

## VALIDATION OF A HPLC METHOD FOR THE SIMULTANEOUS ANALYSIS OF METFORMIN AND GLICLAZIDE IN HUMAN PLASMA

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### Abstract

The study presents the development and validation of a simple HPLC method for the simultaneous determination of metformin (MTF) and gliclazide (GCZ) in the presence of glibenclamide, in human plasma, for the clinical monitoring of MTF and GCZ after oral administration or for bioequivalence studies.

Ion-pair separation followed by UV detection performed on deproteinised plasma samples was chosen for the determination of metformin and gliclazide. The internal standard was glibenclamide. The HPLC method used a Zorbax Eclipse XDB-C18 150x4.6 mm i.d. (5 $\mu$ m) column and analytical guard column 12.5x4.6 mm (5 $\mu$ m), with a gradient elution (1 mL/min) at 40°C column temperature. The mobile phase was acetonitrile: methanol (1:1 v/v) and sodium dodecylsulphate 5mM, pH=3.5 with H<sub>3</sub>PO<sub>4</sub> 85% and gradient elution. The eluent was monitored at 236 nm.

The calibration curve was linear within the range of 0.05-5.00  $\mu$ g/mL ( $r^2=0.99$ ,  $n=6$ ). The lowest limit of quantification (LLOQ) was 50 ng/mL for metformin and 49 ng/mL for gliclazide.

The proposed method was validated and proved to be adequate for metformin and gliclazide clinical monitoring, bioavailability and bioequivalence studies.

### Rezumat

Acest studiu prezintă o metodă HPLC simplă pentru determinarea metforminului (MTF) și gliclazidului (GCZ), în prezența glibenclamidului în plasmă, având ca scop monitorizarea clinică a celor două antidiabetice precum și studii de biodisponibilitate sau bioechivalență a acestora.

Pentru determinarea metforminului și gliclazidului din probele de plasmă s-a ales un mecanism de separare cu pereche ionică și detecție UV, prepararea probelor fiind făcută prin deproteinizare. Standardul intern ales este glibenclamidul. Separarea HPLC s-a realizat pe o coloană Zorbax Eclipse XDB-C18 150x4.6 mm i.d. (5 $\mu$ m) și o pre-coloană de același tip 12.5x4.6 mm (5 $\mu$ m). Faza mobilă a fost acetonitril:metanol (1:1 v/v) și dodecilsulfat de sodiu 5mM; pH-ul fazei mobile a fost ajustat la 3,5 cu H<sub>3</sub>PO<sub>4</sub> 85%. Eluentul a fost monitorizat la 236 nm.

Curba de calibrare a fost liniară în intervalul 0.05-5.00  $\mu$ g/mL ( $r^2=0.99$ ,  $n=6$ ). Cea mai mică limită de cuantificare (LLOQ) a fost de 50 ng/mL pentru metformin, respectiv 49 ng/mL pentru gliclazid.

Metoda propusă a fost validată și s-a dovedit a fi adecvată pentru determinarea plasmatică a celor două antidiabetice, în studii de biodisponibilitate, bioechivalență, monitorizare clinică.

**Keywords:** HPLC-UV; metformin; gliclazide; glibenclamide

### Introduction

Metformin is the first drug of choice for type 2 diabetes mellitus. It is an antihyperglycemic drug which acts by improving the glucose tolerance in patients with type 2 diabetes mellitus [1]. Also metformin reduces hepatic glucose production [2].

Metformin (figure 1) is a small, highly polar compound ( $pK_a = 2.8$ ,  $11.5$ ,  $\log P_{\text{octanol:water}} = -2.6$ ) so it has a great solubility in water and poor solubility in lipids so it is very difficult to extract it from the aqueous plasma matrix. HPLC methods for the determination of metformin in human plasma include ion-exchange, ion-pair or normal-phase extraction [1- 5].

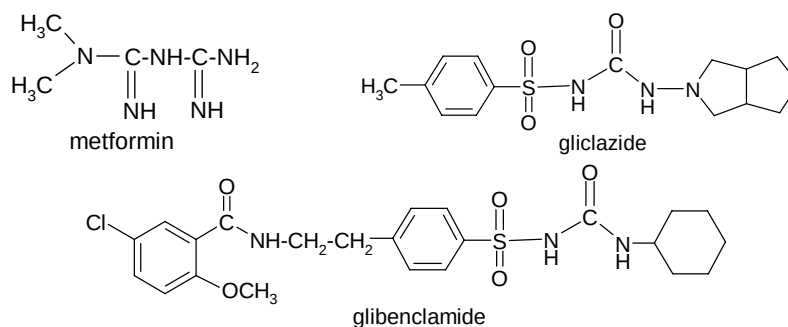
Gliclazide is a second-generation sulphonylurea oral antidiabetic drug, effective in controlling blood glucose in type 2 diabetes mellitus (fig 1). It acts mainly on pancreatic sulphonylurea receptors (SURs), at the surface of  $\beta$ -cells [5], by increasing the secretion of insulin.

Several HPLC procedures have been reported for the determination of gliclazide in biological fluids [6].

Because of the relatively high polarity ( $pK_a = 5.8$ ,  $\log P_{\text{octanol:water}} = 2.1$ ) [7] most of the methods published in literature used liquid-liquid extraction but solid-phase extraction [8] and mild protein precipitation [9,10,11] of the plasma samples were also reported.

Due to a large utility of a combined treatment with metformin and gliclazide, for a better glycaemic control [12] in the treatment of type 2 diabetes mellitus, the presented method must accomplish the best conditions for the simultaneous determination of both metformin and gliclazide, even if they do not have similar formula and similar physico-chemical properties. This method is applicable in therapeutic drugs monitoring, bioavailability and bioequivalence studies.

As internal standard was chose glibenclamide. The first reason for this choice was the chemical structure of glibenclamide, which is a sulphonylurea like gliclazide, the same reason for its utilization in the previous study with gliclazide without metformin [6]. The second reason is that gliclazide, metformin and glibenclamide are widely used as oral antidiabetics, often they are used in association and for this it should be necessary to determine all of them in the same time in plasma samples for routine monitoring.

**Figure 1**

Chemical structure of metformin, gliclazide and glibenclamide

## Materials and methods

### Reagents

Analytes and reagents used for the quantitative determination were: 1,1-Dimethylbiguanid Hydrochlorid 97% (Metformin, MTF), Gliclazide (GCZ), Glibenclamide (GBL) – standards provided by Sigma - Aldrich; methanol and acetonitrile HPLC grade purchased from Sigma - Aldrich; ortho phosphoric acid 85% from Fluka; sodium dodecylsulphate anhydrous 99% from Serva; ultrapure deionized water produced by NANO pure Diamond ultrapure water system. The human blank plasma was supplied by the Local Blood Center – Bucharest.

### Apparatus and chromatographic conditions

For samples preparation it was used a centrifuge-Hettich Universal, a solvent evaporation (under N<sub>2</sub> stream) system using a UHP nitrogen generator Domnick Hunter, Sartorius BP 121S analytical balance, Ultrasonic Cleaner BRANSON - 2510E, vortex. The Hewlett Packard High Performance Liquid Chromatograph, series 1100 equipped with a thermostatted autosampler ALS, binary vacuum degasser. Compounds were screened, identified and quantified in plasma using a diode-array detector (DAD) detector. Chromatographic separations were carried out by a 5µm particle size Zorbax Eclipse XDB-C18 150x4.6 mm i.d. column with analytical guard column 12.5x4.6 mm (5µm). The column temperature was maintained at 40°C. The mobile phase was acetonitrile: methanol (1:1v/v) and sodium dodecylsulphate 5mM pH=3.5 with H<sub>3</sub>PO<sub>4</sub> 85% and gradient elution delivered at a flow – rate of 1.0mL/min. The eluent was monitored at 236±4 nm. The injection volume was 10 µl.

### Preparation of working solutions

Stock solution of metformin (100µg/mL) was prepared in water; stock solutions of gliclazide (650µg/mL) and glibenclamide (200µg/mL) were prepared dissolving the drugs in methanol. Metformin and gliclazide

stock solutions were further diluted with plasma in order to obtain working solutions concentrations ranging from 0.05-5.0 µg/mL. Glibenclamide (the internal standard) (IS) was further diluted in acetonitrile to obtain the working internal standard solution concentration of 10 µg/mL. All solutions were stored at 4°C and were stable for at least 2 months.

#### *Samples preparation*

Metformin and gliclazide working plasma solutions were deproteinated with acetonitrile (1:1) containing internal standard. After mixing (30s) and centrifugation (10 minutes at 3500 rpm), the organic phase (1.5 mL) was removed by evaporation to dryness, at 40°C under a stream of nitrogen. 200 µl mobile phase (10% aqueous solution in organic phase) was added to the residue and after mixing for 30 seconds, 10 µl were injected into the chromatographic system.

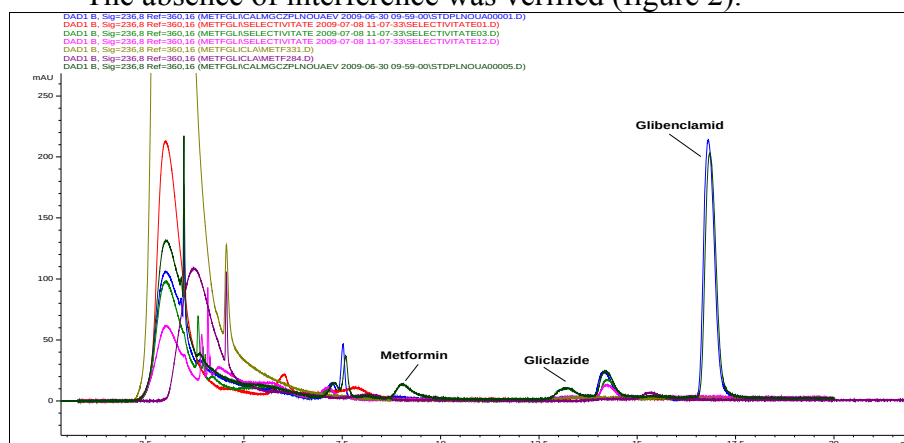
### **Results and discussion**

Validation of the HPLC method was performed according to Food and Drug Administration (FDA) guidelines [13].

#### *Selectivity*

Selectivity is the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample. The procedure is designed to demonstrate the capability of the chosen method to separate metformin, gliclazide and the internal standard (glibenclamide) against the components of the plasma matrix. Six blank plasma obtained from the Local Blood Center (Bucharest) were analysed according to the procedure previously described, in order to evaluate the method selectivity.

The absence of interference was verified (figure 2).



**Figure 2**

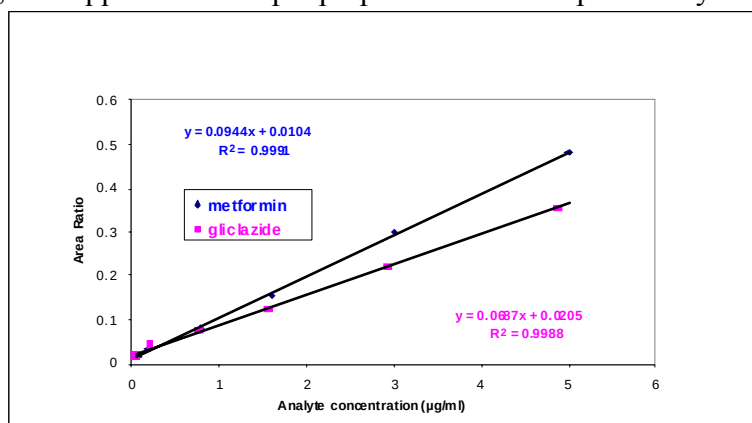
Chromatogram of plasma sample spiked with analytes overlaid with blank plasma samples

The chromatographic method is selective for the separation of metformin, gliclazide and the internal standard (glibenclamide) against the residual matrix components.

### Linearity

The linearity assessment is a procedure designed to measure the capability of both sample preparation and the chromatographic methods, to produce results related in a linear way to the concentrations of the analytes in the plasma samples.

From each spiked plasma sample there were taken three aliquots of 1 mL (except for LLOQ, where 6 aliquots of 1 mL were taken). On these aliquots, was applied the sample preparation method previously described.

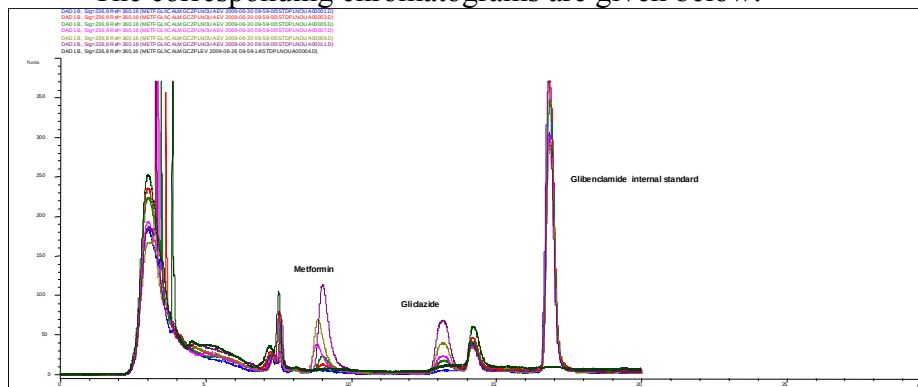


**Figure 3**

Calibration curve for metformin (0.05- 5µg/mL) and gliclazide (0.049-4.875µg/mL)

The lower limit of quantification (LLOQ) was 0.05 µg/mL- metformin and 0.049µg/mL- gliclazide ( $N = 6$ ).

The corresponding chromatograms are given below:



**Figure 4**

Overlaid chromatograms for standards calibrations

*Accuracy and precision*

The accuracy and the precision were evaluated by analysing quality control samples (QC1, QC2 and QC3). Precision was measured using five spiked plasma samples for each concentration level and each day.

The within-day assay validation data are reported in table I for metformin and in table II for gliclazide.

$$\text{Accuracy (\%)} = C_{\text{exp}}/C_{\text{th}} \times 100$$

where:

$C_{\text{exp}}$  was calculated according to the linear regressions for all the investigated analytes determined above:

$$\text{Conc.}(\mu\text{g/mL}) = (\text{Ratio Peak Area} - 0.0104)/0.0944 \text{ (for metformin)}$$

$$\text{Conc.}(\mu\text{g/mL}) = (\text{Ratio Peak Area} - 0.0204)/0.0687 \text{ (for gliclazide)}$$

$C_{\text{th}}$  is the concentration of each analyte in the spiked QC samples.

*Acceptance criteria*

The mean value should be within 15% of the actual value except at LLOQ, where it should not deviate by more than 20%. The deviation of the mean from the true value serves as measure of the accuracy and the precision.

**Tabel I**  
Accuracy for metformin (MTF)

| Theoretical concentration ( $C_{\text{th}}$ $\mu\text{g/mL}$ ) | Ratio $\text{Area}_{\text{MTF}}/\text{Area}_{\text{IS}}$ | Calculated concentration ( $C_{\text{exp}}$ $\mu\text{g/mL}$ ) | Statistic parameters                                                                         |
|----------------------------------------------------------------|----------------------------------------------------------|----------------------------------------------------------------|----------------------------------------------------------------------------------------------|
| <b>QC1</b><br><b>0.15</b>                                      | 0.023737                                                 | 0.1413                                                         | Mean:<br>0.1599 $\mu\text{g/mL}$<br>$s=0.0125$<br>$\text{rsd}=7.82\%$<br>Accuracy<br>102.00% |
|                                                                | 0.026012                                                 | 0.1654                                                         |                                                                                              |
|                                                                | 0.026216                                                 | 0.1675                                                         |                                                                                              |
|                                                                | 0.026044                                                 | 0.1657                                                         |                                                                                              |
|                                                                | 0.025974                                                 | 0.1649                                                         |                                                                                              |
| <b>QC2</b><br><b>1.50</b>                                      | 0.1665                                                   | 1.6389                                                         | Mean:<br>1.5301 $\mu\text{g/mL}$<br>$s=0.1241$<br>$\text{rsd}=8.11\%$<br>Accuracy<br>93.84%  |
|                                                                | 0.1426                                                   | 1.4004                                                         |                                                                                              |
|                                                                | 0.1695                                                   | 1.6853                                                         |                                                                                              |
|                                                                | 0.1486                                                   | 1.4641                                                         |                                                                                              |
|                                                                | 0.1484                                                   | 1.4622                                                         |                                                                                              |
| <b>QC3</b><br><b>4.50</b>                                      | 0.4595                                                   | 4.7574                                                         | Mean:<br>4.6938 $\mu\text{g/mL}$<br>$s=0.2314$<br>$\text{rsd}=4.93\%$<br>Accuracy<br>104.30% |
|                                                                | 0.4893                                                   | 5.0734                                                         |                                                                                              |
|                                                                | 0.4488                                                   | 4.6441                                                         |                                                                                              |
|                                                                | 0.4215                                                   | 4.3551                                                         |                                                                                              |
|                                                                | 0.4529                                                   | 4.6879                                                         |                                                                                              |

$\text{Area}_{\text{IS}}$  = area of the internal standard

**Tabel II**  
Accuracy for gliclazid (GCZ)

| Theoretical concentration<br>( $C_{th}$ $\mu\text{g/mL}$ ) | Ratio<br>$\text{Area}_{GCZ}/\text{Area}_{IS}$ | Calculated concentration<br>( $C_{exp}$ $\mu\text{g/mL}$ ) | Statistic parameters                                                                    |
|------------------------------------------------------------|-----------------------------------------------|------------------------------------------------------------|-----------------------------------------------------------------------------------------|
| <b>QC1</b><br><br><b>0.146</b>                             | 0.029068                                      | 0.124718                                                   | Mean: 0.13 $\mu\text{g/mL}$<br>$s=0.0156$<br>$\text{rsd}=11.98\%$<br>Accuracy<br>89.50% |
|                                                            | 0.030158                                      | 0.140587                                                   |                                                                                         |
|                                                            | 0.031011                                      | 0.152997                                                   |                                                                                         |
|                                                            | 0.028507                                      | 0.116551                                                   |                                                                                         |
|                                                            | 0.02864                                       | 0.118485                                                   |                                                                                         |
| <b>QC2</b><br><br><b>1.46</b>                              | 0.121175                                      | 1.465429                                                   | Mean: 1.39 $\mu\text{g/mL}$<br>$s=0.0745$<br>$\text{rsd}=5.34\%$<br>Accuracy<br>95.51%  |
|                                                            | 0.109181                                      | 1.290851                                                   |                                                                                         |
|                                                            | 0.117612                                      | 1.413559                                                   |                                                                                         |
|                                                            | 0.113002                                      | 1.346459                                                   |                                                                                         |
|                                                            | 0.120521                                      | 1.455913                                                   |                                                                                         |
| <b>QC3</b><br><br><b>4.387</b>                             | 0.27046                                       | 3.638427                                                   | Mean: 3.94 $\mu\text{g/mL}$<br>$s=0.2327$<br>$\text{rsd}=5.89\%$<br>Accuracy<br>89.81%  |
|                                                            | 0.30165                                       | 4.092437                                                   |                                                                                         |
|                                                            | 0.310439                                      | 4.220361                                                   |                                                                                         |
|                                                            | 0.297612                                      | 4.033654                                                   |                                                                                         |
|                                                            | 0.273733                                      | 3.686070                                                   |                                                                                         |

The within-day accuracy and precision of the chromatographic method considering metformin and gliclazide meet the requirements of the acceptance criteria.

#### Stability

The stability of unprocessed plasma samples was studied for 2 months at the storage temperature ( $-20^{\circ}\text{C}$ ), for 24 hours at room temperature, and after three freeze and thaw cycles. The concentration changes related to the nominal concentration were less than 15%, indicating no significant substance loss during the study.

The processed plasma samples proved to be stable for at least 24 hours.

#### Conclusions

An analytical method for the simultaneous determination of metformin and gliclazide, in the presence of glibenclamide (IS), in plasma samples in the 0.05-5  $\mu\text{g/mL}$  concentration range was presented. The method proved to be suitable for pharmacokinetic studies and therapeutic drugs monitoring due to its sensitivity and time- effective.

#### References

1. T.W. Hale, J.H.Kristensen, L.P.Hackett, R.Kohan, K.F.Rett, Transfer of metformin into human milk, *Diabetologia* 2002, 45:1509-1514;

2. Maria-Cristina Ranetti, A.Ranetti, The pharmacokinetics of some oral antidiabetics –a pharmaceutical approach , *Farmacia*, 2006, LIV, 6, 3- 13
3. A.Zarghi, S.M.Foroutan, A.Shafaati, A.Khoddam, Rapid determination of metformin in human plasma using ion-pair HPLC, *Journal of Pharmaceutical and Biomedical Analysis* 2003, 31,197-200;
4. Imre S., Vari C.E., Dogaru M., Curticăpean A., Kelemen L., Muntean D., Determinarea cantitativă a metforminului în plasma umană, *Farmacia*, 2006, LIV(6), 85-93
5. Ali M.Qaisi, Maha F.Tutunji, Charl A. Sahouri, Determination of metformin in human plasma using normal phase high performance liquid chromatography, *Saudi Pharmaceutical Journal*, 2006,14, 2;
6. Maria-Cristina Ranetti, Valentina Anuta, Dalia Miron, F. Radulescu, I. Mircioiu, C. Mircioiu, Study concerning pharmacokinetics of gliclazide after oral administration, *Farmacia* 2007,vol LV3, 257-266;
7. Jennifer R. Pfaffly – Free Radicals in Biology and Medicine, 2001-<http://www.healthcare.uiowa.edu/CoreFacilities/esr/education>;
8. Noguchi H. , Tomita N.,Naruto S. , Nakano S., Determination of gliclazide in serum by high-performance liquid chromatography using solid-phase extraction, *Journal of chromatography. Biomedical applications*, 1992, 583, 2, 266-269;
9. S.M.Foroutan, A.Zarghi, A.Shafaati, A.Khoddam, Application of monolithic column in quantification of gliclazide in human plasma by liquid chromatography, *Journal of Pharmaceutical and Biomedical Analysis*, 2006, 42, 513-516;
10. Zhong Guo-Ping, Bi Hui-Chang, Shufeng Zhou, Xiao Chen, Min Huang, Simultaneous determination of metformin and gliclazide in human plasma by liquid chromatography-tandem mass spectrometry: application to a bioequivalence study of two formulations in healthy volunteers, *Journal of mass spectrometry*, 2005, 40, 11, 1462-1471;
11. Ching-Ling Cheng, Chen-Hsi Chou, Determination of metformin in human plasma by high-performance liquid chromatography with spectrophotometric detection, *Journal of Chromatography B*, 2001, 762, 51-58;
12. Maria -Cristina Ranetti, Camelia Stecoza, Aurelian-Emil Ranetti, Actualitati și perspective în tratamentul diabetului zaharat de tip 2, *Farmacia*, 2004, LII, 3, 36-51
13. US Food and Drug Administration. Guidance for Industry; Bioanalytical Method Validation, 2001

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