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Original article

Development and Validation of an Analytical Method for Glyburide and Its Related Compounds in Tablet Formulation by HPLC-UV

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A reverse phase liquid chromatographic method was developed for the determination of purity of glyburide drug substance and drug product in bulk samples and pharmaceutical dosage form in the presence of its impurities and degradation product. This method is capable of separating all the related substance of glyburide along with impurities. This method also can be used for the estimation of assay of glyburide in drug substance as well as in drug product. The method was developed using Inertsil C8 Octyl decane silane (250 x 4.0 mm 5.0µm) column, buffer pH 5.3: acetonitrile in the ratio of 60:40 %v/v used as mobile phase. The elution compounds were monitored at 230 nm. The method was validated as per ICH guidelines demonstrating to the accurate and precise within the corresponding linear range of impurities from LOQ to 200%. The specificity of the method was investigated under different stress conditions including hydrolytic, oxidative, photolytic and thermal as recommended by ICH guidelines. Relevant degradation was found to be significantly in acid, hydrolysis and oxidative condition. Robustness against small modification in column oven temperature, flow rate and percentage of the mobile phase composition was ascertained. Limit of detection and limit of quantification were also determined. The peak purity indices (purity angle < purity threshold) obtained with the aid of PDA detection with satisfactory resolution between related impurities established the specificity of the determination.

Key words: Development, Glyburide, Sulphonamide, Carbamate, Validation.

YPSK-UV ile Tablet Formülasyonunda Gliburid ve İlgili Maddeleri İçin Bir Analitik Yöntem Geliştirilmesi ve Validasyonu

Gliburid'in saflığı, ilaç hammaddesi ve farmasötik dozaj formunun kirlilikleri ve degredasyon ürünleri varlığında miktar tayini için bir ters faz sıvı kromatografi yöntemi geliştirilmiştir. Bu yöntem kirliliklerin yanısıra gliburid ile ilgili maddelerin de ayrımını yapabilmektedir. Bu yöntem gliburidin ilaç üretiminin yanı sıra ilaç hammaddesinin analizlerini de değerlendirmek için kullanılabilir. Bu yöntem Inertsil C8 Oktil dekan silan (250 x 4.0 mm, 5.0 µm) kolon ve mobil faz olarak %60:40 h/h oranında pH 5.3 tampon: asetonitril kullanılarak geliştirilmiştir. Kirlilikler için tayin sınırından %200'üne kadar olan doğrusal aralık içinde doğruluk ve kesinliği gösteren ICH esaslarına göre yöntem valide edilmiştir. Yöntemin spesifikliği, hidrolitik, oksidatif, fotolitik ve termal gibi farklı stress şartlarında ICH tarafından önerilen şekilde araştırılmıştır. İlgili degredasyon ürünleri önemli derecede asit, hidroliz ve oksidatif şartlar altında elde edilmiştir. Kolon sıcaklığı, akış hızı ve mobil faz birleşiminin oranı gibi küçük modifikasyonlara karşı sağlamlık belirlenmiştir. Tayin alt limiti ve teşhis limiti de belirlenmiştir. Pik saflığını gösteren değer (saflık açısı < saflık eşiği), ilgili kirliliklerin miktar tayininin belirlenmiş spesifikliği sınırlarına giren kabul edilebilir ayırım gücü ile PDA dedeksiyonu yardımıyla elde edilmiştir.

Anahtar kelimeler: Yöntem geliştirme, Gliburid, Sülfonamid, Karbamat, Validasyon.

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INTRODUCTION

Glyburide and its impurities namely (RCA) sulphonamide and (RCB) carbamate are chemically known as 5-chloro-N-[2-(4-{[(cyclohexyl carbamoyl) amino] sulfonyl} phenyl) ethyl]-2-methoxybenzamide, 4-[2-(5-chloro-2-methoxybenzamido) ethyl] benzene sulphonamide and Methyl N-4-[2-(5-chloro-2-methoxybenzamido) ethyl] benzene sulphonyl carbamate (Figure 1a-c).

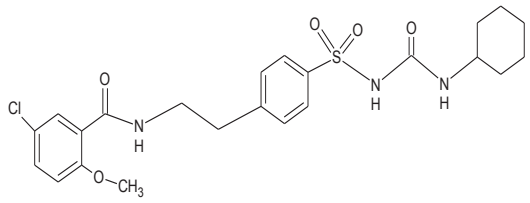


Figure 1a. Structure for glyburide

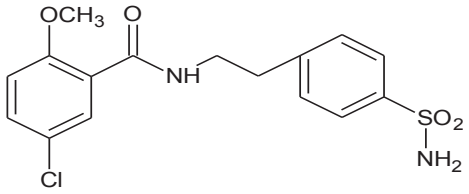


Figure 1b. Structure for glyburide related compound A

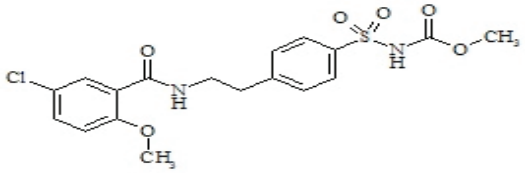


Figure 1c. Structure for glyburide related compound B

Glyburide is a sulfonylurea hypoglycemic. Sulfonylurea bind to adenosine triphosphates sensitive potassium channels on the pancreatic cell surface reduces potassium conductance causing depolarization of the membrane. Depolarization stimulates calcium ion reflux through voltage sensitive calcium channels, raising intracellular concentration of calcium ions, which induces the secretions or exocytosis, of insulin (1, 2). A literature survey reveals that there are number of various analytical methods available for the quantitative individual determination of glyburide or combination with other drugs mainly using ultraviolet spectroscopy, high performance liquid chromatography and liquid chromatography - tandem mass spectroscopy. Determination of Glyburide in human serum with fluorescence detection by high performance liquid chromatography method (3), quantification of glyburide in rat serum by high performance liquid chromatography (4), high performance liquid chromatography with fluorescence detection (5) was reported. Glyburide and its metabolites in plasma sample analyzed by liquid chromatography – mass spectroscopy (6), glyburide combined with metformin in ultraviolet spectroscopy method (7), ultraviolet spectroscopy and high performance liquid chromatography methods (8), studies on forced degradation behavior of glibenclamide and development of a validated stability-indicating method by liquid chromatography-mass spectroscopy (9), determination of metformin and glyburide in human plasma by liquid chromatography-mass spectroscopy (10), quantitative determination of glipizide and glyburide in tablets using high performance liquid chromatography (11) were also reported.

However, to the best of our knowledge, no single stability indicating analytical method for the separation and determination of impurities of glyburide is available in literature. The major objective of the present work is to develop a single method for separation of impurities of glyburide in drug substance. The same method was used for the estimation of assay of the drug substances and drug product.

EXPERIMENTAL

Chemicals

Tablets and standard glyburide and its impurities such as sulphonamide (99.6%) (RCA), carbomate (96.3%) (RCB) were supplied by Edict USV limited, Chennai, India.

The HPLC grade acetonitrile and analytical grade sodium hydroxide, ammonium di hydrogen phosphate was purchased from Renkem and Merck. High purity water was prepared by using Millipore, MilliQ water purification system.

Chromatographic condition

The method was developed using Inertsil C_8 (4.0 x 250mm, 5µm) column, buffer pH 5.3 and acetonitrile (60: 40 %v/v) ratio as mobile phase. The mobile phase was filtered through a PVDF membrane filter (particle size 0.45 µm). The flow rate of the mobile phase was 1.8 mL/min. The column temperature was maintained at 40°C and wave length was monitored as 230 nm. The injection volume was 20µL.

Standard solutions

Standard stock solution preparation (500 µg/mL of glyburide)

50 mg of Glyburide working reference standard was weighed and transferred in to a 100 mL volumetric flask. 50 mL of acetonitrile was added and sonicated to dissolve. Then it was diluted and made up to volume with water and mixed well.

Standard preparation (125 µg/mL of glyburide)

5 mL of Standard stock solution was transferred into 20 mL volumetric flask. Then it was diluted and made up to volume with diluent and mixed well.

Sample preparation

Assay preparation

5 Tablets of glyburide / metformin hydrochloride (containing 1.25 mg glyburide) were accurately weighed to find out the average weight and powdered. The tablet powder equivalent to 1.25 mg of glyburide was transferred in to a 100 mL volumetric flask, diluent was added and sonication for 30 minutes with intermediate shaking. Then it was shaked mechanically for 30 minutes. Then it was diluted and made up to the volume with diluent and mixed well. A portion of sample was centrifuged at 3500 RPM (revolution per minute) for 10 minutes and filtered through 0.45µ poly vinylidene difluoride (PVDF) filter. After discarding first 4 mL of the filtrate, the remaining filtrate was used for the further analysis.

System suitability preparation

System suitability intermediate preparation

1 mg of USP glyburide RCA related substance was weighed and transferred in to a 100 mL volumetric flask. 70 mL of diluent was added and sonicated to dissolve. Then it was diluted and made up to volume with diluent. Further 1 mL of the above solution was transferred in to a 20 mL volumetric flask and made up to volume with diluent. Finally 5 mL of above solution and 5 mL of standard stock solution were transferred into 20 mL volumetric flask and made up to the volume with diluent.

RESULTS and DISCUSSION

Method development and optimization

Method development focuses on identifying buffer type, strength and pH of organic solvent and implementing small changes to optimize selectivity and enhance resolution. The optimization with mobile phase consisted of ammonium dihydrogen phosphate buffer: acetonitrile (70:30%, v/v) (pH 3.4 adjusted with acetic acid), flow rate 1.2 mL/min, Zorbax XDB C_{18} (150 x 4.6mm, 5 µm) and isocratic technique was done. The peak tailing factor was found to be 2.2 and retention time of 23.537 minutes. The optimization was continued by changing the pH of the buffer, same column and mobile phase. As a result glyburide peak shape was distorted, asymmetry was observed. Less USP plate count was found. Next, Symmetry C 8 (150 mm x 4.6 mm i.d, 5 µ) column with mobile phase containing ammonium dihydrogen phosphate buffer: acetonitrile (50:50%, v/v) (pH: 5.50, adjusted with 1 N NaOH) and flow rate 1.2 mL/min was used. The peak tailing factor was found to be 2.8 and retention time was found to be 18.256

minutes. At this stage, the mobile phase ratio, flow rate and pH were changed. Less USP plate count was found and peak tailing was not found. Related compound A peak was found. Finally the sample solution was spiked with known impurities of RCA and RCB. Mobile phase containing ammonium dihydrogen phosphate buffer: acetonitrile (60:40 %, v/v) (pH 5.5 adjusted with 1N NaOH) and flow rate 1.6ml/min was done. Less separation for RCA and RCB peaks were found. To increase the separation, the same mobile phase, flow rate and stationary phase with Inertsil C8 (250 x 4.6 mm i.d, 5 µ) column were used. USP plate count was found to be 4200. Related compound A (sulphonamide impurity) and related compound B (Carbamate impurity) peaks were well separated. The RRT (Relative Retention Time) for related compound A (sulphonamide impurity) was found to be 0.29. Using the above chromatographic conditions resulted in the development of an efficient and reproducible method for the determination of impurities and degradants of glyburide in glyburide and metformin HCl tablet dosage form. The optimized chromatogram was shown in Figure 2.

Method validation

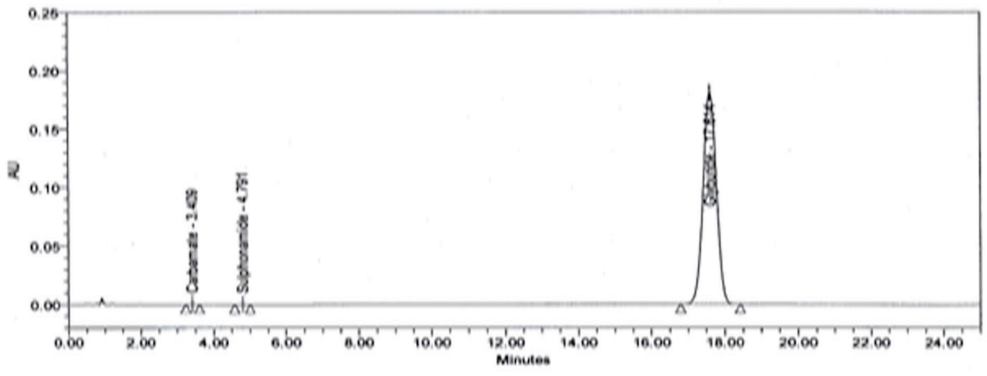
The proposed method was validated as per ICH guidelines (12, 13).

Precision

The precision of the related substances method was verified by method precision and system precision. Method precision was checked by injecting six individual preparations of glyburide and metformin HCl (1.25 mg/250 mg tablet) sample spiked with1.25 µg/mL of its two impurities. Percentage relative standard deviation (% RSD) of area for each impurity was calculated. System precision of the method was evaluated by carrying out five replicate injection of the standard solution (125 µg/mL). The chromatograms were recorded and system suitability was calculated. The % RSD for the area of impurities A & B in method precision study was found to be 0.99 % and 1.68 % respectively. The % RSD of the assay results obtained in the system precision study was found to be 0.05 % for glyburide. The % RSD values were found to be not more than 2.0 %. It indicated good precision of the method. System suitability parameter values were found to be within the limit. The values were presented in Table 1.

Linearity

Linearity test solutions for the assay method were prepared from glyburide stock solution of eight concentration levels from 50 to 200 % of assay analyte concentration (61, 74, 99, 123, 148, 173, 198 and 247 µg/mL). The peak area



Peak Results

	Name	Vial	Inj	RT (min)	Area (µV*sec)	% Area
1	Carbamate	3	1	3.409	3736	0.08
2	Sulphonamide	3	1	4.791	3003	0.07
3	Glyburide	3	1	17.614	4467819	99.85

Figure 2. Optimized chromatogram for glyburide and its impurities

verses concentration data was treated by least square regression analysis. Linearity test solutions for related substance method were prepared by diluting stock solution to the required concentrations. The solutions were prepared at eight concentration levels from LOQ to 200 % of the specification level (LOQ, 40, 50, 80, 100, 120, 160 and 200 %). The correlation coefficient was found to be 0.999. The results showed that an excellent correlation existed between the peak area and concentration of the analyte. The correlation coefficient was found to be 0.999. The results indicated that an excellent correlation existed between the peak area and concentration of the impurities (RCA &RCB). The results were shown in Table 2.

Table 1. Data for system precision

System suitability Parameter	Values	USP limit
Capacity factor	17	Not less than 7
USP plate count	11381	Not less than
RRT for RC-A		3000
impurity	0.19	-
RRT for RC- B		-
impurity	0.27	Not more than
%RSD for Area	0.05	1.5

% RSD: Relative standard deviation,
RRT: Relative retention time

LOD and LOQ

LOD and LOQ for impurities A, B were determined by using the following formula LOD = 3.3 x RSD of Y intercept / slope and LOQ = 10 x RSD of Y intercept / slope respectively, by injecting a series of dilute solutions with known concentration from 0.001 µg/mL to 0.249 µg/mL for glyburide, impurities A & B. Precision study was also carried out at LOQ level by injecting six individual preparations of impurities and calculating the %RSD of the area. The determined limit of detection and limit of quantification and precision at LOQ values for glyburide and its impurities were shown in Table 2. The LOD and LOQ values as shown demonstrated that the method was sensitive for the determination of glyburide related substance.

Accuracy

Accuracy of the assay method was evaluated in triplicate using four concentration levels 50, 100, 150 and 200% on sample (glyburide in glyburide and metformin HCl tablet). Standard addition and recovery experiments were conducted on sample to determine accuracy of the related substance method. Study was carried out in triplicate using four concentration levels LOQ, 50, 100 and 200 %. The percentages of recoveries for glyburide and its

Table 2. Regression and precision data

Parameters	Glyburide	Impurity A	Impurity B
Regression Equation	Y= 36716X-84674	Y=46200X+76.42	Y=42712X+184.1
Correlation coefficient (r^2)	0.999	0.999	0.999
Slope	36716	46200	42712
Intercept	-84674	76.42	184.1
Precision (% RSD)	0.29	0.99	1.68
Intermediate precision (%	1.011	1.023	1.023
RSD)	0.022	0.010	0.007
LOD (µg/mL)	0.067	0.023	0.022
LOQ (µg/mL)			

% RSD: Relative standard deviation, LOD: Limit of detection, LOQ: Limit of quantification

impurities were calculated.

The percentage recovery of glyburide from drug product was ranged from 98.37% to 99.32%. The percentage recovery of impurities in glyburide and metformin tablet samples varied from 97.39% to 100.90%. The % recovery values for impurities and glyburide were presented in Table 3. The % RSD values were found to be not more than 2%. Therefore, the method was considered accurate.

Robustness

To determine the robustness of the developed method, experimental conditions were deliberately altered and system suitability parameters for glyburide and its impurities were recorded. The flow rate of the mobile phase was 1.8 mL/min. To study the effect of flow rate on the resolution, flow was changed by 0.2 units from 1.6 to 2.0 mL/min. The effect of column temperature on resolution was studied at 35 °C and 45 °C instead of 40 °C. The effect of the percent acetonitrile strength on resolution was studied by varying acetonitrile from -2 to +2%. The effect of pH of the buffer on resolution was studied by varying from -2 to +2. In all the deliberate varied chromatographic conditions (flow rate, column temperature and composition of organic solvent), the resolution between all pairs of compounds was greater than 2.0 and tailing factor for glyburide and its impurities was less than 2.0.

Solution stability

Solution stability of glyburide in the assay method was carried by leaving both the test solutions of sample and reference standard in tightly capped volumetric flask at room temperature for 48 hours. The same sample solutions were assayed for 12 hour interval up to the study period. Solution stability of glyburide and its impurities in the related substance method was carried out by leaving spiked sample solutions in tightly capped volumetric flask at room temperature for 48 hours. Content of impurities A and B were determined for every 12 hour interval up to the study period. Percentage relative standard deviation (% RSD) for assay of glyburide during solution stability experiments were found to be within 1%. No significant changes were observed in the content of two impurities during solution stability experiments. The solution stability experiments data confirms that the sample solution used during assay and the related substance determination were stable for 48 hrs.

Ruggedness

Ruggedness is a measure of reproducibility of test results under normal variation of the operating condition, for e.g. analyst to analyst, system to system, column to column and day to day. The method precision ruggedness (reproducibility or intermediate precision) of the assay method was determined by injecting six individual preparation of glyburide (125 µg/mL) level against reference standard. The intermediate precision of the assay method was evaluated by different analyst. Intermediate precision of the impurities method was determined by injecting six individual preparation of glyburide and metformin (1.25 mg / 250 mg tablet) spiked with 1.25 µg/mL of its known impurities by using different analyst and different day. Percentage

Table 3. Evaluation of accuracy studies

Amount spiked	Percent Recovery		
	Glyburide	Impurity A	Impurity B
LOQ	-	99.69±6.28	97.39±3.20
50	98.53±0.33	100.71±0.29	99.23±1.30
100	98.37±0.38	100.90±0.34	98.42±0.53
150	99.17±0.46	-	-
200	99.32±0.11	100.24±0.17	97.17±0.75

LOQ: Limit of quantification

relative standard deviation (% RSD) was calculated. The percentage of assay values obtained from six samples was found to be 94.97 to 95.57 % for analyst 1 and 95.90 to 96.84 % for analyst 2. The % RSD value of six samples was found to be not more than 2.0 %. The ratio between the intermediate precision assay average and method precision assay average value was found to be 1.012. The % RSD values for impurities were found to be 0.99 % for impurity A and 1.08 % for impurity B (analyst 1). The % RSD values for impurities were found to be 0.24 % for impurity A and 0.39 % for impurity B (analyst 2). The ratio for the average values obtained from two analysts was found to be 1.038 for impurity A and 0.896 for impurity B (Acceptance criteria 0.80 and 1.20). Therefore the method was found to be rugged.

Specificity (Forced degradation study)

Specificity is the ability of the method to means the analyte response in the presence of its potential impurities. The specificity of the developed reversed phase liquid chromatographic method for glyburide was carried out in the presence of its impurities namely A and B. Stress studies were performed for glyburide and metformin tablet to provide an indication of the specification of the proposed method. Degradation was attempted with a stress condition of UV light, acid (1N HCl), base (1N NaOH), oxidation (3% H_2O_2) and heat (105°C) to evaluate the ability of the proposed method to separate glyburide from its degradation product. For heat and light studies, study period was 3 days whereas for acid, base and oxidation it was 3 hours. Glyburide was found to degrade significantly in acid, hydrolysis and oxidation. Mild degradation was observed in thermal stress condition. Glyburide was found to be stable under photolytic and base hydrolysis condition. This was confirmed no secondary peaks arising from degraded samples interfered with the elution of the glyburide peak. Peak purity analysis was carried using the PDA detector it demonstrated glyburide peak homogeneity. All known impurities did not interfered with the glyburide peak. The study validated that the method was specific and stability indicating. The reports of analysis were shown in Table 4. The chromatograms were shown in Figure 3a-e.

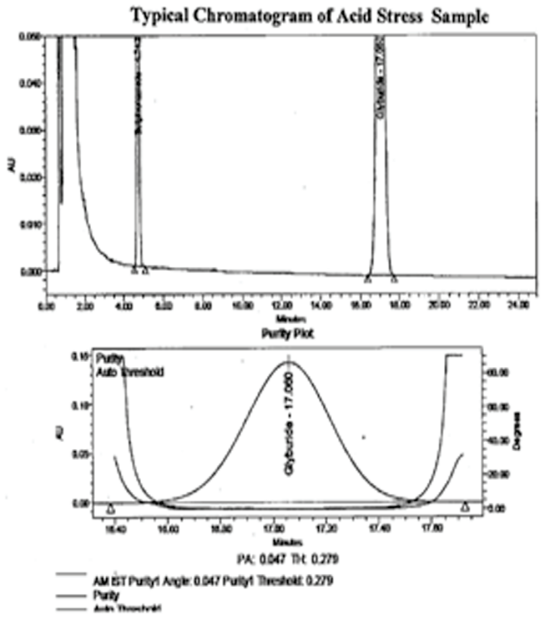


Figure 3a. Chromatogram for acid stress

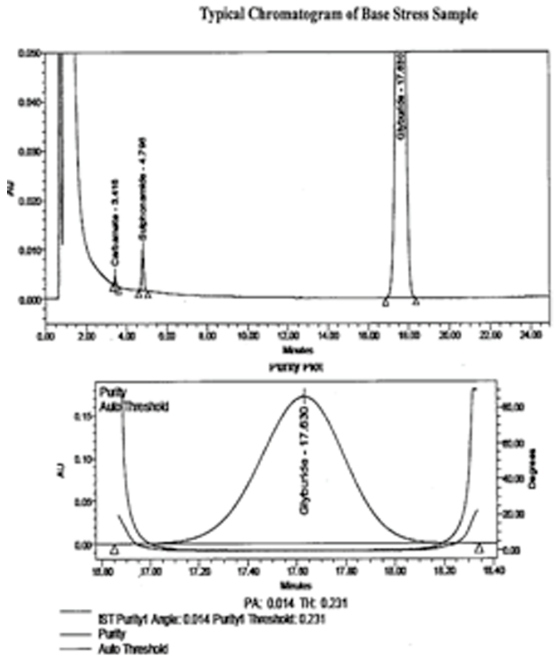


Figure 3b. Chromatogram for base stress

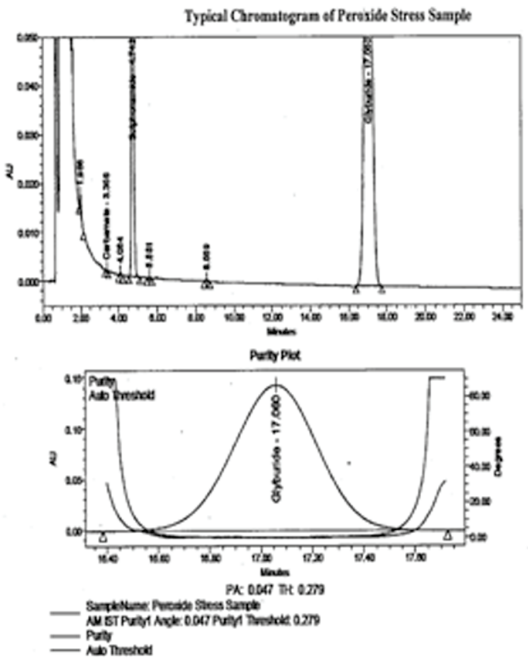


Figure 3c. Chromatogram for peroxide stress

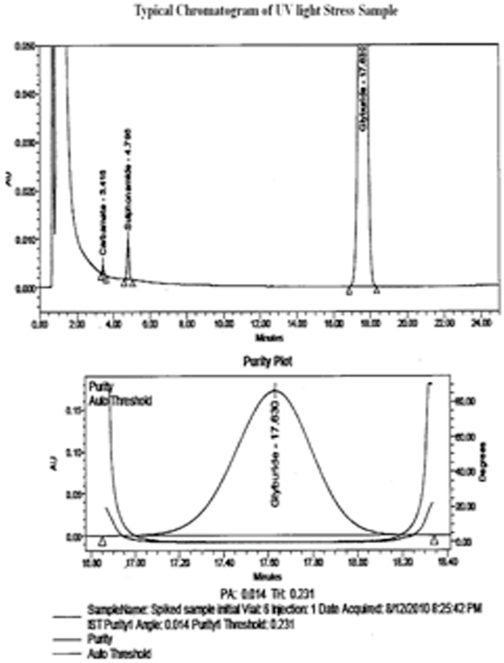


Figure 3e. Chromatogram for UV light stress

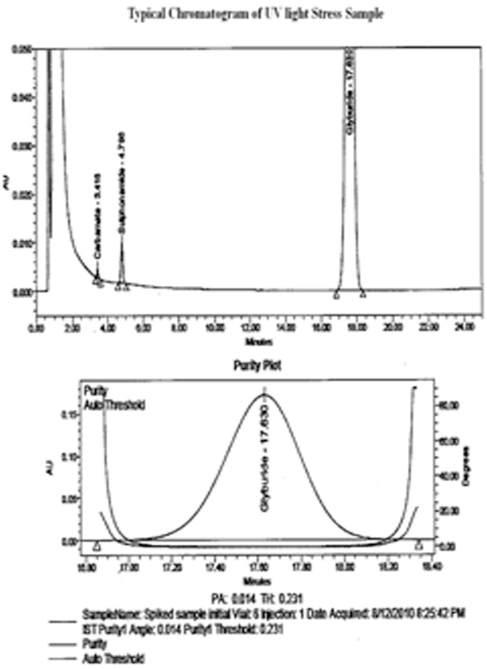


Figure 3d. Chromatogram for heat stress

Filter study

A filter study was performed to determine the suitability of filter used and to determine the amount of filtrate to be discarded before a sample solution is collected for analysis. Accuracy sample at 100 % solution was used for the filter study. This sample was divided in to six portions. One portion of the prepared sample was centrifuged (3500 rpm for 10 minutes), the clear supernatant from the centrifuge tube injected and analysed. The centrifuged sample was used as a control for the filter study. Second portion of sample was filtered through 0.45µ nylon filter by discarding the first 1mL, 2mL, 3mL and 5mL of the filtrate. Accuracy 100 % sample has been considered as 0.45 µ poly vinylidene difluoride filter (PVDF). The assay and percentage of impurities found in the filtered fractions of sample were comparable to the assay and the impurities found in the centrifuged portion of sample, there was no significant difference in assay and percentage impurities between the different volumes filtered. Therefore, the 0.45µ PVDF and nylon filter were suitable for use in

the validation and discarding of 4mL of the sample solution as filtrate, as stated in the method, was a suitable volume to discard before collecting for analysis by HPLC. The percentage difference reports were shown in Table 5.

CONCLUSION

The simple isocratic reversed phase LC method developed for quantitative analysis of glyburide and related substance in bulk and pharmaceutical dosage form was precise, accurate, linear, robust and specific.

Table 4. Summary of forced degradation studies

Sample name	Stress condition	%Degradation	Purity angle	Purity threshold
Control sample	NA	NA	0.017	0.231
Acid stress	5ml of 1N HCL heated on a water bath at 80˚C for 3 hours	19.99	0.045	0.234
Base stress	5 ml of 1N NaOH heated on a water bath at 70℃ for 1 hour	0. 01	0.033	0.237
Peroxide stress	5 ml of 3% H_2O_2 Heated on a water bath at 80˚C for 60min.	13.49	0.047	0.279
UV light stress	Stress under UV light for 72 hrs	0.03	0.026	0.246
Heat stress	Heated in a oven at 105˚C for 72 hrs	12.53	0.030	0.241

NA ------ not applicable

Table 5. Filter studies for 0.45µm PVDF and nylon filter

Sample	Percentage differences		
	Glyburide	Impurity A	Impurity B
Centrifuged (10 min @ 3500rpm)	NA	NA	NA
0.45µ Nylon filtered, 4ml discarded	0.03	1.34	-0.27
0.45µ PVDF filtered, 1ml discarded	-0.52	1.34	-0.63
0.45µ PVDF filtered, 2ml discarded	-0.18	0.89	-0.09
0.45µ PVDF filtered, 3ml discarded	-0.22	0.44	-0.63
0.45µ PVDF filtered, 4ml discarded	-0.24	-1.34	-0.82
0.45µ PVDF filtered, 5ml discarded	-0.09	2.69	-0.54

RPM – Revolution per minute, PVDF - poly vinylidene difluoride filter

Satisfactory results were obtained from the validation of the method. The method was stability indicating and can be used for routine analysis of production samples and so check the stability of glyburide in bulk drugs and in pharmaceutical dosage form.

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