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A new HPLC method for determination of repaglinide in human plasma and its application in bioequivalence studies

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ABSTRACT

A simple, rapid and sensitive isocratic reversed-phase high performance liquid chromatography method for the determination of repaglinide in human plasma has been developed. After protein precipitation with acetonitrile chromatographic analysis of repaglinide in plasma was achieved on a μ-bondapack C_{18} column using acetonitrile- methanol- potassium dihydrogen phosphate 0.01 M (51: 11: 38) mixture, pH 2.5, as mobile phase. The flow rate was set at 1.5 ml/min and the emission and excitation were 348 and 244 respectively. The lower limit of detection was 1 ng/ml and lower limit of quantization was 5ng/ml. The intra and inter-day precisions (CV %) of the quality control samples were 1.43- 3.29 and 1.14- 4.11% respectively. The recovery of method was %98.3 ± 1.08. The method was applied to a bioequivalence study in human.

Key words: Repaglinide; Human plasma; HPLC; Bioequivalence.

INTRODUCTION

Repaglinide is a novel, fast-acting oral hypoglycaemic agent developed for the treatment of patients with type 2 diabetes whose disease cannot be controlled by diet and exercise alone. Although it binds to the sulphonylurea binding sites on pancreatic β-cells and has a similar mechanism of action, repaglinide exhibits distinct pharmacological properties compared with these agents. Following administration, repaglinide is absorbed rapidly and has a fast onset of dose-dependent blood-glucose lowering effect. The drug is eliminated rapidly via the biliary rout, without accumulation in the plasma after multiple doses. Repaglinide is well tolerated in patients with type 2 diabetes, including elderly patients with hepatic or renal impairment[1]. Repaglinide is rapidly absorbed after oral doses and Co-administration with food has only minor effects on its pharmacokinetics. Repaglinide is cleared rapidly from the circulation and eliminated almost entirely by hepatic metabolism, with predominantly biliary or faecal extraction of mainly inactive metabolites; only 8% of an administered dose was excreted in the urine. Repaglinide is primarily metabolized via oxidative biotransformation involving the hepatic microsomal cytochrome P450 system, particularly the CYP3A4 isoform. The major metabolites identified were dicarboxylic acid, which constitutes 66% of the administered dose, an aromatic amine and acyl glucuronide[2]. No significant differences in the pharmacokinetic variables (AUC, C_{max}, and T_{max}) of serum repaglinide were observed between elderly and young adult subjects[3]. Several HPLC, voltammetry, ELISA and LC/MS/MS methods have been reported for the analysis of repaglinide in pharmaceutical dosage forms[4-8], and in human biological liquids (plasma, urine)[9-13]. The aim of this study was to develop a simple, rapid, sensitive and reliable HPLC method with fluorescence detection for quantization of repaglinide in human plasma samples after protein precipitation with acetonitrile. The method was validated according to procedures

and acceptance criteria based on FDA guideline and recommendations of ICH, to provide enough selectivity, sensitivity and reliability in pharmacokinetic and bioequivalence studies.

MATERIAL AND METHODS

Acetonitrile, methanol (HPLC grade), potassium dihydrogen phosphate, sodium chloride and phosphoric acid (analytical grade), were purchased from Merck. Repaglinide and celecoxib were USP reference standard.

Sample and standard solutions preparation

To a 0.5 ml aliquot of plasma, 30 µl celecoxib (10 µg/ml) as Internal standard, 500 µl acetonitrile and 100 mg sodium chloride were added and vortexed for 30 s to precipitate proteins. The mixture was centrifuged at 8000 rpm for 10 min at 20 ^{0} C and 70 µl of the supernatant was injected to the instrument. Stock solution of repaglinide and internal standard was prepared as 1 mg/ml for both. Working solutions were prepared by diluting the stock solution with mobile phase. The final concentration of internal standard in plasma was 300 ng/ml and the spiked plasma samples were subjected to the sample preparation procedure and injected to HPLC

Instrument and chromatographic conditions

Analyses were performed on a younglin model ACME-900 pump equipped with a Lab science USA fluorescent detector. Chromatography was performed at room temperature on a µ-bondapack C_{18} column (5 µm particle size, 25 cm × 4.6 mm I.D.). The mobile phase consisted of acetonitrile- methanol- potassium dihydrogen phosphate 0.01 M (51:11:38) mixture, pH 2.5 which was adjusted using phosphoric acid. The flow rate was set at 1.5 ml/min and the emission and excitation were 348 and 244 nm respectively.

Method validation

Validation was accomplished through determination of specificity, recovery, linearity, quantization limit, precision and accuracy.[14] Specificity was investigated by analyzing six drug-free plasma samples for interference of endogenous compounds. For calibration curve five different concentrations of repaglinide (5-250 ng/ml) in plasma were prepared by adding required volume of working solutions to blank plasma. Plasma calibration curve was prepared by taking area ratio of analyte to internal standard as Y-axis and concentration of analyte (ng/ml) as X-axis. Linearity of the standard curve was evaluated using least-squares linear regression analysis. Quantization limit was defined as the lowest repaglinide concentration that could be determined with mean value deviation and coefficient of variation less than 20%, using five plasma samples. The intra and inter-day precisions (CV %) of the assay procedure were determined by trice analysis of quality control plasma samples (20, 80 and 180 ng/ml) at the same day and three different days. Recovery was determined by comparing the response of three pre-treated quality control plasma samples in three levels (20, 80, 180 ng/ml) with the absolute peak area of un-extracted samples containing the same concentration of the drug as 100%.

Application

The validated method was used in bioequivalence study of repaglinide. It was an open, randomized crossover study to asses relative bioavailability of repaglinide in twelve healthy volunteers following single dose administration of repaglinide as 2 mg tablet (All subjects gave informed consent to the work). Test preparation was repaglinide 2 mg tablet manufactured by an Iranian pharmaceutics Co. The tablet containing 2 mg of repaglinide, manufactured by Novo Nordisk was used as reference preparation. The blood collecting times were 0, 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 4, 6 h after oral administration of 4 mg repaglinide reference and test. The plasma samples were analyzed by the described method. The pharmacokinetic parameters like area under the plasma-concentration-time curve from time zero to the last measurable repaglinide sample time and to infinity ($AUC_{0\text{-}t}$ and $AUC_{0\text{-}inf}$), maximum concentration (C_{max}), time to maximum concentration (T_{max}) were determined for the period of 0 to 6 h.

Statistical analysis

The analysis of variance was performed on data for differences between and within the subspecies using the ANOVA (SPSS ver. 10). Mean separations were determined by least significant difference (LSD) at $P \leq 0.05\%$.

RESULTS AND DISCUSSION

Some HPLC, voltammetry and LC-MS-MS methods have been developed for determination of repaglinide in pharmaceutical dosage form, but few methods have been developed for quantization of repaglinide in plasma. One of the challenging aspects of method development in quantitative analysis is the complexity of the analysis method. The simpler the method the better it could be conducted by different operators and in different labs. However other parameters of a quantitative method such as accuracy and precision demand more complex processes. The proposed method is suitable for repaglinide quantification in plasma samples. It showed specificity, since no interfering peaks from endogenous components of plasma were observed. Representative chromatogram of

Table 1: Precision and Recovery of Repaglinide Assay in Human Plasma (n=3)

Spiked	Intra-day			Inter-day			
(ng/ml)	Found	SD	CV (%)	Recovery%	Found	SD	CV (%)
20	19.66	0.003	3.29	98	19.48	0.004	4.11
80	79.59	0.013	3.14	99.5	80.22	0.014	3.49
180	175.33	0.013	1.43	97.4	175.52	0.011	1.14

blank plasma and spiked plasma with repaglinide and internal standard are shown in figure 1. Retention time for the repaglinide and internal standard were 4.1 min and 9.7 min, respectively. Three dimensional and contour plot views of the chromatograms also confirmed the complete separation. The chromatographic run time was 15 minutes for plasma sample analysis. The method was linear over the range of 5 to 250 ng/ml and the calibration curve could be described by the equation y=0.0054x-0.0152 (r^2=0.998).

The limit of detection (LOD) and quantization (LOQ) were 1, 5 ng/ml respectively. The intra and inter-day precisions (CV %) of the quality control samples were 1.43-3.29 and 1.14-4.11% respectively. The accuracy of this bioanalytical method was %98.3±1.08. (Table1). It can be

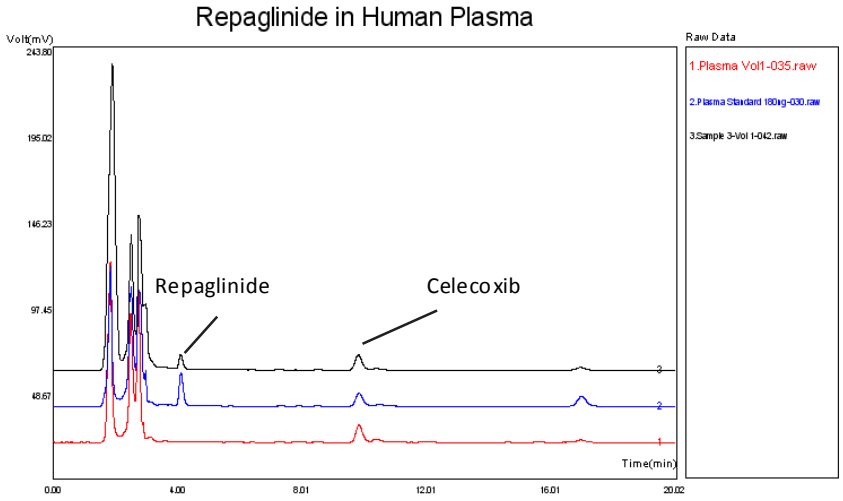

Fig. 1: Chromatograms of (1) Blank human plasma; (2) Plasma spiked with repaglinide and celecoxib ; (3) Human plasma after administration of repaglinide tablet

concluded that the accuracy and precision of repaglinide satisfied the acceptance criteria, and the proposed analytical method gave reproducible intra- and inter-day precision. The above mentioned method was used in the plasma analysis of a bioequivalence study of repaglinide as described earlier. The mean plasma level of repaglinide for test and reference preparation after the oral administration of a 2 mg single dose of repaglinide in 12 health human volunteers are given in Fig 2. Maximum plasma concentration (C_{max}) ranged from 47.41 to 89.93 ng/ml at 30 to 45 min (T_{max}). Also the mean value of area under the concentration time curve (AUC_{0-t}) obtained was 526.64 ng h/ml. AUC_{0-inf} was found to be 774.68 ng h/ml. Statistical comparison of the AUC_{0-inf}, C_{max} and T_{max} clearly indicated no significant difference between test and reference, 2 mg tablets, in any of the calculated pharmacokinetic parameters and these values are entirely within the bioequivalence acceptance range of 80-125%. Therefore, this analytical method is applicable to pharmacokinetic studies.

The main advantage of this method is the use of precipitation for purification, which is easily and fast in comparison with other purification and extraction methods. This HPLC method is reliable, reproducible and sensitive with respect to validation parameters. It can be used as an assay method in the study of repaglinide pharmacokinetics as well as bioavailability/ bioequivalence studies.

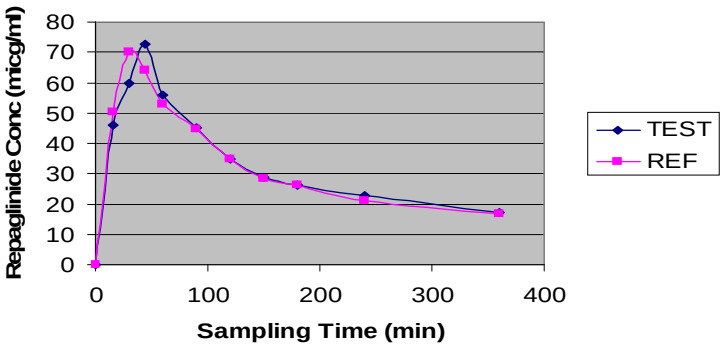

Fig. 2: Mean plasma concentration-time curve for test and reference preparation following single oral administration of repaglinide 2 mg tablet in 12 healthy volunteers

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