In Vitro Metabolic Stability and Permeability of Gymnemagenin and Its In Vivo Pharmacokinetic Correlation in Rats – A Pilot Study

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Abstract

Gymnema sylvestre is traditionally used for diabetes mellitus. A literature survey revealed very few reports, particularly on rat liver microsomal stability, caco-2 permeability and efflux concerns and its correlation with the bioavailability of gymnemagenin, an important component of G. sylvestre. Therefore, the objective of our study was to investigate the in vitro rat liver microsomal stability and caco-2 permeability along with the efflux of gymnemagenin and establish a probable correlation of these in vitro findings with pharmacokinetic parameters after oral and intravenous administration in rats.

Rat liver microsomal stability studies to estimate the in vitro intrinsic half-life, clearance, and Caco-2 permeability after 21 days of culture to determine the apparent permeability from apical to basal and from basal to apical, and efflux ratio of gymnemagenin were performed using liquid chromatography-tandem mass spectrometry. A sensitive, robust bioanalytical method was validated and successfully applied to determine the plasma exposure of gymnemagenin. In vitro rat liver microsomal stability demonstrated that gymnemagenin metabolizes rapidly with a short apparent and intrinsic half-life (~7 min) and high intrinsic clearance, i.e., 190.08 µL/min/mg of microsomes. The results of the Caco-2 study indicated a poor permeability (1.31 × 10⁻⁶ cm/sec) with a very high efflux ratio. The pharmacokinetic study revealed poor oral bioavailability (~14%) of gymnemagenin and it was found to have a short half-life and a high clearance in rats. Our in vitro findings indicated low metabolic stability and poor Caco-2 permeability with high efflux, which might have a role in the observed poor oral bioavailability in rats.

Abbreviations
AUC: area under the curve
CL_app,int: apparent/intrinsic clearance
EFR: efflux ratio
GG: gymnemagenin
IS: internal standard
IV: intravenous
LC-MS/MS: liquid chromatography-tandem mass spectrometry
LY: lucifer yellow
NADP: nicotinamide adenine dinucleotide phosphate
NRS: reduced NADP regenerating system
\( P_{app-A-B/A-B} \): apparent permeability (apical to basal/basal to apical)
P-gp: permeability glycoprotein
QC: quality control
RLM: rat liver microsomes

Supporting information available online at http://www.thieme-connect.de/products

Introduction

Gymnema sylvestre (Retz.) Schult. (Apocynaceae; syn. Periploca sylvestris Retz.) is a traditionally used medicinal plant with reported use as a remedy for diabetes mellitus and stomachic and diuretic problems. The plant extract is also used in folk, Ayurvedic, and homeopathic systems of medicine [1]. G. sylvestre occurs mainly in the Deccan peninsula of western India, Tropical Africa, Vietnam, Malaysia, and Srilanka and is widely available in Japan, Germany, and the USA as a health food [2]. In the last 10 years, several products under brand names such as Body Slatto Tea®, Gymnema®, Gymnema Diet®, Sugar Off®, Glucoset®, Cinnidrome X®, and Pilisoft® have
flooded the global markets as health foods and cosmetics [3]. Gymnemic acid and GG are believed to be the major active compounds of G. sylvestre, and GG, which enters the circulation after hydrolysis of gymnemic acids, is a common genin for most of the gymnemic acids [4–6]. It has been a clinical concern that herbal products containing a number of natural compounds can cause pharmacokinetic interactions with modern medicines, particularly in combination. Therefore, detailed studies of the commonly consumed herbal products with a particular interest to safety and related pharmacokinetics need to be done. Among these issues the pharmacokinetic parameter for herbal products having numerous components can probably be addressed with the help of phytomarker as a representative molecule. So far, only a few programs have been established to study the pharmacokinetics and pharmacodynamics of herbal medicines, as was originally proposed by the WHO Guidelines for the assessment of herbal medicines. Evaluation of in vitro pharmacokinetic properties has become mandatory in industrial drug discovery research to speed up the discovery process, reduce the failure rate at the final stage, minimize time and cost, and guide medicinal chemists to modify the compound’s structures to get pharmacokinetically acceptable compounds [7]. Usually by using the "in vitro T1/2 method", CLapp,int is determined by measuring the first order rate constant for consumption of the substrate at a low concentration. This preclinical in vitro drug metabolism data is considered to have a good correlation with in vivo pharmacokinetics data, which is further used to predict human pharmacokinetic parameters [8]. Evaluation of the drug transport mechanism is becoming increasingly important in drug delivery and pharmacokinetics research. Thus, the Caco-2 cell monolayer has a lot of importance as a reliable and high-throughput in vitro model to evaluate intestinal passive permeability as well as P-gp efflux. P-gp has been identified in rats and human tissue such as the intestines, liver, brain, and kidneys, suggesting that P-gp function may contribute to drug absorption, distribution, and elimination [9]. For orally administered compounds, permeability through the Caco-2 cell monolayer correlates well with in vivo absorption in humans [10, 11].

A comprehensive knowledge of in vitro pharmacokinetics, permeability, the role of P-gp, and their correlation with the in vivo data of GG is important for the interpretation of the pharmacology and toxicology of this herb, G. sylvestre, and its different formulations containing the same.

In the present work, the oral bioavailability of GG in terms of Caco-2 permeability (Papp,A→B) and the P-gp efflux ratio (Papp,B→A/Papp,A→B) was studied after 21 days of cell culture. The in vitro intrinsic half-life (T1/2,int) and apparent intrinsic clearance (Clint,app) of GG was also measured after incubation with RLM at 37.5 °C. Finally, attempts were made to develop a simple, sensitive, and robust LC-MS/MS method, and our method is believed to be advantageous compared with previously published methods [12] and has been validated according to the US Food and Drug Administration’s Guidance for Industry [13] for the determination and quantification of GG in rat plasma following a single oral dose and intravenous administration of GG using dexamethasone as an internal standard, and finding a correlation of WinNonlin calculated pharmacokinetic parameters.

Results

In positive electrospray ionization, GG and dexamethasone showed intense Q1 [M + H]+ ions at m/z 507.4 and 393.2, respectively. The daughter ions were at m/z 471.4 and 147.2 and observed after fragmentation for GG and IS, respectively, which were monitored in MRM (multiple reaction monitoring) to quantify GG in the plasma. The line equation of the standard curve was obtained by regression analysis of the peak area ratio of analyte to internal standard versus nominal concentration with a weighting factor of 1/x^2. The calibration curve was linear in the concentration range of 0.98–1000.00 ng/mL with an average regression coefficient, slope, and intercept of 0.9974 ± 0.0014, 0.0011 ± 0.0004, and 0.0004 ± 0.0003, respectively.

The back-calculated concentration values for all QC samples in six triplicates at each concentration level, i.e., lower limit of quantitation (LLOQ, 0.98 ng/mL), lower quality control (LQC, 2.94 ng/mL), medium quality control (MQC, 400 ng/mL), and higher quality control (HQC, 800 ng/mL) on three different occasions, were used to assess the accuracy and precision of the method. The inter-run and intra-run precision and accuracy for the various concentrations ranged from 1.76–6.75% and 97.74–106.75%, and 3.11–5.57% and 98.74–103.87%, respectively. The mean extraction recovery and matrix effect in the plasma at the LQC, MQC, and HQC levels were 87.41%, 98.81%, and 90.52%, and 2.76%, 3.67%, and 3.06%, respectively.

All the QC samples stored at –70 °C were found to be stable for at least one month. The back calculated concentration values for all QC samples at each concentration level after 24 h in the autosampler at 4 °C as well as the plasma samples standing at room temperature for 8 h and three freeze-thaw cycles of GG showed a good accuracy (92.86–104.43%), which indicated that the compound was stable in specified conditions in the rat plasma. In the RLM stability study, there was a 60 min sample without a cofactor for the test and QC samples to determine non-metabolic degradation. The parent area detected in T = 60 min without cofactor samples by LC-MS/MS for GG, desipramine, metoprolol, and verapamil was comparable to the T = 0 min sample. This indicated that GG, desipramine, metoprolol, and verapamil did not undergo chemical (non-metabolic) degradation. The T1/2,int of GG, desipramine, metoprolol, and verapamil were 7.31 ± 0.51, 5.87 ± 0.54, 37.57 ± 1.53, and 4.26 ± 0.13 min, respectively, and the Clint,app were 190.08 ± 13.01, 237.33 ± 22.48, 36.93 ± 1.50, and 3.87 ± 0.54 min, respectively.

Atenolol is a low permeable compound without efflux and is transported by passive diffusion. Furosemide is also a low permeable compound with a high efflux ratio and a transporter(s) is involved for permeation through the Caco-2 cell monolayer. Carbamazepine is transported by passive diffusion due to it having high permeability, whereas verapamil also belongs to the high permeable group, but blocks P-gp with an efflux ratio > 2. So, considering the permeability rank and efflux ratio, the above compounds were selected for Caco-2 as quality controls.

The Papp values across the Caco-2 cell monolayer for A to B of GG, atenolol, furosemide, carbamazepine, and verapamil were determined. In this study, the Papp for QC compounds as well as the permeability rank were comparable with the literature values [10, 15–17]. The permeability rank of the test and QC compounds were done according to “low”, if Papp < 2.5 × 10^-6 cm/sec, and...
"high", if $P_{app} \geq 2.50 \times 10^{-6}$ cm/sec. The $P_{app,A-B}$ and $P_{app,B-A}$ were determined based on the following equation [14]:

$$P_{app} = \left[ \frac{V_a}{(\text{area} \times \text{time})} \right] \times \left( \frac{\text{area of acceptor well}}{\text{area of donor}} \right)$$

where $V_a = \text{volume of acceptor well (in mL)} = 0.25$, area = surface area of the membrane (cm$^2$) = 0.0804, and time = time of incubation (seconds) = 9000.

The $E_{FR}$, or the ratio of effective permeability for a drug, was calculated based on the following equation:

$$E_{FR} = \frac{P_{app,B-A}}{P_{app,A-B}}$$

It was found that GG showed poor permeability (1.31 ± 0.19 × 10$^{-6}$ cm/sec) across A to B and a high $P_{app,B-A}$ (31.89 ± 0.76 × 10$^{-6}$) with a high $E_{FR}$ 24.49 ± 3.05. The permeability values along with the efflux ratios of GG, atenolol, carbamazepine, verapamil, domperidon, and quinidine are presented in Table 2.

A significant increase of the TEER value was observed during the cell culture, indicating a good integrity of the Caco-2 cell monolayer. A little increase in the TEER value (328.67 ± 11.56 Ω cm$^2$) was found between days 15 and 20. Assessment of the integrity of the Caco-2 cell monolayer was also determined by the LY permeability test using a fluorescence measurement. If the $P_{app}$ of LY exceeds $1 \times 10^{-6}$ cm/sec, then it is assumed that the integrity of the Caco-2 cell monolayer has been improper and the test compound will be retested by another experiment. Wells having more than 1% fluorescence intensity with respect to 0.1 mg/mL of the initial donor solution of LY were not considered for the permeability calculation because of poor membrane integrity.

Different pharmacokinetic parameters were determined by non-compartmental analysis using WinNonlin 6.3 from plasma concentration versus time profile for oral and IV administration at 5 and 1 mg/kg dose, respectively (Fig. 1). The results of the pharmacokinetic study revealed poor oral bioavailability (14.18 ± 2.38%) of GG with a $C_{max}$ of 45.91 ± 5.89 ng/mL and a $T_{max}$ of 0.44 ± 0.13 h following oral administration. GG showed short terminal half-lives ($T_{1/2}$) of 0.41 ± 0.03 and 1.33 ± 0.12 h following IV and oral administration, respectively. It was also observed that GG is rapidly metabolized and quickly eliminated from the body with a high clearance (CL) of 53.49 ± 9.23 and 7.49 ± 0.81 L/kg/h, while the total exposure (AUC$_{0-\infty}$) was found to be 95.53 ± 16.02 ng.h/mL and 134.74 ± 15.77 ng × h/mL after oral and IV administration, respectively (Table 3). For IV and PO, the plasma exposure beyond 4 h and 8 h, respectively, was below quantitation levels.

**Discussion**

G. sylvestre is a potent antidiabetic herb that is used for many polyherbal formulations. Gymnemic acid as well as GG are the two major phytoconstituents having antidiabetic activity [4] and are the most accepted phytomarkers used for characterization as well as validation of G. sylvestre. Although G. sylvestre is a clinically well-accepted herb, correlation of its RLM stability, Caco-2 permeability and P-gp efflux ratio of GG.

### Table 1 Summary of observed intrinsic half-life and in vitro clearance in rat liver microsomes along with scaled in vivo clearance.

<table>
<thead>
<tr>
<th>Compound</th>
<th>$T_{1/2,\text{int}}$ (min)</th>
<th>$CL_{int,\text{app}}$ (µL/min/mg)</th>
<th>$CL_{int}'$ (L/h/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gymnemagenin</td>
<td>7.31 ± 0.51</td>
<td>190.08 ± 13.01</td>
<td>24.12 ± 1.65</td>
</tr>
<tr>
<td>Desipramine</td>
<td>5.87 ± 0.54</td>
<td>237.33 ± 22.48</td>
<td>30.11 ± 2.83</td>
</tr>
<tr>
<td>Metoprolol</td>
<td>37.57 ± 1.53</td>
<td>36.93 ± 1.50</td>
<td>4.76 ± 0.16</td>
</tr>
<tr>
<td>Verapamil</td>
<td>4.26 ± 0.13</td>
<td>325.33 ± 10.07</td>
<td>41.59 ± 1.27</td>
</tr>
</tbody>
</table>

### Table 2 Caco-2 permeability (A to B and B to A) and P-gp efflux ratio of GG.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Avg $P_{app} \times 10^{-6}$ cm/sec; n = 6</th>
<th>Permeability rank</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gymnemagenin</td>
<td>1.31 ± 0.19 B to A, 31.89 ± 0.76 A to B</td>
<td>Low</td>
<td>24.49 ± 3.05</td>
</tr>
<tr>
<td>Furosemide</td>
<td>0.07 ± 0.02 B to A, 11.68 ± 0.24 A to B</td>
<td>Low</td>
<td>167.71 ± 16.94</td>
</tr>
<tr>
<td>Atenolol</td>
<td>0.31 ± 0.05 B to A, ND A to B</td>
<td>Low</td>
<td>ND</td>
</tr>
<tr>
<td>Verapamil</td>
<td>9.02 ± 0.44 B to A, 63.34 ± 8.91 A to B</td>
<td>High</td>
<td>7.03 ± 0.34</td>
</tr>
<tr>
<td>Carbamazepine</td>
<td>33.34 ± 1.49 B to A, 40.10 ± 1.58 A to B</td>
<td>High</td>
<td>1.20 ± 0.05</td>
</tr>
<tr>
<td>Domperidon</td>
<td>2.56 ± 0.12 B to A, 50.14 ± 3.15 A to B</td>
<td>High</td>
<td>18.90 ± 0.31</td>
</tr>
<tr>
<td>Quinidine</td>
<td>4.03 ± 0.21 B to A, 32.28 ± 2.57 A to B</td>
<td>High</td>
<td>8.02 ± 0.11</td>
</tr>
</tbody>
</table>

Fig. 1 Mean ± SD plasma exposure profile of GG following oral and IV administration at 5 mg/kg and 1 mg/kg, respectively, in rats.
meability, and efflux with its bioavailability following oral and IV administration of GG in rats is yet to be investigated. The aim of the in vitro kinetic study was to determine the in vitro \( \text{CL}_{\text{int}} \) by the substrate depletion approach, since formal kinetic characterization and quantification of the specific metabolites are not required [18]. Accordingly, the in vitro intrinsic half-life and clearance were found to be 7.31 ± 0.51 min and 190.08 ± 13.01 µL/min/mg of protein, respectively. The \( \text{CL}_{\text{int}} \) was calculated based on the dose/AUC\(_{\text{inf}}\) instead of the more rigorous approach using enzyme kinetics data that consider maximum enzyme velocity, \( V_{\text{max}} \), and \( K_m \). This simplified approach is probably more appropriate and sophisticated, since the substrate concentration (1 µM) is much below the apparent \( K_m \) for substrate turnover and thus no significant product inhibition- or mechanism-based activation of the enzyme can be expected [19]. To determine assay specificity and enzyme activity, verapamil, desipramine, and metoprolol were used as quality control samples that formed an oral and IV pharmacokinetic study of GG in rats. Generally, an EfR value of < 1.20 or 1.50 indicates a mere in-
100% B; this was continued for the next 60 s and the total run time was 4.0 min.
Samples after the Caco-2 and RLM study were analyzed in LC-MS/MS using a Luna phenyl hexyl cartridge (2 x 10 mm, 5 µm) with a flow rate of 0.80 mL/min. The mobile phases were water containing 0.1% v/v formic acid (A) and a mixture of acetonitrile and water (80:20) containing 0.1% formic acid and 2 mM ammonium acetate (B).

In vitro kinetic study using rat liver microsomes

GG along with the quality control compounds desipramine, metoprolol, and verapamil were incubated for 0, 5, 10, 20, 30, and 60 min in a temperature controlled water bath at 37°C at a 1 µM concentration in the absence of the blank and with another 60 min sample without the cofactor (WC) for chemical degradation control. We followed the experimental procedure/protocol that has already been reported, with minute modifications [22]. Briefly, 100 µL of incubation mixture contained RLM (0.5 mg/mL), NADP (1.3 mM), glucose-6-phosphate (3.3 mM), and glucose-6-phosphate dehydrogenase (0.4 U/mL) containing MgCl₂ (3.3 mM) solution in phosphate buffer pH 7.4. All reactions were started by the addition of NRS in a 96 deep-well plate and were carried out for the abovementioned time points. The reaction was terminated by the addition of 300 µL of ice-cold acetonitrile and mixed, followed by centrifugation at 4000 rpm for 15 min at 15°C. One hundred and twenty µL of supernatant were diluted with 120 µL of water, and 25 µL were injected into the LC-MS/MS.

Time on the X axis and ln (area of analyte) on the Y axis were plotted in a semi-log plot to calculate the slope. Intrinsic T₁/₂ was calculated by dividing −0.693 with the slope. In vitro Clₘ was scaled to an in vivo Clₘ (in units of mL/min/kg) using microsomal protein (mg/g of liver) and average liver weight (g of liver/kg of body weight) according to the following formula:

\[
Cl_{\text{int}} = \frac{(0.693/\text{in vitro } T \text{₁/₂}) \times (\text{mL of incubation volume/mg of microsomal protein}) \times (45 \text{ mg microsomal protein/g of liver}) \times (20^a \text{ g of liver/kg of body weight})}{a: 20 \\text{ and 45 g of liver/kg of body weight were used for human and rat respectively}[19].}

Caco-2 permeability study (A-B and B-A)

A to B cell plating and culture: The Caco-2 cell line was obtained from ATCC and cells were seeded at 6.3 x 10⁴ cells/cm² and grown in a medium containing Dulbecco’s modified Eagle’s medium (DMEM) comprised of 4.5 g/L glucose and supplemented with 10% (v/v) fetal bovine serum (FBS), 1% (v/v) glutamine, penicillin (100 U/mL), streptomycin (100 µg/mL), and 1% (v/v) Minimum Essential Medium (MEM) nonessential amino acids. Cultures were maintained at 37°C in an atmosphere of 95% air and 5% CO₂ with controlled humidity. The medium was changed in every 2-3 days. Routine passing of all cell stocks was carried out in 75 cm² flasks, and experiments were done on passages #25-30. Seventy-five µL of 2.5 x 10⁵ cells/mL and 40 mL of media were transferred to the apical wells and to the feeder tray, respectively. The plates were placed in an incubator (37°C, 5% CO₂, and controlled humidity). The first media change was given between 40–72 h and cells were grown for 21 days with a media change every alternate day. B to A cell plating and culture: 25 µL of 7.5 x 10⁵ cells/mL were transferred to the bottom side (keeping the plate upside down) of apical wells. The plate (upside down position) was kept in an incubator (37°C, 5% CO₂, and controlled humidity) for 2 h. The plate was turned right side up. Seventy-five µL of media/well to the apical plate and 40 mL of media to the feeder tray were added.

Transport study

Permeability assay A to B: The apical wells were washed with buffer pH 6.5 and basal wells were washed with buffer pH 7.4. Two hundred and fifty µL of buffer pH 7.4/well were added to the basal plate. Seventy-five µL of GG, atenolol, furosemide, carbamazepine, and verapamil solutions (2 µM) in buffer pH 6.5 were added to the apical wells (n = 6).

Permeability assay B to A: The apical wells were washed with buffer pH 7.4 and basal wells with buffer pH 6.5. Two hundred and fifty µL of buffer pH 6.5/well were added to the basal plate. Seventy-five µL of GG, domperidon, furosemide, carbamazepine, quinidine, and verapamil solutions (2 µM) in buffer pH 7.4 were added to the apical wells (n = 6). The apical plate was placed on to the basal plate with a lid to prevent evaporation. The assembly was incubated at 37°C for 2.5 h under 95% air and 5% CO₂ with controlled RH. The apical plate was separated after incubation and aliquots from the acceptor wells were taken, diluted, and quantified by LC-MS/MS along with the initial donor samples.

Membrane integrity

To optimize the membrane integrity of the Caco-2 monolayer, the TEER was determined during the cell culture. A little increase in the TEER value was found between days 15 and 20. Another membrane integrity test post the Caco-2 experiment was performed using lucifer yellow by fluorimetry. Solutions in the apical wells were discarded by inverting the plate and soaking them on tissue paper very carefully. Phosphate buffer pH 7.4 250 µL/well was added to the basal plate. Seventy-five µL of LY (0.1 mg/mL) in buffer pH 7.4 per well were added to the apical plate. The apical plate was placed on to the basal plate with a lid. The assembly was incubated at 37°C for 1 h under 5% CO₂ and 95% air. The apical plate was separated after incubation, and 100 µL of solution from the basal wells were transferred for the fluorescence (Ex: 432 nm, Em: 530 nm) measurement. A fluorescence of 100 µL buffer pH 7.4 only and 100 µL LY (0.1 mg/mL) were also measured.

Animal study

Pharmacokinetic study: The experiments were conducted using male Wistar rats under the care and use of laboratory animals in accordance with the guidelines prescribed by the Institutional Ethical Committee (constituted under the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals, CPCSEA, Reg. No. 367). The study was approved by the ethical committee on 11/12/2014 and the approval number is AEC/PHARM/1407/2014. Animals were acclimatized individually in a cage under a 12/12-h light dark cycle, 22 ± 2°C temp, 50 ± 20% RH 5 days prior to the studies and maintained on an 18% casein-containing semisynthetic diet with free access to food and water. Pharmacokinetic studies with GG were carried out in male (180–200 g) Wistar rats after oral and intravenous administration of GG at a dose of 5 mg/kg and 1 mg/kg, respectively. A solution formulation of GG was made in 10% DMSO, 30% propylene glycol, and the rest was 5% glucose solution with a dose volume of 5 mL/kg for oral and 2 mL/kg for IV. Animals were divided into two groups – Gr-I (n = 6) for oral and Gr-II (n = 6) for IV. About 130 µL of whole blood were collected at predetermined time points (Pre-dose, 0.08, 0.25, 0.5, 1, 2, 4, 8, and 24 h) via jugular
vein cannulation, and normal saline solution was supplemented in each time point after sample collection. The collected samples were centrifuged at 2500 rpm for 10 min at 4°C and the collected plasma was stored at -70°C until bioanalysis. 

**Method validation:** The validation of the bioanalytical method in plasma was carried out for selectivity, linearity, precision, accuracy, recovery, matrix effect, and stability according to the principles of the Food and Drug Administration [13] industry guide. An 11-point calibration curve covering a range of 0.98–1000 ng/mL of GG was prepared in duplicate and analyzed in three different runs on three separate days with six replicates of the LLOQ (0.98 ng/mL), LQC (2.94 ng/mL), MQC (400 ng/mL), and HQC (800 ng/mL) samples. The curves were fitted using a linear regression method with weighting 1/x².

The selectivity, specificity, and intraday and interday precision and accuracy of the method were assessed. The extraction recovery (ER) and matrix effect of GG after protein precipitation was determined at three concentration levels, and the extraction recovery of IS was also carried out at a single (70 ng/mL) concentration. The ME was evaluated in the present study as proposed by Matuszewski et al. [23].

Recovery (%) = (peak area of the extracted analyte × 100/peak area of the non-extracted analyte mixed with blank matrix extract)

ME (%) = 1 - (response for post-extraction spiked drug)/(response in solvent)) × 100

The stability of GG was evaluated as part of the method validation. The processed sample stability in the autosampler at 4°C for 12 h, stability of GG in the biomatrix after 8 h exposure on the bench-top, long-term stability (30 days) of the spiked QC samples stored at -70.00°C, and freeze-thaw stability (3 cycles) were evaluated.

The standard stock solution of GG (analyte) and dexamethasone (IS) were prepared in DMSO (2.00 mg/mL) for each. Then an intermediate stock (100.00 µg/mL) for GG and IS were prepared in DMSO. The working stock solutions 50000 µg/mL, 25000 µg/mL, 12500 µg/mL, 6250 µg/mL, 3125 µg/mL, 1562.5 µg/mL, 781.25 µg/mL, 390.63 µg/mL, 195.31 µg/mL, and 97.66 µg/mL were prepared for the calibration curve (CC), and 146.48 µg/mL, 20000 µg/mL, and 40000 µg/mL for QCs were prepared from intermediate stock in DMSO. CC and QC samples were prepared by spiking 2 µL from the working stock to 98 µL blank matrix to get final concentrations of 1000.00 µg/mL, 500.00 µg/mL, 250.00 µg/mL, 125.00 µg/mL, 62.50 µg/mL, 31.25 µg/mL, 15.63 µg/mL, 7.81 µg/mL, and 3.91 µg/mL. Five µL of 0.98 ng/mL IS were spiked in each sample. All standard and IS stock solutions were stored in polypropylene vials at 2–8°C for further use.

**Sample preparation for plasma:** All plasma samples along with linearity and QC samples were analyzed using the validated LC-MS/MS method. The plasma sample aliquot (30 µL) was taken into a V-bottom shallow 96-well plate and direct precipitation of the matrix was done by adding an ice-cold mixture (70/30, v/v) of acetonitrile and methanol (3 x sample volume). The mixture was mixed in a thermomixer for 8 min and centrifuged at 4000 rpm for 15 min at 15°C. Sixty µL of clear supernatant was mixed with 50 µL of water, and 20 µL of aliquot was injected into the LCMS/MS.

**Pharmacokinetic analysis:** Pharmacokinetic parameters were calculated from the plasma concentration data following oral and IV administration (Table 3) by a noncompartmental method using WinNonlin 6.3. The area under the plasma concentration-time curve, AU(t-last), was calculated from time 0 h to the last quantifiable time point by the linear trapezoidal method. The time at which Cmax was achieved (Tmax), the apparent maximum plasma concentration (Cmax) and the terminal elimination rate constant (Ke) terminal half-life (T1/2), clearance (CL), volume of distribution (Vd), total exposure AUC0–last, and AUC0–∞ were determined (Table 3).

**Supporting information**

Mass spectrometry and chromatography data of GG and dexamethasone, as well as intraday and interday precision and accuracy data of GG, and stability data are available as Supporting Information.

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**Conflict of Interest**

The authors declare that there is no conflict of interest.

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