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Determination of gymnemagenin in rat plasma using high-performance liquid chromatography–tandem mass spectrometry: application to pharmacokinetics after oral administration of *Gymnema sylvestre* extract

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ABSTRACT: A sensitive and rapid high-performance liquid chromatography–tandem mass spectrometry (HPLC-MS/MS) method has been developed and validated for the determination of gymnemagenin (GMG), a triterpene sapogenin from *Gymnema sylvestre*, in rat plasma using withaferin A as the internal standard (IS). Plasma samples were simply extracted using liquid–liquid extraction with tetra-butyl methyl ether. Chromatographic separation was performed on Luna C_{18} column using gradient elution of water and methanol (with 0.1% formic acid and 0.3% ammonia) at a flow rate of 0.8 mL/min. GMG and IS were eluted at 4.64 and 4.36 min, ionized in negative and positive mode, respectively, and quantitatively estimated using multiple reaction monitoring (MRM) mode. Two MRM transitions were selected at *m/z* 505.70 → 455.5 and *m/z* 471.50 → 281.3 for GMG and IS, respectively. The assay was linear over the concentration range of 5.280–300.920 ng/mL. The mean plasma extraction recoveries for GMG and IS were found to be 80.92 ± 8.70 and 55.63 ± 0.76%, respectively. The method was successfully applied for the determination of pharmacokinetic parameters of GMG after oral administration of *G. sylvestre* extract. Copyright © 2012 John Wiley & Sons, Ltd.

Keywords: gymnemic acids; Gymnema sylvestre; HPLC-ESI-MS/MS; pharmacokinetics; anti-diabetic

Introduction

Gymnema sylvestre (commonly named as Gurmar, family Asclepiadaceae) is a well-known plant used in diabetic conditions and is officially mentioned in Indian Pharmacopoeia (Indian Pharmacopoeia Commission 2007). Traditionally, it has also been used as stomachic, a remedy for cough and an anti-diuretic. *G. sylvestre* is the one of the major ingredients of various single as well as multi-herb formulations used for diabetic conditions globally. Several preclinical studies on polar/nonpolar extract of roots and leaves of *G. sylvestre* suggested anti-hyperglycemic (Shanmugasundaram *et al.*, 1990), anti-hyperlipidemic (Rachh *et al.*, 2010), anti-microbial (Chodisetti *et al.*, 2012), anti-oxidant (Kang *et al.*, 2012), anti-inflammatory (Saneja *et al.*, 2010) and anti-cancer (Srikanth *et al.*, 2010) activities. Mechanistic studies suggested that *G. sylvestre* exert its hypoglycemic effects through increase in insulin secretion, regeneration of islets cells, peripheral utilization of glucose and inhibition of glucose absorption from intestine (Kanetkar *et al.*, 2005). Further, clinical reports validate the use of *G. sylvestre* in type 1 and 2 diabetic conditions (Baskaran *et al.*, 1990; Shanmugasundaram *et al.*, 1990).

Chemically, *G. sylvestre* contains triterpene saponins, gymnemic acids, considered as active constituents, and the quality of extracts and its formulations is assessed by the content of gymnemic acids (Yoshikaua *et al.*, 1989). Gymnemic acids are a complex mixture of several closely related compounds and those compounds are not available commercially. Therefore, direct quantitative estimation of gymnemic acids is difficult and several analytical methods have been developed on the basis of gymnemagenin, the hydrolyzed product of gymnemic acids (Fig. 1). Analytical methods including high-performance thin layer chromatography (HPTLC) and high-performance liquid chromatography (HPLC) were reported for quantitative indirect estimation of gymnemic acids in *G. sylvestre* crude and its samples (Toshihiro *et al.*, 1994; Valivarthi *et al.*, 2006;

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Abbreviations used: GMG, gymnemagenin; MRM, multiple reaction monitoring; TBME, tetra-butyl methyl ether.

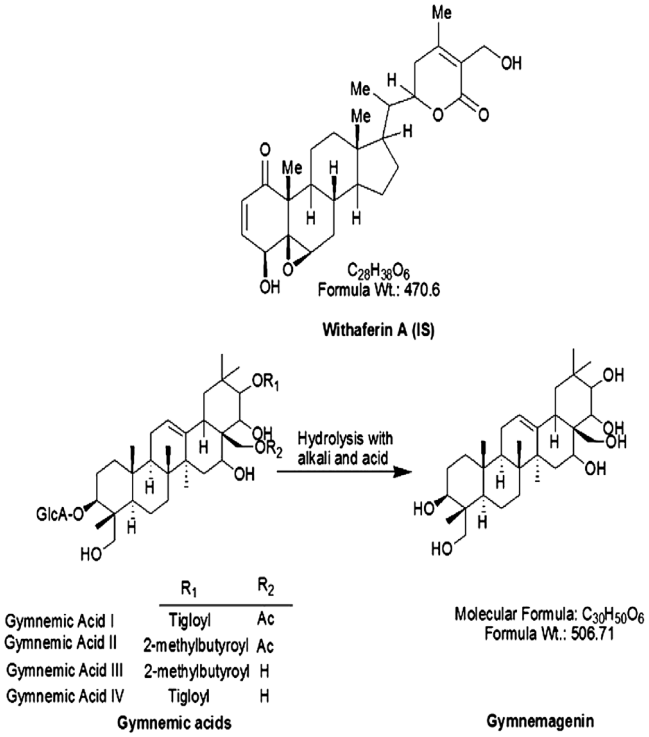

Figure 1. Structures of withaferin A (internal standard) and reference standard gymnemagenin obtained after hydrolysis of gymnemic acids.

Trivedi *et al.*, 2011). HPLC coupled with mass spectrometry (HPLC-MS) method was used for online identification of 18 different gymnemic acids in *G. sylvestre* extracts (Imoto *et al.*, 1991). Recently, we have developed sensitive HPLC-ESI-MS/MS based method for quantitative analysis of gymnemagenin in *G. sylvestre* extract and its various marketed formulations (Kamble *et al.*, 2012). Despite of extensive mechanistic studies and phytochemical investigations on *G. sylvestre*, pharmacokinetics of these constituents after oral administration of extract is lacking. Pharmacokinetic data can provide valuable information to aid practitioners in prescribing herbal drugs safely and effectively. In this investigation, a sensitive and rapid HPLC-ESI-MS/MS method was developed for quantitative estimation of GMG in rat plasma samples. Subsequently, the method was applied for the evaluation of pharmacokinetic parameters of GMG after oral administration of standardized alcoholic leaf extract of *G. sylvestre*.

Experimental

Chemicals and solvents

Standard gymnemagenin (GMG; purity ≥ 95%) was purchased from Natural Remedies Pvt. Ltd, Bangalore, India. Withaferin A (purity ≥ 99%) was purchased from Chromadex (Laguna Hills, CA, USA) and used as internal standard (IS). Acetonitrile, methanol, ethanol and potassium hydroxide were procured from Merck, Mumbai, India. HPLC-grade water was used from J.T. Baker, Mumbai, India. All other chemicals and solvents otherwise used were of analytical grade.

Plant extract and chemical standardization

Alcoholic extract of *G. sylvestre* leaves was received as gift sample from Natural Remedies Pvt. Ltd, Bangalore, India. GMG content was estimated in the extract using HPLC-ESI-MS/MS method and expressed as percentages w/w (Kamble *et al.*, 2012).

LC-MS instruments and conditions

Analysis were performed on an HPLC system LC-10AD (Shimadzu, Japan) coupled with a triple quadrupole mass spectrometer (API-4000; Applied Biosystem/MDS Sciex, USA). Chromatographic separation was achieved on Luna C_{18} column (50 × 4.6 mm, 5 μm; Phenomenex, India) using mobile phase consisting of water (with 0.1% formic acid and 0.3% ammonia) as component A and acetonitrile (with 0.1% formic acid and 0.3% ammonia) as component B. The following gradient elution program was used: from 0 to 1 min, 60% component B; from 1 to 2 min, 95% component B; from 2 to 4.5 min, 95% component B maintained; from 4.5 to 5.5 min, 60% component B; and from 5.5 to 7.0 min, component B maintained at 60%. The flow rate of 0.8 mL/min was used. The quantification of the GMG was performed using multiple reaction monitoring (MRM) acquisition in dual ionization mode. GMG and IS were operated in negative and positive mode respectively. Two MRM transitions were selected: m/z 505.70 → 455.5 for GMG and m/z 471.50 → 281.3 for IS. The optimized mass parameters for GMG (ion source voltage, declustering potential, entrance potential, collision cell energy and collision exit cell), were −4500, −144, −9, −54 and −22 V, respectively. The same optimized mass parameters for withaferin A were 5500, 84, 7, 24 and 5 V, respectively.

Preparation of calibration standards and quality control samples

Primary stock solution of GMG (1 mg/mL) and IS (1 mg/mL) was prepared in methanol–water (60:40) and methanol, respectively. Secondary working stock solutions of 4.78 and 150 ng/mL for GMG and IS, respectively, were prepared by diluting their primary stock solutions. Calibration standards (CS) of GMG (5.28, 10.55, 30.16, 60.33, 120.67, 180.11, 240.14 and 305.92 ng/mL) were prepared by spiking appropriate amounts of working stock solutions into pooled rat plasma. Quality control (QC) samples for GMG (18.32, 64.76, 163.33 and 214.90 ng/mL) were prepared freshly by diluting appropriate amounts of working stock solutions into drug-free rat plasma and used for method validation.

Plasma sample preparation

A 150 μL aliquot of blank plasma, CS, QC samples and animal study samples were mixed with 20 μL of IS solution. The samples were extracted with 2.0 mL of tetra-butyl methyl ether (TBME) by vortex mixing for 5 min at high speed and centrifuged at 600***g*** for 5 min. The organic layer was transferred and evaporated to the dryness under nitrogen vacuum concentrator. The residues were dissolved in 150 μL of mobile phase and transferred to the injection vials. 20 μL of each sample was injected into HPLC-MS/MS system.

Method validation

The developed method was validated for selectivity, linearity, accuracy, precision, recovery and stability as per USFDA guidelines on bioanalytical method validation (US Food and Drug Administration, 2001).

Selectivity. The selectivity of the method was evaluated by comparing blank plasma samples collected from six different rats with the corresponding spiked plasma samples to investigate the potential interferences at the LC peak region for GMG and IS.

Linearity and lower limit of quantitation. Three sets of calibration curves with eight concentrations ranging from 5.280 to 300.92 ng/mL for GMG were constructed by plotting the peak area ratio of the analyte/IS (y) vs analyte concentration (x) in drug-free rat plasma samples. The regression parameters of slope, intercept and correlation coefficient (r^2) were calculated by weighted ($1/x^2$) least squares linear

regression analysis of y vs x. Lower limit of quantitation (LLOQ) was measured for GMG as the lowest analyte concentration with signal-to-noise ratio greater than 10 (S/N ≥ 10) that can be determined with an accuracy and precision <20%.

Precision and accuracy. The precision and accuracy of this method were evaluated using QC samples. For the evaluation of precision (intra- and inter-day) and accuracy of the method, five replicates of four different QC samples (18.327, 64.761, 163.331 and 214.909 ng/mL) were analyzed on three different days. The precision was expressed by the relative standard deviation (RSD) and the accuracy by the relative standard error (RE).

Recovery. The extraction recoveries of GMG from the rat plasma after the extraction procedure was assessed in three QC samples (18.327, 163.331 and 214.909 ng/mL). The detector response (peak area) of processed QC samples (R_2) was compared with the responses of GMG spiked in post-extracted samples at equivalent concentrations (R_1). Percentage extraction recovery was calculated using the formula

$$\%\text{extraction recovery} = (R_2/R_1) \times 100$$

Stability studies. Two different QC samples (18.327 and 214.909 ng/mL) were subjected to the evaluation of three freeze–thaw cycles, room temperature storage (6 h) and autosampler stability of processed samples (12 h).

Pharmacokinetic study

The pharmacokinetic study was performed to show the applicability of the newly developed method and the validated method. Wistar rats (male) in the weight range 250–300 g were used in this study. The animals were housed in standard conditions at a temperature of 23 ± 2°C, relative humidity 55 ± 10% and 12 h light and dark cycle throughout the experiment. Animals had free access to standard laboratory feed and water *ad libitum*. The experimental protocol was approved by the Institutional Animal Ethics Committee constituted in accordance with the rules and guidelines of the Committee for the Purpose of Control and Supervision on Experimental Animals, India. *G. sylvestre* extract at 400 mg/kg dose was administered orally to the animals ($n = 6$) and blood samples were collected at 0, 0.5, 1, 2, 4, 6, 8, 12 and 24 h after the treatment. Plasma samples were separated at 3000***g*** for 10 min at 4°C and stored at −70°C until analysis.

The pharmacokinetic parameters such as area under the plasma concentration–time curve (AUC_{0-t}) and terminal elimination half-life ($T_{1/2}$) were calculated by non-compartmental analysis using software WinNonlin version 3.0 (Pharmasight Corporation, Mountain view, CA, USA). The maximum plasma concentration (C_{max}) and the time to reach C_{max} (T_{max}) were obtained directly from the plasma concentration-time curve. All values are expressed as means ± SD except the T_{max}, which is expressed as the median.

Results and discussion

Plasma sample preparation

Plasma sample preparation is an important step, employed with an aim to remove interferences from biological samples

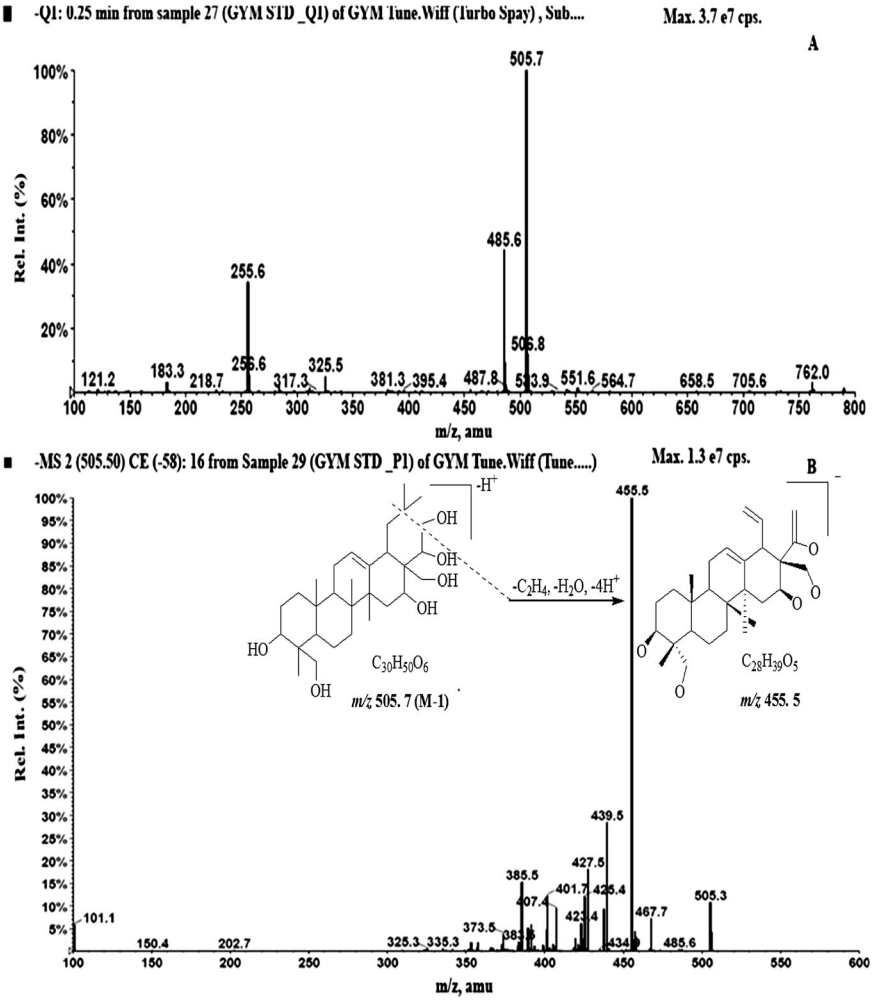

Figure 2. ESI-MS full scan (A) and product ion (B) spectra of GMG showed *m/z* 505.7 and 455.5, respectively, in negative ionization mode.

using simple procedure having suitable recovery. Liquid–liquid extraction, protein precipitation and solid-phase extraction are the most widely used sample preparation techniques during the development. In the present study, three methods were investigated and compared at different conditions individually. It was shown that liquid–liquid extraction with TBME provided higher recovery compared with the other two methods (>80%). The advantage of using liquid–liquid extraction was that it minimizes chances of errors, saves time and simplifies the sample preparation methodology. In quantitative analysis of analytes in biological samples, an appropriate IS is needed. Several compounds were investigated to find a suitable IS. Withaferin A was chosen because of similar chromatographic behaviors and extraction characteristics. The results of method validation using withaferin A as the IS was acceptable in this study.

Optimization of mass spectrometry and chromatography conditions

To optimize ESI conditions for detection of analytes, both the positive and negative ion modes were investigated. GMG showed the maximum response in negative ionization mode whereas withaferin A showed the maximum response in positive mode. The full-scan ESI spectra revealed higher signals at *m/z* 505.7 (Fig. 2a). The product ion spectra of deprotonated molecular ions are shown in (Fig. 2b) in which the most abundant ions were observed at *m/z* 455.5. Additional tuning of ESI source and collision-induced dissociation parameters onto the transition *m/z* 505.7 → 455.5 improved the sensitivity. The full-scan ESI spectra of withaferin A showed the presence of protonated molecule $[M+H]^+$ at *m/z* 471.5 in the positive ion mode (Fig. 3a). The product ion spectra of molecular ion showed the presence of abundant ions at *m/z* 281.3 (Fig. 3b). These mass spectrometric data was found to be consistent with previously published report (Lavie *et al.*, 1965). The major fragment ions at *m/z* 505.7 → 455.5 (GMG) and *m/z* 471.5 → 281.3 (withaferin A) were chosen in the MRM mode for quantitative analysis.

The chromatographic conditions were investigated to optimize sensitivity, speed and peak shape. Methanol was chosen as the organic solvent because it provided a higher sensitivity over acetonitrile. It was also found that the presence of a small amount of formic acid and ammonia in the mobile phase improved the sensitivity by promoting the ionization of the analytes. Optimized gradient elution program containing methanol and water with formic acid (0.1%) and ammonia (0.3%) achieved symmetrical peak shapes and a short

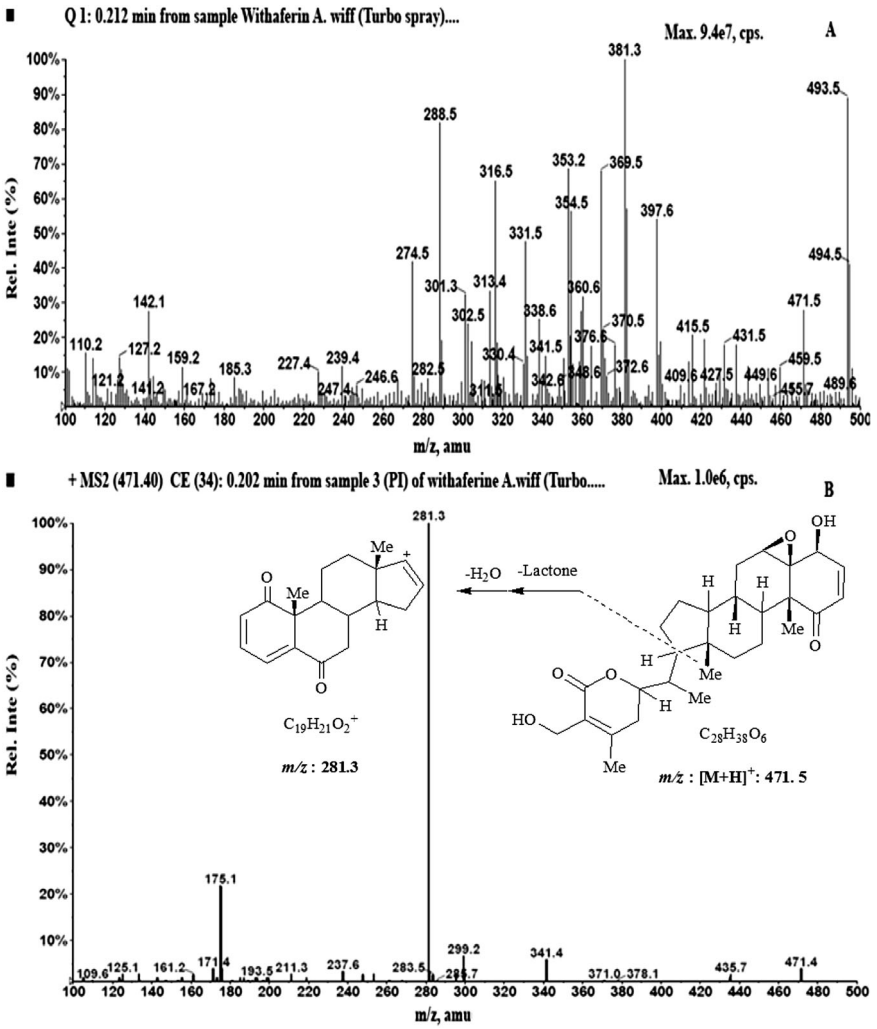

Figure 3. ESI-MS full scan (A) and product ion (B) spectra of withaferin A showed *m/z* 471.5 and 281.3, respectively, in positive ionization mode.

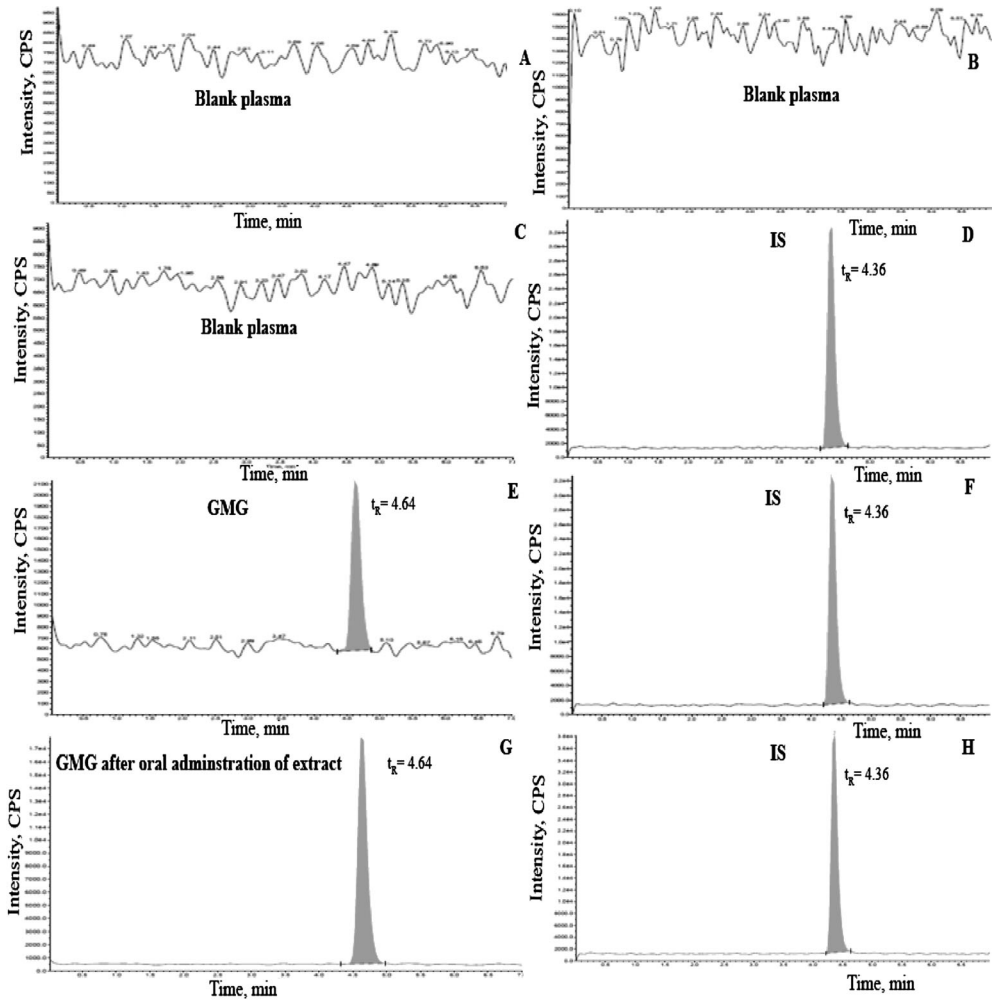

Figure 4. Representative HPLC-ESI-MRM chromatograms of gymnemagenin (GMG) and withaferin A (IS) spiked in blank plasma and detected in study samples. Blank plasma did not show the presence of any peak at retention times of GMG (t_R = 4.64 min) as shown in chromatograms A and B. Thus method was found to be selective. Chromatogram C represents GMG standard spiked at LLOQ (5.280 ng/mL) in blank rat plasma and chromatogram G represents the presence of GMG in rat samples after 1 h of administration of *G. sylvestre* at a dose of 400 mg/kg.

chromatographic analysis time, and eliminated the matrix effect. Representative HPLC-ESI-MS/MS chromatograms of GMG and withaferin A in spiked plasma and experimental animal samples are shown in Fig. 4. Retention times (t_R) for GMG and withaferin A were found to be 4.64 and 4.36 min, respectively. The optimized method was validated for selectivity, linearity, accuracy, precision, recovery and stability.

Method validation

Selectivity. Selectivity was assessed by comparing the chromatograms of six different batches of blank rat plasma with the corresponding spiked plasma. Under the optimized HPLC-ESI-MS/MS conditions, the representative chromatograms of blank plasma, blank plasma spiked with GMG and IS, and plasma sample obtained at 1 h after oral administration *G. sylvestre* extract are shown in Fig. 4. No significant interferences from endogenous substances were observed at the t_R of GMG and IS in the matrix.

Linearity and lower limit of quantitation. The calibration curves were established over on three replicates experiments with GMG spiked in rat plasma, suggesting good linearity ($r^2 \geq 0.995$) over the concentration range of 5.280–300.920 ng/mL. The LLOQ for GMG in rat plasma was 5.280 ng/mL.

Precision and accuracy. The precision (intra and inter-day) and accuracy data of GMG spiked QC samples is summarized in Table 1. The intra- and inter-day coefficient of variation (CV) values were 2.13–4.04 and 3.21–5.14%, respectively. The accuracy values were found to be within 89.30–115.5% at four different QC levels. The CV and percentage accuracy values were within the acceptable limits. This suggested that the method is precise and accurate.

Recovery. Mean extraction recovery of GMG from the rat plasma samples after the extraction procedure was assessed in three different QC samples and was found to be 80.92 ± 8.70% (Table 2). The recovery of the IS was 55.63 ± 0.76% at a concentration of 84.80 ng/mL (n = 6). The data suggested that the extraction recoveries of GMG and IS using liquid–liquid extraction were consistent, precise and reproducible.

Stability study. The stability of GMG in rat plasma under different storage conditions is summarized in Table 3. GMG was

Table 1. Precision and accuracy of the method

	Intra-day ($n = 5$)				Inter-day ($n = 15$)			
QC (ng/mL)	18.32	64.76	163.33	214.90	18.32	64.76	163.33	214.90
Mean (ng/mL)	21.16	68.33	162.22	188.70	21.05	68.33	163.50	191.98
CV (%)	4.04	2.66	3.85	2.13	5.14	3.65	3.21	3.21
RE (%)	15.5	5.50	−0.68	−12.19	14.9	5.60	0.10	−10.70

Coefficient of variation (CV) and relative error (RE) were considered as measures of precision and accuracy of the method respectively. Four different QC samples were prepared and analyzed within the same day ($n = 5$) and on different days ($n = 15$) for the measurement of precision and accuracy. CV and RE were found within the acceptable limits.

Table 2. Recovery of the method

Spiked concentration (ng/mL)	Aqueous drug area (R_1) (mean ± CV)	Extracted drug area (R_2) (mean ± CV)	Recovery (%)
18.32	55,744.66 ± 9.93	40,644.66 ± 2.41	72.91
163.33	379,055 ± 4.90	317,363 ± 2.40	83.72
214.90	457,952.66 ± 7.21	10,987.72 ± 2.78	86.14
Mean recovery (% CV)		80.92 ± 8.70	

Mean extraction recovery of gymnemagenin (GMG) from the rat plasma after the extraction procedure was assessed in three QC samples ($n = 5$). The detector response (peak area) of processed QC samples (R_2) was compared with the response of directly injected aqueous QC samples (R_1). Mean extraction recovery was calculated by using formula: mean recovery (%) = $(R_2/R_1) \times 100$.

Table 3. Stability of GMG in plasma samples

Parameters	Three freeze–thaw cycles		Short- term stability (6 h)		Autosampler stability (12 h)	
Theoretical concentration (ng/mL)	18.32	214.90	18.32	214.90	18.32	214.90
Nominal concentration (ng/mL)	17.01	208.67	16.60	209.27	17.75	201.10
CV (%)	8.3	11.0	3.90	9.60	6.90	5.80
RE (%)	−7.16	−2.10	−9.39	−2.62	−3.22	−6.43

Two different QC samples (18.32 and 214.90 ng/mL) were evaluated for stability testing at three different conditions such as freeze–thaw cycles, storage at room temperature and storage at autosampler. CV and RE were estimated and found within acceptable limits.

found to be stable in rat plasma with the acceptable accuracy (92.84 and 97.10%) and precision (3.7 and 9.6% CV) at 18.32 and 214.90 ng/mL, respectively, after three freeze–thaw cycles. The compound was also shown to be stable after 6 h room temperature storage and 12 h of storage in autosampler conditions. The concentrations of GMG were within 10% deviation of the initial values.

Pharmacokinetics of GMG after oral administration of G. sylvestre extract

The validated method was applied for quantitative estimation of GMG in plasma samples obtained after oral administration of *G. sylvestre* extract at a dose of 400 mg/kg. The method detected the presence of GMG, the hydrolyzed product of gymnemic acids, in extract-fed rat plasma samples. This could be because of hydrolysis of gymnemic acids from *G. sylvestre* extract in the gastrointestinal tract or first-pass hepatic metabolism. Further studies are ongoing in our laboratory to elucidate the underlying mechanisms of this biotransformation. Similar observations were reported on another triterpene saponin, glycyrrhizin, from *Glycyrrhiza glabra* (Takeda *et al.*, 1996). Glycyrrhizin undergoes hydrolysis in the gastrointestinal

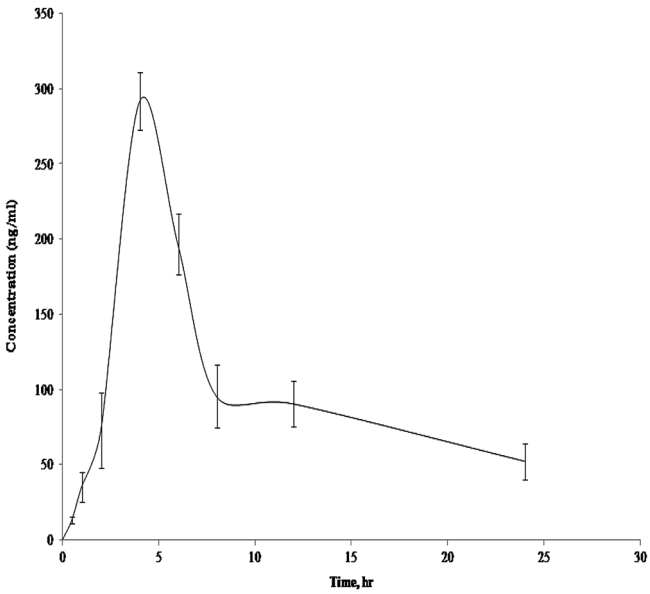

Figure 5. Mean plasma concentration vs time curve for GMG after oral administration of *G. sylvestre* at dose of 400 mg/kg. Values are represented as means ± SD.

Table 4. Pharmacokinetic parameters of GMG after oral administration GE at dose of 400 mg/kg

PK parameters	Values (mean ± SD)
C_{max} (ng/mL)	291.81 ± 18.94
AUC_{0-24h} (ng min/mL)	2450.35 ± 373.87
T_{max} (h)	2–4
$T_{1/2}$ (h)	8.88 ± 1.15

The values were expressed as mean ± SD ($n = 3$) except T_{max}. C_{max} (ng/mL), peak plasma concentration; T_{max} (min), maximum time required to reach C_{max} mentioned as median; AUC_{0-24h} (ng min/mL), area under the curves from time zero to 24 h; and $T_{1/2}$ (min), terminal elimination half-life.

tract and formed an aglycone moiety, glycyrrhetic acid. The mean plasma concentration–time curve for GMG was plotted (Fig. 5). The main pharmacokinetic parameters of GMG are summarized in Table 4. Pharmacokinetic parameters such as C_{max}, AUC_{0-24h}, T_{max} and $T_{1/2}$ were found to be 291.81 ± 18.94 ng/mL, 2450.35 ± 373.87 ng min/mL, 2–4 h and 8.88 ± 1.15 h, respectively, after oral administration of extract. The presence of double peaks in the plasma concentration–time profile was observed. This could be because of enterohepatic recirculation, selective and differential absorption from gastrointestinal tract, formation of a deposit on the intestinal wall and/or variation in gastrointestinal motility (Han *et al*., 2012). This observation was consistent with pharmacokinetic profiles on other triterpene and steroidal saponins, such as madecassoside, asperosaponin VI and timosaponin B-II, published elsewhere (Han *et al*., 2012; Shakya *et al*., 2012; Cai *et al*., 2008).

Conclusion

In the present work, a sensitive, rapid and selective HPLC-ESI-MS/MS method was developed and validated over the concentration range of 5.280–300.920 ng/mL. The lower limit of quantification was obtained and simple liquid–liquid extraction was employed for plasma sample preparation. The present method showed high sample throughput (total analytical run 6 min) and greater sensitivity (5.280 ng/mL). The method was successfully applied for analysis of GMG in plasma samples after oral administration of 400 mg/kg of standardized *G. sylvestre* extract.

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