International Journal of PharmTech Research
CODEN (USA): IJPRIF, ISSN: 0974-4304
Vol.7, No.3, pp 516-527, 2014-2015

Simultaneous Estimation and Analytical Validation of L-Carnitine and Metformin for Type II Diabetes using LC-MS/MS

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Abstract: An Ultra Flow liquid chromatography mass spectrometric simultaneous method for the estimation of L-Carnitine and metformin, in human plasma was validated over a concentration range of 2.289 µg/mL to 33.675 µg/mL for L-Carnitine and 43.483ng/ml to 639.450ng/ml for Metformin. Sample extraction was carried by Protein precipitation and chromatography using Princeton C18, 50 x 4.6 mm, 5µm column with gradient mobile phase consisting of (80:20, v/v) acetonitrile and 2 mm ammonium formate. This method was validated as per the regulatory requirements for specificity and selectivity, matrix effect, carryover, and quality control samples data, precision and accuracy, and recovery. This method can be applicable for Bioequivalence, Therapeutic drug monitoring and as a prognostic tool for Type 2 Diabetes (T2DM), if proved.
Keywords: L-Carnitine, Metformin, Type 2 Diabetes, LCMSMS (Liquid Chromatography Mass Spectrometer), ICH (International Conference of Harmonisation) Guidelines.

Introduction

Diabetes Mellitus Type II, the most common form of diabetes is a metabolic disorder that is characterized by high blood sugar levels in the body. It is caused when the pancreas either produces less insulin to maintain a normal blood glucose level or the human body could not use the insulin that is produced. This increases the level of glucose in blood and glucose will not available for metabolism. The primary cause of Type II diabetes is thought to be obesity in people who are predisposed to the disease. The first line of treatment for the disease is making proper dietary changes, exercise and weight control. But, if these measures do not bring down the blood sugar levels under control, drugs like Insulin or Metformin is prescribed for the patient.

Currently glucose level is studied using A1C level in blood and an A1C level above 6.5% indicates diabetes. In diabetes, the glucose which is taken up by the tissues is utilized as fuel since the oxidation of glucose is increased with the administration of L-Carnitine [1].

L-Carnitine, an essential amino acid is the biologically active stereoisomer of the non-essential amino acid carnitine and is bio-synthesized from two amino acids namely methionine and lysine. β -hydroxy-γ-N-trimethyammonium butyric acid is the chemical name of L-Carnitine which is a quaternary ammonium compound. L-Carnitine is involved in the transport of certain fatty acids into cells where they go through a process of oxidation. As a result of this process, energy is released. It plays an important function in transporting LCFA's (long chain fatty acids) as cylcarnitine esters into the matrix mitochondrialis from the cytosol, where β -oxidation takes place. L-Carnitine has a protective effect on lipid peroxidation by reducing the formation of hydrogen peroxide. It is also known to improve the antioxidant status in rats and demonstrated free radical scavenging activity and inhibits the build-up of free fatty acids and their toxic intermediates, thus counteracting their damaging effects on the cell membranes and the mitochondria. L-Carnitine supplementation improves the use of fat as a source of energy, lowers triglyceride and cholesterol levels and may minimize the

risk of health problems in diabetics, who have impaired fat metabolism. Also, studies shows that L- Carnitine levels decrease in T2DM patients [2-4].

Metformin, a biguanide medication is used alone or in combination with other medications in the treatment of Type II diabetes. It is an FDA approved drug for the treatment of Diabetes Mellitus II in the year 1994. Metformin is the first medication often prescribed by physicians if the blood sugar levels in the body is not controlled by diet, exercise and weight control.

Till date a single method which can be used for identifying diabetes, TDM studies, for clinical trials is not available. Considering this, the study aims to develop a simultaneous method for L-Carnitine and Metformin to study the method as a prognostic tool [5-7].

Bio-analytical methods by LCMSMS are very accurate and precise, specific to the molecule of interest. So a simultaneous method with considered Biomarker of T2DM and drug used for diabetes was developed and validated in this study. Here simultaneous method was developed considering L-Carnitine as biomarker and Metformin as T2DM drug and validated as per USFDA and ICH guidelines using LCMSMS [8,9].

Materials and Methods

Instrumentation:

Liquid chromatography - UFLC (Ultra Fast Liquid Chromatography) XR from Shimadzu, Mass spectrometer - MS/MS (API 4000) from MD SCIEX, Software - Analyst Software version 1.5.1

Reagents / Materials:

Standards:

Analyte 1 - L-Carnitine (Sigma Aldrich, Purity), Analyte 2 – Metformin, Sigma Aldrich, Purity.

Chemicals:

Ammonium Formate – Sigma Aldrich [AR (analytical reagent) Grade], Acetonitrile – JT Baker [HPLC (High Performance Liquid Chromatography) Grade], Methanol – Merck [HPLC Grade], Ethanol – Ranchem [HPLC Grade], Purified water - Milli-Q Water.

Experimental:

The bio-analytical method used for measurement of analyte(s) [drug(s) and /or its metabolite(s)] content in biological matrix (like blood, plasma, serum, or urine) should be demonstrated to be reliable and reproducible. Bio-analytical method validation is demonstrating that the performance characteristics of the method are reliable and suitable for the intended application through the use of specific laboratory investigations. These investigations include the parameters such as accuracy, precision, sensitivity, selectivity, recovery, reproducibility, and stability.

Method validation was carried out as per USFDA and ICH guidelines and the parameters validated are as below.

- Specificity and selectivity
- Matrix effect
- Carry over test
- Ruggedness
- Precision and Accuracy
- Recovery
- Reinjection Reproducibility
- Dilution Integrity
- Stability [FT (Freeze Thaw), BT (Bench Top), DE (Dry Extract), WE (Wet Extract), LT (Long Term)]

Solution preparation

Preparation of L-Carnitine Stock solution

Weighed accurately 20.880 mg of L-Carnitine working standard and transferred into a 5 mL of volumetric flask, dissolved in methanol and made up the volume with the same to make up a solution of 4.09248 µg/mL. Corrected the above concentration of L-Carnitine accounting for its potency, molecular weight and the actual amount weighed

Preparation of Metformin Stock solution

Weighed accurately 69.000 mg of Metformin working standard and transferred into a 5 mL of volumetric flask, dissolved in methanol and made up the volume with the same to make up a solution of 5.526304 µg/mL. Corrected the above concentration of Metformin accounting for its potency, molecular weight and the actual amount weighed.

Mobile Phase

Mobile Phase A - Buffer (2mM Ammonium Acetate):

About 77.08 mg of ammonium Acetate was weighed and dissolved in milli-Q-water to obtain approximately 500 mL of the buffer. Mixed well, sonicated and filtered. Appropriate proportion used to prepare different volumes of solution. This solution was stored at room temperature and used within two days.

Mobile Phase B – Acetonitrile:

Mobile phase gradient is shown in Table 1.

Table No:1 Mobile Phase gradient

Time	Mpa	Mpb
0.01	70	30
0.40	70	30
1.50	40	60
1.60	40	60
1.75	30	30
2.00	30	30

Diluent Solution / Rinsing Solution (Water : Acetonitrile :: 50:50, v/v)

Prepared a mixture of milli-Q water and acetonitrile in the ratio of 50:50. Mixed well, and sonicated. This solution was stored at room temperature and used within seven days. Prepare the diluent solution / rinsing solution as and when required.

Selection of bio-matrix for spiking

Selectivity is the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample.

Minimum of six different lots were taken containing same anticoagulant and from these two aliquots were taken. Spike one aliquot of each blank sample with the analyte at LLOQ (Lowest Limit of Quantification) level.

Take the other aliquot as blank sample. Analyze all spiked and blank samples using the method being validated.

Six human plasma lots (PL12/10, PL13/10, PL14/10, PL15/10, PL16/10 and PL17/10) were screened for specificity and selectivity test` by injecting processed blank matrix and LLOQ samples spiked in each blank matrix lot.

As L-Carnitine is endogenously present Blanks were injected repeatedly and averaged for correction. All the six human plasma lots were free of any significant interference after correction.

And for Metformin all the six human plasma lots were free of any significant interference

Calibration Curve Standards and Quality Control Samples:

Calibration curve standards that consist of a set of 6 non-zero concentrations were prepared ranging from 2.289 µg/mL to 33.675 µg/mL for L-Carnitine and 43.483ng/ml to 639.450ng/ml for metformin. Prepared quality control samples consist of L-Carnitine concentrations of 2.694 µg/mL [QCLLQ (Quality control Lowest Limit of Quantification)], 10.776 µg/mL [QCL (Quality control Low)], 18.858 µg/mL [QCM (Quality control middle)]and 32.328 µg/mL [QCH (Quality control High)] and 51.156ng/mL (QCLLQ), 204.624ng/mL (QCL), 358.092ng/mL (QCM) and 613.872ng/mL (QCH) for metformin. These samples were stored below -50 °C until used. Six sets of QCL and QCH were stored to below -20 °C freezer for generation of below -20 °C stability. Ranges were selected based on the normal values for L-Carnitine and Cmax for Metformin.

Preparation of CC and QC Standards:

Table 2 and 3 shows the Calibration Curve (CC) standards and Quality Control (QC) Samples prepared for L-Carnitine. Table 4 and 5 shows the Calibration Curve standards and Quality Control Samples prepared for Metformin.

Table No: 2. Preparation of CC standards for L- Carnitine

Carnitine Cc Dilutions							
Stock Id	**Stock**	**Ex**	**Ex**	**Ex**	**Ex**	**Ex**	**Ex**
Stock Conc (ng/mL)	**5526304.569**	**5388146.954**	**5388146.954**	**5388146.954**	**5388146.954**	**5388146.954**	**5388146.954**
Spike Volume (mL)	1.950	0.125	0.100	0.075	0.050	0.025	0.017
Volume Made Up TO (mL)	2.0	1.0	1.0	1.0	1.0	1.0	2.0
Conc (ng/mL)	**5388146.954**	**673518.369**	**538814.695**	**404111.022**	**269407.348**	**134703.674**	**45799.249**
Stock Id	**EX**	**AQS F**	**AQS E**	**AQS D**	**AQS C**	**AQS B**	**AQS A**
Conc In Plasma (ng/mL)		**33675.918**	**26940.735**	**20205.551**	**13470.367**	**6735.184**	**2289.962**
Stock Id		**STD F**	**STD E**	**STD D**	**STD C**	**STD B**	**STD A**

Table 3. Preparation of Quality Control Samples for L-Carnitine

Carnitine Qc Dilutions					
Stock Id	**Stock**	**Ex**	**Ex**	**Ex**	**Ex**
Stock Conc (ng/mL)	**5526304.569**	**5388146.954**	**5388146.954**	**5388146.954**	**5388146.954**
Spike Volume (mL)	1.950	0.120	0.070	0.040	0.020
VOLUME MADE UP To (mL)	2.0	1.0	1.0	1.0	2.0
Conc (ng/mL)	**5388146.954**	**646577.635**	**377170.287**	**215525.878**	**53881.470**
Stock Id	**EX**	**AQS D**	**AQS C**	**AQS B**	**AQS A**
Conc In Plasma (ng/mL)		**32328.882**	**18858.514**	**10776.294**	**2694.073**
Stock Id		**QCH**	**QCM**	**QCL**	**QCLL**

Table No: 4. Preparation of CC standards for Metformin

Metformin Cc Dilutions							
Stock Id	**Stock**	**Ex**	**Ex**	**Ex**	**Ex**	**Ex**	**Ex**
Stock Conc (ng/mL)	**4092480.000**	**102312.000**	**102312.000**	**102312.000**	**102312.000**	**102312.000**	**102312.000**
Spike Volume (mL)	0.050	0.125	0.100	0.075	0.050	0.025	0.017
Volume Made Up To (mL)	2.0	1.0	1.0	1.0	1.0	1.0	2.0
Conc (ng/mL)	**102312.000**	**12789.000**	**10231.200**	**7673.400**	**5115.600**	**2557.800**	**869.652**
Stock Id	**EX**	**AQS F**	**AQS E**	**AQS D**	**AQS C**	**AQS B**	**AQS A**
Conc In Plasma (ng/mL)		**639.45**	**511.560**	**383.670**	**255.780**	**127.890**	**43.483**
Stock Id		**STD F**	**STD E**	**STD D**	**STD C**	**STD B**	**STD A**

Table No:5 Preparation of QC samples for Metformin

Metformin Qc Dilutions					
Stock Id	**Stock**	**Ex**	**Ex**	**Ex**	**Ex**
Stock Conc (ng/mL)	**4092480.000**	**102312.000**	**102312.000**	**102312.000**	**102312.000**
Spike Volume (mL)	0.050	0.120	0.070	0.040	0.020
Volume Made Up To (mL)	2.0	1.0	1.0	1.0	2.0
Conc (ng/mL)	**102312.000**	**12277.440**	**7161.840**	**4092.480**	**1023.120**
Stock Id	**EX**	**AQS E**	**AQS C**	**AQS B**	**AQS A**
Conc In Plasma (ng/mL)		**613.872**	**358.092**	**204.624**	**51.156**
Stock Id		**QCH**	**QCM**	**QCL**	**LLOQ**

Analytical conditions

Liquid chromatographic conditions

Column Name : Princenton C18, 50 X 4.6 mm, 5µm
Mobile Phase : Gradient
Rinsing Solution : Acetonitrile: Water: 50:50 v/v
Column Oven : 35°C
Auto-injector Temperature : 10°C
Injection Volume : 10µL
Flow Rate : 0.6 mL/min
RT of L-Carnitine : 0.8 (± 0.5) mins
RT of Metformin : 1.2 (± 0.5) mins
Run Time : 2 mins

A summary of the Mass Spectrometric conditions is as follows:

Ion Source : Turbo Ion Spray Positive Ion Mode
m/z : 162/84 (L-Carnitine)
130/71 (Metformin)
Gas 1 : 50

Gas 2	:	50
Temperature	:	500
Ion Spray Voltage	:	5500
Curtain Gas	:	30
CAD	:	4
DP	:	33 (L-Carnitine), 40 (Metformin)
EP	:	10
CE	:	33 (L-Carnitine), 27 (Metformin)
CXP	:	8 (L-Carnitine), 11(Metformin)

Sample preparation

Withdrew a set of calibration curve standards and quality control samples from the deep freezer and allowed them to thaw at room temperature followed by vortexing. Aliquot 100 µL of plasma from the pre-labelled polypropylene tubes into pre-labelled polypropylene tubes and added 500 µL of ethanol and vortexed for 5 minutes in Shaker at 2000rpm. Samples were then centrifuged at 5000rpm for 5 Minutes at 4^0C. From this 100µl Supernatant was diluted with 900µl of reconstituting solvent and mixed well.

Specificity/selectivity

Selectivity is the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample.

In six different lots containing same anticoagulant, take two separate aliquots of blank samples and in one aliquot, spiked with the analytes at LLOQ level.

Considered the other aliquot as blank sample, analyzed all spiked and blank samples.

As L-Carnitine is endogenously present, Blanks were injected repeatedly and averaged for correction. All the six human plasma lots were corrected to free of interference.

Matrix effect

Blank plasma was processed from six independent sources of matrix and then spiked just before injection into the LC-MS/MS with analytes at QCL level. An aqueous solution of analyte was prepared at QCL level in Dilution solvent. Peak areas of the test samples were compared with that of reference solution to ensure that the matrix factor was consistent for different sources of matrix.

IS-normalized matrix factor should ideally be close to one. Even if it is not close to one, if the value is similar (CV <15%) in all the matrices tested the method is acceptable.

Signal to noise ratio

Signal to Noise ratio was obtained at the (LLOQ) lower limit of quantification (LLOQ) from the chromatogram by comparing the area obtained at LLOQ for each lot used in the specificity / selectivity experiment with area obtained in respective blank samples. Refer Figure 1&2.

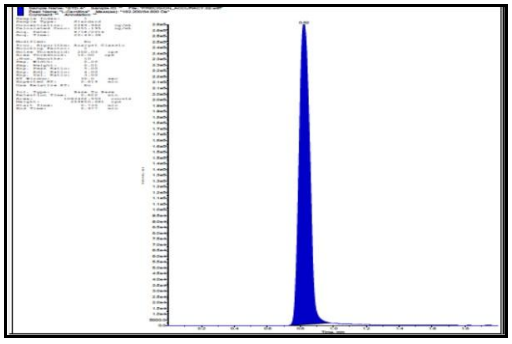

Fig 1. Chromatogram of L-Carnitine LLOQ level

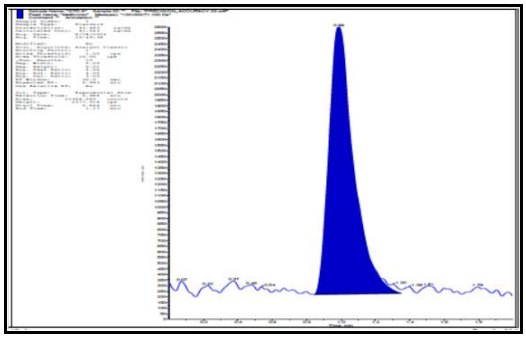

Fig 2. Chromatogram of Metformin LLOQ level

The signal-to-noise ratio obtained for the samples were greater than 5 for blank corrected for L- Carnitine and normally for metformin in all the plasma lots tested.

Carry over

Processed two blank samples, two samples of LLOQ, two samples of ULOQ.
Perform the test by injecting the following sequence:
Processed blank samples (A)
Processed samples of LLOQ
Processed samples of ULOQ
Re-inject step (A)

Linearity

A regression equation with a weighting factor of $1/x^2$ of drug produce the best fit for the concentration-detector response relationship for L-Carnitine and Metformin in human plasma. The representative calibration curves for regression analysis are illustrated in Figure 3&4.

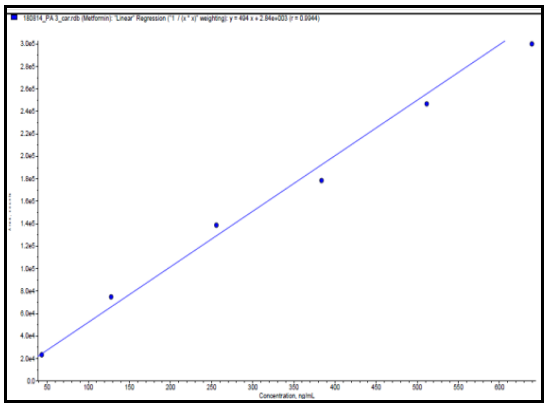

Fig 3. Linearity of L-Carnitine

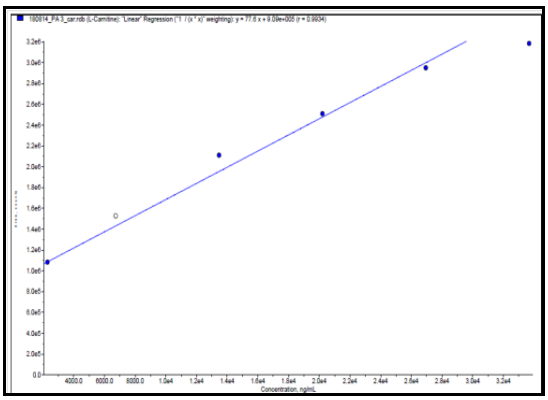

Fig 4. Linearity of Metformin

Precision and Accuracy:

The precision of the assay was measured by the percentage coefficient of variation above the concentration range of Quality Control LLOQ, Quality control Low, Quality control middle and Quality control high samples respectively during the course of validation.

The accuracy of the assay is the absolute percentage of the ratio of calculated mean values of the LLOQ, low, middle and high quality control samples to their respective nominal values.

Used the simplest workable regression equation with weighting factor 1/X2, which was established in method development.

Analyzed 3 PA (Precision and Accuracy) batches each containing the following samples spread in two days:

- Aqueous standard (concentration nearer to the middle QC concentration) to check the RT of the analyte and IS.
- One set of calibration curve standards comprising of one blank sample and at least six non-zero standards including STD A and ULOQ.
- Six replicates of QC samples at a minimum of four concentration levels were spread over the entire range of calibration curve (QCLLQ, QCL, QCM and QCH) whose concentration ranged as below

    - L-Carnitine - 2.694 µg/mL (QCLLQ), 10.776 µg/mL (QCL), 18.858 µg/mL (QCM) and 32.328 µg/mL (QCH)
    - Metformin - 51.156ng/mL (QCLLQ), 204.624ng/mL (QCL), 358.092ng/mL (QCM) and 613.872ng/mL (QCH)

Linearity

Correlation coefficients (r^2) were greater than 0.9923 in the concentration range of 2.289 µg/mL to 33.675 µg/mL for L-Carnitine.

Metformin Correlation coefficients (r^2) were greater than 0.9944 in the concentration range of 43.483ng/ml to 639.450ng/ml.

Recovery

Recovery refers to the extraction efficiency of an analytical method within the limits of variability.

Prepared aqueous Quality Control samples (unextracted) with concentrations close to QCL, QCM and QCH concentrations.

Injected six replicates of unextracted QCs along with 6 sets of processed QC samples (QCL, QCM and QCH) and compared the mean peak areas of the respective QCs.

The recovery need not have be 100% and should not be more than 115% but the %CV at each level should be ≤ 15% and variation between different levels should be ≤ 15%.

Dilution integrity

Twelve sets of DI samples were prepared by spiking approximately 1.7 times ie Metformin 1043.582ng/mL and L-Carnitine 3.459µg/mL of the higher quantification level standard concentration of Metformin 613.872 ng/mL and L-Carnitine 3.232µg/mL. Then six sets were diluted twice and another six sets were diluted four times using blank plasma.

These DI samples were processed and analysed along with a freshly spiked calibration curve standards and concentrations were calculated using dilution factor.

Ruggedness

One P&A batch was processed by different person and injected using a different column.

Stabilities

Stability experiments were conducted to reflect the situations likely to be encountered during actual sample handling and analysis. While preparing samples for stability tests freshly made stock dilutions of the analytes were used. These stock dilutions were prepared from fresh stock solutions.

The biological matrix was analyte-free and interference-free.

Conducted the following stabilities:

- Wet Extract Stability (Autosampler Stability)
- Bench Top Stability
- Reinjection reproducibility
- Long Term Stability
- Freeze Thaw Stability
- Stock Solution Stability and Stock dilution

Bench Top Stability

6 replicates of each QCL and QCH level were withdrawn from deep freezer.

These samples were kept at room temperature for at least 4 hours or more based on the expected duration that the intended study samples may be retained at room temperature.

Prepared freshly spiked QC samples (QCL and QCH) and the freshly spiked Calibration Curve standards and analysed.

Calculated the QC concentrations of the processed samples against the freshly spiked CC standard and corrected the concentration of the stability QC samples by multiplying with the Correction Factor.

Bench top stability was determined for 24 hours, using six sets each of QCL and QCH. The quality control samples were calculated against the freshly spiked CC standards.

Freeze-Thaw Stability

Freeze thaw (FT) stability is carried out to assess the stability of the analytes in biological fluids during repeated freezing and thawing cycles. Establish the influence of a minimum of 4 freeze thaw cycles.

- Stored the QCL and QCH samples at the intended storage temperature in a freezer (below -20ºC) or deep freezer (below -50ºC) for at least 24 hours.
- Withdrew the samples (6 samples each of QCL and QCH) from deep freezer / freezer and allowed the samples to thaw unassisted at room temperature and replace the samples back to the freezer / deep freezer. This completes one Freeze and Thaw cycle (FT3).
- After 12 hours took out the FT3 samples (12 samples) along with a new set of six samples each at QCL and QCH (FT2) from deep freezer / freezer, thawed unassisted at room temperature and replace the samples back to the deep freezer / freezer.
- After 12 hours took out the FT4 (12 samples) from deep freezer / freezer, thawed unassisted at room temperature and analyzed with freshly prepared calibration curve standards.
- Prepared freshly spiked QC samples (QCL and QCH) and the freshly spiked CC standards.
- Calculated the QC concentrations of the processed samples against the freshly spiked CC standard.

Long Term Stability

To assess the stability of the analytes in the biological matrix under the same conditions of storage as that of the study samples the following test was performed.

6 samples of each QC sample at low and high concentrations were stored for 4 days below -20°C in the freezer. These samples were then calculated by comparing against the freshly prepared linearity standards. L-Carnitine and Metformin stability were evaluated against the Quality Control samples.

Stock Dilution Stability

Stock dilution stability for L-Carnitine and Metformin were evaluated at room temperature and between 2-8°C by keeping one portion in Room temperature and another in refrigerator for 24 hours .

Stock Solution Stability

Stability of stock solution was established for 4 days by running diluted stock in six replicates and then refrigerated between 2 - 8° C. Freshly prepared stock dilutions were used as Comparison standard.

Result and Discussion

Parameters	Results		Acceptance criteria
	L- Carnitine	**Metformin**	
Calibration Curve Range	2.289 µg/mL to 33.675 µg/mL for L-Carnitine.	43.483ng/ml to 639.450ng/ml	-
Specificity / Selectivity	All the 6 plasma lots and the pooled plasma lot met the acceptance criteria	All the 6 plasma lots and the pooled plasma lot met the acceptance criteria	Peak area of Analyte: ≤20%
Matrix Effect	%CV - 1.001 Matrix factor was close to unity for all matrix lots	%CV - 0.991 Matrix factor was close to unity for all matrix lots	Matrix factor should be close to unity %CV: <15%
Signal to Noise ratio	All the 6 plasma lots lot met the acceptance criteria	All the 6 plasma lots lot met the acceptance criteria	S/N ≥ 5:1

Carry Over	0.00%	0.00%	Peak area of Analyte: <20%
Intra-Run Accuracy & Precision	PA 1: 1.27 % - 6.93% - Within batch precision and 89.73% - 104.06% - accuracy	PA 1: 0.83 % - 3.69% - Within batch precision and 91.28% - 98.71% - accuracy	Accuracy: 85-115% (LLOQ:80-120%) Precision: ≤15%((LLOQ≤20%)
	PA2: 1.59% - 5.23% - Within batch precision and 91.69% - 110.18%- accuracy	PA2: 0.97% - 6.28% - Within batch precision and 93.09% - 99.13% - accuracy	
	PA3: 1.21% - 6.87% - Within batch precision and 86.94% - 102.69% - Within batch precision	PA3: 2.02% - 5.68% - Within batch precision and 88.72% - 98.42% - accuracy	
Inter-day Accuracy & Precision	1st day: 1.27 % - 6.93% - Intraday precision and 89.73% - 104.06% - accuracy	1st day: 0.83 % - 3.69% - Intraday precision and 91.28% - 98.71% - accuracy	
	2nd day: 1.59% - 5.23% - Intraday precision and 91.69% - 110.18% - accuracy	2nd day: 0.97% - 6.28% - Intraday precision and 93.09% - 99.13% - accuracy	
Between Batch Accuracy & Precision	2.63% - 9.64% - precision and 89.48% - 105.64% - between batch	1.04% - 5.04% - precision and 92.19% - 98.92% - accuracy	
Recovery of Analyte	QCL - % Recovery = 51.09	QCL - % Recovery = 28.56	%CV ≤15% (at each level) and variation between different levels should be ≤15%
	QCM - % Recovery = 48.83	QCM - % Recovery = 28.18	
	QCH - % Recovery = 47.85	QCH - % Recovery = 26.81	
	Overall recovery =49.26%	Overall recovery =27.85%	
	Overall %CV =3.38	Overall %CV =3.32	
Dilution Integrity	2 DI - % Accuracy = 105.09 % CV = 1.08	2 DI - % Accuracy = 101.03 % CV = 1.19	Accuracy: 85-115% (LLOQ:80-120%) Precision: ≤15%((LLOQ≤20%)
	4 DI - % Accuracy = 118.09 %CV = 2.66	4 DI - % Accuracy = 105.69 %CV = 1.36	
Freeze Thaw Stability (FT4)	QCL - %Accuracy = 112.24% %CV = 2.48 QCH - % Accuracy =92.88% %CV = 0.45	QCL - %Accuracy = 92.59% %CV = 1.45 QCH - % Accuracy =94.79% %CV = 1.18	
Bench Top Stability (24 hours)	QCL - %Accuracy = 113.49 %CV = 0.40 QCH - % Accuracy = 93.41 %CV = 1.67	QCL - %Accuracy = 92.04 %CV = 1.42 QCH - % Accuracy = 94.57 %CV = 2.09	
Long Term Stability (4 days, below -50°C)	QCL - % Stability = 112.03 %CV = 1.59 QCH - % Stability = 93.59 %CV = 0.79	QCL - % Stability = 90.50 %CV = 3.47 QCH - % Stability = 93.04 %CV = 1.50	
Long Term Stability (4 days, below -20°C)	QCL - % Stability = 112.50 %CV = 1.50 QCH - % Stability =93.51 %CV = 0.79	QCL - % Stability = 96.18 %CV = 2.34 QCH - % Stability =94.02 %CV = 1.58	
Post Preparative Stability of Analyte (30.5 hours at	QCL - %Accuracy = 95.69 %CV = 7.27 QCH - % Accuracy = 97.40 %CV = 1.68	QCL - %Accuracy = 95.69 %CV = 7.27 QCH - % Accuracy = 97.40 %CV = 1.68	

10°C (± 4°C))			
Short Term Stock Dilution Stability of Analyte (24 hours at Room temperature)	%Change = 0.58	%Change = 0.16	% Change: ±10%
Long Term Stock Solution Stability of Analyte (4 days at 2-8°C)	%Change = 0.96	%Change = 0.58	% Change: ±10%

Discussion

The chromatograms were acquired and processed using the Analyst Version 1.5.1 and using peak area method. The concentration of the unknown L-Carnitine and Metformin were calculated using regression analysis of spiked standard with the reciprocal of the (drug concentration)^2 and weighing factor ($1/x^2$):

$y = ax + B$
Here,
y is the peak area of analyte
a is the slope of cc (calibration curve)
x is the concentration of the analyte
B is the y-axis intercept of the cc

The results of specificity, selectivity, carryover, matrix effect, linearity, stabilities, recovery, precision and accuracy, and ruggedness presented in this report are within the acceptance criteria as per USFDA acceptance range and as per 'Guidance for Industry –Bio-analytical Method Validation' given by CDER other than Dilution integrity for four times for L-Carnitine. As this is a simultaneous method DI is proved up to 2 times only and should not be used for higher dilutions.

Conclusion

The Bio-analytical method described above is valid for the simultaneous estimation of L-Carnitine and Metformin, in human plasma over a range of 2.289 µg/mL to 33.675 µg/mL for L-Carnitine and 43.483ng/ml to 639.450ng/ml for metformin and is stability proven, linear, rugged, precise and accurate This method can be applied for simultaneous quantification in Human plasma for Drug discovery, Therapeutic monitoring as a prognostic tool, if proved.

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