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Research Article

Gliclazide Microcrystals Prepared by Two Methods of *In Situ* Micronization: Pharmacokinetic Studies in Diabetic and Normal Rats

Roya Talari,[1,3] Jaleh Varshosaz,[1,5] Saied Abolfazl Mostafavi,[1] and Ali Nokhodchi[2,4]

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Abstract. The low water-solubility of gliclazide (GL) leads to a low dissolution rate and variable bioavailability. The aim of this study was to investigate the effect of micronization on the absorption and pharmacokinetics of GL after oral administration in rats. GL microcrystals were prepared using solvent-change and pH-shift methods. Scanning electron microscopy showed considerable changes in the shape and size of crystals using both methods. In the optimized formulation of each method, the particle size of treated GL was reduced about 30 (from 290 to 9.9 μm) and 61 times (to 4.76 μm) by solvent-change and pH-shift methods, respectively. Recrystallized samples showed faster dissolution rate than untreated GL particles. Glucose-lowering effect, C_{max}, and area under the drug concentration-time profile (area under the curve (AUC)) were compared in diabetic and normal rats. AUC and C_{max} were increased by microcrystals in both groups of animals. Administration of 40 mg/kg of GL in the form of untreated drug and microcrystals obtained by solvent-change and pH-shift methods caused 12.49% and 21.04% enhancement in glucose-lowering effect of GL in diabetic rats, respectively.

KEY WORDS: gliclazide; *in situ* micronization; microcrystals; pharmacokinetics.

INTRODUCTION

Low solubility of drugs in water results in poor bioavailability because the solubility of a drug is an important factor in determining its absorption rate (1). According to the biopharmaceutics classification system (2), for class II drugs, the dissolution rate is the limiting factor for the drug absorption rate. An improvement in the dissolution rate of these drugs can increase the blood levels to a clinically suitable level. One way to enhance the dissolution rate is to reduce particle size, which increases the total surface area (3). Several methods of reducing particle size have been suggested. Physical methods such as milling and grinding (4) are successful in particle size reduction; however, the particle size uniformity is not achieved. A common particle size reduction method for hydrophobic drugs is microcrystallization (5). The process is usually used to obtain small particles of the disruption of large crystals. Chaumeil describes the improvement in dissolution rate and in bioavailability by micronization of sparingly water-soluble drugs using jar mills and fluid energy mills (6). *In situ* micronization and microcrystallization are suitable methods for the production of micron-sized drugs (5–7). Compared with milled products, drug properties are optimized, the particle size is more uniformly distributed, and the powder is less cohesive (5–13). Gliclazide (GL), 3-(7-azabicyclo [3.3.0] oct-7-yl)-1-(4-methylphenyl)sulfonylurea is a second-generation sulfonylurea, widely used for the treatment of non-insulin-dependent diabetes mellitus. It has a low solubility in water (55 mg/L) and gastric fluids, a common characteristic of this group (14) which determines a low dissolution rate and hence inter-individual variability on its bioavailability (15,16). To enhance the dissolution rate of this drug complexation of GL with beta-cyclodextrin in the presence of hydroxypropylmethylcellulose (HPMC) has enhanced its dissolution 2.5-fold (17,18). Partially methylated beta-cyclodextrin has been also used for this purpose, and the results indicate that it could be useful for the solid GL formulations as it may result in a more rapid and uniform release of the drug. Experimental evidence of the complexation between drug and methylated cyclodextrin was reported for the co-ground and spray-dried systems (19). As discussed by Rasenak and Müller (7), *in situ* micronization by the presence of a stabilizing polymer covers the hydrophobic surfaces of the precipitated substances, and consequently, the steric hindrance caused by the polymer prevents the crystal growth. In this study, the oral absorption and pharmacokinetic parameters of microcrystals prepared by *in situ* micronization techniques based on solvent and pH-shift method were compared with each other and the untreated GL.

[1] Department of Pharmaceutics, School of Pharmacy and Pharmaceutical Sciences, and Isfahan Pharmaceutical Sciences Research Center, Isfahan University of Medical Sciences, Isfahan, Iran.
[2] Medway School of Pharmacy, The Universities of Kent and Greenwich, Central Ave., Chathman Maritime, Kent, ME4 4TB, UK.
[3] Food and Drug Laboratories Research Center, Tehran, Iran.
[4] Drug Applied Research Center and Faculty of Pharmacy, Tabriz Medical Sciences University, Tabriz, Iran.
[5] To whom correspondence should be addressed. (e-mail: Varshosaz@pharm.mui.ac.ir)

MATERIALS AND METHODS

Materials

Gliclazide and ibuprofen (used as internal standard) (IS) were purchased from CFM Co. (Italy) and Sekhasari Co. (India), respectively. HPMC from (Aldrich, UK) and Brij35 was obtained from Fluka (Germany). High-performance liquid chromatography (HPLC) grade of acetonitrile and methanol (Merck, Darmstadt, Germany) and double-distilled water were used throughout the analysis. All other chemicals and reagents were of analytical grade. Male Wistar rats were purchased from the animal house of Isfahan University of Medical Sciences. All the animals were cared for according to the rules and regulations of the Institutional Animal Ethics Committee (IAEC) guidelines of the Health Ministry, Iran.

Micronization

In preliminary studies, two different stabilizing agents (HPMC and Brij 35) with different concentrations of GL (0.5–1%) and stabilizers (0.1–0.05%) were studied by a full-factorial design to give the most stable microcrystals with the least particle size (20). Details of the preparation methods of microcrystals are reported previously (20,21). Briefly, in solvent-change method, 0.5 g of GL was dissolved in 30 ml of acetone. One hundred milliliters of water (as the non-solvent of GL) containing 0.05% of Brij35 (as stabilizer) was poured rapidly into the drug solution under stirring at 26,000 rpm by an ultra-homogenizer at ice bath temperature. A micron-sized dispersion was formed spontaneously. The mixture was allowed to be mixed for further 15 min, and then it was transferred to the vacuum oven at 40°C while stirring for 3 h. The aqueous suspension was freeze dried (20). In pH-shift method, 0.3% of GL was dissolved in an aqueous solution with pH 11 that contained 0.05% of Brij35, a stabilizing agent. The pH of the solution reached 5 by the addition of hydrochloric acid during 5 min in ice bath while stirring in 26,000 rpm (21).

Dissolution Studies

Dissolution rates of prepared microcrystals were studied by paddle method at 37°C, in 250 ml HCl 0.1 N containing 0.25% Tween 20 that was stirred at 100 rpm. Five milliliters of dissolution medium was withdrawn from the dissolution vessels at selected time intervals and analyzed for GL content at 230 nm spectrophotometerically (Shimadzu, Japan).

HPLC Method

A simple and rapid method was used to analyze GL in plasma by a high-performance liquid chromatographic method. The HPLC method was the modification of the previously published method by Rouini *et al.* (22). In the modified method reported previously by the authors (Varshosaz *et al.*, under consideration for publication in Scientia Pharm), only 100 µl of plasma is required. Briefly, GL and ibuprofen (as internal standard) were extracted from a mixture of 100 µl of plasma, 100 µl of 0.07 *M* phosphate buffer (pH 4.5), and 100 µl of the internal standard working solution using 3 ml of toluene following by centrifugation for 15 min at 10,000 rpm (8,500×*g*) (Universal Hettich D7200 Tuttlingen, Germany). A 2,500-µl aliquot of the upper organic layer containing GL and IS was transferred to a clean glass tube and evaporated under air stream to dryness at 50°C. The residue was redissolved in 100 µl of mobile phase, and a 50-µl aliquot was injected into the HPLC column. The GL peak was separated from endogenous peaks on a C_{18} column by a mobile phase of acetonitrile/phosphate buffer pH 3.3 (65:35, *v*/*v*) and the flow rate was 1 ml/min. Waters 2487 Dual UV absorbance detector was used to detect GL at 230 nm.

Animal Studies

Male Wistar rats weighing 180–220 g housed in a light–dark cycle and temperature-controlled environment were used for *in vivo* studies. Food was withheld during the experiment and the night before. Water was accessed *ad libitum*.

Induction of Diabetes

Diabetes was induced in rats by an intraperitoneal injection of streptozotocin solution (16 mg/ml) (Zanosar, 45 mg/kg) in citrate buffer pH 4.5. The animals were diabetic 72 h after injection. Rats were considered diabetic and included in the study when fasted glycemia (by Glucose-SL Kit, Zeistchem, Iran) was higher than 300 mg/dl (23).

Dosing and Sampling

The study was performed on day 3 following streptozotocin injection after an overnight fasting. Forty-two diabetic and 30 normal rats were divided into seven and five groups, respectively (n=6).

Different formulations were intragastrically administered to each group (n=6) as suspended in normal saline by a feeding tube. Normal saline was preferred to any other vehicle because it did not make any change on blood glucose level.

1. Normal saline to diabetic and normal control group of rats.
2. Untreated GL (40 mg/kg) to diabetic and normal rats.
3. Twenty, 30, and 40 mg/kg of microcrystals of GL ($D_{0.3}B_{0.05}$) in pH-shift method to diabetic rats. Dose–response study of the prepared crystals was performed on only diabetic rats.
4. Microcrystals $D_{0.5}$ $B_{0.05}$ (40 mg/kg) in solvent-change method to diabetic and normal rats.
5. Insulin (2 IU/kg) to diabetic and normal rats.

At specified time intervals (15, 30, 45, 60, 90, 120, 240, 480, 720, and 1,440 min) 300-µl blood samples were taken by retro-orbital blood sampling (24) and collected in 0.5-ml micro-centrifuge tubes. About 180 µl plasma was obtained. One hundred microliters was used for GL concentration determination and 10 µl for glucose estimation. The samples were immediately centrifuged in 8,000 rpm for 15 min, and the plasma was collected, and glucose concentration was determined. The remaining plasma was frozen at −20°C for further analysis of GL.

Pharmacokinetic Parameters

Absorption rate constant (k_a) and elimination rate constant (k_e) were calculated using the Wagner–Nelson method (25) and the slope of elimination part of logarithmic concentration-time curve after oral absorption, respectively. The area under the curve (AUC) of plasma GL concentration-time profile was calculated by linear trapezoidal method.

Data Analysis

In order to compare the results, ANOVA and Student's *t* test (SPSS program; version 12.0) were used.

RESULTS

Micronization

As can be seen from Table I, size reduction by the two methods of micronization are significant compared with the untreated powder of GL. The microcrystals of $D_{0.3}B_{0.05}$ obtained from pH-shift method are also significantly smaller than those prepared by the solvent-change method. Figure 1 also compares the differences in the morphology of the microcrystals compared with the untreated GL.

Dissolution Studies

Different dissolution profiles of microcrystals are compared in Fig. 2. As this figure indicates the smaller particle size of microcrystals has caused higher dissolution rate compared with untreated drug or physical mixtures of GL and the stabilizers.

HPLC Method

Gliclazide and IB that were used as internal standard were eluted at 2.8 and 4.1 min, respectively (Fig. 3). As reported elsewhere (Varshosaz *et al*., under consideration for publication in Scientia Pharm), the limit of quantification of microcrystals and untreated GL (LOQ; signal to noise ratio of 5:1, CV%< 10%) and the limit of detection (signal to noise ratio of 3:1) in plasma were 0.12 and 0.06 μg/ml, respectively. The recovery was obtained by comparing area under the peak of known plasma samples spiked with GL and IB to those of their respective aqueous solutions, correcting for volume. Mean recovery percent for GL and internal standard were 80% and 82%, respectively. The intra- and interday variations were obtained by repeating the standard curves three times each day and in three different days, respectively. The method was linear over the range of 0.12–200 μg/ml with r^2=0.999 (Varshosaz *et al*., under consideration for publication in Scientia Pharm). Estimation of GL plasma concentration is indicated in Fig. 4.

Table I. Particle Size of Gliclazide Microcrystals Prepared by Solvent-Change and pH-Shift Methods, and Untreated Drug

Formulation	Particle size (μm)	Span
Untreated drug	290.00±36.05	2.10±0.20
$D_{0.5}B_{0.05}$	9.90±0.26	1.24±0.02
$D_{0.3}B_{0.05}$	4.76±0.15	1.52±0.02

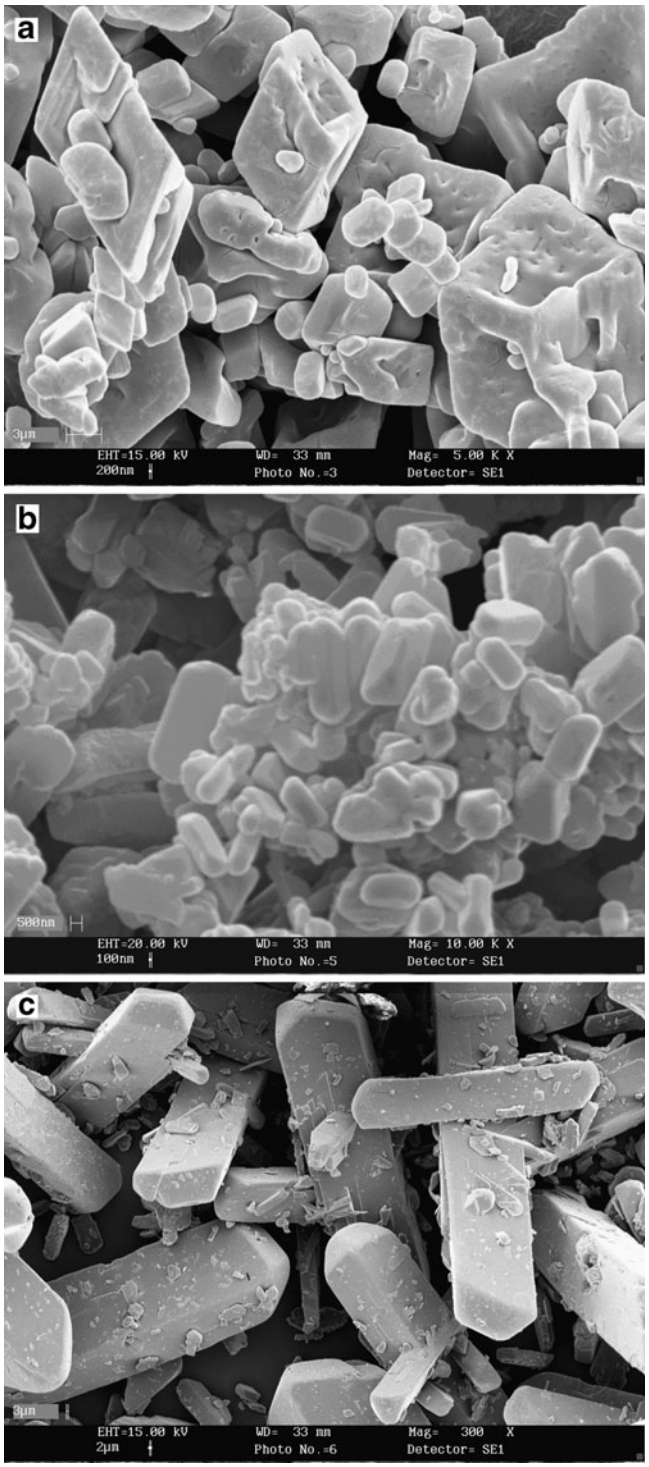

Fig. 1. Scanning electron micrographs of microcrystals. **a** $D_{0.5}B_{0.05}$ (magnification, ×5,000), **b** $D_{0.3}B_{0.05}$ (magnification, ×10,000), and **c** untreated GL (magnification, ×300)

In Vivo Studies

In order to investigate dose–response relationship in diabetic rats, 20, 30, and 40 mg/kg of pH-shift microcrystals

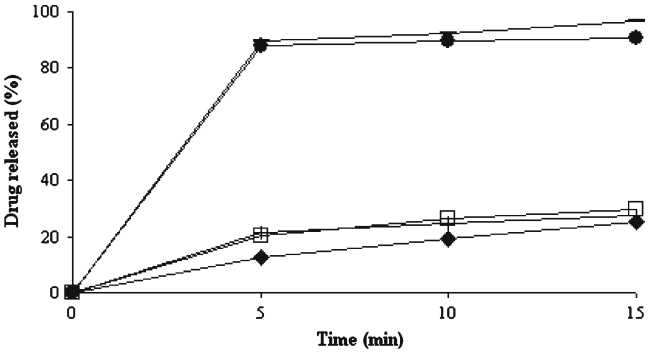

Fig. 2. Dissolution profiles of microcrystal of $D_{0.5}B_{0.05}$ (*filled squares*), physical mixture of $D_{0.5}B_{0.05}$ (*empty squares*), microcrystal of $D_{0.3}B_{0.05}$ (*dashed lines*), physical mixture of $D_{0.3}B_{0.05}$ (*plus signs*), and untreated GL (*filled diamonds*)

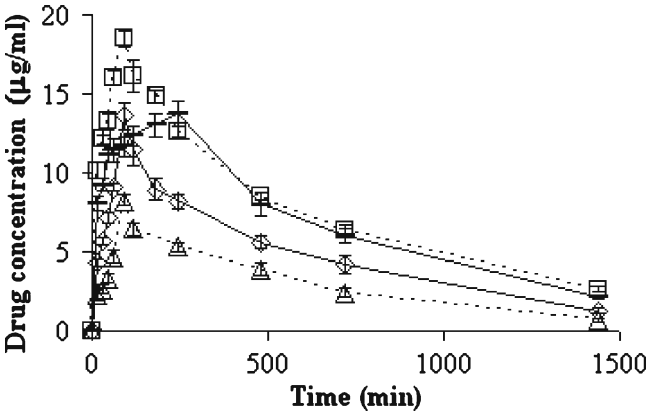

Fig. 4. Plasma concentrations of GL after oral administration of 40 mg/kg of untreated GL (*dashed line*) and 20 mg/kg (*triangles, empty line*), 30 mg/kg (*diamonds*), and 40 mg/kg (*squares, empty line*) of GL microcrystals prepared by pH-shift method (D0.3 B0.05) in diabetic rats (n=6)

were compared, and there was a linear relationship between C_{max} and glucose-lowering effect of microcrystals (r^2=0.9998, p<0.05). Untreated GL has longer t_{max} and lower C_{max} in comparison with the same dose of microcrystals that have shown more significant hypoglycemic effect than untreated GL in normal and diabetic rats (P<0.05) (Fig. 5). The effect of insulin (2 IU/kg) in normal rats appeared and finished faster than diabetic ones. As a result, insulin (2 IU/kg) has glucose-lowering effect in normal and diabetic rats, but the effect was more than GL microcrystals in administered dose (40 mg/kg). In both of normal and diabetic rats, k_a and k_e were approximately the same, and the difference was in C_{max} and AUC (P<0.05) (Table II).

Regarding the *in vivo* biological assays for hypoglycemic activity in pH-shift method (Table II), the oral treatment with 40 mg/kg of GL microcrystals ($D_{0.3}B_{0.05}$) in normal rats led to a significant reduction (46.45±0.66%) in the blood glucose levels after 90 min of treatment, and it was 24.33% more than the effect of untreated GL with the same dose in 240 min. In the treatment with 40 mg/kg of the same microcrystals on hyperglycemia induced by streptozotocin in rats, blood glucose levels were significantly reduced (29.39±0.54%) after 90 min of treatment, and it was 21.04% more than the effect of untreated drug with the same dose in 240 min. Saline, used as negative control group, did not display any hypoglycemic activity.

Injection of insulin to diabetic and normal rats decreased the blood glucose level to 40.55±2.17% and 52.9±4.4%, respectively (Table II, Fig. 5). The results showed that both the normal and diabetic animals are sensitive to insulin, and in contrast to other studies (26), GL microcrystals have glucose-lowering effect on normal rats more than diabetics with approximately the same plasma GL concentration and pharmacokinetic parameters (P<0.05) (Table II).

DISCUSSION

HPLC Method

The HPLC method used in this study is simple and easy for the detection of the drug (Varshosaz *et al.*, under consideration for publication in Scientia Pharm). Ibuprofen used in this study as IS is more available than 4-hydroxybenzoate and nadoxolol, which were reported before (27,28). The LOQ of 0.5 µg/ml in previous studies is far above the sensitivity, which is needed in these investigations

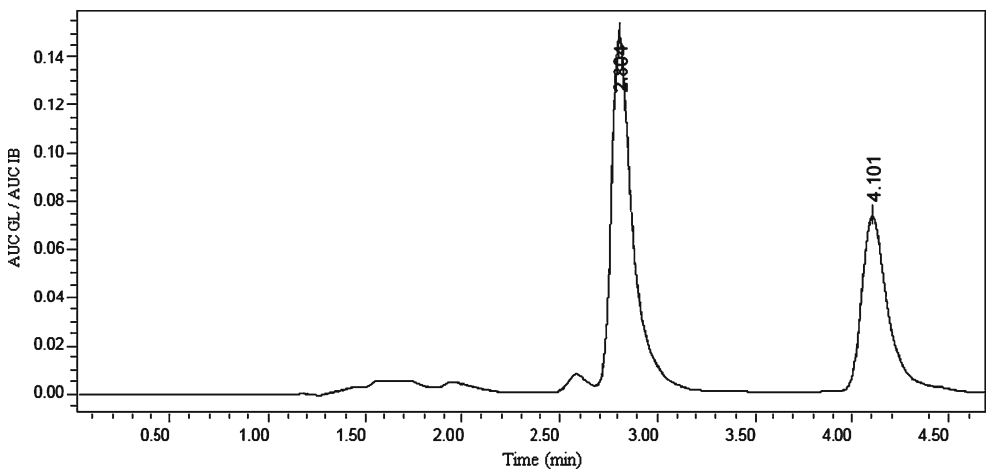

Fig. 3. HPLC chromatogram of GL (2.8 min) and IB (4 min) 90 min after administration of microcrystals ($D_{0.3}B_{0.05}$) containing 40 mg/kg GL to diabetic rats (n=6)

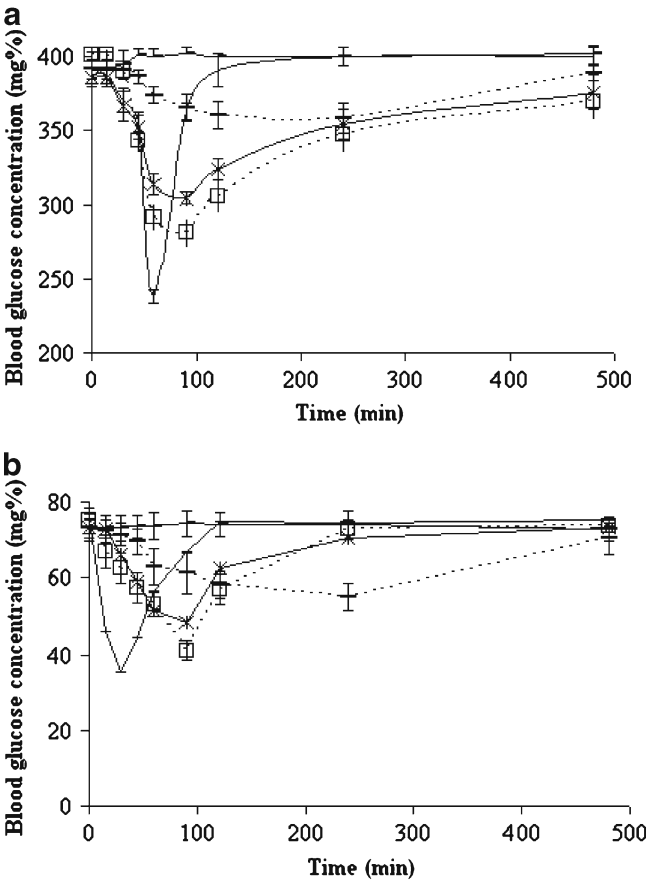

Fig. 5. Blood glucose levels in control group (*dashed line*) and after oral administration of 40 mg/kg of untreated GL (*dashed empty line*), microcrystals of solvent-change method (D0.5 B0.05) (*stars*), pH-shift method (D0.3 B0.05) (*squares, empty line*), and insulin (2 IU/kg) (*simple line*) in **a**) diabetic and **b**) normal rats (n=6)

(29). The method used by Poirier *et al.* (27) needs 250 µl of plasma samples compared with 100 µl, which was used in modified method. The solid phase extraction procedure reported by Yu *et al.* (30) for sample preparation is an expensive sample clean-up method while the method used in the present study is based on a liquid–liquid extraction and has a shorter run time.

In Vivo Studies

As it is indicated in Table II, C_{max}, $AUC_{(0-\infty)}$, and k_a show significant differences between treated and untreated crystals using the same dose ($p<0.05$). For example, in diabetic rats, the administration of microcrystals prepared by pH-shift method showed that the values of C_{max} and k_a were 18.33±0.24 µg/ml and 0.016±0.001 min^{-1}, respectively, whereas the values were 13.43±0.22 µg/ml and 0.008± 0.00 min^{-1} for untreated GL. Increased and faster effect of microcrystals in comparison with untreated drug should be explained by increased dissolution rate, solubility, and specific surface area and decreased crystallinity of the drug because of the decreased particle size (31). There is a linear relationship between dose and effect (blood glucose reduction), and it can be concluded that there is no nonlinearity in dose–response in this range of doses.

It is approved that the insulin-increasing effect of GL is an important route of drug effect (32). As it is shown in Fig. 5, insulin acts more and faster than GL in normal and diabetic rats, but the blood glucose level returns faster to the levels as it was before treatment. Therefore, it should be concluded that GL effect remains longer than insulin that is an important advantage of the drug. The activity of insulin is considered negligible in streptozotocin-induced diabetic rats. Streptozotocin treatment damages the insulin-secreting beta cells, which are located in the pancreas responsible for secreting insulin (33). This can explain more affectivity of

Table II. Pharmacokinetic Parameters After Oral Administration of Untreated Gliclazide and Different Types of Gliclazide Microcrystals in Different Doses to Diabetic and Normal Rats (n=6)

Intervention	T_{max} (min)	C_{max} (µg/ml)	$AUC_{(0-\infty)}$ (µg h/ml)	k_e (min^{-1})	k_a (min^{-1})	Glucose reduction[a] (%)
$D_{0.3}B_{0.05}$ (pH shift) 40 mg/kg in normal rats	90.23±0.87	18.32±0.29	14,968.27±66.9	0.0015±0.0003	0.0150±0.0004	46.45±0.66
$D_{0.3}B_{0.05}$ (pH shift) 40 mg/kg in diabetic rats	90.2±1.9	18.33±0.24	14,162.3±71.4	0.0015±0.0003	0.0160±0.0010	29.39±0.54
$D_{0.5}B_{0.05}$ (solvent change) 40 mg/kg in normal rats	90.13±1.2	16.27±0.17	13,630.33±32.9	0.0015±0.0004	0.0150±0.0010	33.43±1.12
$D_{0.5}B_{0.05}$ (solvent change) 40 mg/kg in diabetic rats	90.44±0.7	16.27±0.18	13,445.07±46.6	0.0016±0.0006	0.0150±0.0010	20.79±0.53
$D_{0.3}B_{0.05}$ (pH shift) 30 mg/kg in diabetic rats	90.8±1.3	13.48±0.36	9,062.14±22.6	0.0015±0.0002	0.0160±0.0010	22.26±0.61
$D_{0.3}B_{0.05}$ (pH shift) 20 mg/kg in diabetic rats	90±1.5	8.2±0.19	2,332.6±41.9	0.0015±0.0004	0.0150±0.0005	14.78±1.11
Untreated GL 40 mg/kg in normal rats	240.2±2.6	13.08±0.31	12,414.56±15.6	0.0016±0.0002	0.0090±0.0002	21.12±2.05
Untreated GL 40 mg/kg in diabetic rats	240.9±1.9	13.43±0.22	12,946.51±49.4	0.0016±0.0002	0.0080±0.0003	8.35±0.98
Insulin (2 IU/kg) in normal rats	30.8±2.1					52.9±4.4
Insulin (2 IU/kg) in diabetic rats	60.1±2.4					40.55±2.17

[a] Glucose reduction % is the maximum glucose reduction that was reached after 90 min for microcrystal formulations, but for untreated GL, it was reached after 240 min

GL in normal rats in reducing blood glucose. The Cunha *et al*. investigations (34) also showed faster hypoglycemic effect in normal rats as a result of the hypoglycemic effect of *Leandra lacunosa* in comparison with alloxan-induced diabetic rats. Varshosaz *et al*. (31) also reported that sustained release chitosan beads containing GL had blood glucose-lowering effect on the normal rats while no effect was seen on the diabetic rats. The authors concluded that a possible reason was that the rats were chronically administered sulfonylureas with foods, which might induce secondary failure to sulfonylureas. These reports can explain the glucose-lowering effect of GL microcrystals in normal rats but not in the streptozotocin-diabetic ones.

CONCLUSION

In conclusion, the microcrystallization, which has an effect on GL crystal habit, can change the drug absorption characteristics. Microcrystallization of GL using both methods resulted in increased pharmacodynamic effect. $D_{0.5}B_{0.05}$ and $D_{0.3}B_{0.5}$ microcrystals from solvent-change and pH-shift methods, respectively, have shown more significant hypoglycemic effect than untreated GL in normal and diabetic rats ($P < 0.05$), which could be attributed to the enhanced dissolution rate of GL. Decreasing blood glucose level specially in microcrystals prepared by pH-shift method seems to be due to the dysfunction of insulin secretion in diabetic rats in comparison with normal ones.

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