A novel, simple and sensitive LC-MS/MS method for simultaneous quantification of insulin glargine and its metabolites (M1 and M2)

Avinash Gaikwad[1], Chaitanya Krishna[1], Yogesh Arote[1], Sujit Patil[1], Ajit Datar[2] , Pratap Rasam[2] , Jitendra Kelkar[2]

[1] ADC - Shimadzu Analytical (India) Pvt. Ltd., Nerul, Navi Mumbai-400706, Maharashtra, India.

[2] Shimadzu Analytical (India) Pvt. Ltd., 1 A/B Rushabh Chambers, Makwana Road, Marol, Andheri (E), Mumbai-400059, Maharashtra, India.

1. Overview

Glargine and its active metabolites (M1 and M2) were analyzed in human plasma using a solid phase extraction method in MRM mode on LCMS-8060. The developed method is novel, simple and sensitive for simultaneous estimation of glargine and its active metabolites in human plasma.

2. Introduction

Insulin Glargine is a recombinant human insulin analog with long-acting, blood glucose-lowering activity [1]. Insulin glargine differs from human insulin by replacing asparagine with glycine in position 21 of the A-chain and by carboxy-terminal extension of B-chain by 2 arginine residues. After subcutaneous injection, glargine undergoes an enzymatic removal of the basic arginine pair at positions 30B and 31B to yield 21A-Gly-human insulin (metabolite 1 [M1]), analogous to prohormone activation, with some further loss of threonine to 21A-Gly-des-30B-Thr-human insulin (metabolite 2 [M2]) [2].

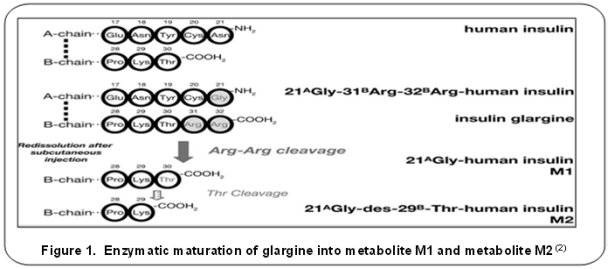

Figure 1. Enzymatic maturation of glargine into metabolite M1 and metabolite M2 [2]

Subcutaneous dose of glargine results in very low plasma levels and thus requires a sensitive method for estimation of glargine and its metabolites (M1 and M2) in human plasma. Structural similarities of glargine metabolites with insulin and insulin analogues will not permit simultaneous determination of glargine metabolites. In addition, the critical challenges associated with the method development of glargine and its metabolites (M1 and M2) are poor ionization, non-specific adsorption and carryover issues.

There are variety of traditional methods for quantifying insulin glargine (along with M1 and M2) like ELISA and LC-MS/MS using sample preparation kit and have significant drawbacks. This motivated us for the current study, aiming at developing a novel, simple and highly sensitive method on LCMS 8060, to quantify insulin glargine and its active metabolites M1 and M2 in a single run.

3. Materials and methods

3-1. Sample preparation

• Preparation of calibration curve standards and quality control (QC) samples

Calibration standards of Insulin glargine, M1 and M2 were prepared in human plasma at concentration levels ranging from 75 to 10000 pg/mL. Quality control samples were prepared at concentration levels between 100 to 2000 pg/mL pg/mL for glargine and its metabolites (M1 and M2) respectively.

• Sample extraction

Spiked calibration standards and quality control samples in plasma were diluted with extraction buffer in 2.0 mL micro centrifuge tubes. Samples were vortexed and centrifuged at 12000 rpm for 15 minutes and further processed using solid phase extraction as per the protocol mentioned below:

Extraction protocol:

Conditioning and equilibration (1mL methanol followed by 1 mL water)
Sample loading
Wash 1 (1 mL wash solution 1 x 2 times)
Wash 2 (1 mL wash solution 2 x 1 time)
Elution (1 mL of elution solution)

SPE eluent was evaporated at 40 ^{0}C till dryness in nitrogen evaporator. The residue was reconstituted in 100 µL of reconstitution solution, vortexed and filled in HPLC vials for injection.

3-2. LC-MS/MS analysis

Figure 2. Nexera X2 with LCMS-8060

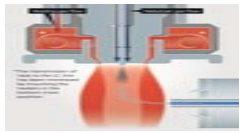

Figure 3. Heated ESI probe

The details of LCMS conditions are given in Table 1.

Table 1. Instrument parameters for analysis of glargine and its metabolites (M1 and M2)

UHPLC condition (Nexera X2)		MS parameters (LCMS-8060)	
Column	Shim-pack velox biphenyl column 100 mm×2.1 mm, 2.7 um (Shimadzu)	MS interface	Electro Spray Ionization (ESI)
Mobile phase	A: 0.1 % formic acid in water B: 0.1 % formic acid in acetonitrile	Nitrogen gas flow	Nebulizing gas- 3 L/min; Drying gas- 10 L/min
Flow rate	0.2 mL/min	Zero air flow	Heating gas- 10 L/min
Elution mode	Flow gradient mode	MS temp	Desolvation line- 250 °C; Heating block- 400 °C; Interface- 300 °C
Column temp	40 °C		

4. Results

4-1. Selectivity

Selectivity of the method was evaluated by analyzing 6 different lots of blank human plasma and blank plasma spiked with glargine, M1 and M2 at LLOQ level. No significant interference was observed at the retention time and MRM transition of analytes (refer figure 4A, 4B & 4C).

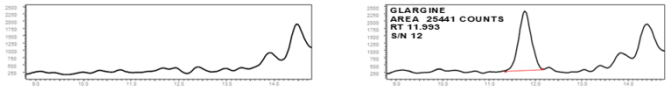

Fig. 4A. Chromatograms of glargine (extracted blank and extracted LLOQ)

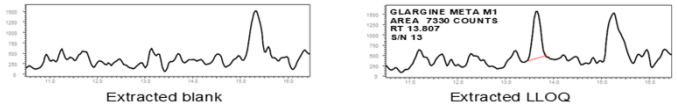

Fig. 4B. Chromatograms of metabolite M1 (extracted blank and extracted LLOQ)

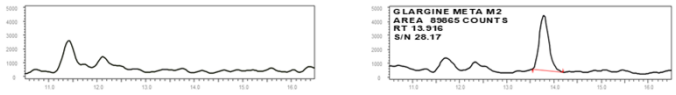

Fig. 4C. Chromatograms of metabolite M2 (extracted blank and extracted LLOQ)

4-2. Recovery

Recovery experiments was studied at LQC, MQC and HQC level. Mean recovery for glargine, M1 and M2 were 33.31% , 51.52% and 50.75% respectively. Recovery was found precise, consistent and reproducible at all levels

4-3. Linearity

Calibration curves were linear from 75 to 10000pg/mL with correlation coefficient r2 > 0.98 for glargine, M1 and M2. The observed mean back calculated concentration of calibration standards with accuracy and precision from 3 linearity are given in Table 2.The accuracy and precision for the calibration standards were found within acceptance criteria.

Table 2. Calibration curve data of glargine and its metabolites (M1and M2)

Calibration curve data of glargine									
Level	CC1	CC2	CC3	CC4	CC5	CC6	CC7	CC8	CC9
Nominal conc.	76.53	203.74	381.09	563.31	716.82	1026.36	2546.35	5463.53	10626.39
Mean (n=3)	81.58	190.35	371.45	614.08	762.90	1127.77	2545.41	5672.77	10979.63
SD	4.51	15.39	10.24	66.86	49.94	159.17	84.32	750.19	372.50
% RSD	5.53	8.09	2.76	10.89	6.55	14.11	3.31	13.22	3.39
% Nominal	108.73	93.87	90.91	100.20	98.19	112.61	102.70	114.44	110.75
Calibration curve data of metabolite M1									
Level	CC1	CC2	CC3	CC4	CC5	CC6	CC7	CC8	CC9
Nominal conc.	75.68	204.53	412.11	618.17	783.70	1010.13	2500.00	5000.00	10000.00
Mean (n=3)	77.60	227.56	375.58	568.79	825.03	1029.27	2710.17	5500.28	10603.58
SD	5.46	30.33	31.94	31.65	92.80	46.76	73.87	217.16	822.41
% RSD	7.04	13.33	8.50	5.56	11.25	4.54	2.73	3.95	7.76
% Nominal	102.54	111.26	91.14	92.01	105.27	101.89	108.41	110.01	106.04
Calibration curve data of metabolite M2									
Level	CC1	CC2	CC3	CC4	CC5	CC6	CC7	CC8	CC9
Nominal conc.	75.68	204.53	412.11	618.17	783.70	1010.13	2500.00	5000.00	10000.00
Mean (n=3)	74.92	207.28	372.68	604.37	784.99	1083.92	2822.83	5426.41	10552.30
SD	3.16	25.69	25.15	54.23	73.77	145.50	308.88	285.01	1535.84
% RSD	4.22	12.39	6.75	8.97	9.40	13.42	10.94	5.25	14.55
% Nominal	99.00	101.34	90.43	97.77	100.16	107.31	112.91	108.53	105.52

4-4. Intra-day and inter-day precision and accuracy

Intra-day and inter-day precision were within acceptance criteria of <20% at LLOQ QC and < 15% at LQC, MQC and HQC. Percent accuracy for both intra-day and inter-day were within acceptance criteria of 80 – 120% at LLOQ QC and 85% - 115% at LQC, MQC and HQC for glargine, M1 and M2. Refer figure 5 & 6 for trend plot of intra-day and inter-day precision and accuracy.

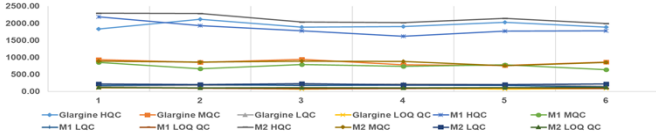

Figure 5. Trend plot of intra-day QC – over lay of insulin glargine and its metabolites M1 and M2

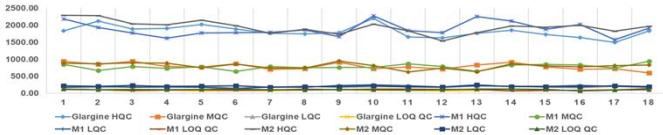

Figure 6. Trend plot of global QC – over lay of insulin glargine and its metabolites M1 and M2

5. Conclusion

To best of our knowledge this is the first report of fully validated method which is simple, sensitive and that can rapidly and reliably quantitate glargine, M1 and M2 in human plasma. Ultra-high speed and high-separation analysis was achieved on Nexera X2 UHPLC by using a simple mobile phase at a minimal gradient flow rate of 0.2 mL/min. By providing this ready to use solutions, we partner with your labs to achieve desired results in your scientific endeavors.

6. References

1. https://pubchem.ncbi.nlm.nih.gov/compound/Insulin-glargine (accessed July 31, 2019)
2. Geremia B. Bolli, et al., Plasma Exposure to Insulin Glargine and Its Metabolites M1 and M2 After Subcutaneous Injection of Therapeutic and Supratherapeutic Doses of Glargine in Subjects With Type 1 Diabetes. Diabetes Care. 35(12): 2626–2630: Dec 2012.