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Research article

METHOD DEVELOPMENT AND VALIDATION OF CHROMIUM PICOLINATE AND METFORMIN BY RP-HPLC

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ABSTRACT

The method is said to be sensitive if small changes in concentration cause large changes in response function. The sensitivity of an analytical method is determined from the slope of the calibration line. The limits of quantification (LOQ) or working dynamic range of bio analytical method are defined as the highest and lowest concentrations, which can determined with acceptable accuracy. It is suggested that, this be set at ± 15% for both the upper and lower limit of quantitation respectively. Any sample concentration that falls outside the calibration range cannot be interpolated from the calibration line and extrapolation of the calibration curve is discouraged. If the concentration is over range, the sample should be diluted in drug-free matrix and re-assayed.

Keywords: Reserpine, Chromium Picolinate, RP-HPLC, Metformin, Method validation.

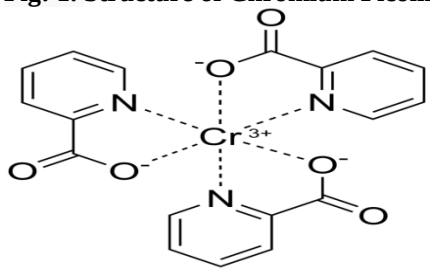
INTRODUCTION

Chromium Picolinate a Low-molecular-weight chromium-binding substance (LMWCr; also known as chromodulin) is an oligopeptide that seems to bind chromium(III) in the body. It consists of four amino acid residues; aspartate, cysteine, glutamate, and glycine, bonded with four (Cr^{3+}) centers. It interacts with the insulin receptor, by prolonging kinase activity through stimulating the tyrosine kinase pathway, thus leading to improved glucose absorption. It has been confused with glucose tolerance factor. Despite recent efforts to characterize chromodulin, the exact structure is still relatively unknown.

Although chromodulin's exact mechanism of action on the insulin receptor is currently unknown, one commonly described mechanism is presented below. This proposed mechanism has the highest amount of agreement with various experiments involving chromodulin.

IUPAC Name: chromium(3+);pyridine-2-carboxylate
Molecular Framework: Aromatic homomonocyclic compounds
Molecular formula: $C_{18}H_{12}CrN_3O_6$
Molecular weight: 418.305 g/mol
Physico Chemical Properties
Appearance: White crystals or fine white powder.
Solubility: Practically insoluble (0.583 mg/L) in water.
Boiling point: 292.5 °C at 760 mmHg.
pKa : Strongest Acidic - 14.49

Fig: 1. Structure of Chromium Picolinate



Metformin's mechanisms of action differ from other classes of oral antihyperglycemic agents. Metformin decreases blood glucose levels by decreasing hepatic glucose production, decreasing intestinal absorption of glucose, and improving insulin sensitivity

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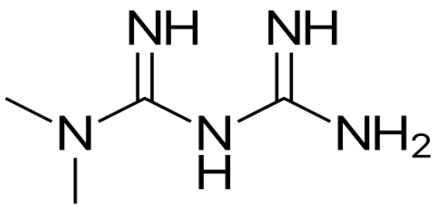
by increasing peripheral glucose uptake and utilization. These effects are mediated by the initial activation by metformin of AMP-activated protein kinase (AMPK), a liver enzyme that plays an important role in insulin signaling, whole body energy balance, and the metabolism of glucose and fats. Activation of AMPK is required for metformin's inhibitory effect on the production of glucose by liver cells. Increased peripheral utilization of glucose may be due to improved insulin binding to insulin receptors. Metformin administration also increases AMPK activity in skeletal muscle. AMPK is known to cause GLUT4 deployment to the plasma membrane, resulting in insulin-independent glucose uptake. The rare side effect, lactic acidosis, is thought to be caused by decreased liver uptake of serum lactate, one of the substrates of gluconeogenesis. In those with healthy renal function, the slight excess is simply cleared. However, those with severe renal impairment may accumulate clinically significant serum lactic acid levels. Other conditions that may precipitate lactic acidosis include severe hepatic disease and acute/decompensated heart failure.

Fig: 2. Structure of Metformin



IUPAC Name: 1-carbamimidamido-N,N-dimethylmethanimidamide
Molecular Framework: Aliphatic acyclic compounds
Molecular formula: $C_4H_{11}N_5$
Molecular weight: 129.1636 g/mol
Physico Chemical Properties
Appearance: White crystals or fine white powder.
Solubility: Practically insoluble (0.583 mg/L) in water.
Boiling point: 224.1 °C at 760 mmHg.
pKa: Strongest Acidic - 12.4

MATERIALS AND METHODS

Instruments

- HPLC –Waters Model NO.2690/5 series Compact System Consisting of Inertsil-C18 ODS column
- Electronic balance (SARTORIOUS)
- Sonicator (FAST CLEAN)

Chemicals

- Methanol HPLC Grade
- Water Hplc Grade

Method Development

The objective of this experiment was to optimize the assay method for simultaneous estimation of Chromium Picolinate and Metformin on the literature survey made. So here the trials mentioned describes how the optimization was done.

Mobile Phase: Degassed Methanol and Water in the ratio of 45:55 V/V.

Preparation of stock solution

Reference solution: The solution was prepared by dissolving 25.0 mg of accurately weighed Chromium Picolinate and 20.0 mg Metformin in Mobile phase, in two 100.0 mL volumetric flasks separately and sonicate for 20min. From the above solutions take 10.0 mL from each solution into a 50.0 mL volumetric flask and then makeup with mobile phase and sonicate for 10min.

Preparation of working standard solution

The stock solutions equivalent to 20ppm to 80ppm with respect to both drugs were prepared in combination of Chromium Picolinate and Metformin above, sonicated and filtered through 0.45µ membrane.

Optimized chromatographic conditions

Parameters	Method
Stationary phase (column)	Inertsil -ODS C_{18}(250 x 4.6 mm, 5 µ)
Mobile Phase	Methanol : Water (45:55)
Flow rate (ml/min)	1.0 ml/min
Run time (minutes)	8 min
Column temperature (°C)	Ambient
Volume of injection loop (µl)	20
Detection wavelength (nm)	254nm
Drug RT (min)	3.005min for Chromium Picolinate and 5.291 for Metformin.

Method Validation

System Suitability

A Standard solution was prepared by using Chromium Picolinate and Metformin working standards as per test method and was injected Five times into the HPLC system. The system suitability parameters were evaluated from standard chromatograms by calculating the % RSD from five replicate injections for Chromium Picolinate and Metformin, retention times and peak areas.

Acceptance Criteria

1. The % RSD for the retention times of principal peak from 5 replicate injections of each Standard solution should be not more than 2.0 %
2. The % RSD for the peak area responses of principal peak from 5 replicate injections of each standard Solution should be not more than 2.0%.

3. The number of theoretical plates (N) for the Chromium Picolinate and Metformin peaks is NLT 3000.
4. The Tailing factor (T) for the Chromium Picolinate and Metformin peaks is NMT 2.0

Observation

The %RSD for retention times and peak areas were found to be within the limit. Refer table: 1.

Specificity

Chromium Picolinate and Metformin

Solutions of standard and sample were prepared as per the test method are injected into chromatographic system.

Acceptance Criteria: Chromatograms of standard and sample should be identical with near Retention time.

Observation: The chromatograms of Standard and Sample were same identical with same retention time.

Precision

Repeatability

a. System precision: Standard solution prepared as per test method and injected five times.

b. Method precision: Prepared six sample preparations individually using single as per test method and injected each solution.

Acceptance Criteria: The % relative standard deviation of individual Chromium Picolinate and Metformin, from the six units should be not more than 2.0%. The individual assays of Chromium Picolinate and Metformin should be not less than 98% and not more than 102.0%.

Observation: Test results are showing that the test method is precise. Refer tables 2 and 3 for system precision and for method precision.

Intermediate precision (analyst to analyst variability)

A study was conducted by two analysts as per test method

Acceptance Criteria: The individual assays of Chromium Picolinate and Metformin should be not less than 98% and not more than 102% and %RSD of assays should be NMT2.0% by both analysts.

Observation: Individual %assays and %RSD of Assay are within limit and passes the intermediate precision, Refer table: 4.

Accuracy (Recovery)

A study of Accuracy was conducted. Drug Assay was performed in triplicate as per test method with equivalent amount of Chromium Picolinate and Metformin into each volumetric flask for each spike level to get the concentration of Chromium Picolinate and Metformin equivalent to 50%, 100%, and 150% of the labeled amount as per the test method. The average % recovery of Chromium Picolinate and Metformin were calculated.

Acceptance Criteria: The mean % recovery of the Chromium Picolinate and Metformin at each spike level should be not less than 98.0% and not more than 102.0% for both the drugs separately.

Observation: The recovery results indicating that the test method has an acceptable level of accuracy. Refer table: 5.

$$\% \text{ Recovery} = \frac{\text{Amount found}}{\text{Amount added}} \times 100$$

Linearity of Test Method

A Series of solutions are prepared using Chromium Picolinate and Metformin working standards at concentration levels from 20ppm to 80 ppm of target concentration .Measure the peak area response of solution at Level 1 and Level 6 six times and Level 2 to Level 5 two times.

Acceptance Criteria: Correlation Coefficient should be not less than 0.9990. % of y- Intercept should be ±2.0. % of RSD for level 1 and Level 6 should be not more than 2.0%.

Observation: The linear fit of the system was illustrated graphically. The results are presented in table 6.

Ruggedness of Test Method

a) System to system variability:

System to system variability study was conducted on different HPLC systems, under similar conditions at different times. Six samples were prepared and each was analyzed as per test method. Comparison of both the results obtained on two different HPLC systems, shows that the assay test method are rugged for System to system variability.

Acceptance Criteria: The % relative standard deviation of Chromium Picolinate and Metformin from the six sample preparations should be not more than 2.0%. The % assay of Chromium Picolinate and Metformin should be between 98.0%-102.0%.

Observation: The % RSD was found within the limit. Ref tables: 3 &7.

Robustness

Effect of variation of flow rate

A study was conducted to determine the effect of variation in flow rate. Standard solution prepared as per the test method was injected into the HPLC system using flow rates, 1.0ml/min and 1.2ml/min. The system suitability parameters were evaluated and found to be within the limits for 1.0ml/min and 1.2ml/min flow. Chromium Picolinate and Metformin and was resolved from all other peaks and the retention times were

comparable with those obtained for mobile phase having flow rates 1.0ml/min.

Acceptance Criteria: The Tailing Factor of Chromium Picolinate and Metformin standards should be NMT 2.0 for Variation in Flow.

Observation: The tailing factor for Chromium Picolinate and Metformin was found to be within the limits. As shown in table 8.

Limit of Detection and Quantitation (LOD and LOQ)

From the linearity data calculate the limit of detection and quantitation, using the following formula.

$$LOD = \frac{3.3\ \sigma}{S}$$

σ = standard deviation of the response

S = slope of the calibration curve of the analyte

$$LOQ = \frac{10\ \sigma}{S}$$

σ = standard deviation of the response

S = slope of the calibration curve of the analyte

RESULTS AND DISCUSSION

Table 1(a). Data of System Suitability for Chromium Picolinate

Injection	RT	Peak Area	USP Plate count	USP Tailing
1	3.009	9438247	1023.845712	1.14721
2	3.007	9436021	1010.547812	1.13384
3	3.006	9431581	1036.874214	1.18742
4	3.004	9432036	1027.254178	1.16547
5	3.007	9433819	1084.658952	1.17485
Mean	3.0066	9434755	1036.825471	1.1852313
SD	0.001817	3358.178	-------	-------
% RSD	0.06042	0.270438	-------	-------

Table 1(b). Data of System Suitability for Metformin

Injection	RT	Peak Area	USP Plate count	USP Tailing
1	5.301	323209	8325.874512	1.284572
2	5.302	323181	8384.547862	1.254872
3	5.301	323028	8314.875424	1.278451
4	5.296	323915	8372.784518	1.287451
5	5.299	324059	8392.084512	1.298745
Mean	5.2998	3238476	8358.8754210	1.255471
SD	0.002387	1588.8	-------	-------
% RSD	0.045048	0.289823	-------	-------

Table 2(i). Data of Repeatability (System precision) for Chromium Picolinate

	Injection	Peak Areas of Chromium Picolinate	%Assay
Concentration 40ppm	1	9437784	99.74
	2	9437412	99.14
	3	9430257	99.62
	4	9438431	99.72
	5	9438754	99.42
Statistical Analysis	**Mean**	94367079	99.13
	SD	10475.12	0.24746
	% RSD	0.784591	0.31713

Table 2(ii). Data of Repeatability (System precision) for Metformin

	Injection	Peak Areas of Metformin	%Assay
Concentration 40ppm	1	323112	99.98
	2	323452	99.30
	3	323742	99.60
	4	323047	99.84

	5	323087	99.72
Statistical Analysis	**Mean**	3231472	99.71
	SD	7452.47120	0.425
	% RSD	0.752411	0.240

Table 3(i). Data of Repeatability (Method precision) for Chromium Picolinate

	Injection	**Peak Areas of Chromium Picolinate**	**%Assay**
Concentration 40ppm	1	9432571	99.25
	2	9438475	99.12
	3	9434752	98.12
	4	9430487	99.52
	5	9436547	98.84
	6	9437841	99.54
Statistical Analysis	**Mean**	9438845	99.56
	SD	1472.05847	0.54213
	% RSD	0.7451	0.412

Table 3(ii). Data of Repeatability (Method precision) for Metformin

	Injection	**Peak Areas of Metformin**	**%Assay**
Concentration 40ppm	1	323584	99.54
	2	323054	99.72
	3	323847	99.31
	4	323751	99.84
	5	323814	99.42
	6	323745	99.32
Statistical Analysis	**Mean**	323875	99.8742
	SD	3240.5412	0.78451
	% RSD	0.54721	0.874654

Table 4(i). Data of Intermediate precision (Analyst 2) for Chromium Picolinate

	Injection	**Peak Areas of Chromium Picolinate**	**%Assay**
Concentration 40ppm	1	9438237	99.45
	2	9438752	99.87
	3	9438754	99.84
	4	9438745	99.25
	5	9435741	99.87
	6	9438754	99.27
Statistical Analysis	**Mean**	9431755	99.875
	SD	4752.5481	1.345
	% RSD	0.8421	0.875

(ii)Data of Intermediate precision (Analyst 2) for Metformin

	Injection	**Peak Areas of Metformin**	**%Assay**
Concentration 40ppm	1	323841	99.92
	2	323874	99.87
	3	323075	99.54
	4	323781	99.74
	5	323412	99.81
	6	323241	99.37
Statistical Analysis	**Mean**	323197.6	99.87241
	SD	1475.21	0.87451
	% RSD	0.34710	0.6341

Table 5(i). Data of Accuracy for Chromium Picolinate

Concentration % of spiked level	Amount added (ppm)	Amount found (ppm)	% Recovery	Statistical Analysis of % Recovery	
50% Injection 1	20	20.01	99.81	**MEAN**	99.783
50% Injection 2	20	19.91	99.48		
50% Injection 3	20	20.08	99.87	**%RSD**	0.68
100 % Injection 1	40	40.03	100.11	**MEAN**	99.97
100 % Injection 2	40	39.98	99.94		
100% Injection 3	40	39.91	99.67	**%RSD**	0.56
150% Injection 1	60	60.02	99.47	**MEAN**	100.023
150% Injection 2	60	60.07	100.02		
150% Injection 3	60	60.04	100.08	**%RSD**	0.27

(ii)Data of Accuracy for Metformin

Concentration % of spiked level	Amount added (ppm)	Amount found (ppm)	% Recovery	Statistical Analysis of % Recovery	
50% Injection 1	20	20.45	99.91	**MEAN**	99.92
50% Injection 2	20	19.84	99.84		
50% Injection 3	20	20.07	100.04	**%RSD**	0.54
100 % Injection 1	40	39.45	99.85	**MEAN**	99.94
100 % Injection 2	40	40.07	100.05		
100% Injection 3	40	39.93	99.89	**%RSD**	0.63
150% Injection 1	60	6.02	99.94	**MEAN**	99.97
150% Injection 2	60	59.98	99.91		
150% Injection 3	60	60.07	100.08	**%RSD**	0.327

Table 6(i). Data of Linearity (Chromium Picolinate)

Concentration (ppm)	Average Area	Statistical Analysis	
0	0	Slope	23306
20	4719376	y-Intercept	77193
30	7079064	Correlation Coefficient	0.999
40	9438751		
50	11798439		
60	14158127		
70	16517815		
80	18477503		

(ii) Data of Linearity (Metformin)

Concentration (ppm)	Average Area	Statistical Analysis	
0	0	Slope	8016
20	161774	y-Intercept	1929
30	242661	Correlation Coefficient	0.999
40	323547		
50	404434		
60	485321		
70	566208		
80	637095		

Table 7(i). Data of system to system variability (Chromium Picolinate) System-2

S.No	Peak area	Assay % of Chromium Picolinate
1	9438754	99.98
2	9438741	99.30
3	9437845	99.60
4	9438741	99.84
5	9437841	99.72

6	9437217	98.89
Mean	9438745	99.8794
%RSD	0.768595	0321244

(ii) Data of system to system variability (Metformin) System-2

S.No	Peak area	Assay % of Metformin
1	323845	99.74
2	323754	99.68
3	323407	99.63
4	323984	99.91
5	323157	99.74
6	323274	99.76
Mean	323879.5	99.86
%RSD	0.57840	0.27841

Table 8(i). Data for Effect of variation in flow rate (Chromium Picolinate)

Flow 0.8 ml	Std Area	Tailing factor	Flow 1.0 ml	Std Area	Tailing factor	Flow 1.2 ml	Std Area	Tailing factor
	9417845	1.178452		9438754	1.145478		9451247	1.125784
	9415201	1.168453		9435784	1.126589		9458762	1.152314
	9417843	1.178456		9433578	1.152341		9458762	1.143419
	9410874	1.157801		9437862	1.148763		9456384	1.135289
	9413784	1.127861		9438074	1.135201		9458014	1.138427
Avg	9417294	1.137841	**Avg**	9437845	1.1417864	**Avg**	9458473	1.178456
SD	3784.852	0.0875474	**SD**	15784.25	0.0012874	**SD**	7846.8421	0.0087452
%RSD	0.289745	1.18745	**%RSD**	0.6547	1.14874	**%RSD**	0.92547	1.108465

Table 8(ii). Data for Effect of variation in flow rate (Metformin)

Flow 0.8 ml	Std Area	Tailing factor	Flow 1.0 ml	Std Area	Tailing factor	Flow 1.2 ml	Std Area	Tailing factor
	321546	1.086917		323458	1.054213		324872	1.075687
	321857	1.074793		323084	1.087542		324791	1.079875
	321780	1.078752		323874	1.087621		324721	1.078965
	321354	1.077894		323784	1.087621		324789	1.072347
	321784	1.078763		323947	1.087452		324742	1.080478
Avg	321785.6	1.077654	**Avg**	323421	1.078754	**Avg**	324786.5	1.078764
SD	17845.21	0.005782	**SD**	4125.021	0.008754	**SD**	25478.3217	0.008764
%RSD	0.57845	0.8754	**%RSD**	1.047102	0.35471	**%RSD**	0.87945	1.02354

Fig: 3. Chromatogram of standard

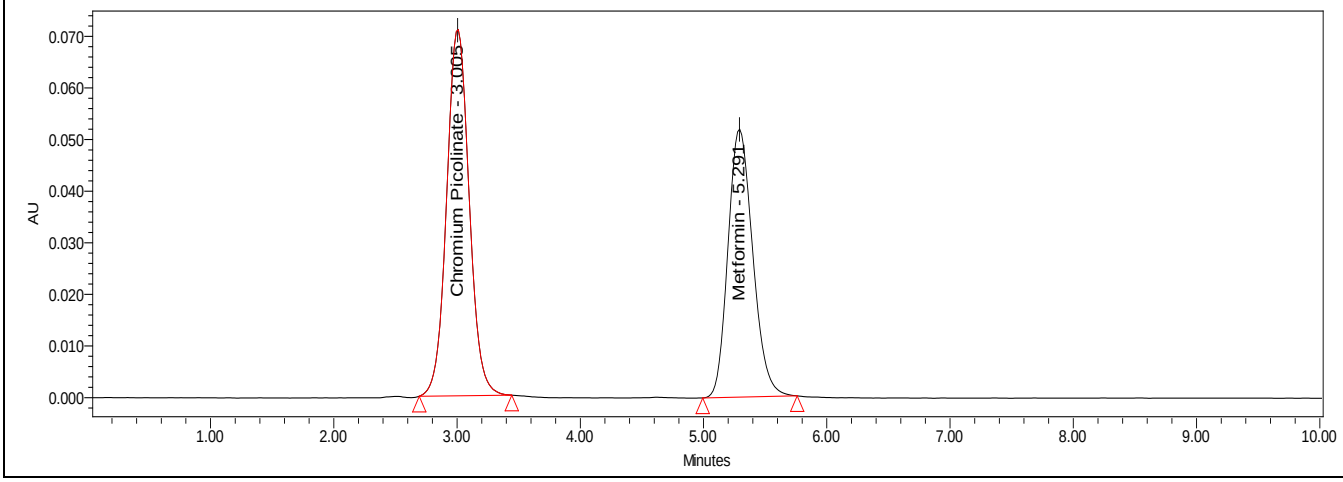


Fig: 4. Chromatogram of sample

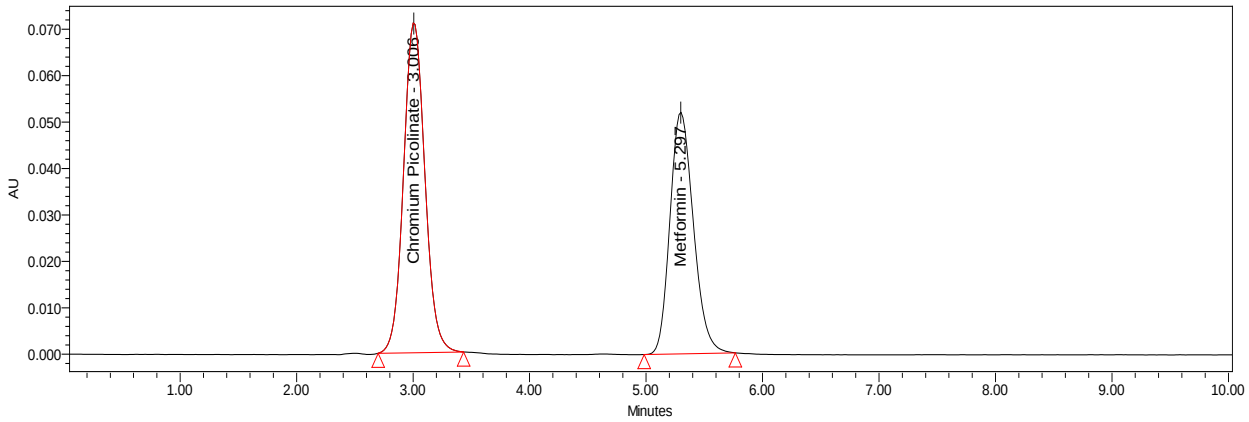


Fig: 5(a) Linearity Plot (Concentration Vs Response) of **Chromium Picolinate**

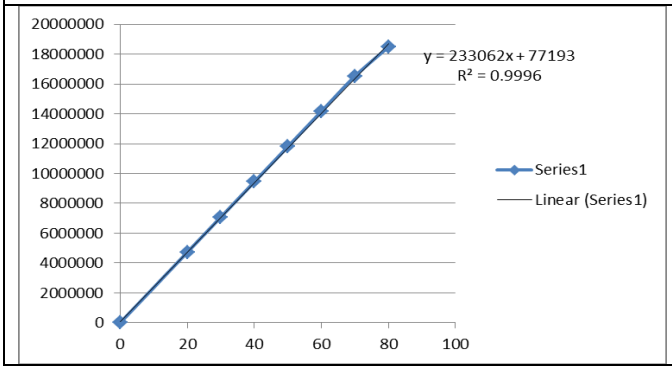
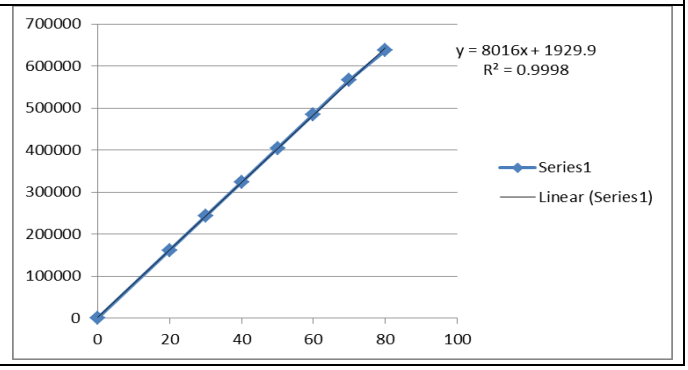


Fig: 5(b) Linearity Plot (Concentration Vs Response) **of Metformin**



Limit of Detection and Limit of Quantitation (LOD and LOQ):

Chromium Picolinate:

From the linearity plot the LOD and LOQ are calculated:

$$LOD = \frac{3.3\,\sigma}{S} = \frac{3.3\times3358.178}{33025} = 0.33$$

$$LOQ = \frac{10\,\sigma}{S} = \frac{10\times3358.178}{33025} = 1.01$$

Metformin:

$$LOD = \frac{3.3\,\sigma}{S} = \frac{3.3\times1588.8}{16169} = 0.32$$

$$LOQ = \frac{10\,\sigma}{S} = \frac{10\times1588.8}{16169} = 0.98$$

CONCLUSION

The analytical method was developed by studying different parameters. First of all, maximum absorbance was found to be at 232nm Chromium Picolinate for and 274nm for Metformin.Common wavelength will be 254nm and the peaks purity was excellent. Injection volume was selected to be 20µl which gave a good peak area. The column used for study was Inertsil C_{18}, ODS chosen good peak shape. Ambient temperature was found to be suitable for the nature of drug solution. The flow rate was fixed at 1.0ml/min because of good peak area, satisfactory retention time and good resolution. Different ratios of mobile phase were studied, mobile phase with ratio of 45:55 Methanol:Water was fixed due to good symmetrical peaks and for good resolution. So this mobile phase was used for the proposed study. The present recovery was found to be

98.0-101.50 was linear and precise over the same range. Both system and method precision was found to be accurate and well within range. Detection limit was found to be 0.0554 Chromium Picolinate and 0.17727 for Metformin. Linearity study was, correlation coefficient and curve fitting was found to be. The analytical method was found linearity over the range of 20-80ppm of the target concentration for both the drugs. The analytical passed both robustness and ruggedness tests. On both cases, relative standard deviation was well satisfactory.

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