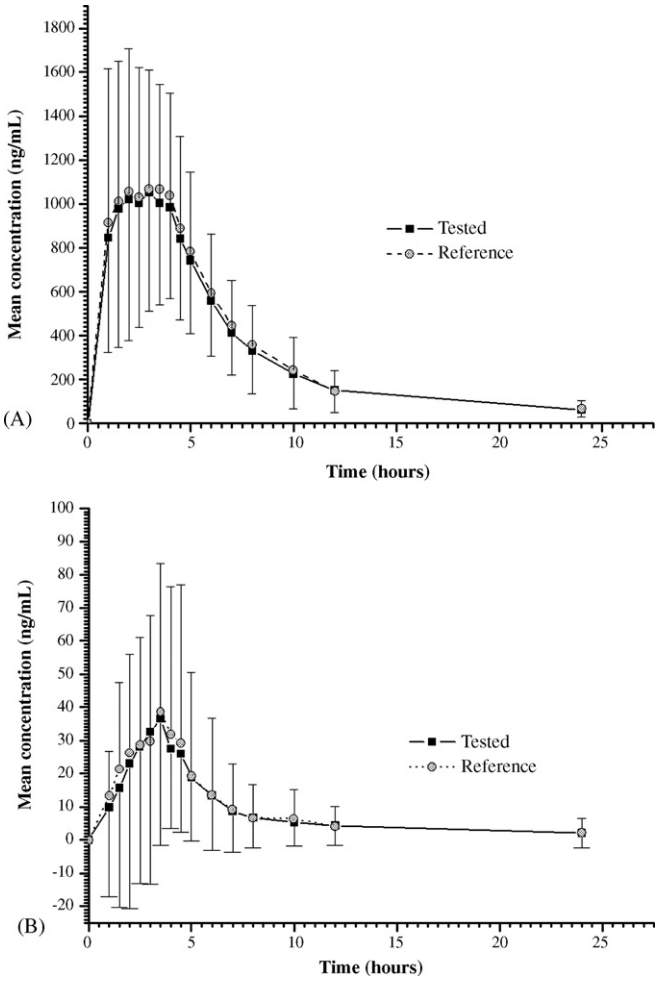

Fig. 5. Mean concentration/time profiles of metformin (A) and glibenclamide (B) in plasma samples, after administration of the reference (R) and the tested (T) products over the bioequivalence study (y bars represent the confidence interval, mean ± 2*s*; upper segments characterize reference product, lower segments the tested product).

dard deviation of a data set in terms of concentration should be lower than 15%). None of the previously detailed procedures failed, proving the stability of the target compounds over the specified intervals and conditions. Data related to the stability of analytes and samples are illustrated in Table 3.

3.3. Pharmacokinetic data

The main pharmacokinetic parameters obtained on study completion are given in Table 4. The mean concentration/time profiles obtained for the reference (R) and tested (T) products are given in Fig. 5 for metformin (A) and glibenclamide (B). Based on the previous results, bioequivalence could be concluded and equal clinical efficacy for the two brands is illustrated.

4. Conclusions

Simultaneous assay of metformin and glibenclamide in human plasma samples was achieved in isocratic conditions using nitrile chemically modified silicagel as stationary phase. Sample preparation was based upon protein precipitation by means of acetonitrile addition. Due to evident discrepancies in terms of polar characteristics, a specific internal standard was used for each of the target compounds. The method is simple, relatively fast, selective and robust. Metformin and IS1 were detected through a single MS stage, while glibenclamide and IS2 were detected through MS/MS using the SRM mode. The method is linear over large concentration intervals. Low limits of quantification are 40 ng/mL for metformin and 4 ng/mL for glibenclamide. The method is precise and accurate, as it results from the validation data. The method was successfully used for the assessment of the bioequivalence of two commercially available pharmaceutical formulations (coated tablets) containing 400 mg metformin and 2.5 mg glibenclamide per dose. Pharmacokinetic parameters were presented.

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