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RESEARCH ARTICLE

Validation of a New High Performance Liquid Chromatography Assay for Glibenclamide in Human Plasma

Ahmed YUSUF*, Muhammad M HAMMAMI*°

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Summary

A simple reversed phase high performance liquid chromatography assay for the determination of glibenclamide in human plasma was developed and validated. Glibenclamide and ketoconazole (internal standard) were extracted with dichloromethane/hexane (50:50, v : v), eluted on a Symmetry RP18 column with a retention factor of 4.6 and 2.1, respectively, and monitored fluorometrically at excitation and emission wavelengths of 235 and 354 nm, respectively. The mobile phase was a mixture of 0.05 M ammonium phosphate (pH 5.5) and acetonitrile (50:50, v : v) delivered at a flow rate of 1.0 ml/min. The limit of detection was 0.005 µg/ml. Calibration curves were linear ($R^2 \geq 0.9993$) over the range of 0.01 (lower limit of quantitation) to 0.60 µg/ml, and intra-and inter-day coefficients of variation were ≤5.9% and 6.5%, respectively. Mean extraction recovery of glibenclamide and ketoconazole were 91% and 93%, respectively. Glibenclamide was stable in plasma for 24 hours at room temperature (≥ 93%), 4 weeks at –20°C (≥ 97%), and after 3 cycles of freeze at –20°C and thaw at room temperature (≥ 97%). In extracted samples, glibenclamide was stable for 24 hours at room temperature (≥ 98%) and 48 hours at –20°C (≥ 102%). Glibenclamide stock solution (1 mg/ml in methanol) was stable for 48 hours at room temperature (98%) and 2 weeks at –20°C (88%).

Key Words: *Glibenclamide, Ketoconazole, Validation, Stability.*

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İnsan Plazmasındaki Glibenklamid İçin Yeni Bir Yüksek Performans Sıvı Kromatografisi Yönteminin Validasyonu

Özet

İnsan plazmasındaki Glibenklamit tespiti için bir basit ters faz yüksek performans sıvı kromatografisi yöntemi geliştirilmiş ve valide edilmiştir. Glibenklamit ve ketokonazol (iç standart) diklorometan/hekzan (50:50, v:v) ile ekstrakte edilmiş, Symmetry RP18 kolonu ile, sırasıyla 4,6 ve 2,1 alıkonma faktörü verecek şekilde ayrıştırılmıştır. Maddeler florometrik olarak, sırasıyla 235 ve 354 nm eksitasyon ve emisyon dalga boylarında gözlenmiştir. Hareketli faz 0,05 M amonyum fosfat (pH 5,5) ve asetonitril (50:50, v:v) içeren bir karışım olup, akış hızı 1,0 ml/dk olarak ayarlanmıştır. Saptanma sınırı 0,005 µg/ml olarak tespit edilmiştir. Kalibrasyon eğrileri, 0,01 (alt nicel sınır) ile 0,60 µg/ml aralığı boyunca doğrusal olup, gün içi ve günler arası varyasyon katsayıları sırasıyla ≤%5,9 ve %6,5 olarak bulunmuştur. Glibenklamit ve ketokonazolün ortalama ekstraksiyon geri kazanımı değerleri sırasıyla %91 ve %93 olarak hesaplanmıştır. Glibenklamit, plazmada oda sıcaklığında 24 saat boyunca (≥%93), –20°C'de 4 hafta (≥%97) ve 3 kez –20°C'de dondurulma ve oda sıcaklığında çözülme döngüsünden sonra (≥%97) stabil kalmıştır. Ekstrakte edilmiş örneklerde, Glibenklamit oda sıcaklığında 24 saat (≥%98) ve –20°C'de de 48 saat (≥%102) stabil kalmıştır. Glibenklamitin stok çözeltisi (metanolde 1mg/ml konsantrasyonda) oda sıcaklığında 48 saat (%98) ve –20°C'de de 2 hafta (%88) stabil kalmıştır.

Anahtar Kelimeler: *Glibenklamit, Ketokonazol, validasyon, stabilite.*

INTRODUCTION

Glibenclamide, 5 - chloro - N - [2 - {4 - (cyclohexylcarbamoyl - sulfamoyl) phenyl} ethyl] - 2 - methoxy - benzamide [C_{23} H_{28} CIN_3 O_5 S; mol wt. 490] (Fig. 1–a), is a second generation oral sulfonylurea that is widely used to treat type II diabetes mellitus (1-3). It circulates 97% bound to plasma albumin (4). Its oral bioavailability is formulation-dependent, about 81% and 100% is absorbed

* Centre for Clinical Studies and Empirical Ethics, King Faisal Specialist Hospital & Research Centre. Riyadh Kingdom of Saudi Arabia.
° Corresponding Author E-mail: muhammad@kfshrc.edu.sa

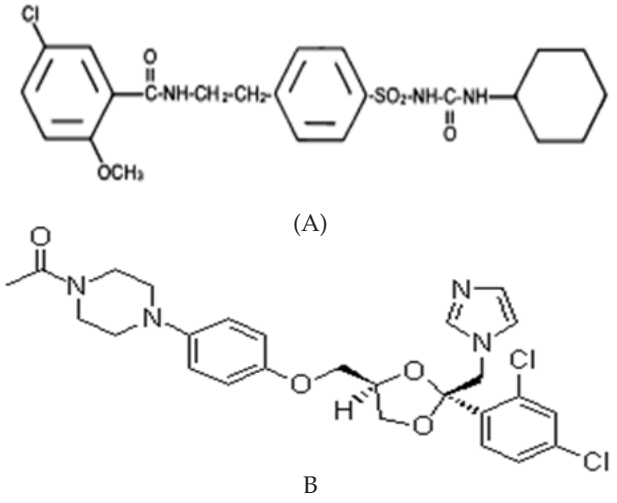

Figure 1: (a) Structure of glibenclamide ($C_{23}H_{28}ClN_3O_5$-S). **(b)** Structure of ketoconazole, the internal standard ($C_{26}H_{28}CL_2N_4O_4$).

when administered as non-micronised form and micronised tablets, respectively (5).

Several high performance liquid chromatography methods with UV (1,2,6-10), fluorescence (11-14) or mass spectrometry (4,15–19) detection have been reported for the determination of glibenclamide in biological samples. However, some of these methods require laborious extraction steps (1), had relatively low sensitivity (6,9,11,14, 15), or used equipment not readily available in most clinical laboratories (4,6,7,16-19). The use of progesterone as internal standard (8), benzene as an extraction solvent (10,12), and low detection wavelength (2) were other significant limitations. Protein precipitation (11,13) can deteriorate the chromatographic column even with low injection volumes due to either high salt content or inadequate precipitation of protein content.

To measure glibenclamide in human plasma, we developed and fully validated a sensitive and precise assay that is based on liquid–liquid extraction, fluorescence detection, and ketoconazole as an internal standard.

EXPERIMENTAL

Apparatus

The HPLC system Waters® (Waters Corporation, Milford, MA, USA) consisted of Alliance 2695 Separations Module and 2475 fluorescence detector with the excitation and emission wavelengths set at 235 and 354 nm, respectively. Empower Software was used for data storage and processing.

Chemicals and reagents

Analytical samples of glibenclamide, the internal standard (IS, ketoconazole Fig. 1–b), and dibasic ammonium phosphate were purchased from Sigma-Aldrich, St. Louis, MO, USA. Acetonitrile, dichloromethane, hexane, hydrochloric acid, phosphoric acid, and methanol (all HPLC grade,) were purchased from Fisher Scientific, Fairlawn, NJ, USA. Water for the HPLC was prepared by reverse osmosis and further purified by passing through a Milli-Q System (Millipore, Bedford, MA, USA).

Chromatographic conditions

The separation of compounds was achieved on Symmetry Shield RP_{18} (4.5 × 150 mm 5-µm) column preceded by a Guard Pak pre-column module with Nova-Pak C_{18} 4-µm insert.

The mobile phase consisted of 0.05 M dibasic ammonium phosphate (pH adjusted to 5.5 with phosphoric acid) and acetonitrile (50 : 50, v : v), and was filtered and degassed under reduced pressure prior to use. Separation was carried out isocratically at ambient temperature at a flow rate of 1.0 ml/min, with the fluorescence detector set at excitation and emission wavelengths of 235 nm and 354 nm, respectively.

Stock and working standard solutions

Stock solutions of glibenclamide and the IS were prepared separately at a concentration of 1 mg/ml in methanol. Aliquots from these stock solutions were transferred into 4.0 ml amber vials and stored at –20°C until used (within two weeks). The working solution of glibenclamide was prepared by diluting 50 µl of the stock solution up to 25 ml in blank human plasma (leftover plasma obtained from the blood bank of King Faisal Specialist Hospital and Research Centre, with the Research Ethics Committee approval) to produce a working solution of 2 µg/ml. 125 µl of the IS stock solution was completed to 50 ml with mobile phase to produce a working solution of 2.5 µg/ml. The working solutions were used within 1 week of their preparation.

Calibration standards and quality control (QC) samples

Nine calibration standards were prepared by mixing blank human plasma with the appropriate volumes of glibenclamide working solution to produce final concentrations in the range of 0.01–0.60 µg/ml. Quality control samples were prepared by mixing four different volumes of glibenclamide working solution with human blank plasma to produce QC samples with final concentrations of 0.01, 0.03, 0.30, and 0.54 µg/ml. Samples were vortex-mixed for 10 seconds, then 0.5 ml aliquots were transferred into (13x100 mm) screw-capped glass culture tubes and stored at –20°C until used.

Sample preparation

Aliquots of 0.5 ml of plasma samples, calibration standards, or QC samples in screw-capped glass culture tubes were allowed to equilibrate at room temperature prior to analysis. To each tube, 100 µl of IS (2.5 µg in mobile phase) and 50 µl of 1 M HCl were added and the mixture was briefly shaken. 5.0 ml of the extraction solvent, dichloromethane–hexane (50 : 50, v : v) were added. After vortex-mixing for 3 minutes, the solution was centrifuged at 4500 rpm for 15 minutes at room temperature. The organic layer was transferred to a clean tube, and dried under a gentle stream of nitrogen at room temperature. The residue was reconstituted in 200 µl mobile phase and 100 µl was injected into the HPLC system.

Stability studies

Plasma samples: The stability of glibenclamide in human plasma was assessed by analyzing QC samples at two concentrations, 0.03 and 0.54 µg/ml. Five aliquots of each QC sample were freshly prepared, extracted, and immediately analyzed (baseline). Counter and long term stability were assessed by placing five aliquots of each QC sample on the bench-top at ambient temperature for 24 hours or at –20°C for four weeks before being processed and analyzed. Further, five aliquots of each QC sample were processed, reconstituted and stored at ambient temperature for 24 hours or at –20°C for 48 hours before analysis (auto-sampler stability). Finally, freeze and thaw stability was estimated by analyzing fifteen aliquots of each QC sample stored at –20°C for 24 hours, and left to completely thaw unassisted at ambient temperature before analysis (with the cycle repeated three times).

Stock and working solutions stability

Stock solution: The stability of glibenclamide and IS (1 mg/ml in methanol) were evaluated for up to 48 hours at room temperature and up to 2 weeks at –20°C. After completion of the desired storage time, the solutions were diluted in methanol and water (50:50, v : v) to 1 µg/ml or 5 µg/ml for glibenclamide and the IS, respectively. 100 µl was injected into the HPLC system in five replicates. Working solution: Stability of the working solutions of glibenclamide (2.0 µg/ml in plasma) and the IS (2.5 µg/ml in mobile phase), were evaluated up to 1 week at –20°C. We assessed stability by comparing peak area of each compound with that of freshly prepared solutions.

Calculations

Precision was calculated as coefficient of variation (standard deviation divided by mean measured concentration × 100) and inaccuracy (bias) as mean measured concentration – nominal concentration / nominal concentration × 100.

RESULTS AND DISCUSSION

Optimization of the chromatographic conditions

A High Performance Liquid Chromatographic assay for the quantification of glibenclamide level in human plasma was developed. The assay was performed at room temperature on a Symmetry Shield RP_{18} (4.5 × 150 mm 5-µm) column, using a Guard Pak pre-column module with Nova-Pak C_{18} 4-µm insert and Waters 2475 fluorescence detector with the excitation and emission wavelengths set at 235 and 354 nm, respectively. The chromatographic conditions were optimized by examining various ratios and pH of the mobile phase. Mobile phase selection was based on peak parameters (symmetry, tailing), run time, ease of preparation, and cost. The best mobile phase consisted of 0.05 M dibasic ammonium phosphate (pH adjusted to 5.5 with phosphoric acid) and acetonitrile (50 : 50, v : v) with a flow rate of 1 ml/min, run time of 11 minutes. Under the described conditions, glibenclamide and the IS were well resolved with a retention factor (K′) of 4.6 and 2.1, respectively.

Assay Validation

The analytical method was fully validated with respect to the parameters described in the US Food and Drug Administration (FDA) bioanalytical method validation guidelines (20).

Specificity

To evaluate specificity, plasma samples from six drug-free plasma samples were screened for the presence of endogenous components, which might co-elute with glibenclamide or the IS. Specificity was also checked for possible interferences arising from nine frequently used medications, namely: aspirin, acetaminophen, ascorbic acid, ibuprofen, caffeine, antipyrine, nicotinic acid, omeprazole and ranitidine. All blank human plasma samples were free from co-eluting peaks, and none of the drugs interfered with glibenclamide or IS. A representative chromatograph of blank human plasma is shown in Figure 2.

Linearity

Linearity of the method was evaluated using nine concentrations over the range of 0.01-0.60 µg/ml. Calibration curves were linear with an $R^2 \geq 0.9993$. Figure 3 shows an overlay of chromatograms of a typical of calibration curve, and Figure 4 depicts the mean of nine representative calibration curves. The mean (SD) of slope, intercept, and (coefficient of variation) [2] of the nine curves were 0.3675 (0.0068), 0000 (0.0013), and 0.9997 (0.0002), respectively. Mean concentrations, back calculated from peak ratios using individual regression equation are given in Table 1 with their respective biases and precisions.

Limits of detection and quantification

The limit of detection, defined as three times the baseline noise, was 0.005 µg/ml. The limit quantification, defined as the lowest measured concentration with a precision and bias of <±10% was 0.01 µg/ml.

Precision and bias (inaccuracy)

The intra-day and inter-day precision and bias of the method were evaluated, by analyzing four different concentrations of glibenclamide over three different days. Intra-day precision and bias (n = 10) ranged from 1.2% to 5.9% and from –2.1% to 6.0%, respectively. The inter-day precision and bias (n = 20) ranged from 2.5% to 6.5% and from 0.5% to 3.0%, respectively (Table 2).

Recovery

The extraction recovery of glibenclamide and the IS from plasma was calculated by comparing the mean

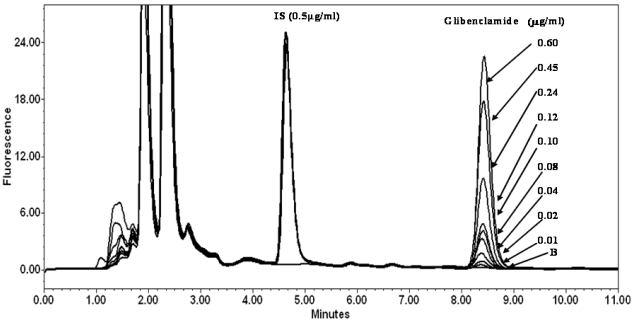

Figure 3: Overlay of calibration curve chromatograms of glibenclamide spiked with ketoconazole (internal standard, IS).

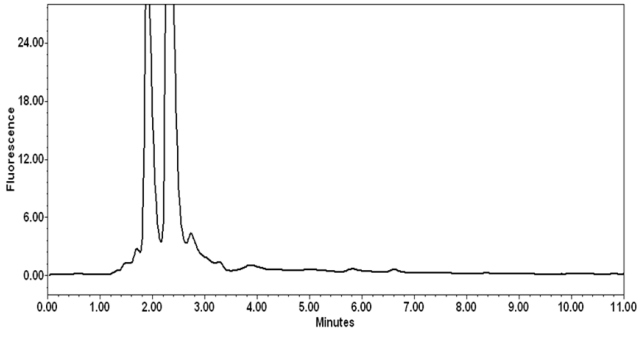

Figure 2: A representative chromatograph of a blank human plasma.

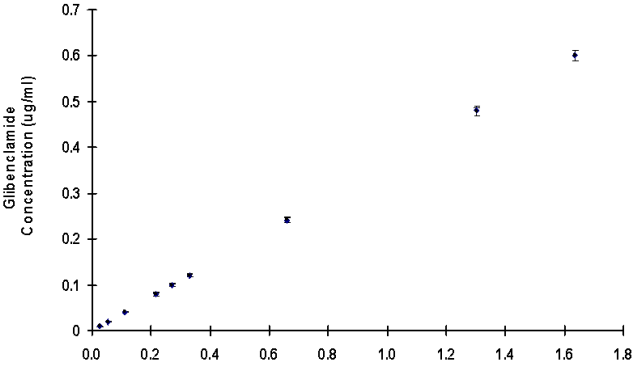

Figure 4: Mean of nine representative calibration curves of glibenclamide assay. Bars represent standard errors.

Table 1: Back-Calculated Glibenclamide Concentrations of Nine Calibration Curves

Nominal Concentration (µg/ml)	0.01	0.02	0.04	0.08	0.10	0.12	0.24	0.48	0.60
Mean	0.0097	0.0195	0.0412	0.0791	0.0988	0.1210	0.2420	0.4780	0.6007
S.D.	0.0007	0.0011	0.0024	0.0025	0.0016	0.0010	0.0061	0.0054	0.0045
*Precision (%, CV)	7.0	5.7	5.9	3.1	1.6	0.9	2.5	1.1	0.7
†Bias	–3.0	–2.5	3.0	–1.1	–1.2	0.8	0.8	0.4	0.1

* Coefficient of variation (CV, %) = Standard Deviation (SD) divided by mean measured concentration × 100.
† Bias = (mean measured concentration – nominal concentration divided by nominal concentration) × 100. The mean (SD) of slope, intercept, and (coefficient of variation) ^{2} of the nine curves were 0.3675 (0.0068), 0000 (0.0013), and 0.9997 (0.0002), respectively.

peak areas of five replicates of four QC samples and five replicates of the IS (0.5 µg/ml) that were prepared in plasma (as described under sample preparation above), to the mean peak areas of five replicates of equivalent concentrations prepared in the mobile phase. The recovery of glibenclamide and the IS was ≥ 84% (mean 91%) and 93%, respectively (Table 3).

Stability

Stability studies were investigated at two concentrations (0.03 and 0.54 µg/ml) in plasma, processed samples, and stock solutions under usual storage conditions. The results revealed no significant decrease in measured concentrations or change in chromatographic behavior of glibenclamide after storage of plasma for 24 hours

Table 2: Intra-day and inter-day precision and bias (inaccuracy) of glibenclamide assay

	Intra-day (n=10)				Inter-day (n=20)			
Nominal Concentration µg/ml	Found Concentration Mean µg/ml	S.D	*Precision (%, CV)	†Bias %	Found Concentration Mean µg/ml	S.D	*Precision (%, CV)	†Bias %
0.01	0.0106	0.0005	4.9	6.0	0.0103	0.0007	6.5	3.0
0.03	0.0313	0.0018	5.9	4.3	0.0305	0.0016	5.4	1.7
0.30	0.3052	0.0035	1.2	1.7	0.3072	0.0076	2.5	2.4
0.54	0.5289	0.0132	2.5	-2.1	0.5426	0.0248	4.6	0.5

* Coefficient of variation (CV, %) = Standard Deviation (SD) divided by mean measured concentration × 100.
† Bias = (mean measured concentration – nominal concentration divided by nominal concentration) × 100.

Table 3: Extraction recovery of glibenclamide and ketoconazole

Nominal Concentration (µg/ml)	* Recovery (%)	
	† Mean	SD
Glibenclamide		
0.01	100.1	5.9
0.03	97.9	6.1
0.30	84.0	1.4
0.54	83.9	0.9
Ketoconazole (IS)		
0.5	93.0	1.1

* Peak area of spiked plasma sample divided by peak area of spiked mobile phase sample × 100.
† Mean of 5 replicates. SD, Standard Deviation.

Table 4: Stability of glibenclamide in human plasma and stock solution

Stability (%)									
	*Plasma sample							†Stock solution	
Nominal concentration (µg/ml)	Unprocessed		Processed		Freeze-Thaw cycle				
	24 hrs RT	4 wks −20°C	24 hrs RT	48 hrs −20°C	1	2	3	48 hrs RT	2 wks −20°C
0.03	93	97	98	102	99	102	103	98	88
0.54	94	97	99	105	99	98	97		

Stability, mean measured concentration (n=5) at the indicated time divided by mean measured concentration at baseline × 100 (n=5).
* Spiked plasma samples were processed and analyzed immediately (baseline, data not shown), after 24 hours at room temperature (24 hrs, RT), after freezing at −20°C for 4 weeks (4 wks, −20°C), or after 1 to 3 cycles of freezing at −20°C and thawing at room temperature (freeze-thaw); or extracted and analyzed after leaving the extract 24 hours at room temperature (24 hrs, RT) or 48 hours at −20°C (48 hrs, −20°C).
† Glibenclamide 1 mg/ml in methanol.

at room temperature or 4 weeks at −20°C, or after three freeze-thaw cycles. Stock solutions of glibenclamide and IS (1 mg/ml in methanol) were stable for at least 48 hours at room temperature (98% and 99% respectively) and for at least 2 weeks at −20°C (88% and 91% respectively.) Further, the working solutions of glibenclamide and IS (2 µg/ml in plasma and 2.5 µg/ml in mobile phase, respectively) were stable for at least 1 week at −20°C (89% and 87% respectively). Finally, glibenclamide was stable in processed samples for at least 24 hours at room temperature or 48 hours at −20°C (Table 4).

Robustness

Robustness of the assay was evaluated by slight deliberate changes in chromatographic conditions (mobile phase composition, buffer strength, and pH) No significant changes were observed, indicating the robustness of the method.

Conclusion

The proposed HPLC method employing simple liquid – liquid extraction and fluorescent detection is sensitive, rapid, precise, specific, and convenient for the determination of glibenclamide in plasma samples. It has been implemented successfully for studying glibenclamide stability, and appears suitable for pharmacokinetic studies of glibenclamide.

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