

## Analysis of Glimepiride in Human Blood and Urine by Thin-Layer Chromatography and UV-Spectrophotometry

### İnsan Kan ve İdrarında İnce Tabaka Kromatografisi ve UV-Spektrofotometrisi ile Glimepiride Analizi

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#### Abstract

**Objective:** An increasing numbers of cases of poisonings by glimepiride, either attempted suicide or accidental, combined with the absence of reliable methods for the detection and quantitation of glimepiride in biological matrices is the basis for the need for the development of new analytical techniques for forensic analysis.

**Materials and Methods:** Analyses were performed using drug-free biological fluids (whole blood and urine). Specimens were spiked with chromatographically pure glimepiride. After hydrolysis with diluted hydrochloric acid at 50-60 °C for 15-20 min and a double extraction into chloroform, glimepiride was identified by thin-layer chromatography. Standard solution of glimepiride (1 mg/mL) and Sorbfil chromatographic plates were used for thin-layer chromatography. The thin-layer chromatography studies showed that the best mobile phase was chloroform:acetone (9:1), Rf value of glimepiride in five examinations was 0.37±0.02. Visualization of glimepiride was achieved by spraying with Dragendorff's, Bushard's, or diphenylcarbazone-chloroform solution followed by mercuric sulphate. The limit of detection of pure glimepiride by thin-layer chromatography was 0.5 µg/mL, 1.5 µg/mL in whole blood and 1.0 µg/mL in urine. For spectrophotometric determinations of glimepiride, a UV/VIS spectrophotometer with 1 cm matches quartz cell was used. Standard solutions of glimepiride in ethanol were prepared at concentrations of 1-50 µg/mL and scanned in full-scan mode between 200-400 nm.

**Results and Conclusion:** The wavelength maxima for glimepiride was found to be 227 nm with molar absorptivity of 3.2685×10<sup>4</sup> l/mol/cm. Beer's law was obeyed in the concentration range of 2-40 µg/mL. The limit of detection and limit of quantification were found to be 0.97 µg/mL and 2.70 µg/mL, respectively. The results have been successfully applied in blood of patients after oral administration and on postmortem blood in an overdose death.

**Keywords:** Glimepiride, Thin-layer chromatography, Spectrophotometry.

#### Özet

**Giriş:** Her geçen gün intihar amaçlı veya kaza sonucu meydana gelen glimepirid zehirlenmelerine ek olarak glimepiridin biyolojik matrislerde tespit ve miktarlandırılmasına yönelik güvenilir bir yöntem olmaması nedeniyle adli incelemeler için yeni analitik tekniklerin geliştirilmesine ihtiyaç bulunmaktadır.

**Gereç ve Yöntem:** Analizler herhangi bir madde içermeyen vücut sıvıları (tam kan ve idrar) kullanılarak gerçekleştirildi. Örneklerle kromatografik olarak saf glimepiride eklendi. Dilüe hidroklorik asitler 50-60 °C'de 15-20 dakika hidrolizden ve kloroformda çift ekstraksiyondan sonra ince tabaka kromatografisi ile glimepiride analizi gerçekleştirildi. İnce tabaka kromatografisi için standart glimepiride solüsyonu (1 mg/mL) ve Sorbfil kromatografi plakaları kullanıldı. İnce tabaka kromatografi analizleri için en uygun mobil fazing kloroform:aseton (9:1) olduğu ve glimepirid Rf değeri beş analizde 0.37±0.02 idi. Glimepirid görüntülenmesi Dragendorff's, Bushard's, veya difenilkarbazon-kloroform ve civa sülfat muamelesi ile sağlandı. İnce tabaka kromatografisi ile saf glimepiride tespit limitinin tam kanda 0.5 µg/mL, 1.5 µg/mL ve idrarda 1.0 µg/mL olduğu saptandı. Glimepiridin spektrofotometrik tespiti için 1 cm quartz UV/VIS kullanıldı. Glimepirid standart solüsyonları etanolde 1-50 µg/mL konsantrasyonlarında hazırlandı ve full-scan modunda 200-400 nm dalga boyları arasında tarandı.

**Bulgular ve Sonuç:** Glimepiride için maksimum dalga boyu 227 nm ve molar soğurganlığı 3.2685×10<sup>4</sup> l/mol/cm idi. İlgili konsantrasyon aralıkları olan 2-40 µg/mL'de Beer's kanununa sadık kalındı. Tespit limiti 0.97 µg/mL iken miktarlandırma limiti 2.70 µg/mL olarak saptandı. Sonuçlar oral alımdan sonra hasta kan analizine ve yüksek doz ölüm olgusunda postmortem analiz için başarılı şekilde uygulandı.

**Anahtar kelimeler:** Glimepiride, İnce tabaka kromatografisi, Spektrofotometri.

## 1. Introduction

Sulfonylurea agents are commonly used in the treatment of diabetes mellitus (1-3). When used appropriately, they promote euglycemia, although hypoglycemia can occur if clearance is impaired or the patient does not eat. Sulfonylureas often cause

hypoglycemia with overdoses or when ingested by nondiabetic patients and may sometimes have forensic or clinical toxicologic relevance (4-7).

Glimepiride (Amaryl) is a second-generation sulphonylurea oral hypoglycemic agent (structures shown in figure-1) used in the treatment of non-insulin dependent diabetes mellitus (8-10).

Today glimepiride (GLM) is widely used in the treatment of type 2 diabetes mellitus in Uzbekistan. The number of registered patients in Uzbekistan on January 1, 2011 amounted

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to more than 122,460 people. 84.2% of these patients have type 2 diabetes (11).

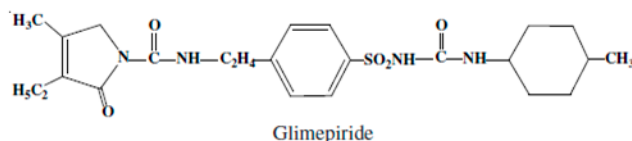


Fig 1. Structure of glimepiride.

Increasing numbers of cases of poisonings by GLM, either as attempted suicides or accidental ingestion, as well as the lack of reliable and simple methods for the detection and quantitation of GLM in biological matrices are the basis for the need for the development of new analytical methods for forensic analysis.

Methods such as high performance liquid chromatography, gas chromatography and high performance thin-layer chromatography etc. are precise with good reproducibility, but the cost of analysis is quite high owing to expensive instrumentation, reagents and need for expertise (12-15). Hence it is worthwhile to develop simpler and cost effective method for qualitative and quantitative analysis.

In forensic chemical laboratories of Uzbekistan thin-layer chromatography and spectrophotometric methods are widely used for routine analysis.

The aim of the present investigation was to use thin-layer chromatographic and UV-spectrophotometric methods for the determination of GLM in biological fluids.

## 2. Material and Methods

### 1. Chemicals and Reagents

Glimepiride (purity 99.30%) was obtained as a gift sample from the Main Directorate of the Quality Control of Medicines and Medical Equipment of the Ministry of Health of the Uzbekistan Republic.

All the reagents were of analytical grade. Glass double distilled water was used throughout the experiment.

### 2. Instrumentation

For the spectrophotometric determination of GLM a UV/VIS spectrophotometer (Model 8453, Agilent Technologies, USA) with 1 cm matches quartz cell was used.

### 3. Preparation of standard stock solution of glimepiride

An accurately weighed 10 mg sample of glimepiride was dissolved in 2 mL of ethanol in a 5 ml volumetric flask and then brought to volume with additional ethanol to obtain a stock solution of 2 mg/mL (for TLC determinations).

An accurately weighed 5 mg sample of glimepiride was dissolved in 10 ml of ethanol in a 50 ml volumetric flask and

then brought to volume with additional ethanol to obtain a stock solution of 100 µg/mL (for UV-spectrophotometric estimation).

All solutions were freshly prepared on the day of analysis.

### 4. Preparation of samples from biological fluids

Analysis was performed using drug-free biological fluids: 5 mL of whole blood and 10 mL of urine. Blood samples were diluted with distilled water (1:1). Specimens were spiked with chromatographically pure GLM. After hydrolysis with diluted hydrochloric acid at 50-60 °C for 15-20 min and a double extraction into chloroform, the serum and urine extracts were passed through coarse filter paper and evaporated to dryness in disposable plastic 25-mL conical beakers. After evaporation to dryness, 3 mL of ethanol were added in conical beakers for dissolution of the dried extracts. The absorbance of sample solution was measured and amount of glimepiride was determined by referring to the calibration curve.

### 5. TLC Analysis

Standard solutions of GLM (2 mg/mL) and Sorbfil chromatographic plates were used for TLC. The solvent mixture was optimized by studying the chromatographic mobility of the GLM. Different solvents such as methanol, ethanol, acetone, acetonitrile, dimethyl formamide, chloroform, diethyl ether, ammonia, cyclohexane, toluene, diethyl were attempted in various proportions. Chromatograms were visualized with Dragendorff's, Bushard's, and diphenylcarbazone-chloroform solution followed with mercuric sulphate. 10 µl of standard solution of GLM and sample extracts were applied to the chromatographic plate using glass capillaries.

### 6. UV-spectrophotometric Analysis

Aliquots of 0.1 to 5.0 mL of stock solution were transferred to a series of 10 ml volumetric flasks and were adjusted to 10 ml with distilled water to obtain a concentration of range of 1-50 µg/mL. Spectral scans of each sample of standard against ethanol were recorded in the range 200 to 400 nm where maxima was observed at 227 nm (figure 2).

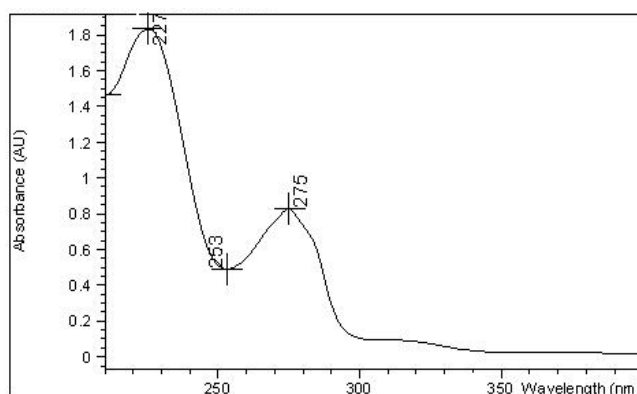


Fig 2. UV spectrum of GLM in ethanol.

The above procedure was repeated three times. Mean absorbance values along with the statistical data for the method are shown in table 1. Tab. 2 represents the optical characteristics and validation parameters of the proposed method for estimation of glimepiride.

**Table 1.** Mean absorbance values and statistical data of the calibration curve for the estimation of glimepiride.

N <sup>o</sup>	Concentration (µg/mL)	Mean ABS <sup>a</sup>
1	4	0.2829
2	5	0.3424
3	6	0.4054
4	7	0.4692
5	10	0.6684
6	15	0.9620

<sup>a</sup>Mean of three values.

**Table 2.** Optical characteristics and validation parameters for standard glimepiride.

Parameter Value	
λ <sub>max</sub> (nm)	227 <sup>a</sup> , 275
Beer's range <sup>b</sup> (µg/mL)	2-40
Molar absorptivity <sup>b</sup> (L/mol/cm)	3.2685 × 10 <sup>4</sup>
Correlation coefficient <sup>b</sup> (r <sup>2</sup> )	0.9995
Regression equation <sup>b</sup>	y = 0.0630x + 0.0301
Y-Intercept	0.0301
Slope (S)	0.0630
Limit of detection <sup>b</sup> (LOD µg/mL)	0.97
Limit of quantification <sup>b</sup> (LOQ µg/mL)	2.70
Precision (% RSD)*	2.58

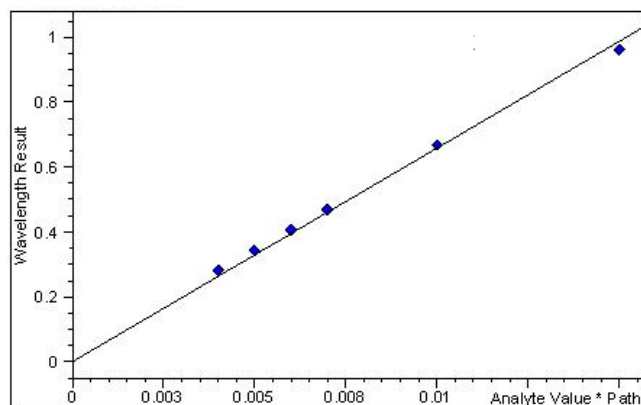
<sup>a</sup>Analytical wavelength for proposed method.

<sup>b</sup>At analytical wavelength.

\*Indicates mean of six determinations (n=6); RSD: Relative standard deviation

## 7. UV-spectrophotometric method validation

The proposed method was validated in the light of FDA Guidelines for linearity, precision, sensitivity and selectivity, stability and recovery (16).



**Fig 3.** Calibration curve of GLM (227 nm, y = 0.0630x + 0.0301).

## Linearity

To establish linearity of the proposed method, six separate series of solutions of the drug (2–40 µg/mL) in ethanol were prepared from the stock solutions and analyzed. A six point calibration curve was constructed and found to be linear (fig 3). Least square regression analysis was used to obtain data (table 2).

## Precision

The precision of an analytical method is the degree of agreement among individual test results when the method is applied repeatedly to multiple samplings of a homogenous sample (United States Pharmacopeia, 2000). In order to determine the precision of the method, solutions containing known amounts of pure drug were prepared and analyzed in three replicates and the absorbances were recorded in six replicates to get the mean. The low %RSD values obtained from the analyses of pure standards of GLM indicated that the method has high precision. The analytical results obtained from these investigations for the methods are showed in table 2.

## Limit of detection and quantitation

The detection limit (LOD) may be expressed as:

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

Where  $\sigma$  is the standard deviation of the response, S is the slope of the calibration curve. The slope, S, may be estimated from the calibration curve of the analyte. A specific calibration curve may be studied using samples containing an analyte in the range of LOD. The residual standard deviation of regression lines or the standard deviation of y-intercepts of regression lines were used as the standard deviation. The Limit of detection of the proposed method was 0.97 µg/mL.

LOQ may be expressed as:

$$LOQ = \frac{10 \sigma}{S}$$

Where  $\sigma$  is the standard deviation of the response and S is the slope of the calibration curve. The slope, S, may be estimated from the calibration curve of the analyte. A specific calibration curve should be studied using samples, containing an analyte in the range of LOQ. The residual standard deviation of regression lines or the standard deviation of y-intercepts of regression lines were used as the standard deviation. The limit of quantitation of glimepiride was 2.70 µg/mL.

## Application of the method in human biological fluids

Analyses were performed using drug-free biological fluids: 5 mL of whole blood and 10 mL of urine. Blood samples were diluted with distilled water (1:1). Specimens were spiked with

chromatographically pure GLM. After hydrolysis with diluted hydrochloric acid at 50-60 °C for 15-20 min and double extraction into chloroform, the serum and urine extracts were passed through coarse filter paper and evaporated to dryness in disposable plastic 25-mL conical beakers. After evaporation to dryness, 3 mL of ethanol were added in conical beakers for dissolution of dried extracts. The absorbance of sample solution was measured at 227 nm and amount of glimepiride was determined by referring to the calibration curve. The above procedure was carried out in triplicate and absorbance readings were recorded three times to get the mean. Results of these determinations are included in tables 3-4.

**Table 3.** Results of the proposed method for estimation of GLM in whole blood

Amount of drug added (mg)	Individual amounts found (mg) mean (S.D.)	Coefficient of variation	Confidence limits <sup>a</sup>
10.20	6.83	1.69	6,46 –7.02 (±0.28)
10.10	6.61		
10.10	6.77		
	6.74 (0.114)		

<sup>a</sup> Confidence limits at P=0.95

**Table 4.** Results of the proposed method for estimation of GLM in urine

Amount of drug added (mg)	Individual amounts found (mg) mean (S.D.)	Coefficient of variation	Confidence limits <sup>a</sup>
10.10	7.42	0.87	7.33-7.65 (±0.16)
10.15	7.55		
10.10	7.49		
	7.49 (0.065)		

<sup>a</sup> Confidence limits at P=0.95

### 3. Results and Discussion

#### 1. TLC Analysis

The TLC studies showed that the best mobile phase was chloroform:acetone (9:1), The R<sub>f</sub> value of GLM in five examinations was 0.37±0.02. In the examination of visualization reagents for GLM, the spraying of Dragendorff's test solution produced a pale yellow-red spot, Bushard's test solution- a brown spot, diphenylcarbazone chloroform solution followed by spraying mercury sulphate – a blue spot (remains after drying the plate) The limit of detection (LOD) of pure GLM by TLC was 0.5 µg/mL, 1.5 µg/mL in whole blood and 1.0 µg/mL in urine.

#### 2. UV-spectrophotometric Analysis

Glimepiride in ethanol yields a characteristic curve when scanned in the ultraviolet wavelength range between 200 and 400 nm. The scan (figure 2) shows absorption maxima at 227 and 275 nm (table 2). The British Pharmacopoeia uses 275 nm

to measure GLM (17). However, the peak response of glimepiride is relatively small at 275 nm as compared to peak at 227 nm. The absorptivity at 227 nm was found to be  $3.2685 \times 10^4$  l mol<sup>-1</sup> cm<sup>-1</sup> and this wavelength was chosen as the analytical wavelength. Regression analysis was performed on the experimental data. The raw data along with the results of regression analysis (method of least squares) is shown in table 2. The regression equation was  $y = 0.0630x + 0.0301$ . The correlation coefficient was found to be 0.9995, signifying that a linear relation existed between absorbance and concentration of the drug. The limit of detection of glimepiride at 227 nm was 0.97 µg/mL and limit of quantitation was 2.70 µg/mL. The precision of the method was carried out using known amounts of pure drug that were subjected to recovery studies in triplicate and evaluated using the S.D. of the results and %RSD - 2.58.

Estimation of glimepiride was also carried out in the human whole blood and urine. The data given in tables 3-4 shows that there is significant difference between the amount of drug spiked in serum and the amount recovered. The proposed method of extraction for glimepiride results in recoveries of more than 65% for blood and 74% for urine. This is due to the complex composition of biological matrix and influence to isolate the co-extractives.

The method has been successfully applied on the blood of patients from the Republican Center for Endocrinology of the Ministry of Health of Uzbekistan after oral administration of GLM and on postmortem blood in overdose cases. The results demonstrate that proposed method for the determination of glimepiride in biological fluids is accurate, precise, and reproducible while being simple and rapid.

### 4. Conclusions

The objective of this work was to develop a simple, rapid, accurate and specific TLC and UV-spectrophotometric method for the estimation of glimepiride in pure substance and biological fluids. The methods were validated for precision, sensitivity and linearity. The limit of detection and limit of quantification were also determined. The results of analysis were validated statistically and by recovery studies. The proposed method is recommended for routine analysis since it is rapid, simple and economical.

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