Biomarker Quantification: Development of Fit for purpose LC-MS/MS Method for Determination of Methyl guanidine in Mice Urine

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ABSTRACT

Introduction: Accurate quantitation of biomarkers is always challenging, it becomes really tedious when biomarker has poor retention on chromatographic column and possess a chemical structure resistant for derivatisation. Methyl guanidine is product of protein catabolism, normally gets excreted in urine. Endogenous methyl guanidine concentrations in urine increases if there is reduce urine production or conversion of creatinine to methyl guanidine as proposed in patients with chronic renal failure. Increased level of methyl guanidine promotes apoptosis of renal proximal tubular cells in vitro, which in-turn could result in renal failure. Therefore Methyl guanidine can be considered as putative biomarker for renal failure studies. **Method:** Artificial urine was used as surrogate matrix for preparation of calibration standards, while quality control standards were prepared in authentic mice urine diluted 50 fold with artificial urine prior to extraction. For determination of basal levels of endogenous methyl guanidine urine samples from naïve mice were quantified. Moreover 50 fold dilution of quality control standards and study samples with artificial urine makes test matrix almost similar to that of calibration standards. **Results:** Developed method was found to linear 2ng/ml to 1000 ng/ml, with R^2 more than 0.98.Basing on the mean endogenous basal levels of methyl guanidine determined in un-treated C57BL/6J mice urine, developed method can accurately quantify up to 10 fold up regulation and up to 20 fold down regulation of methyl guanidine concentrations. **Conclusion:** A fast, robust and cost effective LC-MS/MS method was developed for determination of MG in mice urine. This is the first LC-MS/MS assay for direct quantitation of MG in mice urine samples. Approach followed for quantitation of MG is in-expensive over procurement of stable labeled standards, moreover 50 fold dilution of quality control standards and study samples with synthetic urine makes test matrix almost similar to that of calibration standards.

Key words: Methyl guanidine, Biomarker Quantification, Mice urine, Surrogate matrix, Synthetic urine, LC-MS/MS method.

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INTRODUCTION

Accurate quantitation of biomarkers is always challenging, it becomes really tedious when biomarker has poor retention on chromatographic column and/or possess a chemical structure resistant for derivatisation. Literature suggests use of stable-labeled standard or usage of surrogate matrix for quantitation these endogenous molecules.[1] Procurement/chemical synthesis of stable-labeled standards is expensive, and procedures followed to prepare to surrogate matrix sometimes renders test matrix (surrogate matrix) used for Calibration standards significantly different from that study samples (authentic matrix). Hence to ensure accurate quantitation, it is mandatory to demonstrate parallelism between surrogate matrix and authentic matrix by preparing quality control standards in authentic matrix and quantifying

them against calibration standards prepared in surrogate matrix.[1,2]

Methyl Guanidine (MG) is product of protein catabolism, normally gets excreted in urine. Endogenous MG concentrations in urine increases if there is reduced urine production or there is conversion of creatinine to MG, as proposed in patients with chronic renal failure.[3,4] MG is also an important read out biomarker for Kidney fibrosis model.[5,6]

Very few methods are available for quantitative determination of MG in biological matrices. Marie *et al.* published a method for quantification of MG in 1960 in plasma, that involves removal of protein with Ba $(OH)_2$/Zn SO_4, following by purification by strong cation-exchange resin, methylation with dimethylsulfate and finally sakaguchi reaction producing concentration dependent pink coloration measured by spectrophotometer.[7] In 1973, Israel and coworker also reported a colorimetric method to estimate MG concentrations in serum and urinary excretion in chronic renal failure patients, with a modification of Voges-Proskauer reaction.[8] In 1978, Yamamoto *et al.* developed high performance liquid chromatographic procedure employing fluorometric detection.[9,10] Another method involves post column derivatisation with aqueous ninhydrin solution.[11] Most of these analytical methods[7-11] lack specificity, selectivity and required sensitivity for sample analysis. Further these methods require more sample volume, are time consuming and arduous. Further, we could not find any report utilizing Liquid chromatography Mass spectrometry for determination of MG in biological fluids, which is well-known for its sensitivity, selectivity and precision. Developed method is simple, rapid and highly sensitive (LLOQ-2ng/ml). It is suitable for routine analysis and perhaps the first LC-MS/MS based method to quantify MG levels in mice urine.

Material and method

Chemicals and reagents

Methyl guanidine hydrochloride and Dextromethorphan hydrobromide monohydrate (IS) were purchased from sigma-aldrich (St. Louis, MO, USA). Chemical Structures for both analyte and internal standard are presented in Figure 1. Calcium chloride, sodium chloride, Potassium chloride, ammonium chloride, Disodium sulphate, potassium dihydrogen phosphate, creatinine and urea AR grade required for preparation synthetic urine were also purchased from sigma-aldrich (St. Louis, MO, USA).List of chemicals and their respective concentration used for the preparation of synthetic urine are shown in Table 1. All other solvents used in analysis were of LC-MS grade, Ammonium formate (Fluka), Acetonitrile, meth-
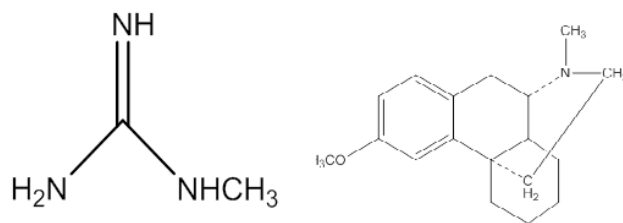

Figure 1: Chemical Structures for methyl guanidine (a) and Dextramethorphan (b) (internal standard)

anol and isopropyl alcohol, dimethyl sulphoxide were obtained from JT Baker (California, USA). Purified water was obtained using water purification installation system Milli-Q from waters. Un-treated C-57/6J mice urine samples were obtained from in-house vivarium.

LC-MS Analysis

The liquid chromatograph (Prominence; Shimadzu, Japan) was coupled to a triple quadrupole mass spectrometer with turbo electrospray ion source (API 3200, Applied Biosystems, Foster City, CA) used in positive ionization mode. Ion source parameters and optimized multiple reaction monitoring (MRM) parameters for MG and Dextramethorphan are shown in Table 2. Chromatographic resolution of MG and IS was achieved on waters HILIC (2.1 × 100mm, 5 μm) column obtained from waters (USA).MG was eluted at 2.3 min and IS at 2.0 min by a gradient mobile phase system consisting of phase A (10mM Ammonium formate) and phase B (acetonitrile). The gradient elution was started with 80% mobile phase B for 0.5 min, after which the % B was linearly decreased from 80 to 20% in 2 min, where it was held constant for 1.5 min. The gradient was then reversed back to initial conditions in 0.1 min, and held for 1.4 min before the next injection. Total run time was 5 min. The mobile phase was pumped at a flow-rate of 0.5 mL/min and splitter was not used. Data acquisition and analysis were performed using the analyst software version 1.5 (Applied Biosystem, Foster City, CA, USA).

Synthetic urine preparation

Synthetic urine was prepared as per composition shown in Table 1. All the listed chemicals were weighed and dissolved in purified water from milliqwater system from water (Type-1). pH value of final solution was adjusted to 6.0 and filtered through 0.22μm filter paper. It was stored at 2-8 °C until use.

Preparation of calibration and quality control standards

Standard stock solutions (2mg/mL) of MG and IS were prepared independently in dimethyl sulphoxide.

Table 1: Composition of artificial urine[12].	
Compound	**Concentration (g/L)**
Calcium Chloride. dihydrate	1.10
Sodium Chloride	2.92
Disodium sulphate	2.25
Potassium dihydrogen phosphate	1.40
Potassium chloride	1.60
Ammonium chloride	1.0
Urea	25.0
Creatinine	1.10

Figure 2: Product ion mass spectra and characteristic daughter ions used to develop MRM transitions of (a) methyl guanidine and (b) dextramethorphan

Separate weighing was made to prepare calibration and quality control stock solutions. Working spiking solutions for calibration and quality controls standards were prepared by appropriate dilution in acetonitrile-water (50:50, v/v, diluent). The stock solutions and the spiking solutions were stored at -20°C. IS working solution (1 μg/mL) was prepared by diluting respective dimethyl sulphoxide stock solution in acetonitrile. 2.5 μL of working spiking dilutions into 47.5 μL of syntheticurine to achieve final concentration of 2, 5,10,25,50,100,250,500 and 1000 ng/mL Quality control samples were made by spiking 5% of working solution in synthetic urine to achieve final concentration of 6,100,400 and 800 ng/mL. Additionally, MG working spiking solutions was spiked at 10, 50, 500 and 1000 ng/mL in untreated mice urine, pre-diluted fifty fold with synthetic urine to validate method performance in authentic sample matrix.

Sample processing

The 150 μL of ice cold acetonitrile containing internal standard solution (1μg/mL of Dextromethorphan) was added to 50 μL of aliquot of spiked Calibration curve/Quality control standards in centrifuge tubes. Tubes were vortexed for 1min on VX-2500 multitube vortexer (VWR Lab product pvt. Ltd, Mumbai) and the centrifuged at 8000rpm for 10 min at 4⁰C (Centrifuge 5810R –Eppendorf Germany). 100 μL of supernatant was transferred to HPLC vials for LC-MS/MS analysis.

RESULT AND DISCUSSION

Mass Spectrometry

Quantification of biomarker in biological matrices by LC-MS/MS is gaining significance due to better selectivity and sensitivity of this technique over conventional radiometric assays.[13] The product ion mass spectrum of MG and Dextromethorphan (internal standard) are shown in Figure 2. [M+H]⁺ was the predominant ion in Q1 spectrum and was used as precursor ion to obtain product ion spectra. Due to presence of ionizable nitrogen, MG could easily be protonated
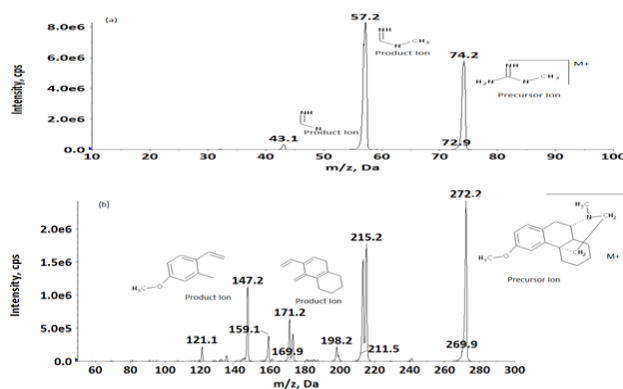
and detected well in positive polarity. The product ion spectrum of MG shows the formation of characteristic daughter ions at m/z 57.2 and 43.1 generated via loss of amine and methyl group respectively Figure 2(a). The product ion mass spectrum of internal standard dextramethorphan shows the formation of characteristic product ions at m/z 147.2, 171.2, 211.5 and 215.2. Tentative structural assignments to daughter ions selected for developing MRM transitions are shown in Figure 2(b). To yield enhanced area counts two MRM transitions were clubbed for both analyte and IS.

METHOD DEVELOPMENT

There are no recent reports on LC-MS/MS based method for quantification of MG in biological samples. Previous published literature on analytical methodologies indicated usage of ion-exchange chromatography/colorimetric techniques/ HPLC with fluorescence detection. Most of the methods based on these techniques lack specificity, selectivity and adequate sensitivity for sample analysis. Further these methods require more sample volume, are arduous andtime consuming. Considering the structure of MG, initially chemical derivatisation was tried with Diethydithiocarbamate (DDTC),5% solution of DDTC was prepared with 0.2M sodium hydroxide to deritivise amine functional group of MG, reaction mixture was incubated at 45°C for 50 min to form adduct which was expected to improve ionization, chromatographic retention on column and extraction efficiency, but MG showed resistance to derivatisation. Hence with intent to quantify MG as such, extensive trials were made with available polar column chemistries, some of the columns those were tried includes Acclaim polar advantage, BDS Hypersil Phenyl, Zorbax PFP, Synergi Fusion and

Kinetex EVO from Phenomenex but best retention of MG with reasonable peak shape was achieved on HILIC silica column from waters (Atlantis, 5 μm, 2.1× 100 mm), employing a gradient programme with mobile phase of 10mM ammonium formate and acetonitrile. Dextramethorphan and verapamil both were tried as internal standard; however dextramethorphan showed more consistent mass spec response in optimized chromatographic conditions.

Selection of surrogate matrix

Initial linearity trials were made in milliqwater, but later on it was replaced with synthetic urine. Synthetic urine devoid of MG was preferred to be used as surrogate matrix over milliqwater because of its close proximity with authentic sample matrix.

Method Performance

The nine point calibration curve for MG was linear over the concentration range 2-1000 ng/mL. The calibration model was selected based on the analysis of the data by linear regression with/without intercepts and weighing factors ($1/x$, $1/x^2$ and none). The best linear fit and least-squares residuals for the calibration curve were achieved with $1/x^2$ weighing factor. Ruggedness of the method was demonstrated set of three calibration curves analyzed on separate day's data shown in Table 3. Intra-day and inter-day precision of the method was demonstrated by quality control samples spiked in synthetic urine at concentration levels 6, 120, 400 and 800 ng/mL. Accuracy, relative standard deviation and % bias is shown in Table 4.

Selectivity and carry over

MRM chromatograms for MG and IS in double blank (a), LLOQ spiked in synthetic urine (b) and authentic urine sample pre-diluted 50 fold with synthetic urine (c) collected from naïve mice is shown in Figure 3. Selectivity of the developed method was performed in surrogate matrix. No interfering peak at retention time of MG and internal standard was observed against LLOQ standard. Further, no quantifiable area counts were seen at retention time of MG as well internal standard when blank sample of synthetic urine was injected after ULOQ calibration standard.

Table 2: Retention times, MRM transitions, and MS parameters for the determination of methyl guanidine and dextramethorphan (IS).		
Compound	**Methyl guanidine**	**Dextramethorphan**
Retention time (min)	2.3	2.0
Parent Ion (M/Z)(Q1)	74.2	272.2
Daughter Ion (M/Z) (Q3)	57.2, 43.2	171.2, 147.2
Dwell time (ms)	200	
Declustring Potential (Volts)	30	53
Entrance Potential (Volts)	10	
Collision energy (Volts)	30	52
Collision cell exit Potential (Volts)	10	

Table 3: Characteristics of optimized calibration curve standards.						
Calibration Samples	**Nominal Concentration (ng/mL)**	**Calculated Concentration (ng/mL)**			**Mean Calculated Concentration (ng/mL)**	**Accuracy (%)**	**Bias (%)**
		Set-1	**Set-2**	**Set-3**			
Standard-1	2.0	2.0	2.01	1.92	2.0	101.3	1.3
Standard-2	5.0	4.4	4.82	5.23	4.6	92.5	-7.5
Standard-3	10.0	10.8	9.73	10.67	10.3	102.7	2.7
Standard-4	25.0	27.6	28.57	27.62	28.1	112.4	12.4
Standard-5	50.0	49.0	50.28	50.32	49.7	99.3	-0.7
Standard-6	100.0	107.1	107.51	106.58	107.3	107.3	7.3
Standard-7	250.0	236.9	237.14	235.89	237.0	94.8	-5.2
Standard-8	500.0	482.9	474.91	458.27	478.9	95.8	-4.2
Standard-9	1000.0	942.9	933.37	890.78	938.1	93.8	-6.2
Correlation coefficient		**0.9967**	**0.9971**	**0.9965**			
Slope		0.000123	0.000128	0.000124			
intercept		2.03e-06	5.02e-05	5.96e-06			

Table 4: Precision and accuracy of the developed method for quality control standards spiked in synthetic urine.

Nominal concentration (ng/mL)	Intra-day				Inter-day			
	Calculated concentration (mean ± SD) (ng/mL)	Accuracy (%)	RSD, % CV	Bias (%)	Calculated concentration (mean ± SD) (ng/mL)	Accuracy (%)	RSD, % CV	Bias (%)
6	5.6 ± 0.1	94.0	2.6	-6.0	5.6 ± 0.2	93.6	3.5	-6.4
120	109.4 ± 1.3	91.2	1.1	-8.8	113.6 ± 4.6	94.7	4.1	-5.3
400	356.7 ±2.7	89.0	0.8	-11.0	366.9 ± 10.5	91.7	2.9	-8.3
800	699.7 ±6.5	97.5	0.9	-12.5	734.1 ± 50.5	91.8	6.9	-8.2

Accuracy (%)= (average measured value/nominal value) × 100, Bias (%) = [(measured value-true value)/true value] × 100.

Table 5: Selection of pre-dilution fold with synthetic urine (surrogate matrix).

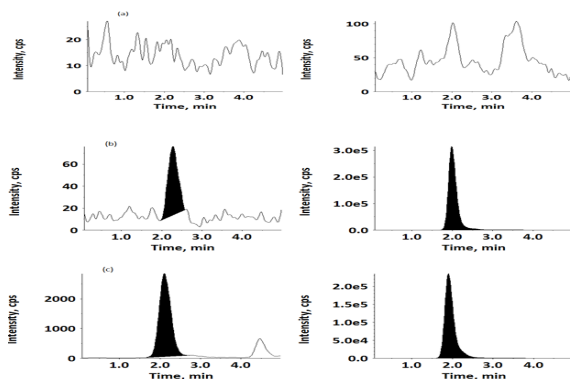| Fold dilution with synthetic urine	mean ± SD	RSD, % CV
10 fold	1701 ± 50	3.0
20 fold	1917 ± 47	2.5
50 fold	2034 ± 27	1.3
100 fold	2044 ± 58	2.9

50 fold dilution was selected based on SD and RSD

Figure 3: MRM chromatograms for methyl guanidine (left pane) and dextramethorphan (right pane). (a) and (b) are chromatograms for double blank and LLOQ prepared with synthetic urine. Representative chromatogram (c) for authentic urine sample pre-diluted 50 fold with synthetic urine
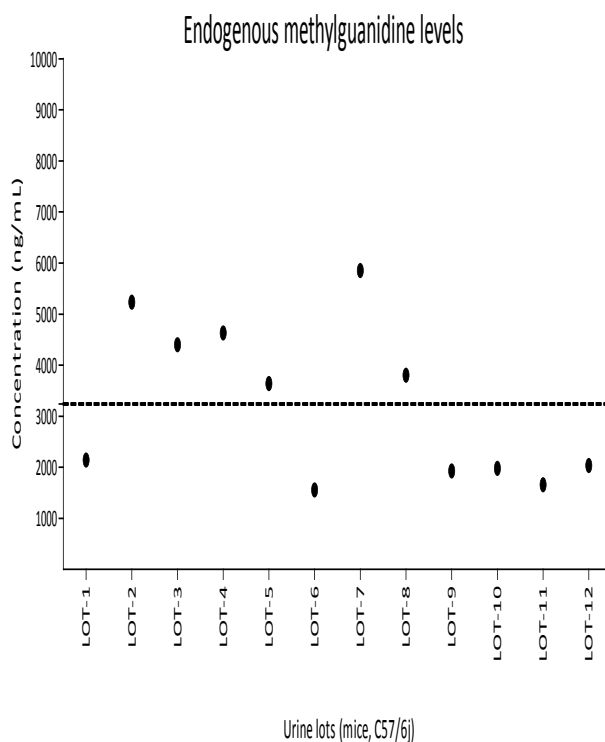

Figure 4: Methyl guanidine concentrations from 12 different lots of urine sample collected from un-treated C57/6j mice. Samples were diluted 50 fold with synthetic urine prior to processing and quantified against calibration curve spiked in synthetic urine alone as surrogate matrix. Dotted line represents mean basal level of methyl guanidine..

Selection of pre-dilution fold with surrogate matrix

One lot of urine sample from un-treated mice was collected and dilution fold selection exercise was performed at 10, 20, 50 and 100 folds. Dilution samples at each fold dilution was executed in triplicate and read against calibration curve spiked in synthetic urine as shown in Table 5. Fifty fold was selected as final pre-dilution factor for processing, since fifty fold dilution showed relatively lower values for standard deviation and relative standard deviation. Further, selection of

fifty fold as pre-dilution step also looks appropriate as it is expected to reduce matrix effect significantly.

Determination of endogenous levels

Endogenous level of MG was established with 12 lot urine samples from 12 different naïve animals. These were processed with 50 fold pre-dilution with synthetic urine and determined against calibration curve prepared in synthetic urine. Endogenous level of MG in naïve mice urine samples was found to be 3.2 ±1.5 µg/mL.

Table 6: Methyl guanidine concentrations from 12 different lots of urine sample collected from un-treated C57/6j mice. Samples were diluted 50 fold with synthetic urine prior to processing and quantified against calibration curve spiked in synthetic urine alone as surrogate matrix.

Mice urine blanks	MG Concentration (µg/mL)
Lot-1	2.1
Lot-2	5.2
Lot-3	4.4
Lot-4	4.6
Lot-5	3.6
Lot-6	1.6
Lot-7	5.9
Lot-8	3.8
Lot-9	1.9
Lot-10	2.0
Lot-11	1.7
Lot-12	2.0
mean ± SD	3 ± 1.5

Spread of MG endogenous levels with a batch of 12 lots is shown in Figure 4, while individual values are listed in Table 6.

Method performance with authentic sample matrix

To asses method precision in quantification MG levels modulations in authentic urine samples, MG standard was spiked in urine sample at four concentration levels covering the entire calibration range and quantified against calibration curve prepared with synthetic urine. Similar exercise was performed three times on different days and results are shown in Table 7. Method showed robust performance at all four tested concentration levels. Developed method can accurately quantify upto 6 fold up regulation and 16 fold down regulation of endogenous MG concentrations of untreated mice.

CONCLUSION

A fast, robust and cost effective LC-MS/MS method was developed for determination of MG in mice urine. This is the first LC-MS/MS assay for direct quantitation of MG in mice urine samples. Approach followed for quantitation of MG is in-expensive over procurement of stable labeled standards, moreover 50 fold dilution of quality control standards and study samples with synthetic urine makes test matrix almost similar to that of calibration standards. Developed method can accurately quantify upto 6 fold up regulation and upto 16 fold down regulation of MG concentrations.

Table 7: Precision and accuracy of the developed method for quality control standards spiked in C57/6j mice urine pre-diluted 50 fold with synthetic urine.

Quality control levels	Spiked Concentration (ng/mL)	Endogenous concentration (ng/mL)	Nominal Concentration (ng/mL)*	Calculated concentration (mean ± SD) (ng/mL) N=6	Accuracy (%)	RSD, % CV	Bias (%)
Level-1	10	98.52	108.52	98.3 ± 3.5	90.6	3.6	-9.4
		103.92	113.92	105.6 ± 3.7	92.7	3.5	-7.3
		271.91	281.91	284.3 ± 7.5	100.8	2.6	0.8
Level-2	50	98.52	148.52	134.0 ± 3.8	90.2	2.9	-9.8
		103.92	153.92	143.8 ± 5.0	93.4	3.5	-6.6
		271.91	321.91	319.2 ± 6.2	99.2	2.0	-0.8
Level-3	500	98.52	598.52	525.4 ± 9.4	87.8	1.8	-12.2
		103.92	603.92	592.2 ± 19.4	98.1	3.3	-1.9
		271.91	771.91	707.4 ± 18.8	91.6	2.7	-8.4
Level-4	1000	98.52	1098.52	1083.2 ± 24.2	98.6	2.2	-1.4
		103.92	1103.92	1178.8 ± 35.6	106.8	3.0	6.8
		271.91	1271.91	1104.7 ± 44.7	86.9	4.0	-13.1

* Nominal concentration= spiked concentration+endogenous concentration, Accuracy (%) = (average measured value/nominal value) × 100, Bias (%) = [(measured value-true value)/true value] × 100.

ACKNOWLEDGEMENT

CONFLICT OF INTEREST

The authors have no conflict of interests to report.

ABBREVIATIONS

MG: Methyl Guanidine, **LC-MS/MS:** Liquid Chromatography and Mass Spectrometry, ng/ml-nano gram per millilitre, **μL:** micro liter, **mM:** milimolar.

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PICTORIAL ABSTRACT

Liquid chromatography and Mass spectrometry method Development
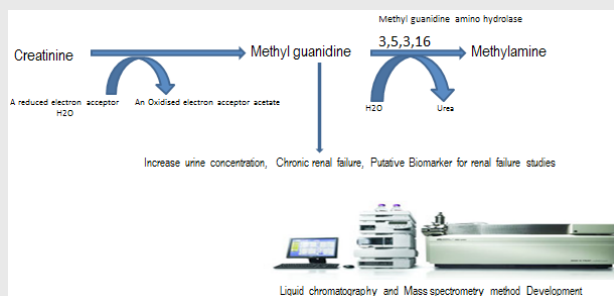
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SUMMARY

• The present work deals development of fit for pu pose A fast, robust and cost effective LC-MS/MS method was developed for determination of MG in mice urine. This is the first LC-MS/MS assay for direct quantitation of MG in mice urine samples. Approach followed for quantitation of MG is in-expensive over procurement of stable labeled standards

• The LC-MS/MS method development and validation parameters were observed and evaluated. All the observations are within the specified limits. Moreover 50 fold dilution of quality control standards and study samples with synthetic urine makes test matrix almost similar to that of calibration standards. Developed method can accurately quantify upto 6 fold up regulation and upto 16 fold down regulation of MG

• Mobile phase consists of 10mM Ammonium formate and Acetonitrile (Gradient programme) at flow rate of 0.5 ml/min. The retention time for methyl guanidine and dextromethorphan was found to be 2.3 and 2.0 respectivly. The nine point calibration curve for MG was linear over the concentration range 2-1000 ng/mL. The calibration model was selected based on the analysis of the data by linear regression r20.9968.

• The validation of developed method shows that the selectivity, accuracy and precision recovery in surrogate and authentic matrix is same and within the acceptance limit,

Mr. Ashwani Gaur: Principal Scientist–II: Dept. of Drug metabolism and pharmacokinetic Advinus Therapeutics Limited Pune. Mr.Ashwani has M.S. degree in Pharmaceutical analysis and has more than twelve years of experience in LC-MS based Bioanalysis, Biotransformation studies of small molecules, Biomarker analysis and ADME-PK of new chemical entities in drug discovery settings.

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