

Simultaneous estimation of six anti-diabetic drugs—glibenclamide, gliclazide, glipizide, pioglitazone, repaglinide and rosiglitazone: development of a novel HPLC method for use in the analysis of pharmaceutical formulations and its application to human plasma assay[†]

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ABSTRACT: This paper describes a convenient method for the separation and simultaneous determination of six anti-diabetic drugs viz., glibenclamide (GLB), gliclazide (GLC), glipizide (GLZ), pioglitazone (PGL), repaglinide (RPG) and rosiglitazone (RGL) in pharmaceutical formulations. Also, the assay has been shown applied to support quantification of the six anti-diabetic drugs in human plasma. The analytes were either injected directly onto the column after suitable dilution (pharmaceutical formulation analysis) or a simple extraction procedure, using acetonitrile, from human plasma spiked with anti-diabetic drugs and internal standard (IS). Ternary gradient elution at a flow rate of 1 mL/min was employed on an Intertisl ODS 3V column (4.6 × 250 mm, 5 μm) at ambient temperature. The mobile phase consisted of 0.01 M formic acid (pH 3.0), acetonitrile, Milli Q water and methanol. Celecoxib was used as an IS. The six anti-diabetic drugs were monitored at a wavelength of 260 nm. The nominal retention times of RGL, PGL, GLZ, GLC, GLB, IS and RGL were 11.4, 13.3, 14.8, 17.6, 20.78, 22.1 and 25.4 min, respectively. The assay developed for formulation analysis was found to be accurate and precise. The calibration curves ranged from 0.1 to 100 μg/mL for all analytes with the exception of GLB, where the range was 0.3–100 μg/mL. The plasma assay was validated for parameters such as specificity, accuracy and extraction recovery. The proposed method is simple, selective and can be extended for routine analysis of anti-diabetics in pharmaceutical preparations and in biological matrices. Copyright © 2006 John Wiley & Sons, Ltd.

KEYWORDS: glibenclamide; gliclazide; glipizide; pioglitazone; repaglinide; rosiglitazone; pharmaceutical formulations; human plasma

INTRODUCTION

Drugs belonging to class such as biguanides (e.g. metformin), sulfonyl ureas (e.g. glipizide, glibenclamide, gliclazide) and thiazolidinedione (TZD) derivatives (pioglitazone, rosiglitazone) are commonly prescribed hypoglycemic drugs for the treatment of non-insulin-dependent type II diabetes mellitus. The use of combination of sulfonyl ureas and TZDs is commonly observed in clinical practice. The six anti-diabetic drugs chosen for this study were glibenclamide (GLB), gliclazide (GLC), glipizide (GLZ), pioglitazone (PGL), repaglinide (RPG) and rosiglitazone (RGL). GLB, GLC and GLZ are sulfonylurea drugs, which act

by increasing the secretion of insulin by the functioning β-cells of the pancreas. RPG, which belongs to the meglitinide class, also acts by stimulating insulin secretion of β-cells, but it binds to sites distinct from the sulfonylurea binding sites (Fuhlendroff *et al.*, 1998). Both PGL and RGL are members of the thiazolidinedione class, which exert their glucose-lowering effect by binding to peroxisome proliferator-activated receptors gamma (PPARγ), thus increasing the receptor sensitivity to insulin (Lehman *et al.*, 1995; Wilson *et al.*, 1996; Young *et al.*, 1998). Ho *et al.* (2004) developed an LC-MS/MS method for simultaneous detection of 10 anti-diabetic drugs comprising mainly sulfonylureas and thiazolidinediones in equine plasma and urine, Aburuz *et al.* (2005) developed an HPLC method in human plasma for the determination of metformin with three sulfonylurea, namely glibenclamide, glipizide and gliclazide. Other literature information is confined to estimation of single components and are not suitable for determination of two or more anti-diabetic drugs (Courtois *et al.*, 1999; Noguchi *et al.*, 1992; Radhakrishna *et al.*, 2002). Except these

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Abbreviations used: GLB, glibenclamide; GLC, gliclazide; GLZ, glipizide; PGL, pioglitazone; RPG, repaglinide; RGL, rosiglitazone; TZD, thiazolidinedione.

reports, to the best of our knowledge there is no analytical method which estimates more than four anti-diabetic drugs simultaneously. With the success of 'combination therapies' using sulfonylurea and thiazolidinedione in the treatment of type II diabetes, it was proposed to develop a generic method that allowed for the determination of anti-diabetic drugs without the need for the development of a separate and distinct method for each anti-diabetic drug exhibiting diversified structural features and associated differences in physicochemical attributes. Hence, we have developed a simple method for the assay of six anti-diabetic drugs, viz. GLB, GLC, GLZ, PGL, RPG and RGL, in a single run for application in pharmaceutical development. Development of an assay that has a generic application for quantitative determinations of anti-diabetic drugs has significant utility, especially if several pharmacokinetic investigations have to be performed. We believe that simultaneous determination of anti-diabetic drugs offers the following additional advantages: (a) ease and convenience of routine clinical monitoring; and (b) simultaneous analysis of pharmaceutical dosage forms. The method has been validated by evaluating the specificity,

selectivity, linearity, recovery, precision and accuracy for quantification in human plasma. Additionally the assay was employed for the formulation analysis of six anti-diabetic drugs.

EXPERIMENTAL

Chemicals and reagents

GLB, GLC, GLZ, PGL, RPG and RGL (Fig. 1) and celecoxib (IS) were synthesized by the Medicinal Chemistry Group, Dr Reddy's Laboratories Ltd (DRL), Hyderabad and were characterized using chromatographic and spectral techniques by the Central Instrumentation Laboratory, DRL, Hyderabad. Purity was found to be more than 99% for all the compounds. Tablets of GLB (Daonil), GLC (Dianorm), GLZ (Glynase), PGL (Pioglit), RPG (Eurepa) and RGL (Rosinorm) were purchased from the local pharmacy outlet. Dimethylsulfoxide (DMSO) was purchased from Sigma Chemical Co., St Louis, MO, USA. Acetonitrile, methanol (HPLC grade) and formic acid (analytical reagent grade) were purchased from Qualigens, Glaxo (India), Mumbai, India. The buffers for the HPLC mobile phase were prepared with Milli Q (Millipore, Milford, MA, USA) grade water.

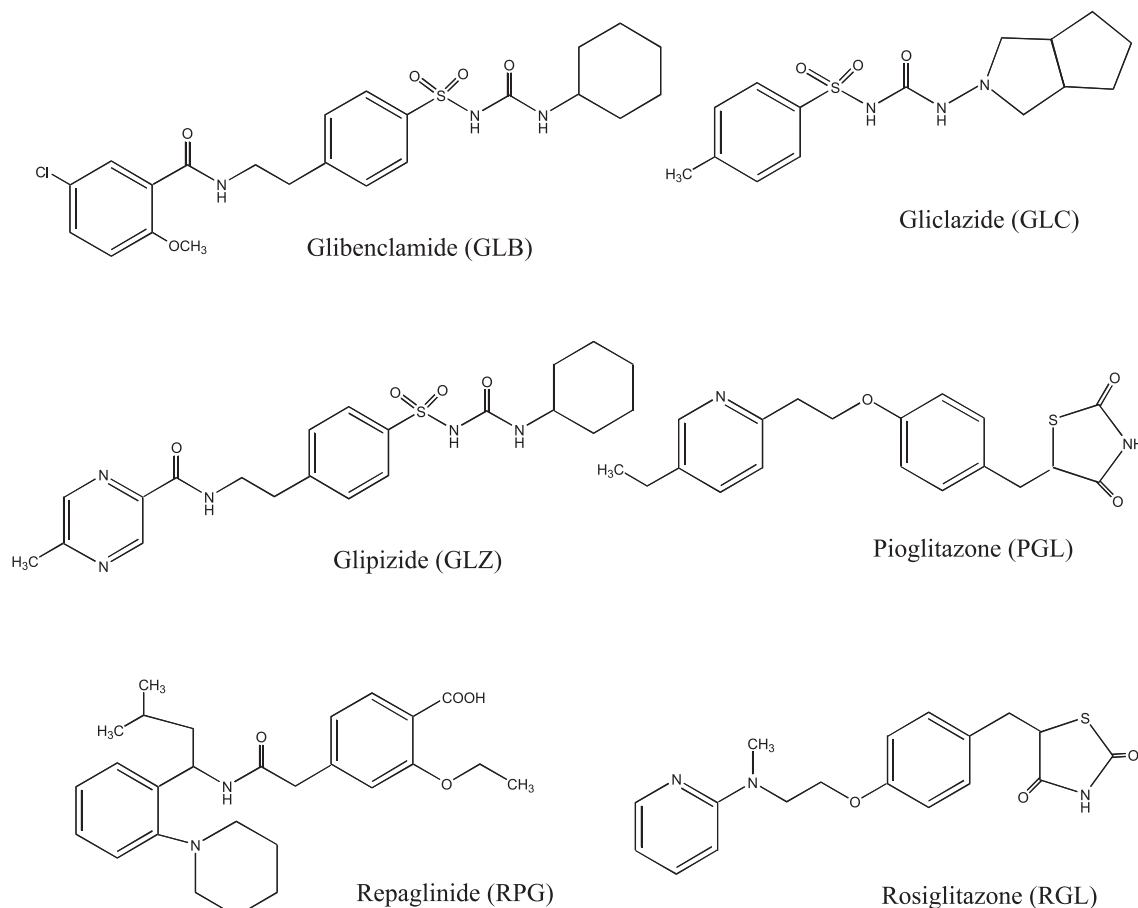


Figure 1. Structural representation of glibenclamide (GLB), gliclazide (GLC), glipizide (GLZ), pioglitazone (PGL), repaglinide (RPG) and rosiglitazone (RGL).

Table 1. The ternary mobile phase system used for the simultaneous estimation of anti-diabetic drugs

| Time (min) | Flow rate (mL/min) | A (%) | B (%) | C (%) |
|------------|--------------------|-------|-------|-------|
| 0.01 | 1.0 | 100 | — | — |
| 1 | 1.0 | 100 | — | — |
| 2 | 1.0 | 90 | — | 10 |
| 6 | 1.0 | 20 | 50 | 30 |
| 12 | 1.0 | 30 | 55 | 15 |
| 22 | 1.0 | 10 | 85 | 5 |
| 24 | 1.0 | 20 | 65 | 15 |
| 25 | 1.0 | 40 | 50 | 10 |
| 27 | 1.0 | 100 | — | — |
| 34 | 1.0 | 100 | — | — |

A, 0.05 M formic acid (pH 3.0); B, Milli Q water:acetonitrile, 5:95; C, Milli Q water:methanol, 10:90.

Chromatography

The HPLC system consisted of a Shimadzu SCL-10A VP system controller (Kyoto, Japan), a Shimadzu LC-10AT VP pump (Kyoto, Japan), a Shimadzu SIL-10AD VP auto injector with sample cooler (Kyoto, Japan), a Shimadzu DGU-14A VP degasser (Kyoto, Japan) and a Shimadzu SPD-10A VP ultraviolet detector (Kyoto, Japan). The data was acquired and processed with Shimadzu Class VP software (version 5.03). The analytical column C₁₈ Inertsil® ODS 3V column (4.6 × 250 mm, 5 μm, GL Sciences Inc., Tokyo, Japan) was used for the chromatographic separation. The ternary mobile phase system consisted of reservoir A (0.05 M formic acid, pH 3.0), reservoir B (Milli Q water:acetonitrile, 5:95 v/v) and reservoir C (Milli Q water:methanol, 10:90 v/v), run as per the gradient program (Table 1) with a flow rate of 1.0 mL/min through the column to elute the analytes. The eluate was monitored by the UV detector set at 260 nm.

Standard solutions

Composite stock solution of GLB, GLC, GLZ, PGL, RPG and RGL (5 mg/mL) was prepared by dissolving appropriate amounts of the compounds in DMSO. Similarly, IS (celecoxib) stock solution (1 mg/mL) was prepared in DMSO. A series of working standard solutions, 200, 150, 100, 50, 20, 10, 5, 3, 2 and 1 μg/mL, were prepared by further diluting the 5 mg/mL stock solution in methanol. Further dilution of the working standards with methanol resulted in the concentration range 0.1–100 μg/mL. A working IS solution (100 μg/mL) was prepared in methanol. These stock solutions were stored at approximately 5°C and were found to be stable for several weeks. In each sample 10 μL of 100 μg/mL of IS were added. The peak area ratio of each anti-diabetic to that of the IS was plotted against the corresponding concentration to obtain calibration graph.

Assay of anti-diabetic drugs in pharmaceutical formulations

To determine the content of specific anti-diabetic drugs (used in the present study) simultaneously in conventional tablets

(label claim: 1 mg of RPG per tablet; 2 mg of RGL per tablet; 5 mg of GLB per tablet, 5 mg of GLZ per tablet; 30 mg of PGL per tablet and 40 mg of GLC per tablet), 10 tablets each of GLB, GLC, GLZ, PGL, RPG and RGL were weighed, their mean weight determined and they were ground to a fine powder using a glass mortar and pestle. An equivalent of 10 mg active ingredient of each anti-diabetic drug was accurately weighed and transferred into a 100 mL volumetric flask. The volume was adjusted with acetonitrile and methanol (1:1 ratio) and the resultant solution was sonicated for 10–15 min and filtered through a 0.45 μm nylon filter (Millipore, Milford, USA). From the resulting solution, an aliquot of 1 mL filtrate solution was transferred to a 10 mL volumetric flask and volume was made up to 10 mL with water. From the final resultant solution, 100 μL solution were mixed with IS (10 μL of 100 μg/mL of celecoxib) and injected directly onto the HPLC column using the allocated condition.

Extraction of anti-diabetic drugs from human plasma

Selection of an extraction solvent. Two sets of standards containing the mixture of six anti-diabetic drugs and IS at a concentration of 50 μg/mL were prepared, one each in human plasma and methanol (neat set). To determine its extraction efficiency of the extraction solvents, each anti-diabetic drug and IS at concentration of 50 μg/mL was spiked into the human plasma and mixed thoroughly by vortexing. This mixture was distributed equally into different tubes. To each tube 2 mL of solvents like methanol, acetonitrile, dichloromethane, ethyl acetate, or a mixture of methanol:dichloromethane (1:1, 1:2, 2:1) or dichloromethane:ethyl acetate (1:1, 1:2, 2:1) was added, vortexed for 2 min, then centrifuged for 3 min at 3600 rpm. The supernatant solution was separated into evaporation tube and dried in Zymark evaporator under nitrogen gas at 50°C. The dried residue was reconstituted with the 150 μL of mobile phase and 100 μL was injected on to the HPLC column.

Recovery of anti-diabetic drugs from human plasma and calibration, specificity experiments. The recovery was determined by comparing peak areas of spiked plasma extracts with those of unextracted neat standards prepared in methanol. Percentage extraction recovery was calculated for the efficiency of extraction of each solvent. The chosen extraction system was employed for the generation of calibration curve to assess linearity for determination of the six analytes in human plasma. The calibration curve was generated over a range of concentrations of 0.1–100 μg/mL for all analytes except for GLB, where the range was 0.3–100 μg/mL, using an IS at the concentration of 1 μg. Specificity experiments were performed to ensure the robustness in the prediction of assay.

RESULTS AND DISCUSSION

Optimization of the experimental conditions

Preliminary experiments were carried out to optimize the experimental parameters affecting both the

Table 2. Maximum UV absorption of anti-diabetic drugs

| Sample no. | Compound | λ_{\max} (nm) |
|------------|----------|-----------------------|
| 1 | GLB | 229 |
| 2 | GLC | 227 |
| 3 | GLZ | 273 |
| 4 | PGL | 266 |
| 5 | RPG | 246 |
| 6 | RGL | 246 |

chromatographic separation of all the anti-diabetic drugs in the pre-selected LC-column and their detection by UV. The UV spectra of the analytes were independently determined. Each anti-diabetic drug has exhibited different maximum UV absorbance (Table 2). At a UV_{\max} range of 227–250 nm, baseline drift towards the negative absorbance was observed in the chromatograms, whereas at wave length 260 nm we could detect all anti-diabetic drugs simultaneously with good separation, sensitivity and consistent baseline. The feasibility of various mixture(s) of solvents such as acetonitrile and methanol using different buffers such as ammonium acetate, phosphate and formic acid with variable pH range of 3–5, along with altered flow-rates (in the range 0.7–1.0 mL/min), was tested for complete chromatographic resolution of the six anti-diabetic drugs and IS. The use of ammonium acetate buffer (pH 5) resulted in peak splitting for RGL and merging of GLB with the IS peak, while the phosphate buffer (pH 3.2) led to a poor resolution of all anti-diabetic drugs along with IS. Finally, the use of formic acid (pH 3)-based buffer provided adequate peak separation, with less tailing, and resulted in the best resolution amongst the buffers tested. Although the employment of formic acid buffer provides a broad pH range of 3–5.5, we fine-tuned pH ~3 using ammonium hydroxide solution. The versatility, suitability and robustness of the method was checked with several C_{18} columns from various manufacturers, viz. Kromasil C_{18} (250 × 4.6 mm, 5 μ m, Hichrom, Berkshire, UK), Symmetry shield RP₁₈ (250 × 4.6 mm, 5 μ m, Waters Corporation, Milford, Ireland) and C_{18} Inertsil® ODS 3V columns (4.6 × 250 mm, 5 μ m, GL Sciences Inc., Tokyo, Japan) by running four replicates of each combination set comprising six anti-diabetic drugs along with IS under identical HPLC conditions. It was found that chromatographic resolution, selectivity and sensitivity were good with the C_{18} Inertsil® ODS 3V column. System suitability parameters like capacity factor (k'), resolution factor (R_s), tailing factor (T_f) and theoretical plates (N) were calculated and recorded in Table 3. The values for system suitability parameters showed feasibility of this method for routine pharmaceutical application.

Table 3. System suitability parameters

| Sample no. | Compound | t_r | k' | R_s | T_f | N |
|------------|----------|-------|------|-------|-------|-------|
| 1 | GLB | 20.7 | 2.56 | 3.70 | 1.23 | 44523 |
| 2 | GLC | 17.6 | 2.32 | 3.11 | 1.14 | 26424 |
| 3 | GLZ | 14.8 | 3.11 | 2.18 | 1.16 | 43671 |
| 4 | PGL | 13.3 | 2.17 | 4.32 | 1.00 | 30818 |
| 5 | RPG | 25.4 | 3.90 | 2.07 | 1.07 | 51334 |
| 6 | RGL | 11.4 | 4.14 | 1.73 | 1.36 | 31912 |

t_r , Retention time; k' , capacity factor; R_s , resolution; T_f , tailing factor; N , theoretical plates.

Specificity and chromatography

Specificity was indicated by the absence of any endogenous interference at retention times of peaks of interest as evaluated by chromatograms of blank human plasma and plasma spiked with GLB, GLC, GLZ, PGL, RPG, RGL and IS. When each single analyte was injected at the highest concentration in the chromatographic system, no interference was observed at the retention times of all analytes. All analytes viz., RGL, PGL, GLZ, GLC, GLB, IS and RPG were well separated with retention times of 11.4, 13.3, 14.8, 17.6, 20.78, 22.1 and 25.4 min, respectively. Figure 2 shows a typical overlaid chromatogram for the blank control human plasma free of analyte and IS, human plasma with LLOQ at 0.5 μ g/mL concentration and synthetic mixture of GLB, GLC, GLZ, PGL, RPG, RGL and IS at 20 μ g/mL concentration.

Calibration curve

Peak-area ratios of each analyte to the IS were measured and used as a surrogate for quantitation over the entire calibration curve range. The lowest concentration with the RSD <20% was taken as the LLOQ and was found to be 0.1 μ g/mL for all anti-diabetic drugs used in the present study except GLB (0.3 μ g/mL). A representative calibration graph of peak-area ratio (each analyte to IS) vs each analyte concentration in the range of 0.1–100 μ g/mL for GLC, GLZ, PGL, RPG, RGL and for GLB (0.3–100 μ g/mL) was found to be linear. The average regression correlation coefficient ($n = 3$) was found to be at least 0.999 for GLB, GLC, PGL and RGL, at least 0.997 for GLZ and at least 0.998 for RPG. The standard curve had a reliable reproducibility for each analyte across the calibration range.

Assay of anti-diabetic drugs in pharmaceutical formulations

The accuracy of the proposed method for application in pharmaceutical formulation analysis was evaluated by recovery assays. Accuracy was determined by applying

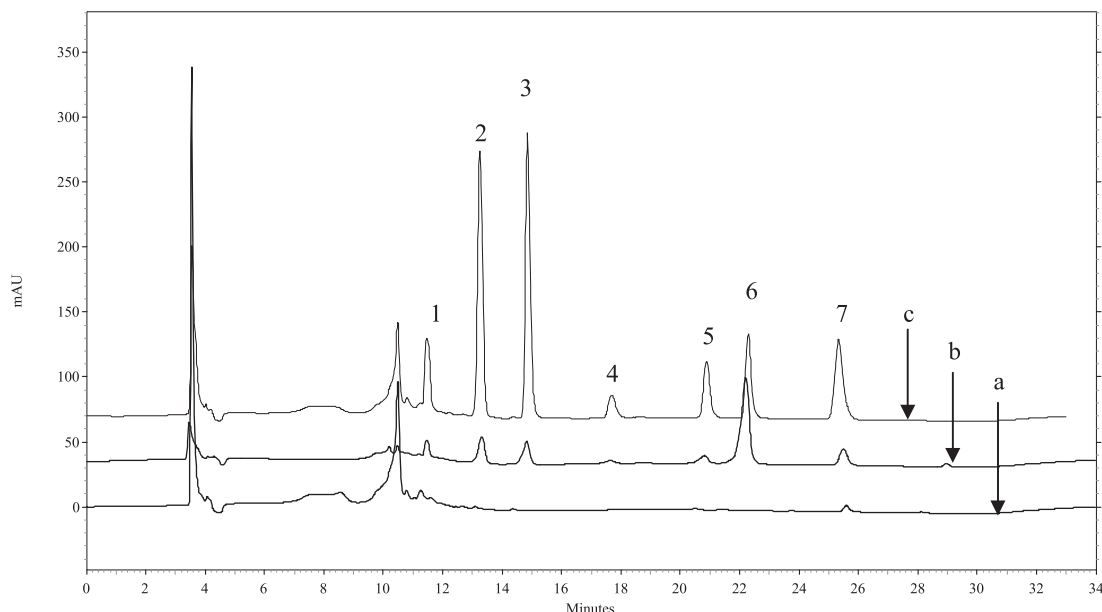


Figure 2. HPLC chromatograms of a 100 μL injection of (a) blank (b) human plasma spiked with GLB, GLC, GLZ, PGL, RPG and RGL at concentrations of 0.5 and 20 $\mu\text{g}/\text{mL}$ of IS. (c) Typical chromatogram of synthetic mixtures containing anti-diabetic drugs and IS (each at 20 $\mu\text{g}/\text{mL}$). 1, RGL; 2, PGL; 3, GLZ; 4, GLC; 5, GLB; 6, celecoxib (IS); and 7, RPG.

Table 4. Precision values obtained with the proposed method for pharmaceutical formulations assay

| Compound ^a | Intra-day precision (SD of areas) | % RSD | Inter-day precision (SD of areas) | % RSD |
|-----------------------|--------------------------------------|-------|--------------------------------------|-------|
| GLB | 3.99 | 3.85 | 1.54 | 2.25 |
| GLC | 4.27 | 4.44 | 6.19 | 7.05 |
| GLZ | 4.36 | 4.62 | 5.41 | 5.46 |
| PGL | 4.55 | 4.57 | 3.53 | 3.80 |
| RPG | 5.73 | 5.53 | 5.17 | 5.67 |
| RGL | 3.42 | 3.80 | 6.81 | 7.04 |

^a Average of three concentrations 15, 20 and 25 $\mu\text{g}/\text{mL}$ for GLB, GLC, GLZ, PGL, RPG and 30, 40 and 50 $\mu\text{g}/\text{mL}$ for RGL.

the described method to synthetic mixtures of excipients to which known amounts of each anti-diabetic drug corresponding to 50, 100 and 150% of the label claim had been added from the respective standard solution.

The accuracy was then calculated as the percentage of each anti-diabetic recovered by the assay. Mean recoveries (mean \pm SD, $n = 4$) for GLB, GLC, GLZ, PGL, RPG and RGL are 101.25 ± 3.06 , 92.30 ± 4.44 , 101.37 ± 2.64 , 97.94 ± 3.46 , 101.84 ± 5.94 and $100.24 \pm 3.28\%$, respectively. The results indicate satisfactory accuracy of the method for simultaneous determination of GLB, GLC, GLZ, PGL, RPG and RGL in the formulation.

The precision of the proposed method was performed by chromatographing four replicate injections of anti-diabetic mixture at 10 $\mu\text{g}/\text{mL}$ on different days. The obtained intra-day and inter-day precision results are depicted in Table 4. The results indicated sufficient

precision of the developed HPLC method. Also, the system suitability factors obtained with the proposed method are presented in Table 3.

The validated HPLC method was applied to the simultaneous determination of GLB, GLC, GLZ, PGL, RPG and RGL in tablets. The formulation assay results, expressed as a percentage of the label claim, are shown in Table 5. The results indicate that the amount of each drug in the tablets corresponds to the requirements of 90–110% of the label claim.

Recovery of anti-diabetic drugs from human plasma

The extraction results were obtained from peak area comparison of neat standards vs plasma-extracted standards at 50 $\mu\text{g}/\text{mL}$ concentration for all the analytes. Acetonitrile was found to be the most efficient

Table 5. Formulation assay results

| Formulation | Dosage form | Labelled amount (mg) | Amount found (mg) | RSD % | Assay (%w/w) ^a |
|-------------|-------------|----------------------|-------------------|-------|---------------------------|
| GLB | Tablet | 5 | 4.82 | 1.85 | 97.03 |
| GLC | Tablet | 40 | 37.56 | 6.81 | 93.91 |
| GLZ | Tablet | 5 | 4.88 | 1.99 | 96.10 |
| PGL | Tablet | 30 | 30.37 | 1.81 | 101.23 |
| RPG | Tablet | 1 | 0.92 | 3.96 | 92.44 |
| RGL | Tablet | 2 | 2.03 | 3.17 | 101.48 |

^a Assay (%) = (found/labelled) × 100.

extraction solvent among all the extraction solvents. The absolute recovery of anti-diabetic drugs from human plasma (with acetonitrile) was in the range 92.37–106.50% and the absolute recovery of internal standard was 95.25%.

CONCLUSION

A HPLC-UV method utilizing optimized gradient elution with single wavelength monitoring has been developed and applied for simultaneous analysis of six anti-diabetic drugs in pharmaceutical formulations. We have used the method to generate standard curve data in human plasma for all analytes due to a very high extraction recovery (92%) for all analytes. The method is simple and can be used for pharmaceutical analysis, therapeutic drug monitoring and pharmacokinetic investigations.

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