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Establishing measurement of uncertainty for simultaneous bio-analytical method by LC-MS/MS

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ABSTRACT

The objective of this activity is to evaluate the expanded uncertainty in the simultaneous bio-analysis of L-Carnitine, an endogenous compound and Metformin a biguanide which is used as treatment for T2DM. This simultaneous method is developed and validated using LCMSMS in human plasma[1]. This method is developed to establish a single method which can be used as the prognostic tool, TDM and for bio-analysis drug Metformin of T2DM. To use this method for bio-analysis and TDM it is validated as per ICH guidelines and found to be within the acceptance criteria. Using the precision data the combined uncertainty was calculated as per the GUM guidelines and the MOU for the method was established. Method was developed using the UFLCMS (Ultra Flow liquid chromatography mass spectrometer) for the simultaneous estimation of Metformin and L-Carnitine in human plasma with LOQ and ULOQ of 43.483ng/ml and 639.450 ng/ml for Metformin and 2.289µg/mL and 33.675 µg/mL for L-Carnitine respectively. Linearity was established by considering Concentration maximum for Metformin and normal value of L-Carnitine[1]. The precision data was used for evaluating the MOU as per GUM guidelines[2]. MOU was found to be ±1.89 for L-Carnitine and ± 0.9 7 for Metformin. MOU is needed to be considered for analysis and the validated data need is evaluated by applying the correction. As the MOU is established this method can be used as a prognostic tool and by calculating the systematic and random error the possible method error is evaluated.

Keywords: L-Carnitine, Metformin, Type 2 Diabetes, LCMSMS

INTRODUCTION

As per the regulatory guidelines any method of analysis should be validated to ensure method goodness, applicability and acceptability. Method validation can be full if it's a new method developed, partial if any changes made in the range or chromatographic conditions or transfer if it's validated in one lab. Before initiating a validation all the instruments and equipment's which will be used for validation should be qualified and calibrated or if glassware used should be of class A. Each validation needs a set of parameters to be proved before accepted for analysis.

Measurement of Uncertainty as per GUM procedure is a requirement for diagnostic laboratories to evaluate the errors and this is the requirement of accreditations as per ISO/IEC 17025 and ISO 15189.

By carrying out Uncertainty, Random and Systematic errors are identified and so method reliability is established. Data generated from conducting the validations are adequate for carrying out the uncertainty calculations. Method validations prove the reliability of the method in actual conditions and by calculating the uncertainty of the method procedure and process are ensured for effective, stable and in control.

N,N-Dimethylimidodicarbonimidic diamide is the IUPAC name for Metformin, which is an oral biguanide medication which is used alone or in combination in the treatment of Type II diabetes.

L-Carnitine**,** (3R)-3-Hydroxy-4-(trimethylammonio)butanoate is a quaternary ammonium compound present endogenously in humans. This is synthesized in liver and kidneys from lysine and methionine.

MATERIALS AND METHODS

Instrumentation
Mass spectrometer – Triple Quadrupole (API 4000) from MD SCIEX
Liquid chromatography - UFLC XR from Shimadzu
Software - Analyst Software version 1.5.1
Volumetric flask - 10mL
Calibrated Thermometer
Millipore
Barometer calibrated
Calibrated analytical balance 210mg
Calibrated Micro balance

Materials/ Reagents
Standards
Metformin (Sigma Aldrich)
L-Carnitine (Sigma Aldrich)
Chemicals
Acetonitrile – JT Baker, HPLC Grade
Ammonium Formate – Sigma Aldrich, AR Grade
Ethanol – Ranchem, HPLC Grade
Methanol – Merck, HPLC Grade
Purified water - Milli-Q Water

EXPERIMENTAL:
This method was developed for simultaneous estimation of L-Carnitine and Metformin in human plasma using LCMSMS and validated as per ICH guidelines. Simultaneous method was developed with an intention of considering the applicability of the method as diagnostic tool, for TDM and drug development for T2DM. To use this method for TDM and bioequivalence, it is validated as per ICH guidelines.

Evaluation of MOU is discussed here and found to be acceptable.
Harmonised Guide to the expression of uncertainty in Measurement ie GUM if followed internationally for calculating the uncertainty measurement. By these guidelines standard uncertainty is calculated by estimating the standard deviation in replicate analysis or by calculating the fit in the calibration curve. Standard uncertainty is evaluated in two ways i.e estimation by statistical means – type A and type B based on the scientific judgement.

Finally Combined Standard uncertainty is got by the root sum of all squares method and then the expanded uncertainty is calculated by including the Factor for agreed coverage probability usually P=95%.

Method was validated as per ICH guidelines for below parameters and range, best fit and method ruggedness was proved during Method development. Range was selected as per the Normal value for L-Carnitine i.e 36.57 micromole/L[3] and concentration maximum for Metformin i.e 1-2.5 micro gram /mL[4,5]. So the range selected was 4.8 to 240.2 ng/mL and 2.5 to 250.3 ng/ml for L-Carnitine and Metformin respectively.

Method validation was carried out as per USFDA[6] and ICH guidelines[7] and the parameters validated were as below.

- Specificity and selectivity
- Matrix effect
- Carry over test
- Ruggedness
- Precision and Accuracy
- Recovery
- Reinjection Reproducibility
- Dilution Integrity

- Stability (FT, BT, DE, WE, LT)

The weighting factor 1/X 2, which was established in method development, is utilized for the Method validation and Sample analysis.

Measurement of uncertainty was carried using the precision data at QCM level from validation. The data was considered from the P&A which was within the acceptance criteria as per Bio-analytical method validation.

CALCULATING THE MEASUREMENT OF UNCERTAINTY:
It was ensured all instruments and equipment were calibrated and glassware used were of class A.

Measurement of uncertainty:
Minimum Six repetitions were carried for any MOU calculation. This is applicable for Method repeatability or Instruments.

- Method repeatability (Type A) is carried at the know concentration for six times using the established conditions and parameters.
- For evaluating the accuracy of analytical instruments like HPLC, LCMSMS six repetitions were carried at known concentration and accuracy is calculated. Mostly analytical instruments were considered in the rectangular distribution.
- For instruments like Weighing Balances, Micropipette working range was considered and repetitions were carried at 50% of maximum capacity or workable range.
- Glassware is done by calculating the weight at filled status Standard uncertainty was calculated as SD/√n=no of repetitions.

Relative standard uncertainty was calculated as

Standard Uncertainty multiplied by Value X, in which,

X=1 for parameters measuring parameters i.e 1 for method repeatability, 10 for 10ml Volumetric flask etc

Then combined uncertainty is calculated by square rooting after adding up all uncertainty squared values.

Finally expanded uncertainty is given as

K X Combined uncertainty, where K is the confidential interval considered.

Here confidential interval considered is 95% so, K is 2.

Type A – Repeatability of Method
Six repetitions at QCM level ie 3.23 Micro gram per mL for L-Carnitine and 358.092 nanogram per mL for metformin was carried out.

Sample was prepared using protein precipitation extraction
Chromatographic and instrument conditions were as per the developed method[1]
Repeatability was calculated as SD/√n. (Refer Table.1)

Table 1: It shows repeatability of six injections at QCM level

Parameter	A	B	C	D	E	F
Repeatability L-Carnitine	91.386	91.631	93.734	91.179	89.385	92.835
Repeatability Metformin	98.023	98.736	97.983	98.701	100.339	100.97

n = 6 n-1= 5

$$\text{Mean } (X) = \frac{A+B+C+D+E+F}{n}$$

L-Carnitine = 91.69
Metformin = 99.13

Standard Deviation

Table 2: It shows the calculation of standard deviation

	$(A-X)^2$	$(B-X)^2$	$(C-X)^2$	$(D-X)^2$	$(E-X)^2$	$(F-X)^2$
L - Carnitine	0.0934321	0.0036804	4.17112544	0.262827111	5.320711	1.307211
Metformin	1.215138778	0.151580444	1.304925444	0.180058778	1.472986778	3.402795111

$$\text{Standard Deviation} = \sqrt{\frac{(A-X)^2+(B-X)^2+(C-X)^2+(D-X)^2+(E-X)^2+(F-X)^2}{n-1}}$$

L- Carnitine = 2.312619434
Metformin = 1.185846264

$$\text{Repeatability, Ua} = \frac{\text{Standard Deviation}}{\sqrt{n}}$$

L-Carnitine = 0.9441229
Metformin = 0.4841197

Type B – Instruments & Glass wares:
Balance:

- Both Micro and analytical balance were used during the validations and analysis
- Weighing range: Analytical balance – 0.05gm to 200 gm and Micro balance – 5 mg to 5 Gms
- Standard and relative standard uncertainty was calculated for 0.05gm, 0.5gm, 10, 50 and 200 gm for analytical balance and for 5mg, 50 mg, 100mg, 1gm and 5 gm for Microbalance.
- For analytical balance 10gms and Micro balance 1gm is considered for MOU calculations[8] Refer Table 3 and 4.

Table 3: It shows Analytical balance MOU calculation

Weight Used	50.0 mg	500.0 mg	10000.0 mg	50000.0 mg	200000.0 mg
Actual weight (mg)	49.9996	499.9991	9999.990	49999.940	199999.950
S.No	**Observed Weight (mg)**				
1	49.99	500.01	10000.12	50000.35	200001.8
2	50.00	500.01	10000.13	50000.35	200001.8
3	50.00	500.02	10000.13	50000.36	200001.7
4	50.00	500.01	10000.13	50000.35	200001.5
5	50.00	500.00	10000.13	50000.32	200001.6
6	50.00	500.03	10000.12	50000.32	200001.6
7	50.00	500.01	10000.12	50000.33	200001.5
8	50.00	500.01	10000.12	50000.33	200001.5
9	50.00	500.01	10000.13	50000.33	200001.4
10	50.00	500.01	10000.13	50000.33	200001.4
Mean	49.999	500.012	10000.126	50000.337	200001.58
S.D.	0.0032	0.0079	0.0052	0.0142	0.148
Systematic Error (SE)	0.0095	0.0237	0.0155	0.0425	0.4427
MOU	0.00019	0.00005	0.00000	0.00000	0.00000

LC-MS/MS (Refer Table 5):

- Six repeats at QCM concentration was considered as system suitability level and considered for the evaluation
- Area of each repetition was considered and Standard deviation was calculated.
- Then standard uncertainty was calculated as RSD/√3, considering rectangular distribution.

Table 4: It shows Micro balance MOU calculation

Weight Used	5.0 mg	50.0 mg	100.0 mg	1000.0 mg	5000.0 mg
Actual weight (mg)	5.0006	49.9996	99.9998	999.999	4999.998
S.No	Observed Weight (mg)				
1	5.003	50.014	100.003	1000.011	5000.004
2	5.003	50.014	100.003	1000.011	5000.003
3	5.004	50.013	100.003	1000.009	5000.003
4	5.004	50.013	100.004	1000.009	5000.003
5	5.004	50.015	100.002	1000.011	5000.002
6	5.005	50.015	100.002	1000.011	5000.002
7	5.002	50.015	100.002	1000.010	5000.002
8	5.002	50.015	100.002	1000.010	5000.003
9	5.002	50.014	100.003	1000.010	5000.003
10	5.002	50.015	100.003	1000.011	5000.002
Mean	5.0031	50.0143	100.0027	1000.0103	5000.0027
S.D.	0.00110	0.00082	0.00067	0.00082	0.00067
Systematic Error (SE)	0.00330	0.00247	0.00202	0.00247	0.00202
MOU	0.00066	0.00005	0.00002	0.00000	0.00000
Tolerance		≤0.001			

Table 5: It shows calculating the standard uncertainty for LCMSMS

S.No.	Area L- Carnitine	Area Metformin
1	2437165.135	170420.768
2	2479311.13800	171639.70500
3	2432810.06000	170351.67700
4	2448934.42900	171581.16900
5	2415114.99900	174382.07600
6	2411612.91900	175461.90100
Mean	2437556.709000	172683.305600
S.D.	27713.38177	2141.65313
% RSD	1.14	1.24
STD Uncert	0.656408459	0.71604143

- Finally Relative standard uncertainty is calculated by multiplying Standard uncertainty with 1 as value X

Micropipette (Refer Table 6):

- Calibrated 1 mL Micropipette as per Anivsa guidelines were used in the validation and analysis
- Collected 10 ml Purified water. Millipore water purification system was used and water used is freshly collected.
- Measured Water temperature using calibrated thermometer and temperature correction was evaluated and recorded.
- Clean dry beaker was tared in balance and 1 ml water dispensed into beaker and noted the weight
- Repeated above steps for six times
- All weighing's are noted and corrected for temperature
- Relative standard deviation was calculated as SD/Mean X 100
- Finally MOU was calculated as SD/√N, where N is number of repetitions.

Table 6: It shows 100ul MOU calculation

Water Temperature:		25.0°C
Temperature Correction:		0.004
S.No.	Weight (g)	Capacity (mL)
1	1.0008	1.00480
2	1.0006	1.00460
3	1.0000	1.00400
4	1.0005	1.00450
5	1.0001	1.00410
6	1.0007	1.00470
Mean		1.004450
S.D.		0.00033
% RSD		0.03
MOU		0.000

Glassware (Refer Table 7):

- Volumetric flask – 10ml and 50 ml
- Used for Sample preparation, Bulk spiking, solution preparation

- Tare the weighing balance
- Collect water, measure and note the temperature
- Fill the volumetric flask with water
- Weigh and note the reading
- Repeat above for six times and include the temperature correction
- Calculate the Standard deviation
- MOU was calculated as SD/√N=6

Table 7: It shows the calculation of MOU for 10 and 50 mL volumetric glassware

Type of Glassware:		**Volumetric Glassware, 10 mL & 50 mL**		
Water Temperature:		25.0°C	**Atmospheric Pressure:**	770 mmHg
Temperature Correction:		0.040	**Pressure Correction:**	0.000
S.No.	**Weight (g)**	**Capacity of Vessel (10 mL)**	**Weight (g)**	**Capacity of Vessel (50 mL)**
1	9.93448	9.97448	49.7044	49.70440
2	9.92015	9.96015	49.6863	49.68630
3	9.92750	9.96750	49.6926	49.69260
4	9.94178	9.98178	49.7151	49.71510
5	9.92348	9.96348	49.7323	49.73230
6	9.93287	9.97287	49.7260	49.72600
Mean		9.97004		49.70945
S.D.		0.00791		0.01829
% RSD		0.08		0.04
MOU = SD/ √N ie 6		0.003		0.007

RESULTS

Type A: Systematic error (Refer Table 8,9)
Instrument Repeatability, Ua = Standard deviation / √n

Type B : Random error (Refer Table 8,9)
Standard Uncertainty for Micropipette, Uvol 1= √ (Up2) = 0.0000
Standard Uncertainty Volumetric flasks by averaging, Uvol 2= √ (Uvf2) = 0.00535
Standard uncertainty Weighing balance, Analytical balance UAb = 0.0000000 & Microbalance UMb = 0.0000000
Combined Uncertainty is measured from the individual uncertainty of Type A and Type B

Table 8: It shows calculation of Relative standard uncertainty of Type A and Type B for L-Carnitine

TYPE OF EVALUATION	**DESCRIPTION**	**VALUE X**	**STANDARD UNCERTAINTY UX**	**RELATIVE STD. UNCERTAINTY U(X)/X**
Type A	**Repeatability, Ua**	1	0.9441229	0.94412293
Type B	**Instrument performance, Uins**	1	0.006350853	0.006350853
	Micropipette, Up	10	0.000000000	0
	Volumetric Flask, Uvf10	10	0.003000000	0.0003
	Volumetric Flask, Uvf50	50	0.007000000	0.00014
	Analytical Balance	0.05	0.000000000	0.000000000
	Micro Balance	10	0.000000000	0.000000000

Table 9: It shows calculation of Relative standard uncertainty of Type A and Type B for Metformin

TYPE OF EVALUATION	**DESCRIPTION**	**VALUE X**	**STANDARD UNCERTAINTY UX**	**RELATIVE STD. UNCERTAINTY U(X)/X**
Type A	**Repeatability, Ua**	1	0.4841197	0.48411971
Type B	**Instrument performance, Uins**	1	0.006350853	0.006350853
	Micropipette, Up	10	0.000000000	0
	Volumetric Flask, Uvf10	10	0.003000000	0.0003
	Volumetric Flask, Uvf50	50	0.007000000	0.00014
	Analytical Balance	0.05	0.000000000	0.000000000
	Micro Balance	10	0.000000000	0.000000000

The combined Uncertainty,
Uc = √ Ua2 + Uins2 + Up2 + Uv10f2 + Uv10f2+UAbal2 +UAbal2

- L-Carnitine Combined Uncertainty , Uc =0.944144348
- Metformin Combined Uncertainty, Uc = 0.484161478

Expanded Uncertainty,
UE = K X Uc =1.888288697, where k = 2 at confidence level 95.45%

- L Carnitine = ±1.888288697
- Metformin = ± 0.968322956

CONCLUSION

The above simultaneous method for estimation of L-Carnitine and Metformin in human plasma using LCMSMS was accurate and precise which is within the acceptance criteria as per Bio-analytical method validation as per ICH guidelines and the expanded uncertainty is calculated as ±1.888.

So this method's applicability for Therapeutic drug monitoring, as prognostic tool, for pharmacokinetic studies or using biomarker in drug discovery studies is validated.

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