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Full Length Research Paper

The effect of fenugreek on the bioavailability of glibenclamide in normal beagle dogs

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Fenugreek (*Trigonella foenum-graceum*) is an herbal medicine widely used in the traditional medicine to alleviate many diseases including diabetes. Many studies proved its efficacy in reducing blood sugar in diabetic patients. Concurrent administration of fenugreek and glibenclamide may affect bioavailability of glibenclamide. For this reason this study was designed to clarify effect of fenugreek ingestion on bioavailability of glibenclamide in beagle dogs. 4 beagle dogs were administered either glibenclamide alone or glibenclamide with fenugreek and bioavailability of glibenclamide in each of these groups was estimated utilizing HPLC-fluorescence detector method. The method was validated and the results were compared by paired t-test. The method of analysis was linear in the range from 5 to 400 ng/ml and possessed highly specificity and high intra and inter-day precision {1.80 to 9.16% and 5.82 to 10.40% respectively as C.V(%)}. Recovery of glibenclamide (relative to warfarin as IS) was 98.7 to 105.1%. Fenugreek ingestion increased bioavailability of glibenclamide significantly ($p < 0.05$) compared to control group. The exact mechanism of increased bioavailability of glibenclamide was not studied although literature review of fenugreek constituents points to possibility of increased absorption and/or displacement of glibenclamide from protein binding sites.

Key words: Fenugreek, glibenclamide, Beagle dogs, HPLC, bioavailability.

INTRODUCTION

Diabetes mellitus is a chronic disease characterized by deficiency in insulin release and/or insulin receptors insensitivity resulting in increased blood glucose levels and glucose intolerance. The main signs and symptoms of this disease are polyphagia, polydepsia and polyuria (McPhee et al., 2007). This disease affects 24 to 28% of the population in Saudi Arabia. Prognosis of the disease includes neuropathy, nephropathy, retinopathy, blood coagulability and increased infection chances and all are due to ineffective treatment or poor patient compliance (McPhee et al., 2007). Drug treatment – if effective- delays prognosis of the disease and improves patient's life quality but none is able to completely cure the disease. Oral hypoglycemic drugs are used extensively and successfully in the treatment of this disease, but despite the good pharmacological profile of these agents (sulfonylureas, biguanides, glitinides or thiazolidindiones), their uses are limited by decreased action in the long run (to decreased insulin production by the body or insulin-receptors down regulation) and side effects (Hardman et al., 2006). Therefore, patients may need to take insulin injection in different stages of disease progress. Insulin, though show great extent of efficacy, is not devoid of complications and is not as convenient as oral hypoglycemic. For this reason some patients tend to use an adjunctive herbal treatment either alone or concurrently with oral hypoglycemic agents to increase their efficacy. One of the most extensively used herbs as hypoglycemic is fenugreek.

Fenugreek seeds are the dried mature seeds of *Trigonella foenum-graceum* (Leguminosae). It is indigenous to Western Asia and Southern Europe, but is now cultivated in India, Pakistan, France, Argentina and North African countries. Its seeds and leaves are used not only as food but also as an ingredient in traditional medicine. In ancient times it was used as an aphrodisiac by the Egyptians and, together with honey, for the treatment of rickets, diabetes, dyspepsia, rheumatism,

anemia and constipation. It has also been described in early Greek and Latin pharmacopoeias for hyperglycemia and was used by Yemenite Jews for type 2 diabetes (Yeh et al., 2003). In India and China it is still widely used as a therapeutic agent for treatment of diabetes as well. In the United States, it has been used since the 19th century for postmenopausal vagina dryness and dysmenorrhea (Ulbricht and Basch, 2005). The main chemical constituents of fenugreek are fiber, tannic acid, fixed and volatile oils and a bitter extractive, steroidal saponins, flavonoids, polysaccharides, alkaloids, trigonelline, trigocoumarin, trigomethyl coumarin, mucilage, seven essential amino acids and vitamins A, C, B_1, B_2 and B_3 (Shang et al., 1998; Zia et al., 2001; Bin-Hafeez et al., 2003). It was found to possess hypoglycemic effect in mice, rats, dogs, rabbits and humans (Sharma et al., 1996). Aqueous extract of the seeds was found to have antiulcerogenic effect (Pandian et al., 2002). It was found to possess hypocholesterolaemic (Bin-Hafeez et al., 2003), anti-inflammatory and antinociceptive activities (Ahmadiani et al., 2001).

Several clinical studies conducted in people with and without diabetes have identified significant lipid-lowering activity (Sharma et al., 1996; Bordia et al., 1997; Gupta et al., 2001) and positive blood sugar regulation (Sharma et al., 1996; Gupta et al., 2001). Though fenugreek proved efficient in regulating blood sugar levels, its effect on the bioavailability of oral hypoglycemic-particularly those frequently prescribed such as glibenclamide - is not yet experimentally elucidated. For this purpose this study is designed to figure out the effect of fenugreek ingestion on glibenclamide bioavailability and consequently blood glucose modulation. Glibenclamide (glyburide) is chosen because it is a potent, second generation oral sulfonylurea antidiabetic agent widely used to lower blood glucose levels in patients with type II non-insulin-dependent diabetes mellitus. It acts mainly by stimulating endogenous insulin release from beta cells of the pancreas (Montvale, 2007). Glibenclamide is rapidly and completely absorbed from the gastrointestinal tract. As there is no significant first pass metabolism, 100% of the oral dose is bioavailable (Neugebauer et al., 1985).

Glibenclamide concentration-time curves in plasma exhibit biphasic elimination (Montvale, 2007) with a terminal elimination rate of 1.4 to 5 h (Marchetti et al., 1991).

MATERIALS AND METHODS

Chemicals and reagents

Fenugreek (*T. foenum-graecum* Rosc.), family Leguminosae, was purchased from a local market and authenticated by experts in the college of agriculture, KSU, Riyadh, SA. Glibenclamide and Warfarin (as internal standard, IS) authentic powders, were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Acetonitrile, dichloromethane and methanol, obtained from BDH Co., were of HPLC grade. All other chemicals and solvents used were of analytical grade. De-ionized Milli-Q water was prepared at our laboratories using a Milli-Q apparatus (Millipore, Bedford, MA, USA).

Stock solutions and standards

Standard solutions preparation was conducted at room temperature under subdued light. The solutions were protected from light with aluminum foil wrapping and stored at –70°C. An amount of 10.0 mg of glibenclamide powder was weighed accurately and dissolved completely in 100 ml of methanol to give a stock standard solution of 100 $\mu g ml^{-1}$. This solution was diluted 10-folds in methanol to give a working standard solution of 10 $\mu g ml^{-1}$. Warfarin (IS) stock standard solution was prepared by dissolving an accurately weighed 50 mg warfarin in methanol up to 100 ml to produce a concentration of 0.50 $mg ml^{-1}$ (500 $\mu g ml^{-1}$). This stock solution was diluted in methanol to give a working standard solution of 2.0 $\mu g ml^{-1}$. All solutions were stored at -70°C.

Preparation of calibration curve and quality control standards

For calibration standards, eight 10 ml volumetric flasks were labeled as: blank, 5, 15, 30, 50, 100, 200 and 400 ng ml^{-1}, respectively. For quality control standards, four 10-ml volumetric flasks were labeled as lower limit of quantitation (LLOQ, 5 ng ml^{-1}), low quality control (LQC, 15 ng ml^{-1}), medium quality control (MQC, 150 ng ml^{-1}) and high quality control (HQC, 320 ng ml^{-1}) standards, respectively. Into each flask 5 ml of controlled blank Beagle dog plasma were added. Then appropriate volumes of glibenclamide working standard solution (10 $\mu g ml^{-1}$) were pipetted into each flask. Volumes were then completed with controlled blank plasma up to the mark and flasks were shaken very well to give the aforementioned glibenclamide concentrations. Volumes of 1.0 ml of each of the above standard samples were transferred into a prelabeled polypropylene microcentrifuge eppendorf 1.5 ml tube (Eppendorf AG, Hamburg, Germany); all standard calibration curve and quality control samples were then stored at –70°C, pending analysis.

Procedure for sample preparation

Sample preparation and analysis were performed at room temperature under subdued light. A 50 µl aliquot of warfarin (IS, 2.0 $\mu g ml^{-1}$), was added to one ml plasma sample (standard, quality control or Beagle dog's) in 15 ml glass stopper red tube. Samples were vortex-mixed for 10 s and 8 ml of dichloromethane were added to the tube, vortexed for 30 s, then shaken on a rotary mixer at 30 rpm for 10 min, and then centrifuged at 4000 rpm for 10 min. 5 ml of clear supernatant of organic layer were transferred to glass centrifuge tube and evaporated to dryness under a stream of purified nitrogen gas at 40°C. The residue was reconstituted with 100 µl of mobile phase and 50 µl was injected onto the HPLC column.

Instrumentation and chromatographic condition

HPLC was performed using a Waters-Alliance liquid chromatography system (Waters Associates, USA) containing the following units: model 2695 separation module consisting of solvent delivery pump, an autosampler and a column oven, a Model 2475 Multi λ Fluorescence detector. The chromatographic system and peak data handling was managed by Empower software package version

4.0, and a Hewlett-Packard LaserJet 1200 series printer. The stationary phase, giving satisfying resolution and run time, was a Novapak C_{18} (3.9 x 150) mm, 5 µm particle size HPLC stainless steel analytical column, protected by a sentry guard column, Novapak RP C_{18} (3.9 x 20) mm, 5 µm particle size HPLC column. Polypropylene microcentrifuge Eppendorf 1.5 ml tubes (Eppendorf AG, Hamburg, Germany). Glass-stoppered 15 ml glass tubes. The mobile phase consisted of 65% ammonium dihydrogen phosphate buffer (0.05 M adjusted to pH 3.7 with orthophosphoric acid) and 35% acetonitrile (ACN). The solvents were filtered prior to use and degassed, under vacuum, using 0.22 and 0.45 µm membrane filters (Millipore, Milford, MA), respectively and subsequent sonication. A flow-rate of 1.2 ml min^{-1}, at ambient temperature, was used for separation of glibenclamide and internal standard with fluorescence detector operated at an excitation and emission wavelengths of 308 and 360 nm, respectively, at attenuation of 16 and gain x 100.

A column temperature of 30°C was used with an injection volume of 50 µl.

Standardization and calculation

The method linearity for glibenclamide determination in Beagle dog plasma was confirmed for a range of concentrations from 5 to 400 ng ml^{-1}, suitable for bioequivalence studies to determine the sample concentrations in the unknown dog samples. Calibration curves were determined by least square linear regression equation (y = bx + a), of the best-fit peak area ratios vs. concentration, where 'b' represents the slope, 'a' represents the intercept, 'y' represents the peak area of glibenclamide/peak area of IS and 'x' represents the concentration (ng ml^{-1}). Glibenclamide concentrations in plasma samples were determined by comparing peak areas obtained when analyzing these samples with the standard curve.

Treatment protocol

Four adult male Beagle dogs (25 to 30 kg body weight) recruited from animal care house (College of Pharmacy, King Saud University, Riyadh) were used for the experiment under the permission and approval of the ethical committee in the animal care house. Before the experiment, dogs were fasted for at least 12 h, but water will be given *ad libitum*. Blood samples (2 ml) were withdrawn as zero treatment reading before giving the treatment. The dogs were separated into two groups (each consisting of 4 animals). Group I animals were each orally administered a 5 mg glibenclamide tablet (as control group). The dogs of the other group were each orally administered concomitantly Fenugreek 5 + 5 mg glibenclamide. Freshly ground fenugreek was triturated with little amount of water and formed into small balls of 5 gm each and administered orally. Ingestion was assisted by administering 50 ml water, with close inspection to assure complete ingestion of the medications. Blood samples were then drawn (-0.5 h) before drug(s) administration and at 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0, 8.0, 12.0 and 16 h after drug(s) administration. Blood samples were then centrifuged at 2500 rpm for 5 min and the separated plasma was aspirated and transferred to eppendorf tubes and immediately stored in freezer at a nominal temperature of -70°C, pending analysis.

Assay method validation

All samples used for the validation tests were prepared by spiking interference-free pools of heparinized Beagle dog plasma with prepared standards to give the specified final concentrations.

Linearity

Seven non-zero concentration over the range of 5.0 to 400 ng ml^{-1} plasma standards were used. Standards were analyzed in eleven replicates. The slope, intercept and correlation coefficient values were determined by the method of least-squares linear regression analysis.

Precision

Replicate samples spiked at four quality control standard concentrations (15, 75, 150 and 350 ng/ml) were used to assess intraday and interday precisions of glibenclamide assay in plasma. Selection of concentrations for analysis was made to allow for definition of precision at low limit of quantitation (LLOQ), low (LQC), medium (MQC) and high (HQC) concentrations of the linear range. Precision is expressed as the percent coefficient of variation (%C.V.). The results of the intraday precision of glibenclamide were reported as mean of twelve replicates, whereas those of interday were the mean of twenty four replicates of the four different concentrations.

Recovery

Recoveries (relative and absolute) studies has been performed for glibenclamide and internal standard stock solutions, and in heparinized dog plasma spiked with LQC (15 ng ml^{-1}) and HQC (350 ng ml^{-1}). The relative analytical recovery was measured in the following way: the drug and internal standard were added to drug-free plasma (six replicated for each standard). The spiked plasma was then analyzed by the developed method. The relative recovery was calculated by comparing the concentrations obtained from the drug-supplemented plasma with actual added amounts. The absolute recoveries were obtained by comparing the peak height ratios of the processed standard samples to that of stock solutions prepared at concentrations which represented 100% recovery.

RESULTS

Method development results

Glibenclamide and warfarin were freely soluble in methanol and the mobile phase. Method development started with the modification published method (Niopas and Daftsios, 2002). The experiment was started with acetonitrile: buffer (1:1) and percentage of buffer was increased gradually by 5% till good separation was achieved. The pH was adjusted at 3.5 at the beginning and was increased to yield sharp peaks. The optimum pH was found to be 3.7. Flow rate was started with 1.5 ml/min and decreased according to the chromatogram picture till resolved and well separated peaks arose in suitable retention times. The perfect flow rate was found to be 1.2 ml/min.

Validation of results

Specificity

The specificity of the method was evidenced by the lack

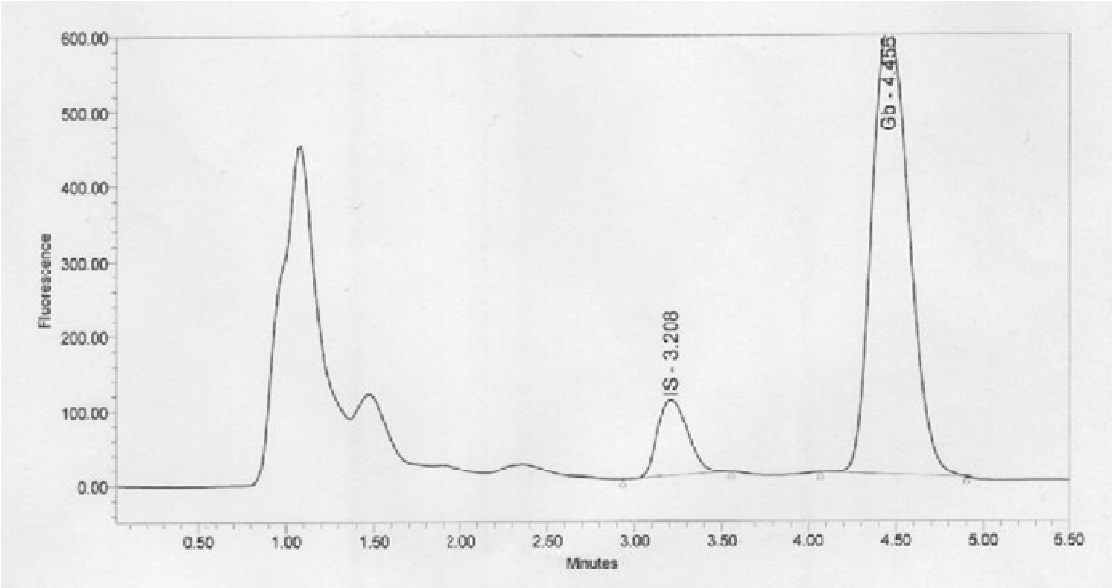

Figure 1. Chromatogram representing glibenclamide and internal standard peaks apart from plasma peak interferences.

Table 1. Linearity and precision of glibenclamide calibration curve assay in plasma (intercept, slope and correlation coefficient and r values).

No.	Intercept (a)	Slope (b)	r
1	0.0003	0.0106	0.9996
2	0.0069	0.0105	0.9999
3	0.0364	0.0103	0.9987
4	0.0388	0.0091	0.9993
5	0.0225	0.0092	0.9991
6	0.006	0.0087	0.9997
7	-0.0297	0.0082	0.9993
8	-0.039	0.0097	0.9988
9	-0.0311	0.0088	0.9981
10	0.0204	0.0092	0.9997
11	0.02	0.0096	0.9996
Mean	0.0047	0.0094	0.9993
S.D.	0.02588	0.00074	0.00052
C.V.(%)		9.474	

of interfering peaks, at the retention times of drug and IS, in the chromatograms of six different drug-free dog's plasma batches samples, as shown in Figure 1.

Linearity

The calibration standard curve of the method defines a linear range from 5 to 400 ng/ml, using the seven non-zero concentrations of glibenclamide in plasma. The linear regression of glibenclamide assay in plasma was characterized as having a slope of 0.0095±0.0009 and an intercept of (r^2 = 09993±0.0005) (Table 1). Calibration curves were evaluated individually by least squares linear regression equation.

Precision

The intraday precision of the back-calculated

Table 2. Intraday precision of glibenclamide assay in plasma.

Run	10 ng/ml	75 ng/ml	150 ng/ml	350 ng/ml
1	9.44	78.4	146.75	341.07
2	9.99	72.09	148.4	373.59
3	9.99	75.96	146.94	384.79
4	10.08	73.64	148.4	342.92
5	9.34	74.63	147.23	386.32
6	11.27	75.19	143.06	345.64
7	11.82	80.94	145.29	403.17
8	8.89	82.83	146.75	337.16
9	10.35	86.81	152.88	395.45
Mean	10.13	77.83	147.3	367.79
S.D.	0.87	4.56	2.50	24.59
%C.V.	9.16	6.21	1.8	7.09

Table 3. Effect of fenugreek ingestion on bioavailability of glibenclamide (mean amount in plasma in ng/ml, n = 4).

Time (h)	Glibenclamide alone	Glibenclamide+ fenugreek	Increase (%)
0	0	0	0
0.5	11.77	89.78	662.79
1	41.63	199.7	379.70
1.5	109.95	411.75	274.49
2	252.63	520.8	106.15
2.5	362.95	594.95	63.92
3	385.65	591.75	53.44
4	378.93	330.46	-12.79
6	171.58	203.92	18.85
8	131.02	159.86	22.01
10	153.68	124.1	-19.25
12	66.35	69.08	4.11
16	50.75	ND	NA

ND = Not detectable, NA = Not available.

concentrations ranged from 1.80 to 9.16% C.V (Table 2). Interday precision ranged from 5.82 to 10.40% C.V.

Recovery

Relative recovery of glibenclamide from plasma ranged from 98.7 to 105.1%. The absolute analytical recovery of glibenclamide ranged from 86.9 to 103.9%.

Effect of concomitant ingestion of fenugreek on bioavailability of glibenclamide

Table 3 represents concentrations (ng/ml) of glibenclamide alone and after oral administration of fenugreek to Beagle dogs. Administration of glibenclamide to dogs (n = 4) resulted in standard bioavailability curve. C_{max} of glibenclamide 2 h after oral administration of fenugreek was 694.96 ng/ml, that is 30 min earlier than control glibenclamide group. Complete elimination of glibenclamide was not affected by concomitant administration with fenugreek. However, extent of absorption was significantly ($p < 0.01$, n = 4) increased during the first hours of administration by 106 to 662% (n = 4) while the effect was only minimal after C_{max}.

DISCUSSION

Concomitant administration of glibenclamide with fenugreek increased bioavailability of glibenclamide by more than 100% during the first 5 h which constitute duration of action of glibenclamide in the body (Marchetti

et al., 1991). The exact mechanism of action was not elucidated in this study. However, an increase in plasma drug concentration might be due to either an increase in the extent of absorption, displacement from protein binding or decreased biotransformation (Gibaldi, 1984). Food alters absorption of glibenclamide. It may increase glibenclamide absorption by minimizing the dissolution problems of glibenclamide. Computation of the hydrophobicity parameter, octanol/water partition constant (log*K*o,w), by means of the fragment methodology (Melyan, 1995) revealed a relatively high value, that is log *K*o,w = 4.79, that is poorly water solubility. Consequently glibenclamide dissolution may be incomplete during the period of time available for absorption (Melyan, 1995). This is why –at least partly- patients are asked to take glibenclamide with food in addition to maximum sugar control after the meal. Fenugreek was found to enhance bioavailability of “iron” in rats (Ibrahim and Hegazy, 2009). This was interpreted as a result of the effect of amino acids in fenugreek. El-Guindi et al. (1998) reported that certain amino acids (especially cysteine, histidine and lysine) improve iron absorption (El-Guindi et al., 1988). These amino acids may play the same role with glibenclamide. The other possibility includes the inhibitory effect of tannic acid on gut motility which will help increasing absorption of glibenclamid. Relatively high value of hydrophobicity could explain the affinity to plasma matrix. About 99% is bound to plasma albumin as a weak acid anion and hence, it is susceptible to displacement by many weak acid compounds (Martindale extra pharmacopeia, 2002). Fenugreek contains tannic acid and flavonoids which are regarded as weak acids through their phenolic parts (Evans, 2000). Tannic acid can be absorbed and possess some pharmacological activities (Evans, 2000). Tannic acid and flavonoids – as weak acids- may compete with glibenclamide and displace it from albumin protein binding sites resulting in increased free plasma concentration.

Glibenclamide is hepatic enzyme inhibitor (Lacy et al., 2004). Although no strong evidence points to ability of fenugreek constituents to inhibit liver metabolizing enzymes, but the increased free plasma concentration- as a result of displacement of glibenclamide if present- will increase the inhibitory effect on liver metabolizing enzyme -Cytochrome P450- leading to accumulation of the drug in plasma. Elimination occurs via hepatic and renal routes resulting in a half-time of 1.5 to 5 h. Glibenclamide is eliminated via urine (50%) and feces (50%) (Lacy et al., 2004). Fenugreek was reported to be cholagogue (Izzo et al., 2005). This minimizes the chance for retarded elimination as a cause for increased bioavailability of glibenclamide when concomitantly administered with fenugreek. However, there are no definite studies elaborating on effect of fenugreek on kidney function which plays a role in excreting 50% of the glibenclamide plasma amount.

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