A Validated HPLC Method for the Determination of Linagliptin in Rat Plasma. Application to a Pharmacokinetic Study

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Abstract

A sensitive and reproducible HPLC method for the determination of linagliptin (LNG) in rat plasma was developed and validated using pindolol (PIN) as the internal standard. Both LNG and PIN were separated on a Zorbax Eclipse XDB C18 column kept at ambient temperature using as mobile phase a combination of 75% methanol: 25% formic acid 0.1% pH 4.1 at a flow rate of 1.0 mL min\(^{-1}\). UV detection was performed at 254 nm. The method was validated in compliance with ICH guidelines and found to be linear in the range of 5–1,000 ng mL\(^{-1}\). The limit of quantification (LOQ) was found to be 5 ng mL\(^{-1}\) based on 100 µL of plasma. The variations for intra- and inter-assay precision were <10%, and the accuracy values were ranged between 93.3 and 102.5%. The extraction recovery (\(R_0\)% was >83%. The assay was successfully applied to an \(in vivo\) pharmacokinetic study of LNG in rats that were administered a single oral dose of 10 mg kg\(^{-1}\) LNG. The maximum concentration (\(C_{\text{max}}\)) and the area under the plasma concentration–time curve (\(\text{AUC}_{0-72}\)) were 927.5 ± 23.9 and 18,285.02 ± 605.76 ng mL\(^{-1}\), respectively.

Introduction

Diabetes mellitus (Type 2 (presents ~95% of all diabetes cases that shows increasing incidence. The complications of this disease will significantly reduce the life expectancy (1). In May 2011, the US Food and Drug Administration has approved Tradjenta (linagliptin) tablets, used with diet and exercise, to improve blood glucose control in adults with Type 2 diabetes (https://www.hhs.gov/morgan.liscinsky@fda.hhs.gov). Linagliptin (LNG), 8-[(3R)-3-aminopiperidin-1-yl]-7-(but-2-yn-1-yl)-3-methyl-1-[(4-methylquinazolin-2-yl) ethyl]-3,7-dihydro-1H-purine-2,6-dione (Figure 1) belongs to dipeptidyl-peptidase-4 inhibitor class (2, 3). DPP-4 inhibitors represent a new therapeutic approach for the treatment of Type 2 diabetes that functions to stimulate glucose-dependent insulin release and reduce glucagon levels. This is done through inhibition of the inactivation of incretins, particularly glucagon-like peptide-1 (GLP-1) and gastrin inhibitory polypeptide (GIP), thereby improving glycemic control (4). Linagliptin is not extensively metabolized, 90% of dose is excreted unchanged. The small portion of drug that is metabolized, the main metabolite is CD1790 and is pharmacologically inactive with respect to DPP-4 inhibition (5).

High performance liquid chromatography (HPLC) is considered a valuable separation technique for the quantitative determination of analytes in complicated biological matrices. The development of an analytical method for the elimination of matrix interferences is very essential for a successful HPLC analysis of drugs in biological fluids. The literature revealed few methods developed for the determination of LNG by HPLC technique (6–11). Some of these methods were only applied for LNG assay in pharmaceutical tablets with a LOQ not <0.03 µg mL\(^{-1}\) (6–8). Other methods are using HPLC coupled to tandem mass spectrometric technique, (HPLC–MS/MS) which are
a single extraction step of a very small plasma volume (100 µL). It has will be validated in compliance with ICH guidelines (12) and involves termination of LNG with high sensitivity in rat plasma. The method and validate an accurate and reproducible HPLC method for the de-

sophisticated and much more sensitive and selective analytical meth-

ods (5, 9–11). However, these methods are not affordable for most routine laboratories due to their special requirements and high equip-

ment cost. In addition, these methods are mainly designed for human biological samples in a relatively large volume of plasma. Rats could be considered ideal for the preclinical pharmacokinetic studies of drugs because of their small size, low cost, and ease in handling. Under the scope of this view, the aim of this work was to develop and validate an accurate and reproducible HPLC method for the determination of LNG with high sensitivity in rat plasma. The method will be validated in compliance with ICH guidelines (12) and involves a single extraction step of a very small plasma volume (100 µL). It has been successfully applied in a pharmacokinetic study of LNG in rats.

Experimental

Materials and reagents

HPLC grade reagents (methanol, ethylacetate, formic acid, sodium hydroxide) were obtained from BDH Chemicals Ltd, Poole, Dorset, England. LNG analytical standard (99.6%) was obtained from Eli Lilly and Company, Indianapolis, IN, USA and PIN was purchased from SIGMA, Steinheim, Germany.

Equipment

An HPLC system Agilent 1100 series (Agilent Technologies, Santa Clara, USA) was used and was consisted of a G1314A variable wavelength UV detector, G1321A fluorescence detector, G1311A quaternary pump, G1379A microvacuum degasser and G1313A autosampler. The HPLC system control and data processing was performed by Chemstation (Agilent Technologies).

Liquid chromatographic conditions

The analytes of interest were separated on a Zorbax Eclipse XDB-C18, 250 × 4.6 mm, 5 µm columns (Agilent Technologies), kept at ambient temperature and protected by a precolumn (Zorbax Extend-C18). The mobile phase was consisted of methanol:formic acid 0.1% pH 4.1 (75:25 v/v%) and delivered at a flow rate of 1.0 mL min\(^{-1}\). The UV detector was set at 254 nm.

Standard and working solutions

Stock standard solutions of LNG (1 mg mL\(^{-1}\)) and PIN (1 mg mL\(^{-1}\), IS) were prepared by dissolving the appropriate amount of pure substance in methanol. Working standard solutions were obtained by diluting the stock standard solutions with the mobile phase. All standard solutions were stored at 4°C. Matrix calibration curves were daily prepared in drug-free rat plasma and appropriate volumes of the working standard solutions and the drug-free rat plasma were added to test tubes in order to achieve concentrations ranging from 5 to 1,000 ng mL\(^{-1}\). Quality control (QC) samples were independently prepared in the same way to achieve final concentrations of 10, 100 and 1,000 ng mL\(^{-1}\).

QC samples were prepared from a stock solution that was different from the one used to generate matrix calibration curve samples. These QC samples were run in each assay to investigate intra- and inter-run variations.

Sample preparation

Plasma sample (100 µL) was spiked with 100 µL of IS solution 50 ng mL\(^{-1}\) and 100 µL of sodium hydroxide 1 mol L\(^{-1}\) vortex-mixed for 10 s and then 3 mL ethylacetate was added. The samples were vortex-mixed for ~2 min and centrifuged at 2,500 × g for 15 min. The upper organic layer was carefully transferred into another clean glass tube and evaporated to dryness under a gentle stream of nitrogen at 40°C. The residue was reconstituted by adding 75 µL of the mobile phase, vortex-mixed for 10 s filtered through 0.2 µm syringe filter and then a volume of 50 µL was injected to the HPLC system for analysis through an autosampler.

Method validation

Selectivity

The selectivity of the method was assessed by extracting and analyzing control blank plasma samples with each run. The absence of interfering peaks at the analyte retention time was considered as an acceptable selectivity.

Linearity and range

The linearity of the method was evaluated via matrix calibration curves in the range of 5–1,000 ng mL\(^{-1}\). Calibration calculations were performed by a least-square linear regression analysis of the peak-area ratios of the drug to the IS versus the respective standard concentration.

Limit of detection–limit of quantification

The lowest detectable concentration (limit of detection—LOD) as well as the limit of quantification (LOQ) of our validated method were calculated based on the signal/noise (S/N) ratio measurements.

Accuracy, precision and extraction recovery

Accuracy was determined by using our method for the analysis of QC plasma samples to which known amounts of LNG corresponding to 10, 100 and 1,000 ng mL\(^{-1}\) were added. The accuracy was then calculated as the percentage of the analyte recovered by this method. LNG recovery achieved was calculated by comparing the analyte peak areas of six QC samples to those obtained from direct injection of six standard solutions with the same concentrations. The extraction recovery of IS was determined similarly.

Precision is a measurement of the method variability that can be expected for a given analyst performing the analysis. It was
determined by performing six replicate analyses of the same spiked QC drug-free samples at three different concentrations of 10, 100 and 1,000 ng mL\(^{-1}\). Intra- and inter-assay precision were calculated over three consecutive days and gave the corresponding percentage of relative standard deviation (%RSD). Accuracy was expressed as percentage recovery (%R) value where (%R = [measured concentration/added concentration] \times 100\%). For acceptable intra- and inter-assay values, accuracy (%R) should be within 85–115% and %RSD values should be ≤15% over the calibration range, except at the LOQ, where accuracy (%R) should be between 80 and 120% and %RSD should not exceed 20%.

Stability
The stability of QC samples was also investigated at three LNG concentration levels, including (i) stability of the plasma extracts at room temperature for 24 h, (ii) stability after three freeze–thaw cycles with the frozen temperature of –20°C and thawing temperature of 25°C and (iii) stability of plasma samples at –20°C for 20 days. Thereafter, samples were analyzed and the resulting values for these samples were then compared with those of the respective freshly prepared QC samples.

Pharmacokinetic study

Rat study design
White Wistar rats (200–250 g body weight (b.wt.), \(n = 10\) ) were in fasted state, i.e., they did not have access to food for 18 h prior to drug administration, while access to tap water was provided \(ad libitum\). The rats received a single oral dose of LNG (10 mg kg\(^{-1}\) b.wt.) dissolved in saline. Blood samples were collected in EDTA test tubes at 0, 0.25, 0.5, 1, 2, 3, 6, 12, 24, 36 and 72 h after administration. Plasma was separated after sampling by centrifugation at 2,500 \(\times\) g for 15 min and was stored at –20°C until assay.

All the experimental procedures were performed in accordance with Saudi Arabia Research Bioethics and Regulations, which are consistent with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. The experimental protocol was approved by the Unit of Biomedical Ethics Research Committee, King Abdulaziz University (reference number 147-2013).

Pharmacokinetic parameters
The pharmacokinetic parameters were calculated using noncompartmental analysis using Microsoft excel based on the equations of reference (13). The maximum observed plasma concentration \(C_{\text{max}}\) and the time to reach \(C_{\text{max}}\) \(t_{\text{max}}\) were determined directly from the data. The area under the plasma concentration–time curve was calculated using the linear trapezoidal rule to the last measured concentration \(\text{AUC}_{0-\infty}\). The terminal half-life \(t_{1/2}\) was determined by dividing 0.693/\(k_{\text{el}}\). Data were reported as mean ± SD.

Results

Method development and optimization
For the separation of LNG, the best results were obtained when an acid medium was used. The optimum resolution of LNG and PIN (IS) in rat plasma was achieved by isocratic elution using methanol:formic acid 0.1% pH 4.1 (75:25 v/v%) as the mobile phase at a flow rate of 1.0 mL min\(^{-1}\) on an Agilent Zorbax-C\(_{18}\) EclipseXDB 4.6 mm i.d \(\times\) 250 mm length, 5 \(\mu\)m) column that was kept at ambient temperature. UV detection was performed at 254 nm (Figure 2). PIN was chosen as internal standard due to its good resolution and satisfactory validation results under the applied chromatographic conditions.

For the extraction, ethylacetate was selected. Alkalization of the plasma with 1 M NaOH enhanced the extraction recovery of the analyte and proved to reduce interferences from the other plasma organic components. A small plasma volume of 100 \(\mu\)L was sufficient for the analysis and gave satisfactory results.

The method developed showed excellent chromatographic specificity with both analyte and IS being well resolved from other endogenous compounds within a total run time of 8 min. Representative chromatograms for rat blank plasma and rat plasma spiked with LNG (200 ng mL\(^{-1}\)) and the IS (50 ng mL\(^{-1}\)) are shown in Figures 2A and B, respectively. The chromatographic performance parameters of both LNG and PIN are presented in Table I.

Method validation
According to ICH guidelines (12), different validation parameters were verified for the presented method.

Selectivity
There was no significant chromatographic interference around the retention times of LNG and IS in drug-free specimens (Figure 2) indicating good selectivity of the method.

Linearity and range
The calibration was performed by least-square linear regression analysis. Representative linear equation for LNG in plasma was

Table I. Chromatographic Parameters of Linagliptin (LNG) and Pindolol (PIN)

<table>
<thead>
<tr>
<th>Parameter(^a)</th>
<th>Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>LNG</td>
<td>PIN</td>
</tr>
<tr>
<td>Capacity factor, (k')</td>
<td>2.1</td>
</tr>
<tr>
<td>Selectivity, (\alpha)</td>
<td>1.24</td>
</tr>
<tr>
<td>Resolution, (R)</td>
<td>5,324</td>
</tr>
<tr>
<td>Plate count, (N)</td>
<td>1.1</td>
</tr>
</tbody>
</table>

\(^a\)Reference values: \(k' > 1.5, \alpha > 1, R > 1.5, N > 2,000, A_{R} < 2\).
Plasma samples stored at 95.4 ± 3.5% to 100.1 ± 5.8%.

The mean recoveries for LNG at each concentration, and ranged from 95.4 to 97.0%. Postpreparative samples for 24 h 95.6 ± 2.4 96.4 ± 2.1 100.1 ± 5.8.

Regression coefficient = 0.0062 y = 0.0062x – 0.0118 in the range of 5–1,000 ng mL⁻¹ with squared regression coefficient r² = 0.9961. The nonzero standards showed <20% deviation at the 5 ng mL⁻¹ concentration and <15% deviation at all other concentration levels.

Accuracy, precision and extraction recovery
The results of the accuracy and precision are shown in Table II. The intra- and inter-assay accuracy for LNG at 10, 100 and 1,000 ng mL⁻¹ levels in rat plasma were found to be in the ranges of 93.8–99.2% and 93.3–102.5%, and the intra- and inter-assay precision (%RSD) were in the range of 3.2–9.5% and 6.3–9.0%, respectively. The mean extraction recoveries (R%) for LNG from plasma at three QC concentrations were in the range of 82.9 ± 1.9%–99.5 ± 4.6%, while for IS in plasma at 100 µL, it was 82.6 ± 3.0%.

Stability
LNG was found to be stable after three freeze–thaw cycles in plasma. It was also proved that LNG could be stable in the rat plasma at room temperature for at least 24 h and at −20°C for 20 days. Table III lists the mean recoveries for LNG at each concentration, and ranged from 95.4 ± 3.5% to 100.1 ± 5.8%.

Pharmacokinetic study
Plasma concentration–time curve for LNG was shown in Figure 3. After oral administration, LNG gave a maximum plasma concentration (Cmax) of 927.5 ± 23.9 ng mL⁻¹ at 1 h. Thereafter, LNG concentration declined until 72 h after administration. The terminal half-life (t½) of LNG was 27.5 ± 0.75 h. The area under the plasma concentration–time curve (AUC₀→72 h) was 18,285.02 ± 605.76 h ng mL⁻¹ (Table IV).

Discussion
In developing of an analytical method, the elimination of the interfering organic components and other impurities, such as plasma proteins, is a prerequisite for a successful HPLC determination of analytes in biological fluids. In this study, the chromatographic conditions were optimized in such a way to suit the preclinical pharmacokinetic studies for LNG. Two columns were tried including; Agilent Zorbax-C₁₈ Extended (4.6 mm i.d. x 150 mm length, 5 µm particle diameter) and Agilent Zorbax SB-C₈ (4.6 mm i.d. x 150 mm length, 5 µm particle diameter). The best chromatographic separation was achieved using Agilent Zorbax-C₁₈ EclipseXDB) 4.6 mm i.d. x 250 mm length, 5 µm) because this column showed better resolution and better peak shapes.

Different mobile phases composed of an aqueous acidic solution as solvent A and organic solvent as solvent B were tested. Three types of solvent A, 0.1% aqueous acidic solution adjusted to pH 3–5 by phosphate buffer, acetate buffer or formic acid, were tried. Formic acid (pH 4.1) was finally chosen because it produces a better signal–noise ratio and peak shape for both the analyte and IS. For optimization of solvent (B), acetonitrile and/or methanol were tried to provide sufficient resolution between analyte and IS. Methanol was used to give better resolution and symmetric peaks; however, upon using acetonitrile, peak broadening (tailing) with more retardation was observed.

Although the detection wavelength was not monitored at λmax LNG, it was optimized to 234 nm as the response obtained was satisfactory for both LNG and PIN. A quite short run time was achieved with the retention times of PIN and LNG to be 5.2 and 6.4 min, respectively.

PIN was chosen for internal standard as it has shown good resolution and satisfactory validation results under the applied chromatographic conditions. And it was quantitatively measured at the same wavelength.
Various extraction procedures including protein precipitation methods and liquid–liquid partition steps were investigated. Direct protein precipitation with acetonitrile or 50% perchloric acid gave poor recoveries and showed many interfering chromatographic peaks due to plasma endogenous substances. For liquid–liquid extraction several solvents, alone and in combination, were tested like, diethyl ether, isopropanol, ethylacetate, dichloromethane in order to improve recovery as well as to eliminate interferences. Finally, ethylacetate was selected as the best solvent because we got better recovery (>83%) and no interfering peaks. Alkalization of the plasma with 1 M NaOH enhanced the extraction recovery of the analyte with the reduced interferences. A small plasma volume of 100 μL was sufficient and gave satisfactory results.

Finally, it must be noted that the significantly high linearity and the low variability resulted in a sensitive method with such a low LOQ (5 ng mL⁻¹). Selectivity was demonstrated by the absence of any endogenous interferences at retention times of LNG and IS as evaluated by chromatograms of blank rat plasma.

The data of accuracy and precision indicated that the repeatability, intermediate precision, and bias values of the assay were within the acceptance limits of ±20% at LOQ and ±15% at other concentration levels. The mean extraction recoveries for LNG in plasma at three QC concentrations was >83% indicating low matrix effects for the analyte. The mean recovery for IS in plasma was 82.6 ± 3.0%.

LNG was proved to be stable at room temperature in rat plasma for at least 24 h and at −20°C for 20 days.

Based on the above study of LNG in rat plasma, it was expected that the present method would be applicable to pharmacokinetic study of LNG in rat plasma.

In the present study, the pharmacokinetic results agree with previously published reports on pharmacokinetics of LNG which stated that LNG showed an unusual pharmacokinetic profile within the DPP-4 inhibitor class, both in relation to its binding to plasma proteins and its route of elimination (14). It binds extensively to plasma proteins (15, 16). The high affinity binding of LNG to DPP-4 in plasma and tissues produces a long terminal half-life (t½) and a nonlinear pharmacokinetic profile which has been demonstrated in both animal and human.

Conclusion

In conclusion, a sensitive, simple and cost effective analytical HPLC method was developed and validated for the quantitative determination of LNG in rat plasma. The good accuracy and low LOQ of the method were suitable for monitoring the full pharmacokinetic profile of LNG in rats. The main advantages of the method were the sensitivity, small sample volume, single-step extraction procedure and the short time of the analysis.

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