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Ecological HPLC method for analyzing an antidiabetic drug in real rat plasma samples and studying the effects of concurrently administered fenugreek extract on its pharmacokinetics

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Currently, the total number of diabetic people worldwide is constantly increasing. Metformin (MET) is known to be a first-line antidiabetic drug with varied, wide-reaching applications. Concurrent administration of phytochemicals such as fenugreek extract with synthetic drugs is very common. It is reported that concomitant administration of fenugreek extract with metformin maintains lower blood glucose levels than metformin alone. In this work, an ecofriendly RP-HPLC method was established to study and compare the pharmacokinetics of metformin with and without the contemporary administration of fenugreek extract using rat as an animal model. In the developed method, a solvent mixture of 0.5 mM KH_2PO_4 solution : methanol (65 : 35, v/v) was used as a mobile phase and guaiphenesin was used as an internal standard. The plasma concentration–time curve was plotted, and non-compartmental pharmacokinetic analysis was performed using PKSolver. The results of the pharmacokinetic study showed that concurrent administration of fenugreek significantly increased the bioavailability of metformin and doubled the time required to reach the peak plasma concentration (T_{max}). Moreover, the volume of drug distribution decreased by about 70%, while its rate of clearance decreased by about 55.96%. Accordingly, the administration of fenugreek in combination with metformin significantly affected the pharmacokinetics of metformin, and this combination will be very useful in controlling blood glucose levels in diabetic patients. The greenness of the method was assessed using the Analytical Eco-Scale, Analytical Method Volume Intensity (AMVI), and National Environmental Method Index (NEMI), and all results affirmed that the method can be considered to be ecological.

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1. Introduction

Type 2 diabetes mellitus is a non-insulin-dependent metabolic disorder in which the body becomes tolerant to the response of insulin.¹ Management of patients with type 2 diabetes is possible with oral hypoglycemic pharmaceutical drugs. Diverse chemical classes have been developed as oral hypoglycemic agents that have various mechanisms of action, among which biguanides (metformin, MET) are the most widely used drugs.²

MET can be adjudged to be a first line oral hypoglycemic; it does not cause hypoglycemia or weight gain. Maintaining long-term glycemic control in diabetes treatment is a challenge; hence, concurrent administration of metformin and herbal medicines may achieve a synergistic effect and satisfactory blood glucose levels.³ After extensive literature review, different methods have been published for analysis of MET alone in different samples (plasma, urine, liver, brain, kidney, and muscles), including HPLC,^{4–9} hydrophilic interaction liquid chromatography (HILIC),¹⁰ LC/MS/MS,^{11–14} HILIC/MS,^{15,16} and GC/MS.¹⁷ Likewise, MET in combination with different drugs was determined in different plasma samples by HPLC,^{18–23} LC/MS/MS,^{24–31} and HILIC/MS^{32,33} methods.

Recently, scientists have become concerned with applying the principles of green chemistry to different analytical methods in order to reduce hazards to human health and environment. Different metrics are now used for the assessment of the environmental impact of different analytical methods, such as the Analytical Eco-Scale,³⁴ Analytical Method Volume

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Intensity (AMVI),³⁵ and the National Environmental Method Index (NEMI).³⁶

On the other hand, the co-administration of herbal medicines with conventional drugs may alter their pharmacokinetic or pharmacodynamic properties. Therefore, studying the herb-drug interactions has become a necessity.

Trigonella foenum-graecum Linn is an annual plant belonging to the family Fabaceae; it is commonly called fenugreek or methi, and it is used as a condiment in Mediterranean countries and the Indian sub-continent. *Trigonella* seeds have recently been used as a medicinal herb and as a flavoring agent to imitate the taste of maple.³⁷ Moreover, *Trigonella* is known for its hypocholesterolemic, antibacterial, anticancer, hepatoprotective, lactation-aiding, and antidiabetic effects.³⁸

The hypoglycemic effect of fenugreek seeds has been studied in many animal model systems³⁹ as well as in humans, both in insulin-dependent diabetes mellitus (IDDM) patients^{40,41} and in non-insulin-dependent diabetes mellitus (NIDDM) patients.⁴² The therapeutic role of *Trigonella* seed powder in type-1 diabetes can be attributed to changes in glucose and lipid metabolizing enzyme activities to normal values.⁴³ The various antidiabetic properties of *Trigonella* seed powder and extract and their active principles may be attributed to different constituents, such as steroid saponins, the fiber content in the seeds,^{44–46} furostanol saponins called trigoneosides, glycoside D, trigofenoside A,⁴⁷ and steroidal sapogenins such as diosgenin and yamogenin.⁴⁸ Recently, it was reported that 4-hydroxyisoleucine,⁴⁹ a novel amino acid in fenugreek extract, facilitates insulin secretion. It was also published that fenugreek seed fibers slow the rate of postprandial glucose absorption. Fenugreek is known for its emulsifying effect due to its high fiber, protein and gum content.⁵⁰

This work aims to study how the concomitant use of fenugreek extract affects the pharmacokinetics of the oral hypoglycemic MET using rats as the experimental animal model. This pharmacokinetic study was carried out through the development of a green RP-HPLC method after simple sample pretreatment steps. Additionally, the method was validated according to FDA⁵¹ requirements. The most noteworthy feature of this study is that fenugreek extract can be a promising complimentary herbal medicine to the traditional hypoglycemic MET for satisfactory diabetes control; hence, this combination may reduce MET requirements and thus prevent long-term complications. The work in this manuscript was then extended to estimate the greenness of the developed chromatographic method using the previously mentioned tools. All the obtained results confirmed that the method fulfilled the necessary requirements to be an ecological method. The proposed method can be used as an economic alternative tool to previously published LC/MS methods because the required instruments and chemicals are less expensive than those required for the LC/MS method; additionally, no tedious sample purification steps are required for the suggested RP-HPLC method compared to those used in published LC/MS methods. On the other hand, this method possesses the

sensitivity and selectivity required to quantify the studied drug in real plasma samples.

2. Experimental

2.1. Instruments

– For plant extraction

• *For powdering of the plant seeds.* Jet mill machinery, Mill Jet, Mill Classifier (ALPA) (1–1000 V, ISO9001, CE, Shandong, China).

• *For extract evaporation.* A rotary evaporator (Buchi Rotavapor R-300, Cole-Parmer, Vernon Hills, IL, USA).

– For preparation of plasma samples

• *For taking different volumes from the separated plasma samples.* Rongtai variable volume micropipettes (0.1–100 μ L and 100–1000 μ L) (Mainland, Shanghai, China).

• *For mixing the taken plasma samples.* A 250 VM vortex mixer (Hwashin, Seoul, Korea).

• *For centrifugation and removal of the precipitated plasma protein.* A 80-2C low-speed electric centrifuge (Zjzzym, China), 4000 rpm and 12 tubes \times 20 mL.

– For the HPLC method

• *For chromatographic separation.* A Dionex Ultimate 3000 UHPLC equipped with an auto-sampler, quaternary solvent delivery pump and diode array detector (Germany). The software used was Chromeleon software. The stationary phase used was a ZORBAX Eclipse Plus® C18 column with dimensions of 250 \times 4.6 mm, 5 μ m (California, USA). On the other hand, the used mobile phase was a mixture of 0.5 mM KH_2PO_4 solution : methanol (65 : 35, v/v) which was filtered through a 0.45 μ m millipore membrane filter.

• *For sample weighting.* A digital balance (Sartorius, Germany).

2.2. Chromatographic conditions

Separation was performed on a stationary phase of a 250 \times 4.6 mm, 5 μ m ZORBAX Eclipse Plus® C18 column using a mobile phase consisting of 0.5 mM KH_2PO_4 solution : methanol (65 : 35, v/v), maintaining a constant flow rate at 1.7 mL min⁻¹. The UV detector was set at 235 nm. The column temperature was adjusted to 25 °C, and guaiphenesin was the selected internal standard. The injection volume was 30 μ L and the run time was 7.5 min.

2.3. Materials and reagents

– **Metformin pure sample.** It was certified to have a purity of 100.13 \pm 0.89 and was kindly provided by Chemical Industries Development (CID) Co. (Giza, Egypt).

– **Guaiphenesin pure sample.** A sample with purity of 99% was provided by NOVARTIS PHARMA S.A. E Cairo, Egypt.

– **Methanol.** (Fisher, Loughborough, UK), HPLC grade.

– **KH_2PO_4 and ethanol.** El Nasr Pharmaceutical Chemicals Co., Abu Zabaal, Cairo, Egypt.

– **Deionized water.** (SEDICO Pharmaceuticals Co., 6th October City, Egypt).



2.4. Solutions

– **Stock solutions of metformin and guaiphenesin (IS).** Separate pure samples of metformin and guaiphenesin (10 mg each) were dissolved in accurate 10 mL methanol to obtain their respective stock solutions of 1 mg mL⁻¹.

– **Working solutions of metformin and guaiphenesin (IS).** The solutions were prepared by appropriate dilutions of their previously prepared stock solutions with the mobile phase mixture to obtain 10 mL working solutions of 0.1 mg mL⁻¹.

The prepared solutions were then stored at -20 °C until the time of analysis.

2.5. Experimental animals

Mature female Wistar Albino rats (18 rats) with weights ranging from 200 to 250 g were used during this work. These rats were acquired from “Nahda University in Beni-Suef (NUB) Animal House”. For adaption and in order to scrutinize the variations in the pharmacokinetic parameters of the treated groups in comparison to the control group, the rats were set aside in the animal room for 14 days before being used in this work. Guidelines stated by “Nahda University Animal House” and approved by the Pharmacology and Toxicology Department, Faculty of Pharmacy, Nahda University in Beni-Suef (NUB) were followed for handling the experimental animals.

2.6. Extraction of plant material

The plant seeds (1 kg) were obtained from a commercial market (Harrasz store, Beni-Suef, Egypt). Dr Ahmed M. Sayed, Pharmacognosy Department, Faculty of Pharmacy Nahda University in Beni-Suef (NUB), examined and identified the purchased seeds; after that, they were finely powdered by small laboratory jet mill machinery. Extraction was then carried out by maceration without agitation using 70% ethanol (1–1.5 L, 3×, seven days each) at room temperature; then, the extract was concentrated under vacuum at 45 °C using a rotary evaporator to obtain 60 g crude extract. The crude extract was maintained at 4 °C until use for biological investigation.

2.7. Preparation of calibration standards and quality control samples (QC) and construction of the calibration curve

Into a series of test tubes, 0.5 mL of rat blank plasma was spiked with different volumes (2.5–150 µL) of metformin, transferred from its working solution (100 µg mL⁻¹). To each sample, 250 µL of guaiphenesin (internal standard (IS)) was added using its previously prepared working solution (100 µg mL⁻¹), and the volume was then adjusted to 2 mL with methanol. The prepared samples were mixed for one minute using a vortex mixer, and the precipitated plasma protein was removed by centrifugation at for 4000 rpm for 10 min. The net supernatant liquid of each sample was totally transmitted to a new test tube and then evaporated to dryness. The deposit of every sample was then reconstituted in 0.5 mL mobile phase mixture to prepare metformin samples in the concentration range of 0.5–30 µg mL⁻¹ that contained 50 µg mL⁻¹ IS. From each sample, 30 µL was introduced into the HPLC system (three times for each); then,

the chromatographic conditions were followed. The peak area ratio (peak area of metformin/peak area of IS) was recorded for each sample; then, the calibration curve was plotted, from which the regression equation was calculated.

Additionally, three quality control samples were prepared (2, 15, and 28 µg mL⁻¹) by the method adopted for calibration standards and were then used for validation of the proposed method by following the chromatographic conditions mentioned previously.

2.8. Drug administration and collection of plasma samples

Full-grown female Wistar Albino rats (18 rats, 200–250 g) were randomly divided into three groups ($n = 6$). Group (I) was the blank group, which received vehicle only, group (II) received an oral dose of 300 mg kg⁻¹ metformin which was dissolved in saline,⁵² and group (III) received a combined dose of 300 mg kg⁻¹ metformin and 500 mg kg⁻¹ fenugreek extract which was suspended in saline.⁵³ 0.5 mL blood samples were withdrawn from the retro-orbital plexus of each rat into separate heparinized tubes at different time intervals (0.25, 0.5, 1, 2, 4, 7, and 24 h) after administration. The blood was immediately centrifuged at 3000 rpm for 10 min for separation of blood plasma, which was then collected into another clean tube and then maintained at -20 °C until analysis.

2.9. Preparation of the collected plasma samples

The stored frozen plasma samples were thawed to room temperature, and 0.5 mL of each sample was accurately transferred to a clean centrifuge tube. 250 µL of IS working solution (0.1 mg mL⁻¹) was accurately added, and then the volume was set to 2 mL with methanol. The samples were mixed for one minute; after that, centrifugation was carried out for 10 min. The pure supernatant of each prepared sample was accurately transferred to a clean test tube and then evaporated to dryness. The residue was reconstituted with 0.5 mL mobile phase mixture. The instructions of the developed method were then followed, and the concentration of the administered drug in each sample was calculated by substitution in the previously computed regression equation.

2.10. Pharmacokinetic study

The previously calculated concentrations of metformin in the collected plasma samples at different time intervals from group II and III were used to plot the mean plasma concentration–time curves for metformin (300 mg kg⁻¹) and metformin + fenugreek extract (300 mg kg⁻¹ + 500 mg kg⁻¹). Then, non-compartmental pharmacokinetic analysis was performed using PKSolver (a freely available menu-driven add-in program for Microsoft Excel)⁵⁴ to obtain different pharmacokinetic parameters.

3. Results and discussion

This article aims to provide clinical data regarding the effect of concurrent administration of fenugreek extract on the pharmacokinetics of the widely used drug metformin through



development of a RP-HPLC method using rats as an animal model.

3.1. Method optimization

During method optimization, different factors were studied in order to achieve complete separation between metformin and the plasma matrix within a short analysis time. The difficulty in the optimization of the developed method was increasing the retention time of metformin in order to separate it from the plasma peak because it is a highly polar component and thus is poorly retained in the stationary phase. Different columns were tried, including C18 XBridge® (Massachusetts, USA) with dimensions of 25 cm × 4.6 mm, 5 μm particle size, C18 ZORBAX Eclipse Plus® (250 × 4.6 mm, 5 μm, California, USA), C8 XBridge® (Massachusetts, USA) with dimensions of 25 cm × 4.6 mm, 5 μm particle size, and C8 ZORBAX Eclipse Plus® (250 × 4.6 mm, 5 μm, California, USA) columns, in a trial to improve the retention of metformin; no significant difference was observed between the separation efficiencies of these stationary phases. Optimization was then continued using the C18 ZORBAX Eclipse Plus® (250 × 4.6 mm, 5 μm) column. Different mobile phase mixtures were then tested, starting with the previously reported mobile phase solvent mixture of acetonitrile : 10 mM KH₂PO₄ solution with different ratios (from 60 : 40 to 40 : 60, v/v) adjusting the pH of the aqueous phase at different pH values (from 3.8 to 7), and pumping the mobile phase mixture at different flow rates (from 0.5 to 2 mL min⁻¹). In all the trials, the plasma matrix interfered with the early eluted metformin peak. All these trials were then repeated using methanol as an organic modifier that is greener than acetonitrile; unfortunately, unresolved plasma matrix and metformin peaks resulted. The 10 mM KH₂PO₄ solution was then replaced with water. It was noted that the retention time of metformin was significantly increased when using water as an aqueous phase, while very low retention was observed when using 10 mM KH₂PO₄ solution. After that, lower concentrations of KH₂PO₄ solution were tried (5 mM, 1 mM, and 0.5 mM) with different ratios of methanol. Complete separation between the plasma matrix and metformin was observed on using a mobile phase mixture of 0.5 mM KH₂PO₄ solution : methanol (65 : 35, v/v). Additionally, the mobile phase was pumped at different rates (from 0.5 to 2 mL min⁻¹), and a flow rate of 1.7 mL min⁻¹ was selected as the rate at which complete chromatographic separation within a suitable analysis time was obtained. Scanning was carried out at different wavelengths (210, 225, 235, and 254 nm); the optimum sensitivity with minimum noise was attained on scanning at 235 nm.

Using an internal standard during development of a bio-analytical method is very useful to enhance the accuracy, precision, and robustness of the developed method. Additionally, it is very important to compensate for the variability during the preparation and analysis of samples as well as to correct for the loss in the analyte of interest during sample preparation. Selection of a suitable internal standard is a challenge because it should have the same chromatographic behavior as the analyte and at the same time be completely resolved from both the

analyte and the plasma peaks. Additionally, it should have similar chemical and physical character to the analyte of interest to be extracted by the same method. Different internal standards were tested, such as atenolol, metoclopramide, olanzapine, ibuprofen, and guaiphenesin. Guaiphenesin was the most suitable internal standard based on its chromatographic behavior relative to the separated peaks of both the plasma matrix and metformin. Finally, complete separation of the plasma matrix, metformin, and guaiphenesin was achieved within 7.5 min, with retention time values of 2–4, 4.9, and 6.3 min, respectively (Fig. 1).

The method of extraction of the parent drug from the collected plasma samples is very critical because it affects the sensitivity and selectivity of the method. The plasma protein precipitation method was chosen because it is a simple and inexpensive method. Different organic solvents were tested (acetonitrile, ethanol, and methanol). The highest extraction recovery for both the drug and the internal standard as well as the maximum protein precipitation resulted when using methanol.

3.2. Method validation

In order to test the validity of the developed bio-analytical method, guidelines recommended by the US Food and Drug Administration (FDA)³¹ were followed. Three quality control samples (QCs) were prepared and used during testing of the validity of the method (low QC (LQC), middle QC (MQC), and high QC (HQC) samples).

Linearity of the calibration curve and lower limit of quantification. The calibration curve was prepared using the peak-area ratios *versus* the metformin concentrations in the range of 0.5–30 μg mL⁻¹; good correlation was acquired ($r = 0.9999$), and the computed regression equation was found to be:

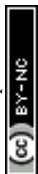
$$y = 0.1937x + 0.0890$$

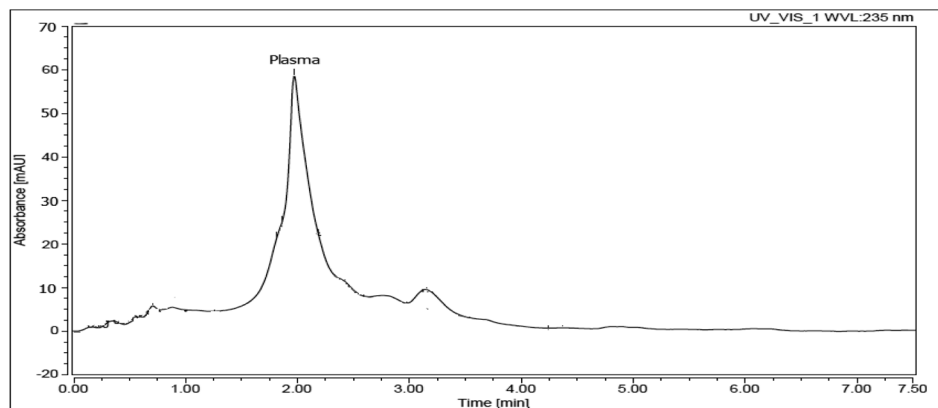
where y is the peak area ratio and x is the concentration of metformin in μg mL⁻¹.

The lower limit of quantitation was selected to be 0.5 μg mL⁻¹, and this limit was chosen on the basis that it was the analyte concentration that gave a reproducible response which was at least five times that of the blank.

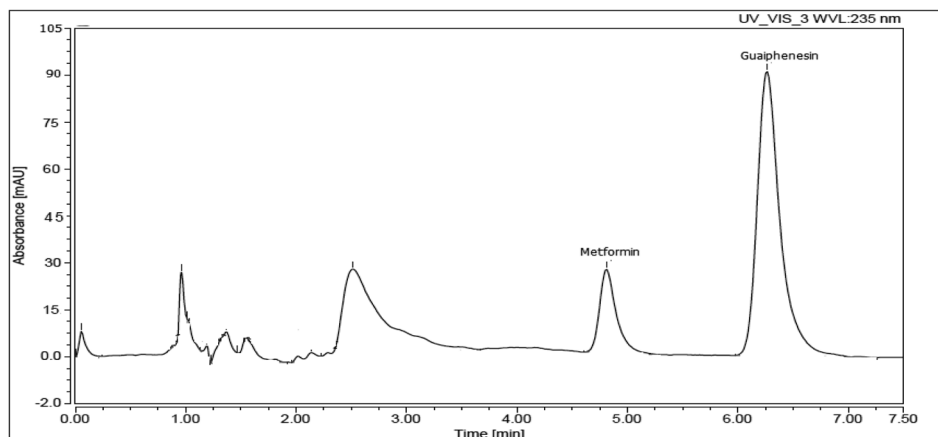
Accuracy and precision. The previously prepared QCs were analyzed ($n = 5$) by following the steps of the developed method. Accuracy was expressed as % recovery, and the acceptable limit was ±15% of the actual concentration. As shown from the results given in Table 1, the calculated % recovery ranged from 93.18% to 100.99%. Furthermore, the precision was tested and presented as % RSD (coefficient of variation (CV)); all the calculated % RSD values ranged from 0.08 to 4.72 (Table 1), which are within the acceptance criteria given by the FDA guidelines. All these results confirmed the accuracy and precision of the developed bio-analytical method.

Selectivity. The selectivity of the method was checked by comparing the recorded HPLC chromatograms of the blank plasma sample, plasma sample spiked with metformin and IS, and a real rat plasma sample taken from group 3 (given

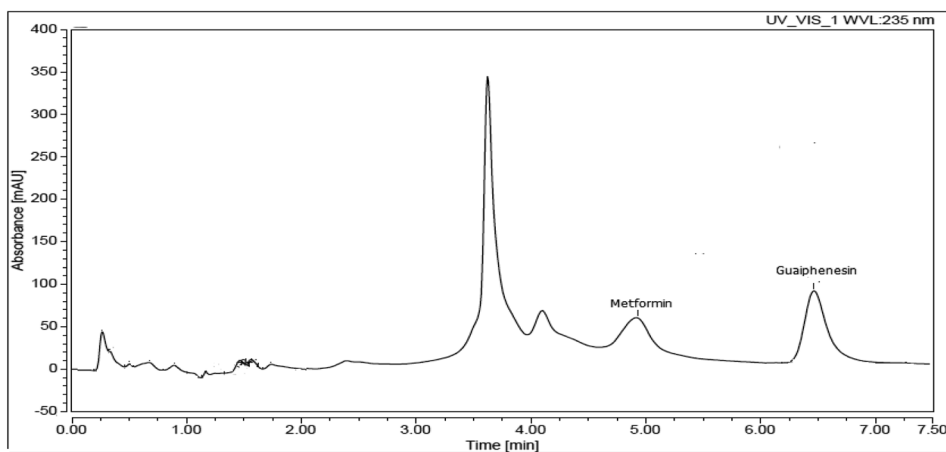




(A)



(B)



(C)

Fig. 1 HPLC chromatograms of (A) blank plasma, (B) plasma spiked with $15 \mu\text{g mL}^{-1}$ metformin and $50 \mu\text{g mL}^{-1}$ guaiphenesin, and (C) a rat plasma sample after 2 hours (from group III) spiked with $50 \mu\text{g mL}^{-1}$ guaiphenesin.

metformin + fenugreek extract) after two hours from the time of drug administration, spiked with the specified amount of the internal standard. As shown in Fig. 1, the endogenous

plasma matrix did not significantly interfere with the separated peaks, confirming the selectivity of this chromatographic method.



Table 1 Intra and inter-assay precision and accuracy of the proposed method

Concentration ^a ($\mu\text{g mL}^{-1}$)	Intraday			Interday		
	Recovery%	RSD%	Bias% ^b	Recovery%	RSD%	Bias% ^b
2.00 (LQC)	100.99	0.27	0.99	100.30	0.57	0.30
15.00 (MQC)	93.18	1.60	-6.82	94.44	4.72	-5.56
28.00 (HQC)	99.30	0.08	-0.70	99.30	0.15	-0.70

^a Average of 5 experiments. ^b Bias = [(measured concentration - nominal concentration)/nominal concentration] \times 100.

Table 2 Extraction recovery results of the studied drugs in spiked human plasma

	Concentration of the analyte ($\mu\text{g mL}^{-1}$)	% Recovery ^a
	2.00	87.56
	15.00	82.90
	28.00	92.22
Mean \pm % RSD		87.56 \pm 5.32
IS	50.00	90.51 \pm 3.82

^a Average of 5 determinations.

Extraction recovery. The recovery was tested in order to check the extraction efficiency of the method. It was evaluated by comparing the peak area obtained upon analyzing the spiked plasma sample (with a certain concentration of the parent drug and the calculated concentration of the internal standard) with those obtained upon analysis of a mixture containing the same concentration of pure drug and the specified amount of the internal standard in the solvent mixture. The results for the analyte and the internal standard should be reliable and precise. Referring to the results in Table 2, it was observed that the extraction recovery ranged from 82.90% to 92.22% and 90.51% for metformin and the internal standard, respectively with relative standard deviations of 5.32% and 3.82%, respectively, ensuring that the extraction efficiency of both the drug and the internal standard was not significantly affected by the endogenous plasma matrix.

Stability. The stability of the drug in biological fluids was tested by exposing QCs to different conditions, such as three

freeze-thaw cycles, benchtop stability under normal laboratory conditions, and autosampler stability (processed sample stability) for one day. The drug was considered stable in plasma if the stability results were within 15% of the existing concentrations. All the obtained results given in Table 3 were within the recommended true values, ensuring that the QCs were stable under all tested conditions.

3.3. Results of the pharmacokinetic study

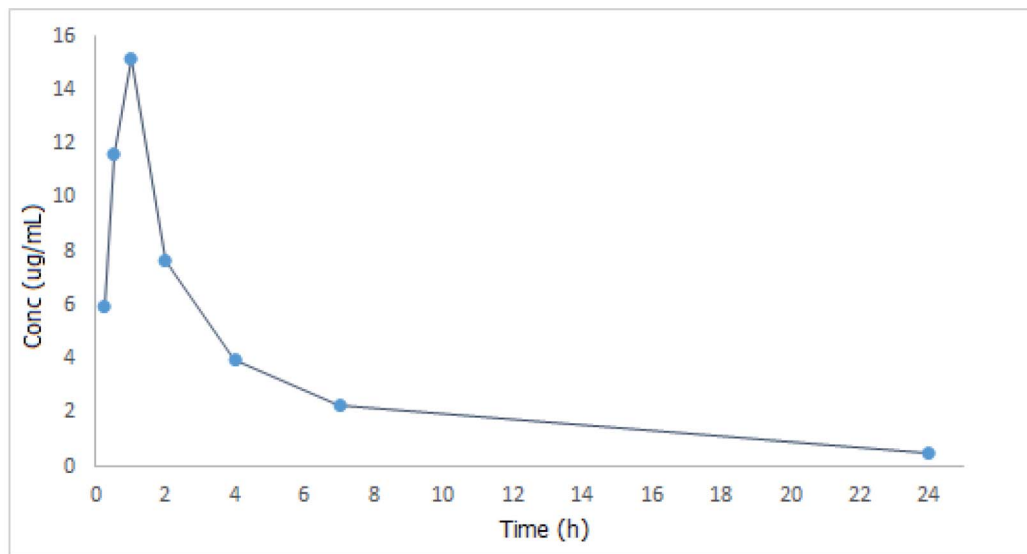
After drug administration either alone (group II) or with fenugreek extract (group III), the concentrations of the administered drug in the collected plasma samples were calculated using the previously computed regression equation; then, the mean plasma concentration-time plot was recorded (Fig. 2). Moreover, PKSolver was then used to perform the non-compartmental pharmacokinetic analysis. As shown in Fig. 2, metformin was rapidly absorbed after oral administration and appeared in plasma with an easily detectable concentration within 15 min. On the other hand, the calculated pharmacokinetic parameters given in Table 4 showed that the bioavailability of metformin was significantly increased by its concomitant administration with fenugreek extract, which was proven by the higher values of the maximum drug plasma concentration (C_{max}) and area under the curve (AUC). It was found that C_{max} of group III increased by 74.68% compared to that of group II, while its AUC_{0-t} increased by about 148.55%. As a result of the increasing metformin absorption, its apparent volume of distribution was significantly decreased on using fenugreek extract. These alternations in the metformin pharmacokinetics may be due to the emulsifying effects of the fiber, protein and gum contents of fenugreek, which may increase the

Table 3 Stability results of the studied drugs in spiked human plasma under different conditions

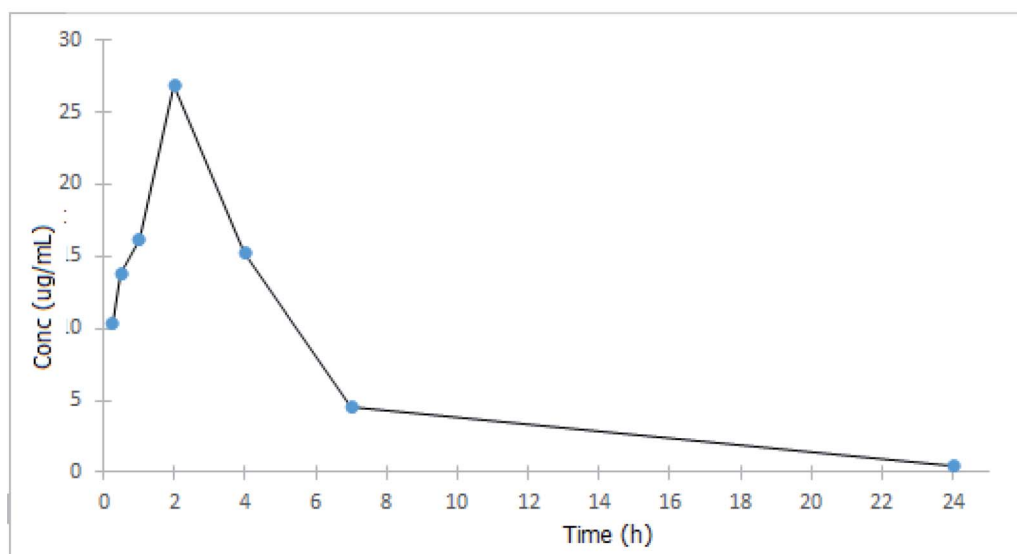
Concentration of the analyte ($\mu\text{g mL}^{-1}$)	% Recovery ^a		
	Three freeze-thaw cycles	Bench top stability	Auto-sampler, for 24 h
2.00	86.48	92.27	99.13
15.00	101.17	99.36	113.77
28.00	97.44	99.82	112.00
Mean \pm % RSD	95.03 \pm 8.04	97.15 \pm 4.35	108.30 \pm 7.38

^a Average of 5 determinations.





(A)



(B)

Fig. 2 Mean plasma concentration–time curves of metformin after oral administration of 300 mg kg⁻¹ metformin (A) and after oral administration of 300 mg kg⁻¹ metformin + 500 mg kg⁻¹ fenugreek extract.

Table 4 Pharmacokinetic parameters of the developed method

Parameter	Unit	Metformin (300 mg kg ⁻¹)	Metformin + fenugreek extract, 300 + 500 (mg kg ⁻¹)
$T_{1/2}$	h	2.95	1.94
T_{max}	h	1.00	2.00
C_{max}	μg mL ⁻¹	15.14	26.90
AUC_{0-t}	μg mL ⁻¹ h ⁻¹	42.82	106.43
AUC_{0-inf}	μg mL ⁻¹ h ⁻¹	52.60	119.32
Mean residence time (MRT)	h	4.01	3.54
Volume of distribution (V_d/F)	L	24.25	7.05
Clearance (Cl/F)	L h ⁻¹	5.70	2.51



Table 5 Penalty points for the determination of the analytical eco-scale score of the proposed method

Parameters	Developed HPLC method ^a	Penalty points
Reagents	PP of solvent = subtotal PP × number of pictogram × signal word	
	0.5 mM KH₂PO₄ solution Consumed volume = 8.29 mL Subtotal PP = 1 [solvent <10 mL] Signal word = 0 none No. of pictogram = 0	0
	Consumed volume = run time × flow rate × solvent percentage in system	
	Methanol Consumed volume = 4.46 mL Subtotal PP = 1 [solvent <10 mL] Signal word = 2 danger [more severe hazard = 2] No. of pictogram = 3	6
Instruments Energy	≤1.5 kW h per sample	1
Occupational hazard	Analytical process hermetization	0
Centrifuge		1
Wastes	>10 mL	5
Total penalty points	12	
Analytical eco-scale total score	87	

^a Mobile phase consisted of 0.5 mM KH₂PO₄ solution : methanol (65 : 35, v/v).

solubility of the drug, leading to a remarkable increase in its bioavailability. On the other hand, the time required to reach the peak plasma concentration of metformin (T_{max}) was significantly influenced by the concomitant administration of the extract (T_{max} of the metformin group = 1 h and of the metformin + extract group = 2 h); meanwhile, the half-life ($T_{1/2}$) and the clearance rate of metformin decreased by 33.90% and 70.93%, respectively, in the metformin and extract co-administered group compared to the metformin-administered group.

From the results of the pharmacokinetic study discussed above, one can conclude that fenugreek can be considered to be a promising complimentary option for diabetes control when concurrently used with the first-line hypoglycemic metformin. It will increase the extent of absorption of metformin, leading to greater hypoglycemic effects and, hence, better blood glucose control.

3.4. Greenness assessment of the developed method

Recently, the concept of green chemistry has grown massively worldwide; it involves the exclusion or the reduction of hazardous, corrosive, toxic, bio-accumulative solvents and the resulting wastes. Different tools are now used to evaluate the greenness of an analytical method. Method greenness assessment can be carried out using the Analytical Eco-Scale, Analytical Method Volume Intensity (AMVI), and National Environmental Method Index (NEMI).

The Analytical Eco-Scale depends on giving penalty points to any element that does not coincide with green analysis. The penalty points concerning different parameters in the developed analytical method (the used reagents and instruments) are calculated; then, the summation of these penalty points is subtracted from 100 to obtain the Eco-Scale score. A method is considered an ideal ecological method if it has an Eco-Scale score of 100, excellent if the score exceeds 75, acceptable if it

Table 6 AMVI calculation of the proposed method

Parameters	Developed HPLC method
Solvent consumption method (mL)	114.75
Flow rate	1.7
Run time	7.5
No. of injection for one full analysis	9
No. of potential analytes	1
Solvent consumption sample preparation (mL)	42.50
Standard prep. volume (mL)	10
No. of standard preps.	2
Sample prep. volume (mL)	2.5
No. of sample preps.	9
Total method solvent consumption	157.25
Analytical method volume intensity	157.25/1 = 157.25
% Consumption method	72.97%
% Consumption preparation	27.03%



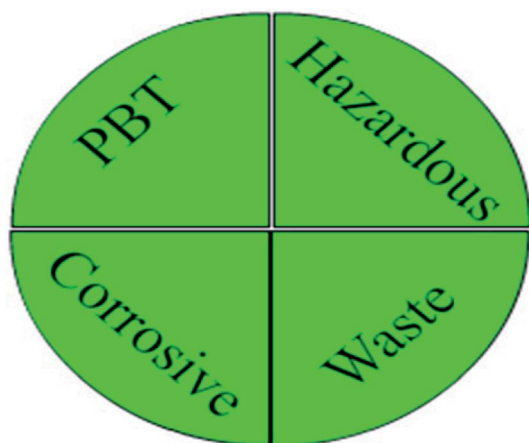


Fig. 3 The NEMI pictogram of the developed RP-HPLC method.

is more than 50, and inappropriate if it is less than 50. As shown from the results in Table 5, the Analytical Eco-Scale of the developed method was calculated and found to be 87, revealing that the method can be classified as an excellent ecofriendly method.

Likewise, AMVI is a method which is based upon measuring the total consumed volume of solvent and the waste created from the proposed method. The AMVI for the proposed method was calculated and was found to be 157.25 (Table 6), ensuring that this method has limited negative impact on both human health and the environment.

Moreover, on evaluating the greenness profile of the developed HPLC method using the National Environmental Method Index (NEMI), it was noted that the chemicals used (KH_2PO_4 and methanol) are considered to be non-PBT (persistent, bioaccumulative, and toxic) and non-hazardous. The pH of the mobile phase was >2 and <12 ; hence, it was not corrosive. Furthermore, the waste produced was calculated and was found to be 12.75 g per sample (<50). Because the developed method succeeded in achievement of the four quadrants of the greenness profile (Fig. 3), the method can be considered to be ecologically harmless.

4. Conclusion

For the first time, a novel ecological RP-HPLC method was developed and optimized for *in vivo* analysis of MET and to study the effect of concurrent administration of fenugreek extract on the pharmacokinetics of the studied drug. The high fiber, protein, and gum contents of fenugreek may act as emulsifying agents that increase metformin solubility, leading to a significant increase in its bioavailability. The combination of fenugreek extract and metformin can be considered as an auspicious treatment for satisfactory diabetes control, which may lead to reduced MET requirements and hence minimize the expected long-term complications of metformin use. Furthermore, the developed RP-HPLC method has the advantage of being a highly sensitive and green method with minimal hazardous environmental effects. Moreover, it requires a short

analysis time and simple protein precipitation method for sample pretreatment.

Ethical committee approval

Ethical committee approval was attained by the Committee of Ethics for Scientific Research on Living Organisms, Faculty of Pharmacy, Nahda University in Beni-Suef (NUB). The approval number was (NUB-019-020).

Author contributions

Nada S. Abdelwahab, Hossam M. Hassan and Asmaa M. AboulMagd contributed to the idea. Yasmine M. Ahmed carried out the *in vivo* study on the rats. Hossam M. Hassan aided the extraction and preparation of the plant. Nada S. Abdelwahab, Amani Morsi, Hossam M. Hassan, and Asmaa M. AboulMagd shared the practical analysis plan and performed the practical analysis. All the authors shared in the writing and revising the manuscript.

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Conflicts of interest

Authors declare that they have no conflict of interest.

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