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*Research Article*

# Simultaneous Quantification of Glibenclamide, Simvastatin, and Quercetin by Using LC-UV Method and Its Application to Pharmacokinetic Study in Rats

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A sensitive, precise, and simple LC method for the simultaneous quantification of glibenclamide, simvastatin, and quercetin in rat plasma has been developed and validated. The chromatographic separation was achieved on a cyano column (250 mm × 4.6 mm, 5 $\mu$m) maintained at room temperature, using isocratic elution with methanol : acetonitrile : 10 mM potassium dihydrogen orthophosphate, pH adjusted to 4.5 with o-phosphoric acid (8 : 32 : 60, v/v) and detected using UV-VIS detector. Plasma samples were deproteinated with 0.1% perchloric acid and acetonitrile for extraction of the glibenclamide, simvastatin, and quercetin which resulted in their high recoveries. LC calibration curves based on the extracts from the rat plasma were linear in the range of 50–1000 ng mL$^{-1}$ for all the three drugs. The limit of quantification was 50 ng mL$^{-1}$. The described method was successfully applied to study the pharmacokinetics of glibenclamide, simvastatin, and quercetin following oral administration, in combination to *Sprague-Dawley* rats.

## 1. Introduction

Diabetes mellitus is one of the most prevalent metabolic syndrome which on progression leads to many secondary complications. One of the major clinical manifestations associated with Type II diabetes is hyperlipidemia which subsequently predisposes the patient to cardiovascular diseases and many other complications [1, 2]. Amongst the various lipid lowering drugs, statins are the most widely accepted therapy [3]. However, with respect to the dose level, efficacy, and tolerability, simvastatin has been found to be the most beneficial. Illingworth et al. (2001) suggested that simvastatin led to a greater increase in HDL cholesterol and apo A-I levels in hypercholesterolemic patients as compared to atorvastatin [4]. Apart from controlling lipid levels, effective management of blood glucose level is also primary concern for the diabetic patients, which generally requires a combination of oral antihyperglycemic drugs. Glibenclamide, a second generation sulfonylurea, has been widely used alone or in combination due to its ability to effectivly control the blood glucose level [5]. Thus a patient with Type II diabetes and hyperlipidemia has to undergo multiple therapies simultaneously which may include concomitant therapy of drugs like glibenclamide and simvastatin.

However, one major concern is the low oral bioavailability of simvastatin (<5%) due to its rapid metabolism by CYP3A4 isoenzymes [6]. Thus, it becomes important to improve its oral bioavailability for attaining better therapeutic efficacy at a lower dose which may also help to reduce dose associated side effects. In order to increase the oral bioavailability,

suitable formulations may be developed and/or different bioenhancers may be tried [7]. However, in this study, an attempt was made to improve the oral bioavailability of simvastatin using quercetin, a CYP3A4 inhibitor. Quercetin is a plant derived flavonoid and has been reported to improve the oral bioavailability of doxorubicin by inhibiting CYP3A4 isoenzyme [8]. Moreover, glibenclamide is also a substrate for CYP3A4 isoenzymes. Thus administration of quercetin to a diabetic patient who is on glibenclamide and simvastatin therapy may be helpful in terms of dose reduction and subsequently their associated side effects.

Thus, a suitable bioanalytical method is required for simultaneous quantification of these drugs in plasma, which can be used for therapeutic drug monitoring. However, no method is available for quantifying these three drugs simultaneously, either in human or rat plasma. The present study was focussed on developing a simple, economical, sensitive, and specific RP-LC method for simultaneous determination of glibenclamide, simvastatin, and quercetin in rat plasma. The developed method was then applied for the pharmacokinetic study in rat for these three drugs dosed in combination. In this method, nimodipine was selected as the internal standard, and chromatographic separation of glibenclamide, simvastatin, and quercetin was completed within 20 min.

## 2. Experimental

*2.1. Chemicals and Reagents.* Glibenclamide (purity ≥ 99.0%) and quercetin (purity ≥ 98.0%) were purchased from Sigma Aldrich, USA. Simvastatin (purity 99.6%) and nimodipine (purity 99.1%) were obtained as gift samples from Cadila Pharma Ltd., Ahmedabad, India, and Astron Research Ltd., Ahmedabad, India, respectively. All the solvents and chemicals used for the study were of chromatographic grade and purchased from Qualigen Fine Chemicals, Mumbai, India. Potassium dihydrogen phosphate (analytical grade) was obtained from Qualigen Fine Chemicals, Mumbai, India. Heparin was purchased from Biological E. Ltd, Hyderabad, India. Deionised water for LC was prepared in-house using a Milli-Q integral water purification system (Millipore Elix, Germany).

*2.2. Chromatographic Conditions.* The LC system consisted of PU-980 LC pump (Jasco, Hachioji, Tokyo, Japan), UV-975 UV-Visible detector (Jasco, Hachioji, Tokyo, Japan), and AS-950 autosampler (Jasco, Hachioji, Tokyo, Japan) and the data were analyzed by Borwin software version 1.5. Chromatographic separation was achieved by using Phenomenex Cyano (250 mm × 4.6 mm, 5 $\mu$m) column maintained at room temperature. The mobile phase consisted of methanol : acetonitrile : 10 mM potassium dihydrogen orthophosphate, pH adjusted to 4.5 with o-phosphoric acid (8 : 32 : 60, v/v). The mobile phase was prepared freshly and degassed before use. The flow-rate was maintained at 1 mL min$^{-1}$. The detection was done at 237 nm for simultaneous detection of all the three drugs. Samples were quantified by determining the peak area ratio (Peak area$_{\text{Drug}}$/Peak area$_{\text{IS}}$).

*2.3. Extraction Procedure.* A liquid-liquid extraction method was followed for efficient recovery of glibenclamide, simvastatin, quercetin, and nimodipine (internal standard) from the rat plasma. Twenty-five microlitres of internal standard (8 $\mu$g mL$^{-1}$) was added to 100 $\mu$L of rat plasma sample and vortex for 1 min. To the above samples, 25 $\mu$L of 0.1% perchloric acid was added and vortex for 1 min. Then, 300 $\mu$L of acetonitrile was added to the above sample and mixed for 2 min on a vortex mixer. The resulting samples were centrifuged at 8000 ×g for 15 min at 4°C. The organic layer (supernatant) were collected and evaporated under nitrogen till dryness. The samples were reconstituted in 100 $\mu$L mobile phase and 50 $\mu$L of it was injected onto LC system.

## 3. Method Validation

*3.1. Calibration Curve.* Ten milligram each of glibenclamide, simvastatin, and quercetin was dissolved in methanol (final adjusted volume 100 mL) to obtain a stock solution of concentration 100 $\mu$g mL$^{-1}$. The working solutions were prepared from the stock solution by dilution with methanol. Spiked calibration curve samples containing 50, 100, 200, 400, 600, 800, and 1000 ng mL$^{-1}$ were prepared using the stock and working solutions in order to plot seven-point calibration curve. The working solution concentration of internal standard was 8 $\mu$g mL$^{-1}$, from which 25 $\mu$L was added to each sample. Protein precipitation of the plasma samples was carried out as per the procedure described above. Standard curves were constructed by plotting ratio of the peak areas of glibenclamide, simvastatin, and quercetin to internal standard (nimodipine), individually versus concentration. The calibration curves were obtained by least square linear regression analysis using weight scheme as $1/c$ ($c$: concentration) using Borwin software Ver. 1.5.

*3.2. Preparation of Quality Control Samples.* The quality control (QC) samples used during the validation and in the pharmacokinetic studies were prepared by spiking appropriate volumes of glibenclamide, simvastatin, and quercetin from the dilution solution into blank rat plasma and stored at −80°C till further use. The QC sample concentrations of all the three drugs were 900, 500, and 150 ng mL$^{-1}$ to represent high, medium, and low QC samples, respectively.

*3.3. Precision and Accuracy.* Intraday precision and accuracy were calculated by taking six replicates of QC samples which were then extracted and analyzed using LC method as described above. Interday accuracy and precision were calculated by taking six replicates of all the three QC sample concentrations for glibenclamide, simvastatin, and quercetin for three consecutive days along with the standard calibration curve. Concentrations of the analytes were calculated from the calibration curve.

*3.4. Recovery.* Recovery of the extraction procedure was calculated by analyzing six extracted samples at concentrations of 900, 500, and 150 ng mL$^{-1}$ for all the three drugs. The peak areas of the extracted samples were then compared

with those of unextracted (nonplasma samples of identical standards prepared in the mobile phase which have not undergone extraction procedure) glibenclamide, simvastatin, and quercetin samples with the same concentration. The recovery value of the IS was determined in the same way at a single concentration of 500 ng $mL^{-1}$.

*3.5. Stability Studies.* Stability of glibenclamide, simvastatin, and quercetin in rat plasma during storage and processing was checked using QC samples. Bench-top stability was determined for the aliquots of each of the low and high QC samples, which were spiked into rat plasma and kept at room temperature for 6 h prior to their analysis. Six replicates of high and low control samples were frozen at −80°C and analysed for three freeze-thaw cycles to determine their freeze-thaw stability. A dry extract stability study was performed for six replicates of high and low controls after extracting the analytes from plasma and storing the dried samples at −80°C for 24 h. The samples were processed and kept at room temperature for 6 h and then analysed after reconstitution with the mobile phase. For checking the autosampler stability, six replicates of high and low QC samples were reconstituted and kept in auto sampler and analyzed at 0 and 12 h. Long-term stability of glibenclamide, simvastatin, and quercetin was checked for six replicates of the high and low control samples after storing them for 30 days at −80°C. The samples were then processed and analysed as per the method described above.

*3.6. Animals.* Male *Sprague-Dawley* rats weighing 280–300 g were obtained from the animal house of B. V. Patel PERD Centre, Ahmedabad. Animal housing and handling was performed in accordance with Good Laboratory Practice (GLP) mentioned in CPCSEA guidelines. Animal house is registered with the Committee for the Purpose of Control and Supervision of Experiments on Animals, Ministry of Social Justice and Empowerment, Government of India, vide registration number 1661/PO/a/12/CPCSEA, dated November 21, 2012. All experimental protocols were reviewed and accepted by the Institutional Animal Ethics Committee prior to initiation of the experiment. The animals were housed in polypropylene cages (three animals per cage) and placed in the experimental room where they were allowed to acclimatize for a week before experiment.

*3.7. Pharmacokinetic Study.* Six animals were used in the study. The first group was dosed with glibenclamide (dose: 30 mg $kg^{-1}$ body weight), simvastatin (dose: 20 mg $kg^{-1}$ body weight), and quercetin (dose: 20 mg $kg^{-1}$ body weight) in combination. The dose was finalized based on their previously reported dose for pharmacokinetics study in rats [9–11]. All the compounds were orally administered as a suspension in 0.2% agar. The jugular veins of all the animals were cannulated for collection of the blood at different sampling time points. The blood samples of 0.3 mL were withdrawn from each rat at 0, 30 min and 1, 2, 3, 4, 5, 6, and 8 h, after dose. The samples were collected into heparinized microcentrifuge tubes and centrifuged at 1500 ×g for 7 min at 4°C. The resulting plasma samples were kept frozen at −80°C prior to LC analysis.

The maximum plasma concentration ($C_{max}$) and the time to reach the maximum concentration ($T_{max}$) was directly determined from the plasma concentration versus time curves. The area under the curve from 0 to $t$ ($AUC_{0-t}$) was calculated following linear trapezoidal rule by summing the area from 0 to $t$ h. Elimination rate constant ($K_{el}$) was determined by taking the absolute value of the slope of any three points lying on a straight line of the curve after the $C_{max}$, that is, during the elimination phase. Elimination half-life ($t_{1/2}$) was determined using the relationship $t_{1/2} = 0.693/K_{el}$. The volume of distribution ($V_d$) was calculated by dividing amount of drug dosed by the total plasma concentration.

## 4. Results and Discussion

A new analytical method for simultaneous estimation of glibenclamide, simvastatin, and quercetin in rat plasma was developed and validated. The method was found to be reproducible and specific. The chromatograms of blank rat plasma, unextracted standards of all three drugs, plasma spiked with standards of glibenclamide, simvastatin, and quercetin at a concentration of 500 ng $mL^{-1}$, and a plasma sample from a rat 3 h after administration of glibenclamide, simvastatin, and quercetin in combination are shown in Figures 1(a)–1(d), respectively. Retention times of quercetin, nimodipine (internal standard), glibenclamide, and simvastatin were 5.14, 10.81, 12.33, and 17.88 min, respectively. Total LC run time was 20 min. Glibenclamide, simvastatin, and quercetin were unambiguously identified in the plasma upon comparison of the retention times with those of their respective standards.

*4.1. Linearity and Lower Limit of Quantification.* Standard curves were constructed by plotting ratio of peak area ratio of drugs to internal standard versus their respective concentration. The standard curves were linear in the range of 50–1000 ng $mL^{-1}$ for all the three drugs. The calibration curves for spiked plasma samples could be described by the equation $y = 0.0015\,(\pm 0.007)x + 0.0996\,(\pm 0.002)$ ($r^2 = 0.9998$) for glibenclamide, $y = 0.0013\,(\pm 0.009)x + 0.1088\,(\pm 0.006)$ ($r^2 = 0.9994$) for simvastatin, and $y = 0.0018\,(\pm 0.005)x + 0.0769\,(\pm 0.005)$ ($r^2 = 0.9995$) for quercetin. The lower limit of quantification was found to be 50 ng $mL^{-1}$ for all the three drugs. The lower limit of detection was 10 ng $mL^{-1}$. No carry-over was observed upon the injection of standard sample of highest concentration.

*4.2. Precision and Accuracy.* Table 1 shows intra-day and inter-day precision and accuracy. The intra-day precisions; that is, coefficients of variation (CV) of high, medium, and low QC samples of glibenclamide were 1.6, 2.8, and 5%, respectively, of simvastatin were 2.4, 3.8, and 2.9%, respectively, and of quercetin were 3.9, 3.6, and 2.6%, respectively. Inter-day precisions (CV) of high, medium, and low QC samples of glibenclamide were 2.6, 3.8, and 2.9%, respectively, of simvastatin were 4.7, 6.6, and 6.4%, respectively, and of quercetin were 5.1, 6.1, and 4.3%, respectively. Intra-day

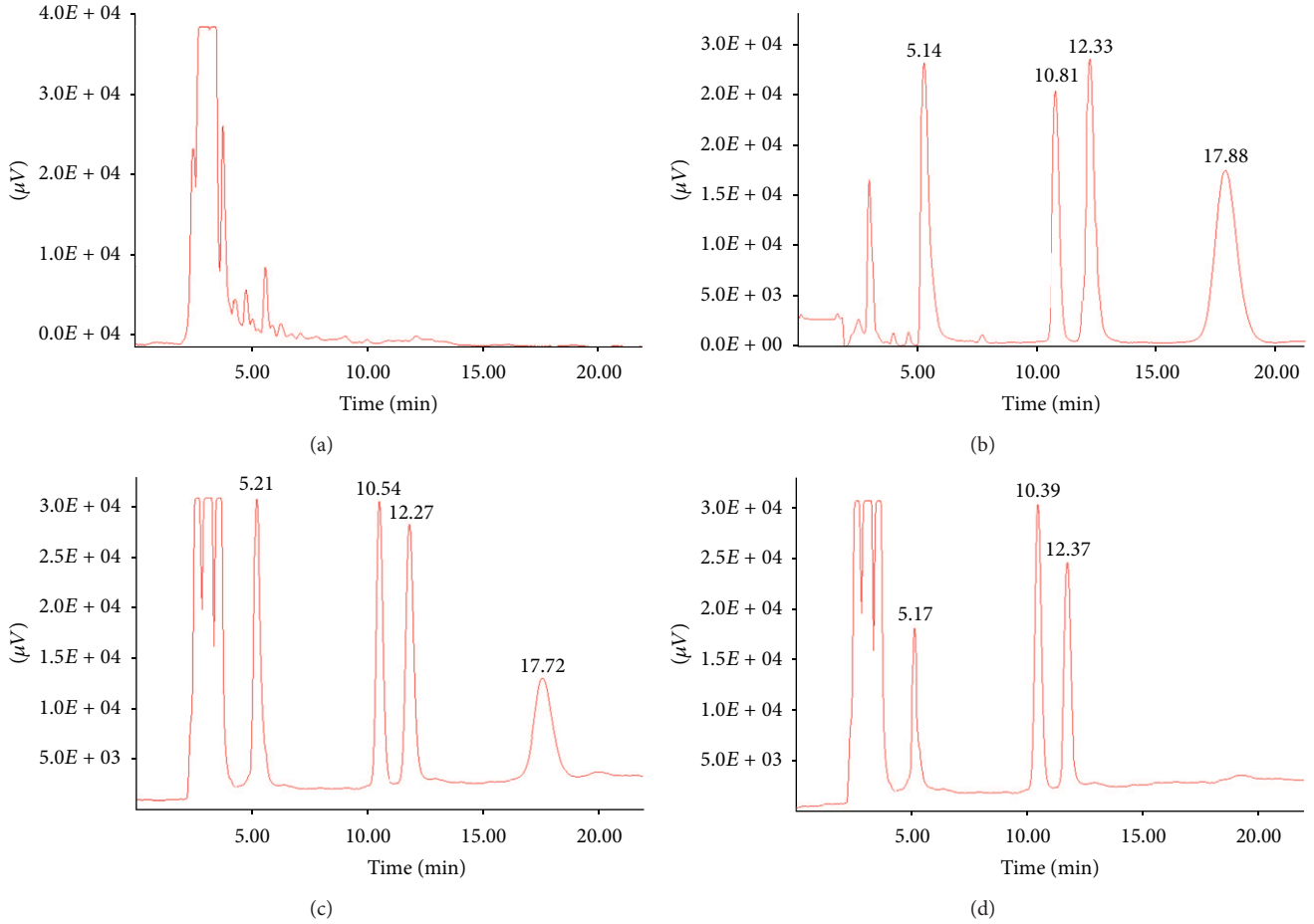


Figure 1: LC chromatograms of (a) blank rat plasma, (b) unextracted standards of quercetin ($R_t$: 5.14 min), nimodipine ($R_t$: 10.81 min), glibenclamide ($R_t$: 12.33 min), and simvastatin ($R_t$: 17.88 min), (c) plasma samples spiked with standards of glibenclamide, simvastatin, and quercetin at a concentration of 500 ng/mL, and (d) plasma sample from a rat 3 h after administration of glibenclamide, simvastatin, and quercetin in combination.

Table 1: Intraday and interday precision and accuracy for glibenclamide, simvastatin, and quercetin.

| QC samples | Precision (CV%) | | | Accuracy (%) | | |
|---|---|---|---|---|---|---|
| | Glibenclamide | Simvastatin | Quercetin | Glibenclamide | Simvastatin | Quercetin |
| | | | [a]Intra-day ($n$ = 6) | | | |
| HQC | 1.6 | 2.4 | 3.9 | 92.2 | 97.5 | 92.3 |
| MQC | 2.8 | 3.8 | 3.6 | 93.9 | 92 | 96.3 |
| LQC | 5 | 2.9 | 2.6 | 97.1 | 94.6 | 97.2 |
| | | | [b]Inter-day ($n$ = 18) | | | |
| HQC | 2.6 | 4.7 | 5.1 | 91.6 | 96.8 | 93.5 |
| MQC | 3.8 | 6.6 | 6.1 | 94.1 | 91.4 | 94.4 |
| LQC | 2.9 | 6.4 | 4.3 | 95.6 | 97.1 | 96.7 |

[a]Intra-day precision: data expressed as means ($n$ = 6).
[b]Inter-day precision: data expressed as means ($n$ = 18).

and inter-day accuracies ranged between 91.6 and 97.1% for glibenclamide, 91.4 and 97.5% for simvastatin, and 92.3 and 97.2% for quercetin. These results inferred that the developed method was accurate and precise.

*4.3. Recovery.* Recovery of glibenclamide, simvastatin, and quercetin was in the range of 81–84%, 85–88%, and 78–82%, respectively. The recovery of internal standard was 91%.

Table 2: Stability data of glibenclamide, simvastatin, and quercetin in rat plasma.

| QC samples | Mean concentration (ng mL$^{-1}$) observed at 0 h | | | Mean concentration (ng mL$^{-1}$) observed at last h | | | CV% | | |
|---|---|---|---|---|---|---|---|---|---|
| | Glibenclamide | Simvastatin | Quercetin | Glibenclamide | Simvastatin | Quercetin | Glibenclamide | Simvastatin | Quercetin |
| Bench top stability ($n$ = 6) (after 6 h) | | | | | | | | | |
| High | 852.1 | 846.3 | 852.9 | 754.9 | 823.8 | 857.9 | 3.6 | 5.3 | 2.8 |
| Low | 46.4 | 48.1 | 45.3 | 45.3 | 46.6 | 48.4 | 6.2 | 4.7 | 3.7 |
| Freeze thaw stability ($n$ = 6) (three cycles) | | | | | | | | | |
| High | 859.2 | 857.2 | 847.9 | 856.4 | 848.4 | 861.2 | 3.7 | 6.2 | 1.8 |
| Low | 45.2 | 46.4 | 45.1 | 48.6 | 46.9 | 46.1 | 5.2 | 4.1 | 5.8 |
| Dry extract stability ($n$ = 6) (24 h) | | | | | | | | | |
| High | 869.3 | 862.8 | 872.2 | 853.6 | 855 | 857 | 2.5 | 2.7 | 3 |
| Low | 46 | 46.8 | 48.2 | 47.9 | 46.7 | 48.3 | 5.5 | 3.9 | 5.7 |
| Auto sampler stability ($n$ = 6) (12 h) | | | | | | | | | |
| High | 841.4 | 866.4 | 851.4 | 864.3 | 852.5 | 868.5 | 2.8 | 1.7 | 2.8 |
| Low | 46.8 | 45.3 | 47.6 | 47.7 | 46.7 | 47.5 | 6.6 | 2.9 | 3.7 |
| Long- term stability ($n$ = 6) (30 days) | | | | | | | | | |
| High | 842.3 | 847.8 | 869.4 | 864.4 | 847.5 | 854.8 | 1.8 | 3.6 | 2.9 |
| Low | 46.8 | 45.7 | 46.9 | 45.8 | 48.7 | 46.8 | 2.8 | 5.4 | 4.4 |

Data is expressed as mean and CV%.

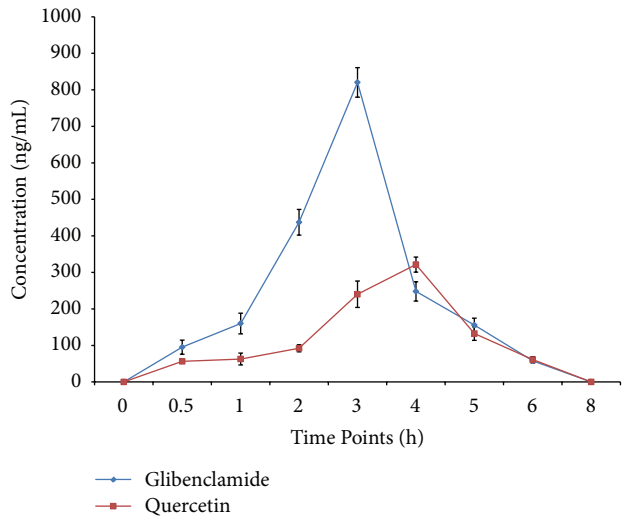


Figure 2: Mean (±SEM) plasma concentration of glibenclamide and quercetin in rats.

*4.4. Stability Study of Plasma Samples.* Table 2 shows the results for bench-top stability, freeze-thaw stability, dry extract stability, and autosampler and long-term stability. The results illustrate that glibenclamide, simvastatin, and quercetin were stable during processing and storage for up to one month.

*4.5. Pharmacokinetic Parameters.* The mean plasma concentration versus time profile is shown in Figure 2. The results of the pharmacokinetic study showed that both glibenclamide and quercetin were detectable in plasma up to 6 h, after dose. However, simvastatin could not be detected in the plasma. This may be attributed to its low bioavailability. Furthermore the dose of simvastatin may not be sufficient to get detected in plasma samples of pharmacokinetic study. Thus, further studies have to be done for all the three drugs administered alone and also in combination to get some concrete results from the proposed hypothesis.

## 5. Conclusion

A simple, precise, specific, and sensitive method for simultaneous quantification of glibenclamide, simvastatin, and quercetin in rat plasma was developed and validated. This is the first reported LC method for simultaneous quantification of these three drugs in rat plasma using protein precipitation technique. Stability studies showed that all the three drugs were stable for short and long-term periods (30 days). This analytical procedure was successfully applied to the pharmacokinetic study of glibenclamide, simvastatin, and quercetin in male *Sprague-Dawley* rats.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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