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Application News

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Multidimensional LC-MS/MS Method for the Quantification of Intact Human Insulin

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❑ Overview

In this study, we developed and validated a novel multidimensional LC-MS/MS method for the analysis of human insulin in serum samples. Insulin was extracted from serum samples by protein precipitation using ice-cold methanol. Stable isotope-labelled human insulin (insulin-d40) was added in the extraction step as internal standard. The method was validated based on FDA guidance for industry on bioanalytical on method validation: selectivity, accuracy, precision, recovery, calibration curve, sensitivity, reproducibility and stability.

❑ Introduction

Insulin is a peptide hormone produced by pancreatic islets that regulated the metabolism of carbohydrates, fats and proteins by promoting the absorption of glucose. Historically, insulin has been analyzed by radioimmunoassay or enzyme-linked immunosorbent assay. These assays could suffer from the lack of standardization or cross reactivity, and this has driven the development of alternative assays using LC-MS/MS which provide greater specificity. Hence, method development was done using Shimadzu NexaraX2 LCxLC coupled to LCMS-8060 for the quantification of intact human insulin in human serum. In this study, we developed and validated a novel multidimensional LC-MS/MS method for the analysis of human insulin in serum samples. The validation results have been evaluated with the FDA guidance for industry on bioanalytical on method validation.

❑ Experimental

Sample preparation and analytical conditions

Insulin was extracted from serum samples by protein precipitation using ice-cold methanol; (1:1; vol/vol, serum:methanol). Stable isotope-labelled human insulin (insulin-d40) was added in the extraction step as internal standard. Chromatographic separation was performed on LC system comprising of a loading column (Kinetex C18, 50 × 2.1 mm, 2.6 µm) on which insulin peak was heart-cut and transferred to the analytical column (Kinetex C18, 50 × 2.1 mm, 1.3 µm) shown in figure 1. A Gradient elution of 7.5mins was used for both the loading and analytical columns. Detection and quantification of intact insulin was performed based on multiple reaction monitoring (MRM) under positive electrospray ionization mode in the Shimadzu LCMS-8060 triple quadrupole mass spectrometer.

Table 1. Analytical conditions and parameters on LCMS-8060

Column 1	Kinetex 2.6µ C18 100A (100 mmL x 2.10mm I.D.)
Column 2	Kinetex 1.3um C18 100A (50 mmL x 2.10mm I.D.)
Mobile Phase A & C	Water with 0.1% FA
Mobile Phase B & D	Acetonitrile:water (9:1) with 0.1% FA
Elution Program 1	B: 25% (0 to 0.5 min) ➔ 50% (3.0 min) ➔ 90% (3.1 to 4.5 min) ➔ 25% (4.6 to 7.5 min)
Elution Program 2	D: 25% (0 to 4.0 min) ➔ 100% (6.5 to 7.5 min)
Flow Rate for column 1	0.25 mL/min
Flow Rate for column 2	0.35 mL/min
Oven Temp.	40ºC
Injection	50 µL

Interface	ESI
Interface temp. & Voltage	350 ^{O}C & 2 kV
MS Mode	MRM, Positive
Heat Block Temp.	500 ^{O}C
DL Temp.	250 ^{O}C
CID Gas	Ar, 270 kPa
Nebulizing Gas	N_2, 2.5 L/min
Drying Gas	N_2, 5.0 L/min
Heating Gas	Dry Air, 10L/min

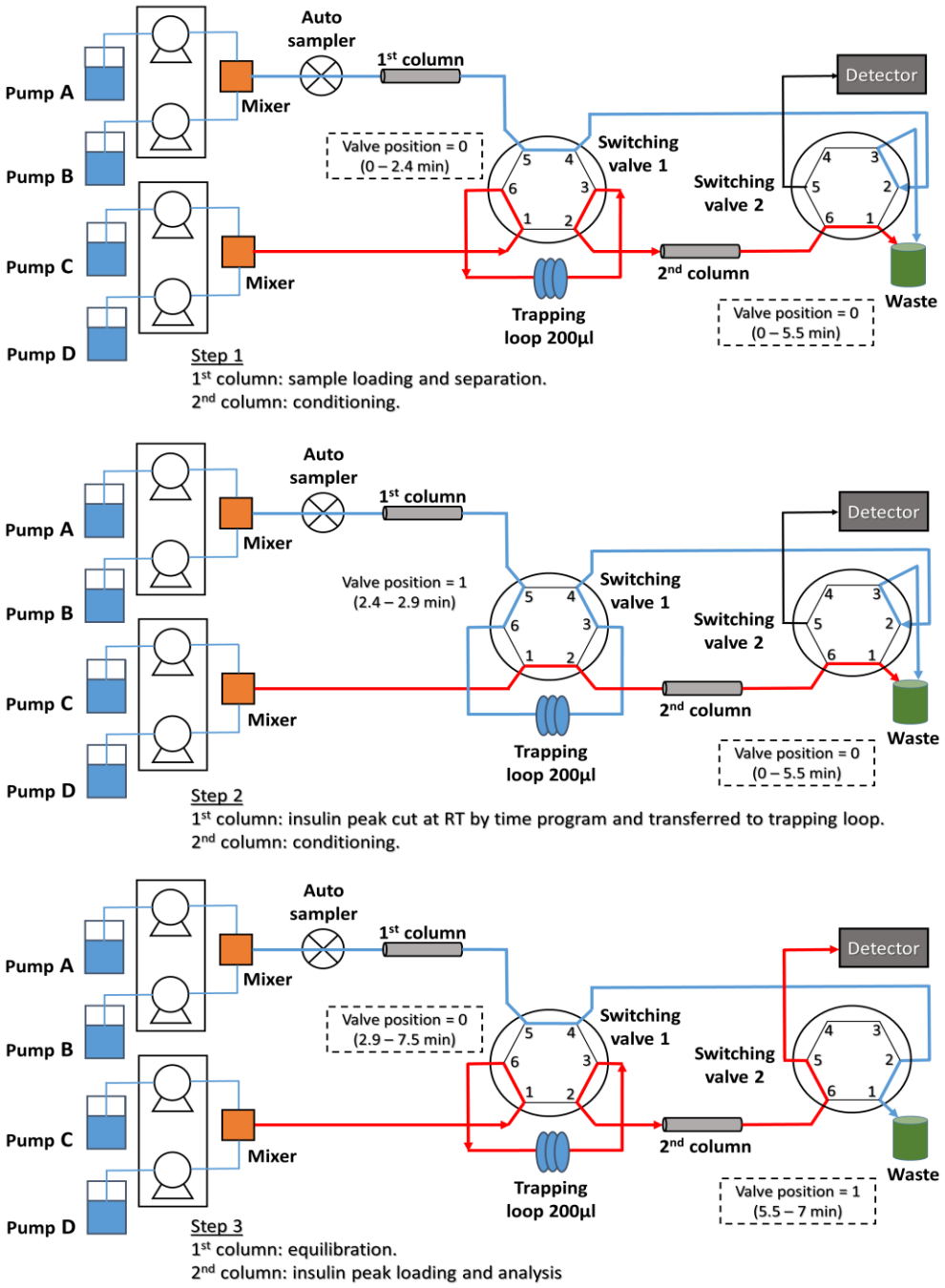

Figure 1. Mutli-dimensional LC-MS/MS schematic diagram and operation scheme

❑ Results and Discussion

Method Development

Insulin and insulin-d40 (internal standard) were used for setting up the MRM quantitation method. Table 2 shows the summarized results of optimized MRM transitions and parameters of the insulin standards and isotope-labelled internal standards. Two MRM transitions were selected for each compound, with one as the quantitation ion and the other for confirmation. Furthermore, a gradient elution MRM method was established with a total run time of 7.5 minutes. The MRM chromatogram of insulin in human serum is shown in Figure 3. The retention time of insulin peak is at 5.8mins

Table 2. MRM transitions and parameters of Insulin on LCMS-8060

Standard				Internal Standard			
Compd.	**R.T (min)**	**MRM (m/z)**	**CE (V)**	**Compd.**	**R.T (min)**	**MRM (m/z)**	**CE (V)**
Insulin	5.8	1162.1>1158.4	-33	Insulin-d40	5.8	1170.3>1166.7	-34
		1162.1>1410.1	-29			1170.3>1419.9	-37

Method validation

Selectivity of insulin in serum was studied. In figure 2 (a) and (b), it shows endogenous compound was found in the blanks. Therefore, insulin standard + endogenous compound shown in figure 2(c) was used to construct the calibration. In addition, the confirmation criteria include the MRM transitions as well as retention time.

Accuracy, Precision and Recovery of the quantitation method was validated within and between-run. The results are shown in Table 3 and 4, which indicate that reliable quantitation accuracy, precision and recovery were obtained. Accuracy and precision was being measured at five determinations per concentration level with four different concentration levels (LLOQ, Low, Med and High). Recovery and matrix effect is studied at three differently concentration levels (Low, Med and High). The accuracy result obtained for intra and inter day are within 15.0% of the nominal value. The precision CV for intra and inter day are <6.8%. The recovery obtained for insulin ranged from 87.5 to 100.5% with CV% of <3.7% for inter day. Furthermore, matrix effect was evaluated with results ranging from 92.6% to 111.4%.

Calibration curve was established using the internal standard method prepared by pre-spiked in serum matrix (see figure 3). It can be seen that good linearity with R^2 greater than 0.99 was obtained for the insulin in the range from 8.6 pmol/L to 1720 pmol/L in serum.

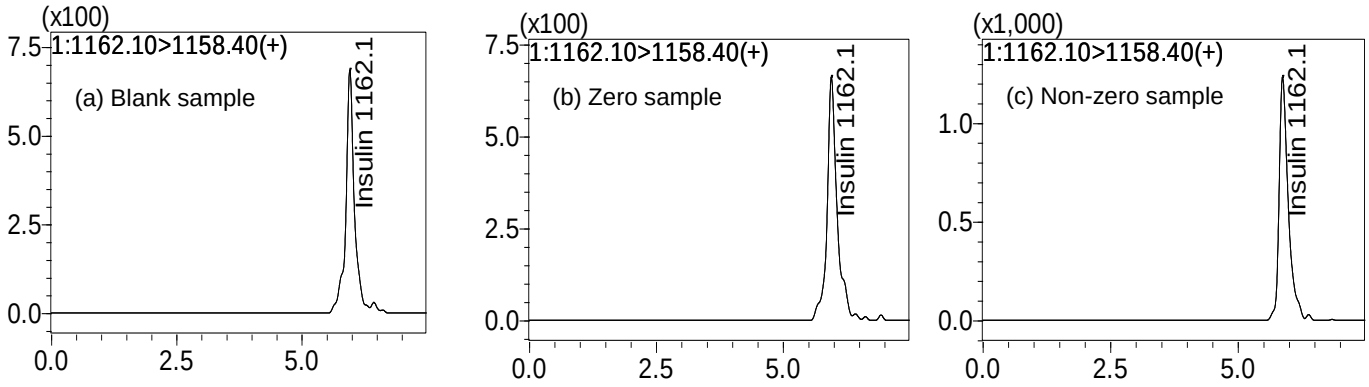

Figure 2. Chromatogram of (a) Blank, (b) Zero and (c) Non-Zero samples (172.2ppt).

Table 3. Intra- and inter-day accuracy and precision of insulin

Nominal Conc. (pmol/L)	Intra-day (n=5)			Inter-day (n=5)		
	Found conc. (pmol/L)	Accuracy (%)	Precision (% CV)	Found conc. (pmol/L)	Accuracy (%)	Precision (% CV)
LLOQ: 103.3 (Endogenous +8.6)	92.9 ± 4.8	89.93	5.21	100.8 ± 6.1	97.62	6.07
Low: 120.5 (Endogenous +25.8)	126.7 ± 8.5	105.13	6.71	126.2 ± 6.7	104.67	5.32
Med: 783.4 (Endogenous +688.7)	825.1 ± 53.5	105.32	6.49	842.3 ± 33.6	107.52	3.99
High: 1472.1 (Endogenous +1377.4)	1306.9 ± 69.1	88.78	5.29	1368.4 ± 90.1	92.95	6.58

Table 4. Matrix effect and recovery of insulin

Nominal Conc. (pmol/L)	Mean peak area (x10^3) (n=5)			Inter-day (n=5)		Matrix Effect (%)
	Set A	Set B	Set C	Mean (%)	CV (%)	
25.8	2.64	2.94	2.17	87.46	3.67	111.36
688.7	24.73	22.89	23.00	100.48	1.24	92.57
1377.4	41.50	46.04	41.93	91.07	1.55	110.93

Set A was neat solution standards. Set B and Set C were standards spiked in extracted serum and serum respectively.

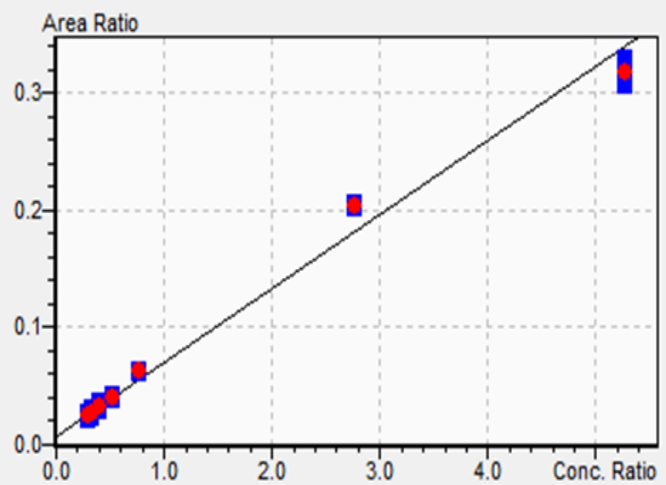

Figure 3. Insulin calibration curve from 8.6 to 1720 pmol/L with r^2>0.99.

Sensitivity of the method for LLOQ of 103.3 pmol/L nominal concentration was evaluated and giving an accuracy of 89.9% for intra day and 97.6% for inter day. The precision for both intra and inter day is < 6.1%. Both accuracy and precision for LLOQ being measured is acceptable.

Reproducibility of the method was assessed. This includes the QCs and incurred samples. Reinjection reproductivity was also evaluated for instrument interruption

Stability of the analyte insulin was assessed after freeze and thaw, at room temperature, cold room and long term stability (see table 5). The mean recovered concentration (%) was within ±15.0% of the nominal concentration except for the low concentration of 25.8 pmol/L which is -17.6%.

Table 5. Stability assessment of insulin

Nominal Conc. (pmol/L)	Room Temp. (25°C, 4h)		Cold-room (4°C, 24h)		Freeze-thaw (-80°C, 3 cycles)		Long term stability (-80°C, 14 weeks)	
	Mean recovered conc. (%)	CV (%)	Mean recovered conc. (%)	CV (%)	Mean recovered conc. (%)	CV (%)	Mean recovered conc. (%)	CV (%)
25.8	105.85	6.96	92.19	3.99	82.42	0.40	96.39	3.58
688.7	97.58	6.63	96.32	2.91	98.74	6.63	99.87	2.90
1377.4	98.08	5.64	97.51	5.64	97.41	1.37	99.79	2.81

Method Application

Table 6. Comparison of insulin sample concentrations assayed by 2D-LC-MS/MS and immunoassay methods.

Sample	2D-LC-MS/MS conc. (pmol/L)	Immunoassay conc. (pmol/L)	% Difference
1	671	656	2.33
2	636	646	-1.49
3	562	590	-4.75
4	647	615	5.10
5	806	786	2.57
6	1261	772	48.15
7	414	610	-38.32
8	404	654	-47.31
9	817	767	6.30
10	932	909	2.45

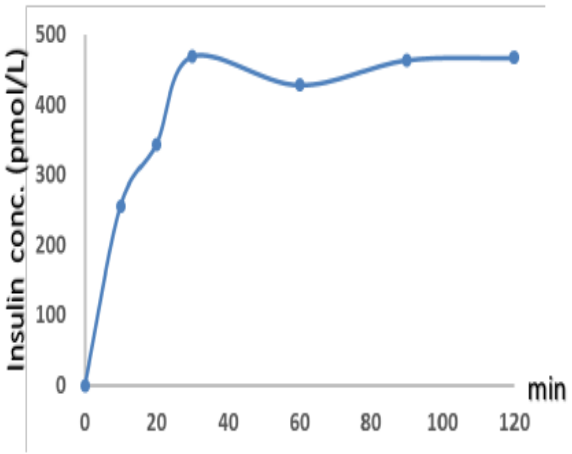

Figure 4: Pharmacokinetic curve of insulin derived from a human subject*.

*Samples were derived from a study of insulin sensitivity via euglycaemic, hyperinsulinaemic clamp technique. After an overnight fast of ten hours, two polythene cannulae are inserted, one into an antecubital vein for infusion of 20% dextrose solution and insulin, and the second into the contralateral antecubital vein for regular blood sampling.

The 2D-LC-MS/MS validated method was assayed and it was compared against immunoassay method. The results are shown in table 6, the difference % is <±6.5% except sample 6, 7, and 8 which is >±38.3%. In figure 4, a pharmacokinetic studies using this method was done for 2 hrs with intervals of 20mins.

❑ Conclusions

A 2D-LC-MS/MS method for quantitation of insulin in human serum was developed and validated. The results data have been evaluated with the FDA guidance for industry on bioanalytical on method validation; based on the selectivity, accuracy, precision, recovery, calibration curve, sensitivity, reproducibility and stability.

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