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Determination of human insulin in dog plasma by a selective liquid chromatography-tandem mass spectrometry method: Application to a pharmacokinetic study

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ABSTRACT

A simple, sensitive and selective LC-MS/MS method for quantitative analysis of human insulin was developed and validated in dog plasma. Insulin glargine was used as the internal standard. After a simple step of solid-phase extraction, the chromatographic separation of human insulin was achieved by using InertSustain Bio C18 column with a mobile phase of acetonitrile containing 1% formic acid (A)—water containing 1% formic acid (B). The detection was performed by positive ion electrospray ionization in multiple-reaction monitoring (MRM) mode. Good linearity was observed in the concentration range of 1–1000 μIU/mL ($r^2 > 0.99$), and the lower limit of quantification was 1 μIU/mL (equal to 38.46 pg/mL). The intra- and inter-day precision (expressed as relative standard deviation, RSD) of human insulin were ≤12.1% and ≤13.0%, respectively, and the accuracy (expressed as relative error, RE) was in the range of −7.23–11.9%. The recovery and matrix effect were both within acceptable limits. This method was successfully applied for the pharmacokinetic study of human insulin in dogs after subcutaneous administration.

1. Introduction

The global incidence of diabetes mellitus is increasing rapidly as the pace of life and eating habits change, and it has become one of the major diseases severely affecting human health. According to the International Diabetes Federation (IDF), > 415 million people worldwide were suffering from diabetes in 2015, and an increase to 642 million people is anticipated by 2040. The discovery and application of insulin and its analogs has allowed numerous patients who suffer from diabetes to improve their living conditions [1]. As yet, insulin and its analogs are still the most effective biotherapeutics for diabetes. The easy availability of insulin has proved to be both a blessing and a curse. Many studies have indicated that insulin is not only used in the treatment of diabetes but also involved in intentional homicide caused by injurious insulin administration [2,3], and it can be used as a doping substance for athletes [4]. Therefore, sensitive, high-throughput and accurate analytical methods are urgently needed for this substance.

Currently, there are two prevailing pathways for quantification of insulin [5]: one is the ligand-binding assay (LBA) such as the radioimmunoassay (RIA) or enzyme-linked immunosorbent assay (ELISA), and the other is liquid chromatography coupled with mass spectrometry (LC-MS). Historically, LBAs were often considered to provide sufficient accuracy and sensitivity in pharmacokinetic studies. However, the shortcomings of LBAs were made clearer with the advent of LC-MS. The disadvantages of LBA compared with LC-MS for the quantification of insulin can be summarized in the following prominent points. First, the process of developing an LBA method includes finding the optimal critical reagents and eliminating endogenous interference, and strict method validation in various matrices is often time consuming and costly [6–8]. Second, the critical reagents generally have high variability due to the complexity of the production processes [9]. Third, a mature method for a PK study must be able to quantify samples from different matrices (e.g., plasma, urine or tissues). Nevertheless, the LBA method developed in one matrix/species cannot readily be transferred to another [10]. Fourth, it is unrealistic to simultaneously quantify multiple compounds in one analysis of the LBA method, but this is easy to carry out when using LC-MS. Such multiplexing capacity can greatly improve the efficiency of experiments and can reduce the costs required for the initial screening and development of biotherapeutics [11]. Since the late 1990s, various LC-MS techniques have been developed, and it has emerged as a promising alternative to LBAs for quantification of insulin. In the past few years, although a few LC-MS/MS based methods have been reported to quantify the insulin in clinical studies [12–15], the quantification of insulin drugs is still based

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on the LBA method [16–19] for use in preclinical studies, and no LC-MS/MS method has been reported until now. Cross-reaction is often observed between exogenous insulin and endogenous insulin when the LBA method is used to quantify insulin in preclinical studies. It is well known that the cross-reaction of the LBA method seriously affects the data accuracy, which will dramatically limit the application of the LBA method and the progress of drug development. Therefore, it is necessary to develop a LC-MS/MS method to quantify the insulin used to support preclinical studies.

In this investigation we developed an analytical method that, for the first time, enables the sensitive quantification of insulin in dog plasma. Additionally, a simple solid-phase extraction method was developed for biological sample preprocessing instead of using traditional immunoaffinity methods, and this is highly sought after by researchers in preclinical studies [12,15,20,21]. The limit of quantification of our method was 1 μIU/mL (equivalent to 38.46 pg/mL). Compared to the current literature on the quantification of insulin using LC-MS/MS, there were significant advantages in this study. In the Chambers EE's study [14], the sample preprocessing was complicated and time consuming, which included protein precipitation (PPT) and solid phase extraction (SPE). Although the sample preprocessing was improved in Zhi's investigation [22], the sensitivity (70 pg/mL) was poorer than that achieved in our study. In addition, the multidimensional LC technology that was used to separate insulin in the above studies was not necessary considering the cost; our results indicate that one analytical column is sufficient to achieve effective separation and analysis of insulin.

2. Experimental

2.1. Chemical and reagents

Both insulin glargine used as the internal standard (IS) and recombinant human insulin (Fig. 1) were kindly provided by Tianmai biotechnology Co., Inc. (Hefei, China). HPLC-grade methanol and acetonitrile were purchased from Concord Co., Inc. (Tianjin, China) and formic acid (FA) for the HPLC was from Tianjin Guangfu Fine Chemical Research Institute (Tianjin, China). Water was prepared in-house with the BM-40 water purification system from ZhongShengMaoYuan Tech. Co. Ltd. (Beijing, China). All other chemicals were of analytical grade. Drug-free heparinized dog plasma was freshly collected from beagle in our laboratory and stored at -20 °C before use.

2.2. Liquid chromatography-tandem mass spectrometry

The analysis was performed on a LC-MS/MS system consisting of a binary LC-30AD delivery pump, a DGU-20A$_{5R}$ vacuum degasser, a CTO-20A column oven, a SIL-30AC auto-sampler, a CBM-20A system controller (Shimadzu, Japan) and an API LCMS-8060 mass spectrometer (Shimadzu, Japan). The LC system coupled with the mass spectrometer through the electro-spray ionization (ESI) source. Chromatographic separation was performed on an InertSustain Bio C18 column (GL Sciences, 100 × 2.1 mm, particle size of 1.9 μm) at a flow rate of 0.4 mL/min for 8 min with column temperature at 40 °C. The gradient elution solvents were 1% FA in acetonitrile (mobile phase A) and 1% FA in water (mobile phase B). The gradient was employed starting at 70% B and held for 0.5 min. The analytes were separated using a linear gradient from 70% B to 65% B within 3.5 min. Mobile phase B was then ramped from 65% to 2% over 0.1 min and held for 1.9 min to clean the column. The gradient ended at initial conditions for 1.9 min to equilibrate the column. Auto-sampler temperature was maintained at 4 °C, and the injection volume was set at 10 μL. For the target peptide and IS, collision induced dissociation (CID) products of multiply charged precursors were detected in positive ion multiple reaction monitoring (MRM) mode by tandem mass spectrometry with a dwell time of 200 ms. The precursor ions for human insulin and IS were the 5+ precursor at m/z 1162.2 and the 6+ precursor at m/z 1011.2, respectively. The mass spectrometry parameters including nebulizing gas flow, heating gas flow, interface temperature, desolventizer (DL) temperature, heat block temperature and drying gas flow, and the compound parameters containing collision energy, Q1 and Q3 voltages were optimized to obtain the highest sensitivity for the monitored transitions. The transitions from precursor ion to product ion were m/z 1162.2 → 143.2 for recombinant human insulin and m/z 1011.2 → 143.2 for IS, respectively. Detailed parameters are summarized in Table 1.

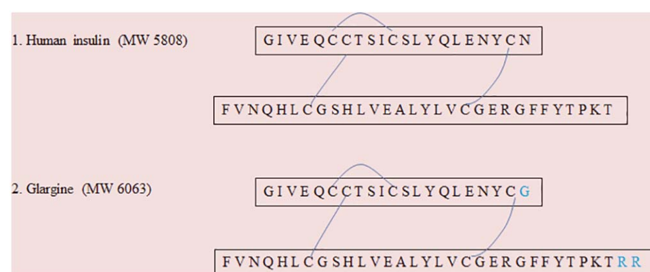

Fig. 1. The structures of human insulin and glargine (internal standard, IS).

2.3. Preparation of calibration curve and quality control samples

The recombinant human insulin and IS were present at 100 IU/mL. If the concentrations of the insulin and IS were converted from IU/mL into mg/mL, 100 IU would be equal to 3.846 mg of human insulin or 3.638 mg of glargine, respectively. Stock solutions for human insulin and IS were prepared with diluent consisting of 50/50 methanol/water plus 2% FA from the original pharmaceutical preparation in polypropylene tubes (low protein binding). Working solutions were prepared from the stock solution with dilution to the following concentrations: 10, 20, 50, 200, 1000, 5000 and 10,000 μIU/mL. Quality control (QC) solutions (25, 500, 8000 μIU/mL) were prepared in a similar manner. The working solution for the internal standard was prepared in the above diluent at a concentration of 5 mIU/mL. All solutions were stored at 4 °C and equilibrated for 10 min at room temperature before use. Calibration curve and QC samples were prepared by spiking 110 μL of either standard or QC working solutions to 990 μL of blank dog plasma in polypropylene tubes. Samples were mixed after addition of 22 μL of FA. The final concentrations of human insulin in calibration curves were 1, 2, 5, 20, 100, 500 and 1000 μIU/mL, and those in QC samples were 2.5, 50, and 800 μIU/mL. All the spiked plasma samples were then treated according to the sample preparation procedures. Both the calibration curve and QC samples were applied in the method validation and the pharmacokinetic studies. All calibration and QC samples were prepared daily to avoid potential degradation or adsorption issues.

2.4. Solid-phase extraction procedure

Samples were extracted using WondaSep® MCX column (size: 60 mg/3 mL, GL SciencesCorp., Japan). The column was activated with 3 mL methanol, and followed by equilibrated with 3 mL water. The 1 mL pretreated calibration curve, QC and blank samples were loaded onto the SPE column and then added 100 μL of IS. Samples were first washed with 3 mL of water followed by 3 mL of methanol. The analytes were eluted with 1 mL of eluent consisting of 30/70/0.5100 mM ammonium acetate/methanol/ammonium hydroxide (v/v/v) into a 10 mL glass tube. The eluates were evaporated to dryness under a stream of nitrogen. The residue was reconstituted in 100 μL of methanol-water solution (50:50, v/v) containing 2% FA and centrifuged for 10 min at 4 °C at 12000g, 10 μL was injected for LC-MS/MS analysis.

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