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PO-CON1645E

Development of 2D-LC/MS/MS Bioanalytical Method for Quantitative Determination of Insulin Glargine in Human Plasma

ASMS 2016 MP 311

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Introduction

Human insulin is an essential factor for regulating the metabolism of carbohydrates. Insulin glargine (Lantus) is one of the recombinant insulin analogues widely-used in diabetic patients to regulate glucose levels. Various bioassays have been used for quantitation of insulin and the analogues in serum/plasma for research and diagnosis. Recently, LC/MS/MS has been applied for quantitative analysis of glargine and other insulin analogues, because it enables to distinguish human insulin and recombinant analogues as well as their metabolites. The American Diabetes Association has recommended the sensitivity of bioassay to achieve an LLOQ of 70 pg/mL [1], which is challenging to the current LC-MS systems. The bioassay reported always require tedious solid phase extraction (SPE) for clean up before injecting into the LC/MS/MS [2, 3]. This motivated this study, aiming at developing a high sensitivity method on the latest LCMS-8060 to determine quantitatively insulin glargine in human plasma with lesser sample preparation steps. We describe a newly developed 2D-LC/MS/MS method for high sensitivity quantitation of insulin glargine spiked in human plasma without SPE clean up.

Methods and Materials

Insulin glargine (MW 6063) was obtained from Sigma-Aldrich. Commercially available pooled human plasma supplied by Innovative Research (USA) was used in this study. A 2D-LC/MS/MS system (see Figure 2) was set up on Shimadzu LCMS-8060 triple quadrupole system coupled to Nexera UHPLC with a column switching valve installed in column oven controlled by LabSolutions workstation. A stock solution of insulin Glargine was prepared with a mixed solvent of water-methanol (80/20) with 0.1% acetic acid. The procedure of sample preparation of spiked human plasma samples is shown in Figure 1. It includes protein precipitation step by adding ACN-MeOH solvent into the human plasma in 3 to 1 ratio followed by vortex and centrifuge at high speed. The supernatant was filtered and blown dry with nitrogen gas. The dried supernatant was reconstituted with mixed solvent of water-methanol (80/20) with 0.1% acetic acid before analysis on 2D-LC/MS/MS. Both pre-spiked and post-spiked plasma samples of various concentrations were prepared freshly from high concentration stock in method development. The details of columns, mobile phases and gradient programs of 1stD and 2nd D UHPLC separations and MS/MS conditions are compiled into Tables 1(a) and 1(b).

Table 1 (a): 2D-UHPLC conditions (on Nexera X2 system)

Column	: 1st D: Kinetex, 2.6um C18 100A (50 x 2.1mm)
	2nd D: Kinetex, 1.3um C18 100A (50 x 2.1mm)
Mobile Phase of 1st D	: A: 0.1% formic acid in Water
	B: 0.1% Formic acid in Acetonitrile: Isopropanol (50:50)
Mobile Phase of 2nd D	: C: 0.1% formic acid in Water
	D: 0.1% formic acid in Acetonitrile
1st D gradient program	: B: 15% (0 to 0.1min) → 90% (2.5 to 4.0min) → 15% (5.0 to 7.0min)
& flow rate	Flow rate: 0.3mL/min
2nd D gradient program	: D: 15% (0 to 2.25min) → 90% (4.0 to 5.0min) → 15% (5.5 to 7.0 min)
& flow rate	Peak cutting: from 1.50 to 1.73
	Flow rate: 0.35mL/min
Oven Temp.	: 40°C
Injection Vol.	: 50 µL

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Table 1 (b): LCMS-8060 MS conditions

Interface	ESI
MS mode	Positive
Block Temperature	300°C
Interface Temp.	400°C
DL Temperature	300°C
CID Gas	Ar (350kPa)
Nebulizing Gas Flow	N2, 2.0L/min
Drying Gas Flow	N2, 10.0L/min
Heating Gas Flow	Zero Air, 10.0L/min

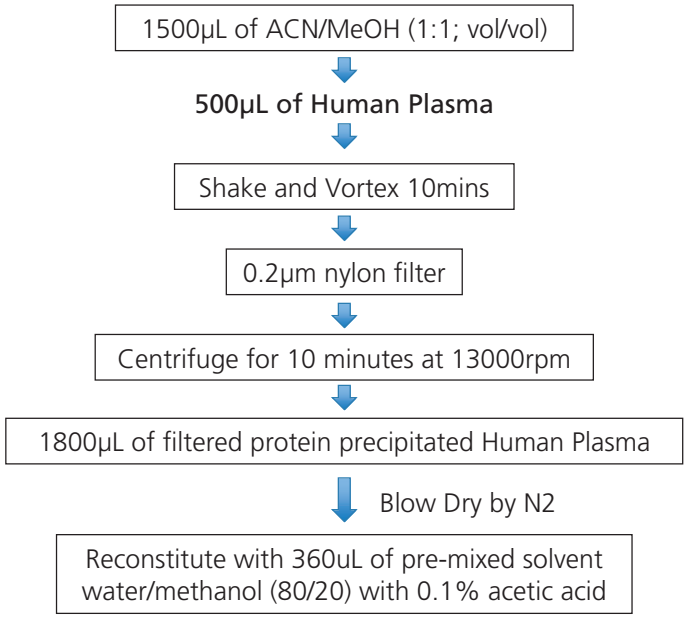

Figure 1: Flow chart of human plasma sample pre-treatment method (dilution factor: 4)

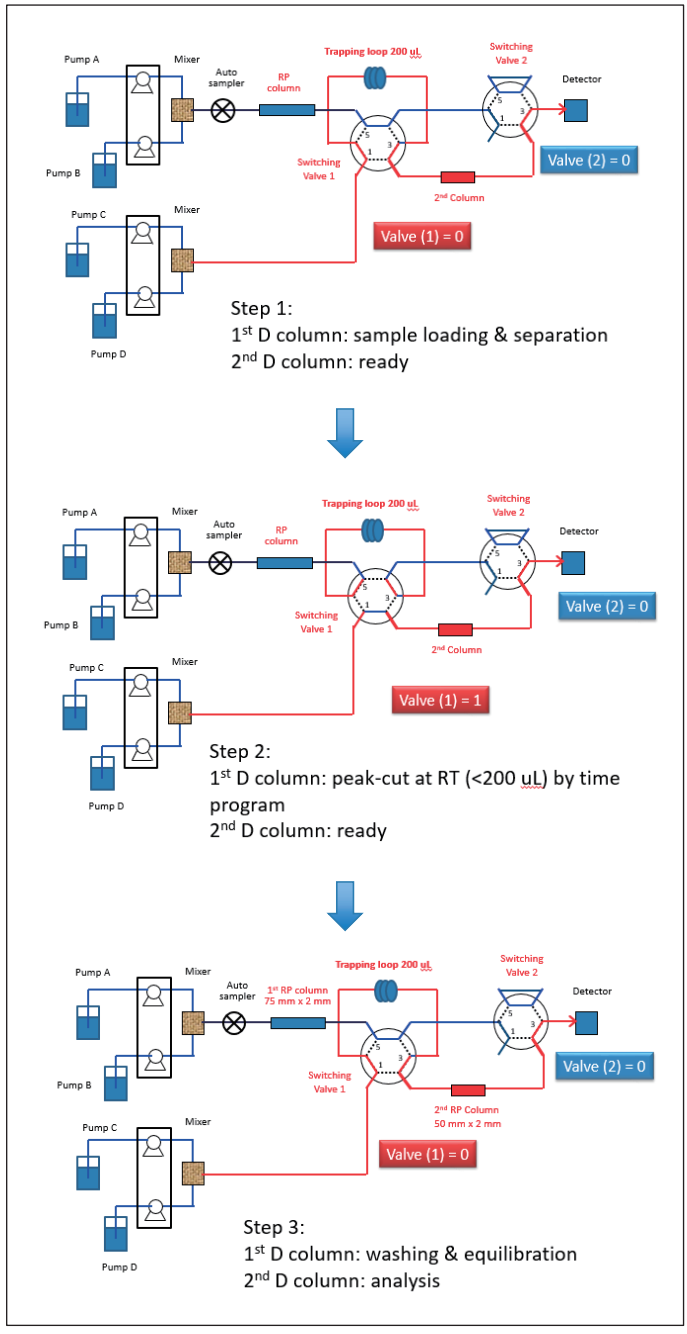

Figure 2: Schematic diagram of 2D-LC/MS/MS and operation scheme

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Results and Discussion

Development of 2D-LC/MS/MS method

An ESI interference was employed for effective ionization of insulin glargine ($C_{267}H_{404}N_{72}O_{78}S_6$, MW 6063). A MRM quantitation method for insulin glargine was developed. MRM optimization was performed using an automated MRM optimization program with LabSolutions workstation.

The insulin glargine molecules form multiply charged ions in ESI ionization and the precursors selected for MRM optimisation were charges +6 and +7. Two MRM transitions were used (Table 2), the first transition for quantitation and the second transition for confirmation.

Table 2: MRM transitions and CID parameters of insulin glargine on LCMS-8060

Name	RT (min)	Transition (m/z)	Voltage (V)		
			Q1 Pre Bias	CE	Q3 Pre Bias
Glargine	3.926	867.00 > 984.00	-20	-20	-22
		1011.50 > 1179.00	-34	-32	-42

The 2D-LC/MS/MS method developed in this study involves "cutting the targeted peak" in the 1st-D elution precisely (1.50~1.73 min) and the trapped elute in a stainless steel sample loop (200 uL) being transferred into the 2nd-D column for separation and MS/MS detection (see Figure 2). The operation was accomplished automatically by a control program with the LabSolutions switching the 6-way valves in and out during an analysis. Both 1st-D and 2nd-D separations were carried out in gradient elution mode (Table 1(a)). The advantages of this 2D-UHPLC separation are firstly to remove effectively the plasma matrix and, secondly, to minimize interferences which occurred in 1D-LC/MS/MS analysis. In addition, the MS interface and lens system are kept clean from contamination, which is very critical when a large amount of plasma sample was injected for obtaining higher detection sensitivity. As such, with 2D-LC/MS/MS, SPE clean-up is not needed to achieve the desired sensitivity (70 pg/mL).

Calibration curve, linearity and accuracy

Seven levels of post-spiked calibrants of insulin glargine from 0.05 ng/mL to 10 ng/mL were prepared freshly for establishment of calibration curve (See Table 3). A linear calibration curve (R2 > 0.9996) with weighting method 1/C was established as shown Figure 3. The accuracy of the method is between 98 % and 105 %. The chromatograms of the 1ng/mL post-spiked glargine standard sample is shown in Figure 4.

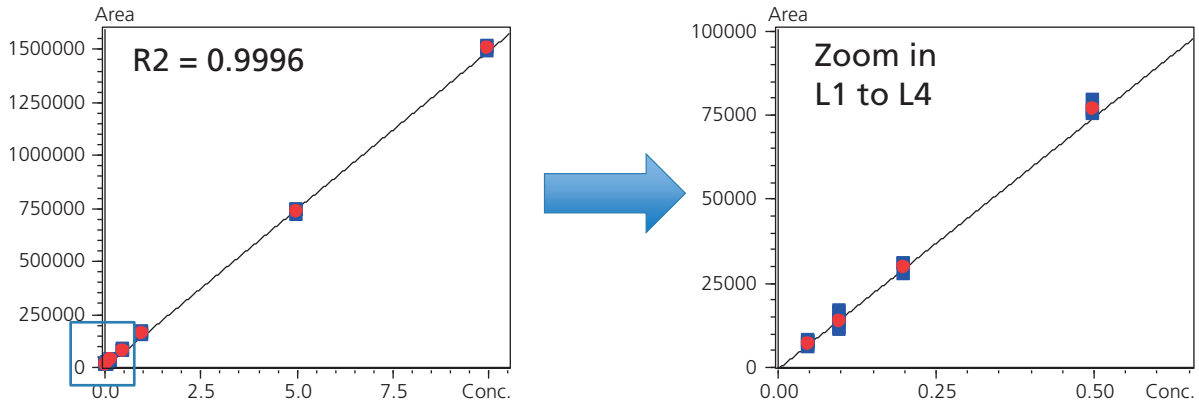

Figure 3: Calibration curve of insulin glargine in human plasma, full range and zoomed

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Table 3: Calibration curve standard samples in plasma

Cali. level [1]	Conc. (ng/mL)	Avg. Area	Avg. Accuracy %
L1	0.05	6827	100.2
L2	0.1	13604	104.5
L3	0.2	29667	100.7
L4	0.5	76704	101.1
L5	1.0	156677	98.9
L6	5.0	732260	98.6
L7	10.0	1501911	100.6

[1] Calibration levels were injected in triplicates (n=3) except for level 1 and level 2 (n=6)

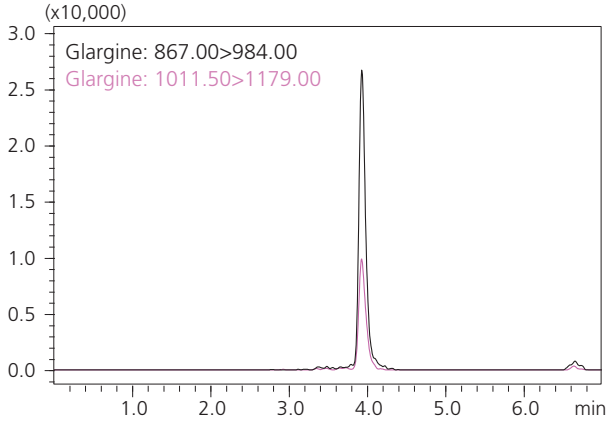

Figure 4: MRM chromatograms of insulin glargine spiked in human plasma (1 ng/mL)

Recovery, repeatability, LOQ and specificity

Recovery of the 2D-LC/MS/MS method was determined by comparison of the peak areas of pre-spiked and post-spiked samples at L1, L2, L4 and L6. As shown in Table 4, the recovery of the sample preparation method are between 63 % and 87 %. The repeatability (RSD%, n=7) of the method obtained is 11.1% at L1 and between 7.8 % and 2.3 % for L2, L4 and L6. It is worth to note that matrix effect was not evaluated due to the non-specific absorption nature of insulins, which may cause significant lost of the molecules in neat solution at low concentrations. The LOQ of the method is estimated to be 0.018 ng/mL from the L1 chromatograms of pre-spiked and post-spiked samples (see Figure 5). This corresponds to a LOQ of ~70 pg/mL of insulin Glargine in plasma before pre-treatment. The specificity of the method relies on several criteria: two MRMs (867.0>984.0 and 1011.5>1179), their intensity ratio and RT in 2nd-D MRM chromatogram as shown in Figures 4 and 5.

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Table 4: Summary of method performance of insulin Glargine spiked in human plasma (n = 3~6)

Sample	Conc. (ng/mL)	Area (Pre-spiked)	Area (post-spiked)	%RSD	Recovery%
L1	0.05	5,944	6,827	11.1	87.1
L2	0.1	10,216	13,604	7.8	75.1
L4	0.5	58,905	76,704	5.1	76.8
L6	5	465,387	732,260	2.3	63.6

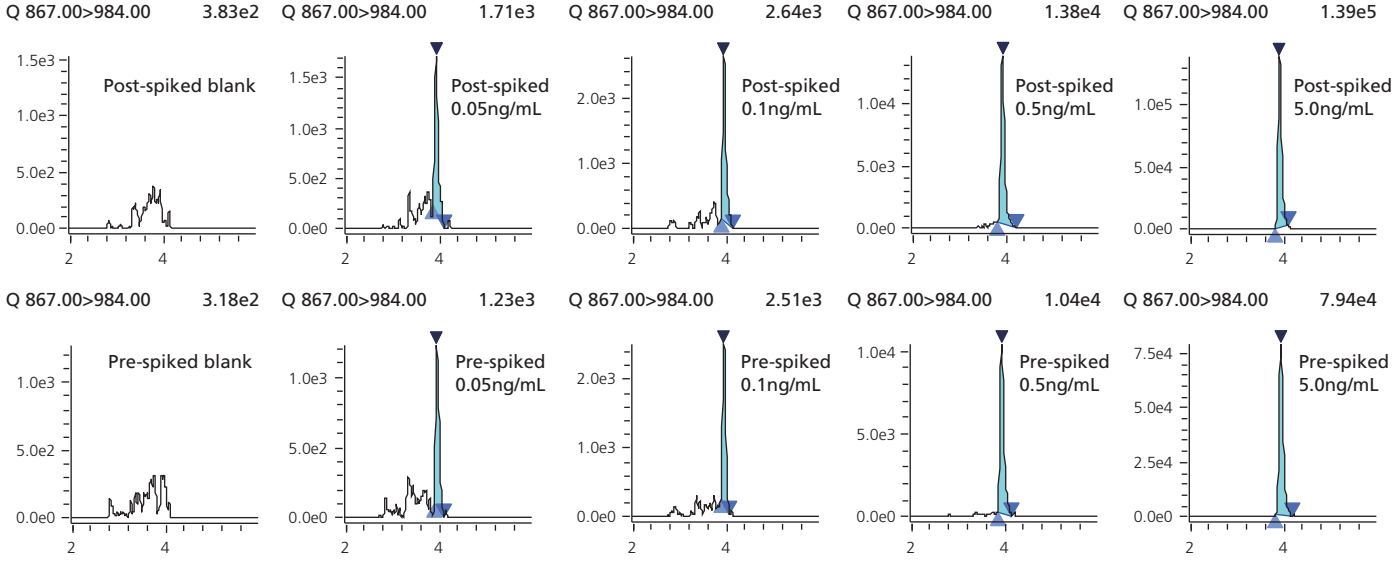

Figure 5: MRM peak of insulin glargine post-spiked (top) and pre-spiked (bottom) in human plasma

Conclusions

A 2D-LC/MS/MS method has been developed on LCMS-8060 for quantitative analysis of insulin Glargine in human plasma without use of SPE extraction and clean-up. The performance of the method was evaluated thoroughly, including linearity, accuracy, repeatability, recovery, sensitivity and specificity. The LOQ of the method is estimated to be 70 pg/mL in human plasma. **The advantages of the 2D-LC/MS/MS method are not only simplifying the sample pre-treatment and enhancing the sensitivity, but also reducing the potential contamination of plasma samples to the interface and lens of MS/MS system.**

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References

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Disclaimer: Shimadzu LCMS-8060, Nexera X2 UHPLC system, and Labsolutions Insight are intended for Research Use Only (RUO). Not for use in diagnostic procedures.

First Edition: June, 2016

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