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CHROMATOGRAPHIC DEVELOPMENT & VALIDATION OF 2–CHLOROMETHYL–4–METHYL QUINAZOLINE FOR QUANTIFICATION OF QUALITY

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Abstract: *Linagliptin is a DPP–4 inhibitor[dipeptidyl peptidase–4 inhibitor] used as antidiabetic drug which is made of three subordinate units (4–methyl quinazoline, purine–2,6–dione and 3R–piperidine–3–amine) which has one chiral point [8–[(**3R**)–3–Aminopiperidin–1–yl]–7–(but–2–yn–1–yl)–3–methyl–1–[(4–methylquinazolin–2–yl)methyl]–3,7–dihydro–1H–purine–2,6–dione] at amino piperidine unit. The quality of starting material 2–(chloromethyl)–4–methylquinazoline has been examined for purity to avoid the unwanted impurities, safety and efficacy by which the active pharmaceutical ingredient (API) linagliptin has to be synthesized. A host of impurities in pharmaceutical ingredients do occur that may be partially responsible for toxicity, chemical interference and general instability. In order to ensure that drugs reaching consumers are effective, safe of good quality regulatory requirement now demand to use standard pure API. Purity of API depends on the synthetic process involving chemical reactions using different reagents under different conditions. Therefore, estimation of purity along with impurity profile is necessary to get the pure API. These can only be achieved by thorough analysis of the precursor (Starting Material) used. Here, the anti–diabetic drug, linagliptin is to be synthesized from 2–(chloromethyl)–4–methyl quinazoline (CMQ). Its standardization i.e. purity and impurity profile has been developed and validated as required by the regulatory authorities. There is no such existing literature reports available for the estimation of CMQ.A key component of the quality of pharmaceutical drugs is the control of impurities. The pharmaceutical analytical chemistry is concerned with new analytical techniques. The main objective of our research work is to develop a RP–HPLC validated method for estimation of the purity of CMQ (KSM) along with the impurities level (known & unknown). Here, linagliptin (API) has the structural similarity with CMQ (2–chloromethyl–4–methyl–quinazoline). The method has been developed & validated should have the related impurities level <0.1% (unknown impurities) and <0.15% (known impurities) as per ICHguidelines[6,27]. This has been carried out in two steps:1.RP–HPLC Method Development.2.RP–HPLC Method Validation.*

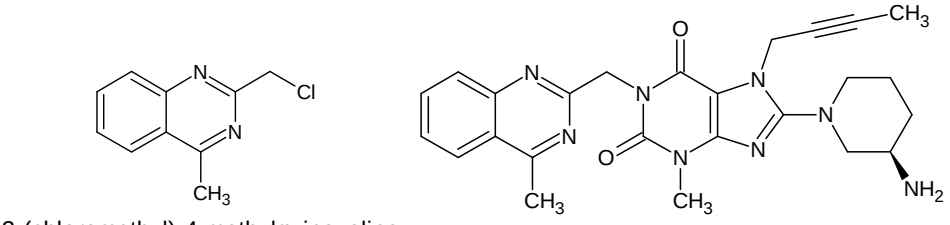
KEYWORDS: CMQ, Linagliptin, API, TLC, UV, RP–HPLC, R_f, R_t, Validation, LOD, LOQ, Robustness, Accuracy, Stability.

INTRODUCTION

Linagliptin is a DPP–4 inhibitor[dipeptidyl peptidase–4 inhibitor] used as antidiabetic drug which is made of three subordinate units (4–methyl quinazoline, purine–2,6–dione and 3R–piperidine–3–amine) which has one chiral point [8–[(3R)–3–Aminopiperidin–1–yl]–7–(but–2–yn–1–yl)–3–methyl–1–[(4–methylquinazolin–2–yl)methyl]–3,7–dihydro–1H–purine–2,6–dione] at amino piperidine unit.[2,15] The quality of starting material 2–(chloromethyl)–4–methylquinazoline has been examined for purity to avoid the unwanted impurities, safety and efficacy by which the active pharmaceutical ingredient (API) linagliptin has to be synthesized. A host of impurities in pharmaceutical ingredients do occur that may be partially responsible for toxicity, chemical interference and general instability.[19,24] In order to ensure that drugs reaching consumers are effective, safe of good quality regulatory requirement now demand to use standard pure API. Since Linagliptin has been found to have a great structural resembles with 2–chloromethyl–4 methyl–quinazoline (CMQ) thus for the method development and validation of API form synthesis of Linagliptin, CMQ has been taken into account as the key starting material (KSM). In this research paper the author has developed a RP–HPLC

validated method for estimation of the purity of CMQ (KSM) along with the impurities level (known & unknown). The method has been developed & validated should have the related impurities level <0.1% (unknown impurities) and <0.15% (known impurities) as per ICH guidelines which has been strictly followed by the author and has been carried out in two steps: 1. RP–HPLC Method Development 2. RP–HPLC Method Validation.[7,21,22]

Chemistry:
2–chloromethyl–4 methyl–quinazoline (CMQ)



2-(chloromethyl)-4-methylquinazoline — Linagliptin

Figure–1: Starting material & final API.

CMQ: CAS Number (109113–72–6), IUPAC (2–chloromethyl–4 methyl–quinazoline): Molecular Formula: $C_{10}H_9ClN_2$, Formula Weight:192.64g, Composition: C(62.35%), H(4.71%), Cl(18.40%), N(14.54%), logP=1.94, mp=61–65ºC, Storage conditions: Store in a tightly closed container below 25ºC, Description: It occurs as a off white to yellow powdered substance (Inhouse specification).

Linagliptin:(Category: Anti–diabetic agent; Type 2 diabetes mellitus, DPP4 inhibitor), CAS Number (668270–12–0), IUPAC (8–[(3R)–3–Aminopiperidin–1–yl]–7–(but–2–yn–1–yl)–3–methyl–1–[(4–methylquinazolin–2–yl)methyl]–3,7–dihydro–1H–purine–2,6–dione): Molecular Formula: $C_{25}H_{28}N_8O_2$, Formula Weight: 472.54g, Composition: C(63.54%), H(5.97%), N(23.71%), O(6.77%), logP=2.62, mp=190–196ºC, water solubility=<1 mg/mL [0.0502 mg/mL], pKa=9.86

AIMS AND OBJECTIVES OF THE RESEARCH

- To estimate the purity along with impurity profile for synthesizing API
- To analyse the precursor or the starting material 2–(chloromethyl)–4–methyl quinazoline (CMQ) for synthesizing the anti–diabetic drug, Linagliptin since CMQ has the structural resembles with Linagliptin
- To develop a RP–HPLC validated method for estimation of the purity of CMQ (KSM) along with the impurities level <0.1% (unknown impurities) and <0.15% (known impurities) as per ICH guidelines

MATERIALS/CHEMICALS:
2–chloromethyl–4–methyl quinazoline: Glenmark Pharmaceuticals
HPLC grade water: Milli Q or equivalent, HPLC grade acetonitrile: Merck, HPLC grade methanol: Merck, GR grade ammonia solution: Merck, HPLC grade triethylamine: Merck, HPLC grade perchloric acid (70%): Merck, GR grade ammonium acetate: Merck, GR grade hydrogen peroxide: Merck, GR grade dimethyl sulfoxide: Merck, GR grade dichloromethane: Merck.

Stationary Phases: Inertsil ODS 3V(250×4.6mm) 5µ [GL Sciences Inc, Japan]

Brand name and dosage available in the market
Table–1: Brand name and dosage available in the market.

Brand Name	Contains	Dosage Form	Manufacturer
TRADJENTA	Linagliptin 5 mg;tablet	Tablet	**Boehringer Ingelheim & Eli Lilly Ltd.**
JANUVIA	Linagliptin 50mg,100 mg;tablet	Tablet	**Merck Sharp &Dohmi Pharmaceuticals Ltd.Palvia,Italy**
JENTADUETO	Linagliptin & Metformin Hydrochloride 5mg/500 mg; 2.5 mg/850 mg; tablet	Tablet	**Boehringer Ingelheim and Lilly**
ONGLYJA	Linagliptin derivative 2.5mg;tablet	Tablet	**Bristol–Myers Sqibb Ltd.**

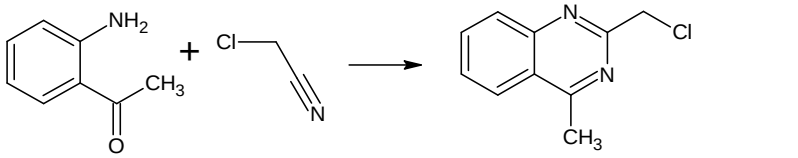
Mode of action: Linagliptin is an oral drug that reduces blood sugar(glucose) levels in patients with type 2 diabetes.[10,12] Linagliptin is a member of a class of drugs that inhibit the enzyme, dipeptidyl peptidase–4(DPP–4) Other member of a class includes sitagliptin and saxagliptin. Following a meal, in such as glucagon–like

peptide–1(GLP–1) and insulinotropic polypeptide (GIP) are released from the intestine, and their levels increase in the blood. GLP–1 and GIP reduce blood glucose by reducing the secretion by the pancreas.GLP–1 also reduces blood glucose by reducing the pancreas hormone glucagon, a hormone that increases the production of glucose by liver. The net effect of increased release of GLP–1 and GIP is to reduce blood glucose levels[2]. Linagliptin inhibit the enzyme, DPP–4, that destroys GLP–1 and GIP in the blood remain higher and blood glucose level fall. In summary, linagliptin reduces blood glucose levels by inhibiting DPP–4 and increasing the levels of GLP–1 and GIP. Linagliptin may be taken with or without food. The recommended dose is 5 mg/day[3]. The most common side effects of linagliptin are stuffy or running of nose and sore throat. Hypoglycemia may occur when linagliptin is combined with insulin or sulfonylurea–type drug.[9,11] Allergic reactions occur when linagliptin is combined with insulin or a sulfonylurea type drug. Allergenic reaction or muscle pain also may occur.

Brief Synthetic Procedure of 2–(chloromethyl)–4–methylquinazoline:[26]

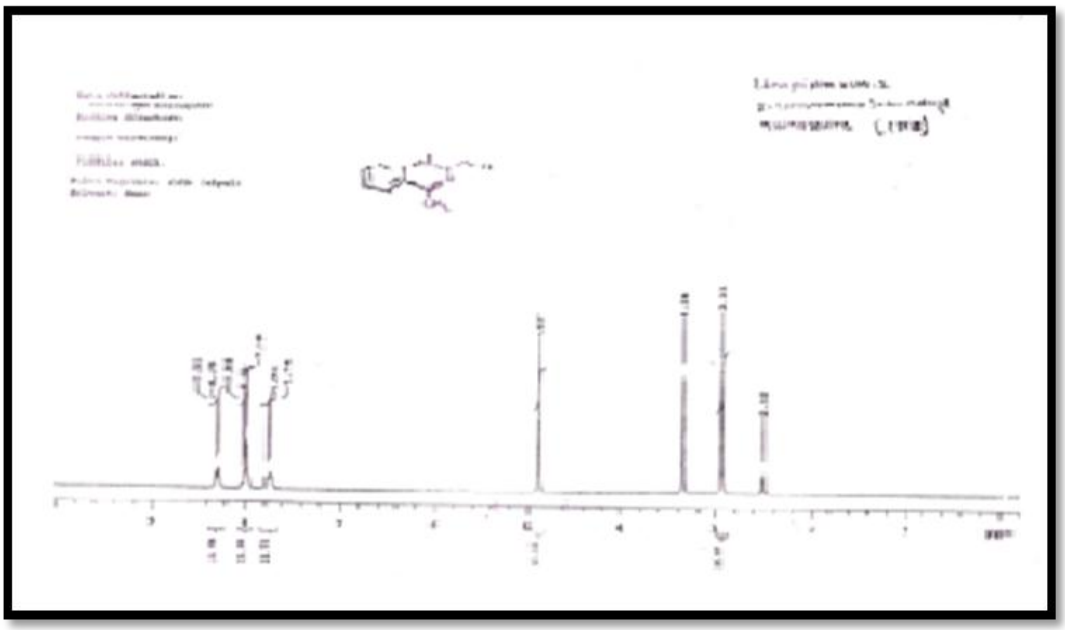
2–Aminoacetophenone charged in a dry RB (Round Bottom flask) and 1,4–dioxane is added. The mixture cooled to 10ºC and hydrogen chloride gas passing through the reaction mixture for 6–8 hours.[17] Then the reaction mixture is cooled to 0ºC and stand overnight at same temperature. A solution of chloro–acetonitrile in 1,4–dioxane is added at 0ºC for one hour. After the reaction mixture is slowly warmed to 5ºC and stirred for a further 3–4 hrs. Then a mixture of 1,4–dioxane and water is added into it. The mixture is stirred for 1 hour. The product is centrifuged and washed with water and dried at 30ºC. Compound is dissolved in hot n–Hexane and add charcoal maintain for 90 minutes and filtered. n–Hexane layer is cooled to 0–5ºC, solid is filtered and wash with cold n–Hexane to get 2–(chloromethyl)–4–methylquinazoline.

Route of Synthesis:

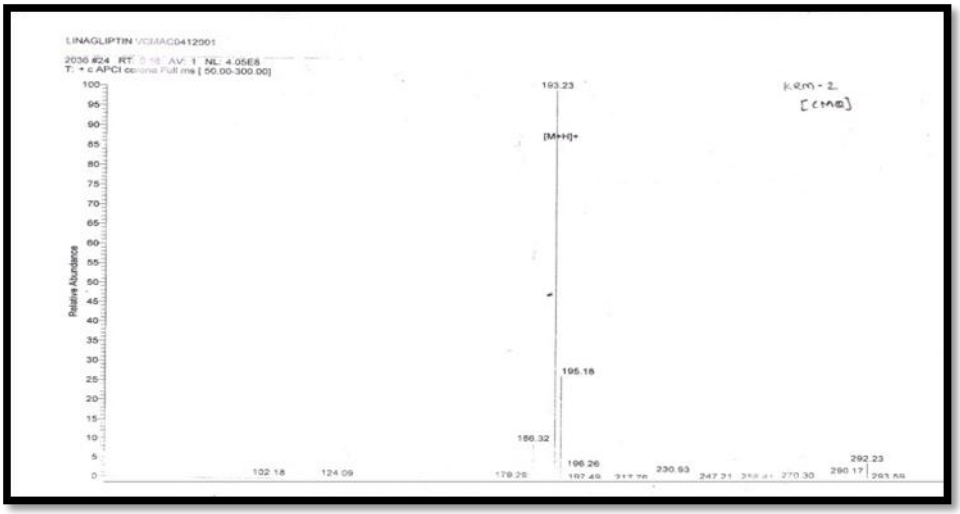


Figure–2: Scheme for CMQ: 2–(chloromethyl)–4–methylquinazoline.

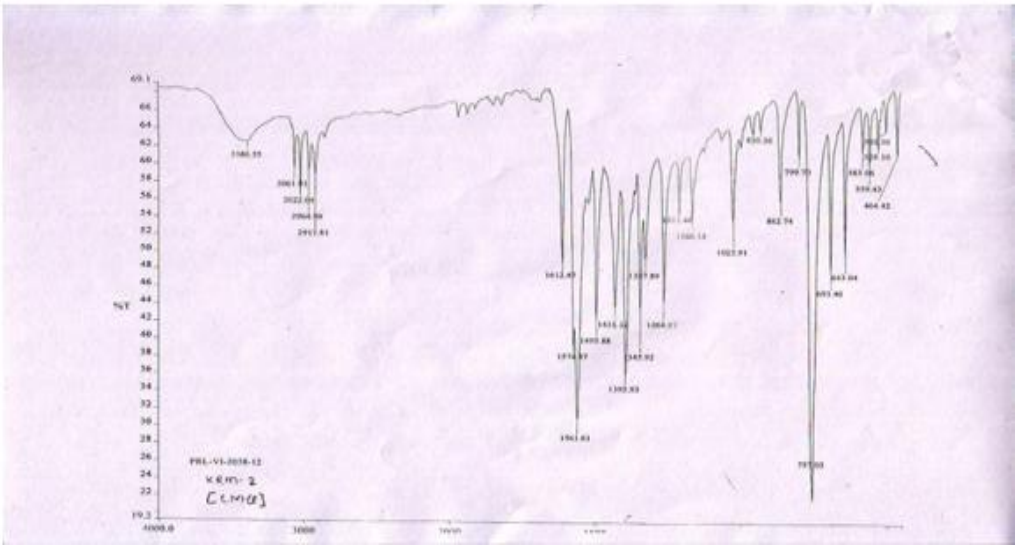
Spectral interpretation data of 2–(chloromethyl)–4–methylquinazoline: $C_{10}\mathbf{H_9}ClN_2$



1. H^1–NMR Spectra:(300 MHz, DMSO–d_6, δ ppm): 2.93(S,–CH_3,**3H**), 4.89(S,–CH_2,**2H**), 7.75–7.78(d,Ar–H,**1H**), 7.98–8.03(t,Ar–H,**2H**), 8.27–8.30 (d,Ar–H,**1H**)



2. Mass Spectrum (m/z):193(M^+)



3. IR Spectrum

Table–2: Instrument specifications.

Name of the Instrument	SpecificationModel /Brand	Company
HPLC	LC–2010AHT auto injectorSPD–M 10–AVP– PDADetector	Shimadzu, Japan
HPLC	LC–2010CHT auto injector with dual λ absorbanceDetector	Shimadzu, Japan
HPLC	Waters 2695 gradientsystem with auto samplerand column oven,2487 dualabsorbance detector	Waters, Alliance
PerkinElmer UV–VIS Spectrophotometer	Lambda 35	PerkinElmer
Weighing balance	BP 211D	Sartorius
Weighing balance	XS 205 DUAL RANGE	Mettler Toledo
pH METER	Orion 3star pH bench top	Thermo electron Corporation
Bath ultrasonicator	Fast clean	Entertech electronics pvt, Ltd Mumbai.
Milli–Q Water treatment system	MilliQ–Liocel	Millipore Ltd, Mumbai

Method Development

Selection of chromatographic method: Proper selection of the method depends upon the nature of the sample (ionic/ionizable/neutral molecule, its molecular weight and solubility).[7,16,18] The drug selected in the present study was polar in nature therefore, reverse phase or ion exchange or ion pair chromatography method can also be used. Here, the reverse phase HPLC method was selected for the initial separation owing to its simplicity, suitability, ruggedness and its wider usage.

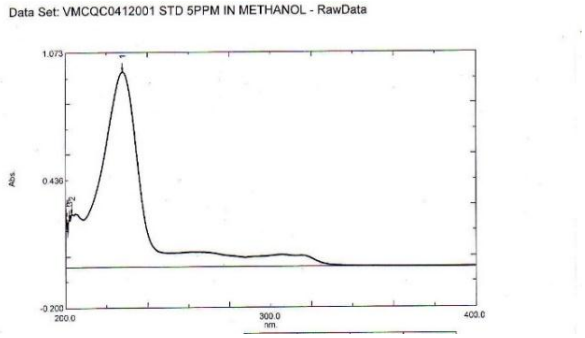
Solubility data:

CMQ is very slightly soluble in dimethyl sulfoxide (DMSO); insoluble in water; soluble in dichloro methane (MDC).[29]

Criteria for Solubility: Freely soluble:100mg/1ml. Soluble:100mg/3ml. Sparingly soluble:100mg/10ml. Slightly soluble:10mg/10ml. Very slightly soluble:10mg/100ml. Insoluble: Still if present.

Wavelength selection: λmax of CMQ is 227(±3)nm.

An UV spectrum of the 2–chloromethyl–4–methyl quinazoline (C.M.Q) and its related substances at a concentration of 10µg/ml, 5 µg/ml in methanol ware recorded by scanning the sample in the UV range of 200–400 nm and then overlaid to determine the detection wavelength. The UV absorption spectrum of the substance being examined should exhibit wavelength maxima at about 227(±3)nm.



Figure–4: UV scan graph of CMQ.

In order to achieve the optimized chromatographic conditions to separate and quantify related substances of CMQ, numbers of trials were done by changing one parameter at each trial and chromatograms were recorded with all specified chromatographic conditions.

Table–3: Properties of HPLC Buffer and Additives

Additive or Buffer	pKa	pH Range	UV Cut off
TFA	<<2(0.5)	1.5–2.5	210 nm(0.1%)
Acetic acid (as Ammonium Acetate)	4.8	3.8 to 5.8	205 nm (10mM)
	9.2	8.2 to 10.2	
Formic acid (as Ammonium formate)	3.8	2.8 – 4.8	200 nm (50mM)
	9.2	8.2 – 10.2	
Phosphate	2.2	1.2 – 3.2	200nm (0.1%)
	7.2	6.2 – 8.2	
	12.3	11.3 – 13.3	
Borate	9.2	8.2 – 10.2	200nm (10mM)
4–Methyl–Morpholine	8.4	7.4 – 9.4	
Ammonium hydroxide / ammonia	9.2	8.2 –10.2	200nm (10mM)
Bicarbonate	10.3	9.3 – 11.3	<200 nm
1–Methyl–Piperidine	10.3	9.3 – 11.3	
Triethylamine (TEA)	10.7	9.7 – 11.7	<200 nm
Pyrrolidone	11.3	10.3 – 12.3	
Glycine	9.8	8.8 – 10.8	

Table–4: HPLC Solvents – Relative Polarity.

Relative Polarity	Compound Formula	Group	Representative Solvent
Nonpolar (Hydrophobic)	R–H	Alkanes	Petroleum ethers, hexane
	Ar–H	Aromatics	Toluene, benzene
	R–O–R	Ethers	Diethyl ether
	R–X	Alkyl halides	Tetrachloromethane, chloroform
	R–COOR	Esters	Ethyl acetate
	R–CO–R	Aldehydes and Ketones	Acetone,methyl ethyl ketone
	$R–NH_2$	Amines	Pyridine, Triethylamine
	R–OH	Alcohols	Methanol, ethanol, isopropanol, butanol
	$R–CONH_2$	Amides	Diethylformamide
Polarity (Hydrophilic)	R–COOH	Carboxylic acids	Ethanoic acid
	H–OH	Water	Water

The trial conditions are mentioned as follows:

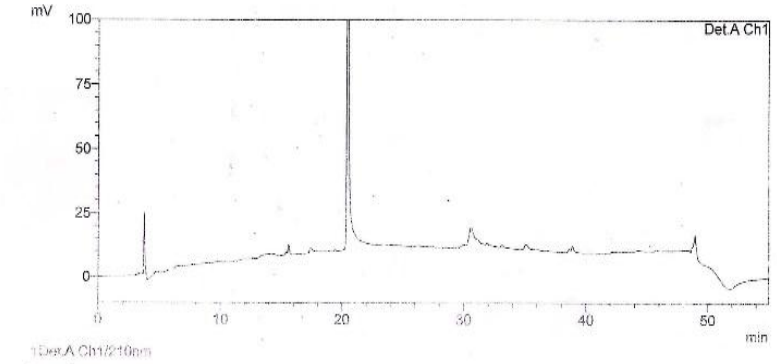
Trial No–1
Aim:–To separate the peak first base to base.

Table–5: Chromatographic Conditions.

Mobile phase–A	0.1% Tri Ethyl Amine in water (Milli Q). Adjust pH to 7.0 with Perchloric acid.
Mobile phase–B	Acetonitrile (100%)
Detection wavelength	UV 210 nm
Flow	1 ml/min
Injection volume	20µl
Column Pressure	1765 psi
Column (Stationary phase)	Inertsil,ODS(C18),3V, (250×4.6mm) 5µ
Column–Temp	30°C
Sample Cooler temperature	25°C
Diluent	ACN: Water (70:30)
Sample Concentration	1000ppm
Time Program	Gradient

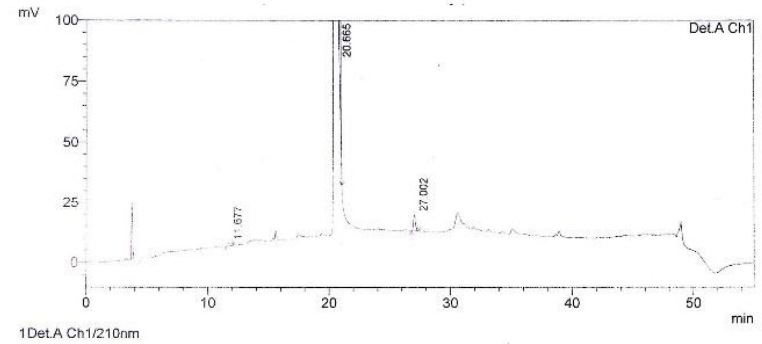
Time (min.)	%MP–A	%MP–B
0.01	90	10
40	10	90
45	10	90
47	90	10
55	9	1

Run time	55 min.



<Results>

Detector A Ch1 210nm



1Det.A Ch1/210nm

<Results>

Detector A Ch1 210nm

Peak #	Name	Ret. Time	Area	Area %	T. Plates	Tailing F.	Resolution	R. R.T.
1	RT11.677	11.677	14970	0.04	29907	1.58	0.00	0.56
2	CMQ	20.665	39549480	99.77	47585	1.15	27.70	1.00
3	RT27.002	27.002	75259	0.19	130708	1.11	18.70	1.31
Total			39639708	100.00				

Figure–5: Chromatogram–I of Blank (Diluent)&Chromatogram–II of CMQ.

Observation: R_t of CMQ=20.66 min.
(1) One peak is obtained in the tailing side of the main peak which is not separated in this chromatographic condition. (2) Resolution is very less. (3) Blank peak is observed at same Retention Time, thus blank peak and the main peak are merged.

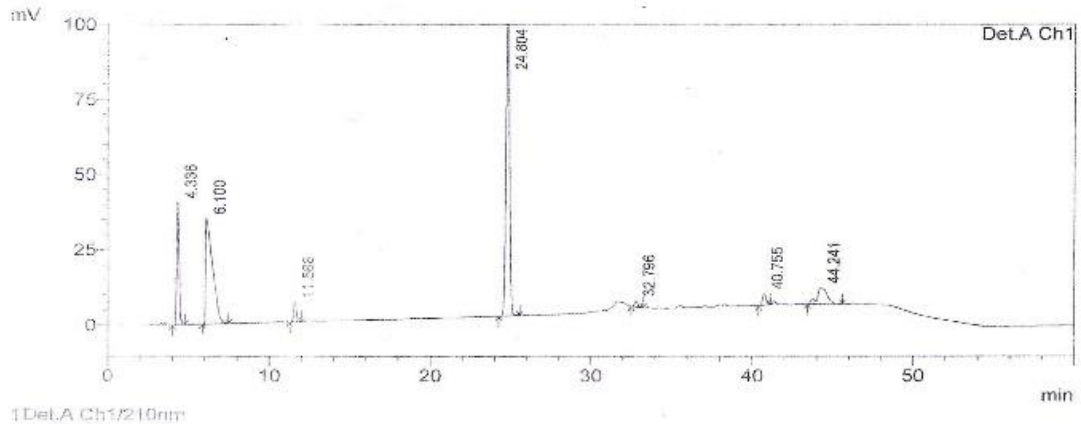
Conclusion: To overcome these problems need to change the buffer composition. Next trial is being taken.

Trial No–2
Aim: To separate the peak merging, changing of buffer has done. 0.1M Sodium perchlorate in water, pH 4.45 as such.

Table–6: Chromatographic conditions.

Mobile phase–A	0.1M Sodium perchlorate in water (Milli Q). Buffer pH will be 4.45 as such.
Mobile phase–B	Acetonitrile (100%)
Detection wavelength	UV 210 nm
Flow	1 ml/min
Injection volume	20µl
Column Pressure	1765 psi
Column (Stationary phase)	Inertsil, ODS (C18), 3V, (250×4.6mm) 5µ
Column–Temp	30°C
Sample Cooler temperature	25°C
Diluent	ACN: Water (70:30)
Sample Concentration	1000ppm
Time Program	Gradient
Run time	60 min.

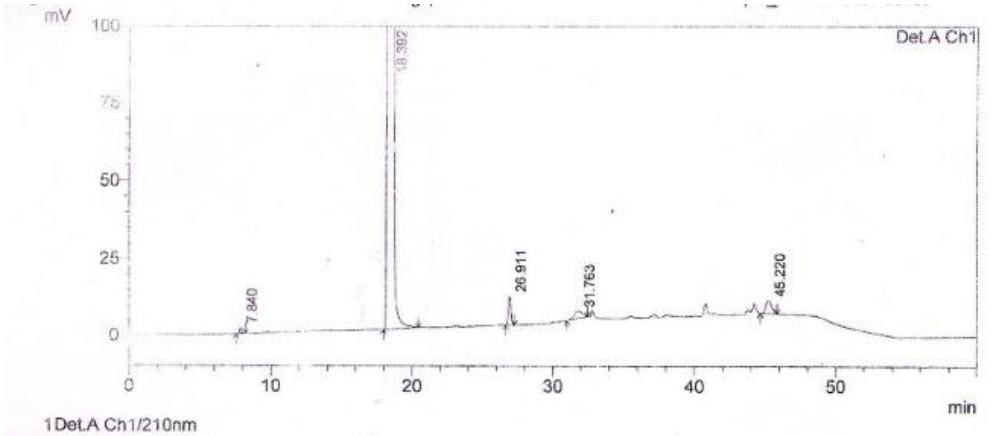
Time (min.)	%MP–A	%MP–B
0.01	80	20
40	25	75
45	25	75
50	80	20
60	80	20



<Results>

Detector A Ch1 210nm

Peak #	Name	Ret. Time	Area	Area %	T. Plates	Tailing F.	Resolution	R. R.T.
1		4.336	403571	11.38	4217	1.15	0.00	0.00
2		6.100	1066731	30.07	729	4.93	3.01	0.00
3		11.588	80405	2.27	20145	1.15	8.92	0.00
4		24.804	1678899	47.33	61745	1.06	36.41	0.00
5		32.796	26939	0.76	159508	1.23	21.96	0.00
6		40.755	59418	1.68	163670	1.21	21.76	0.00
7		44.241	230983	6.51	36483	1.14	5.25	0.00
Total			3546946	100.00				



1Det.A Ch1/210nm

<Results>

Detector A Ch1 210nm

Peak #	Name	Ret. Time	Area	Area %	T. Plates	Tailing F.	Resolution	R. R.T.
1	RT7.840	7.840	18172	0.04	17583	1.29	0.00	0.42
2	CMQ	18.392	47169895	99.25	29794	1.14	31.84	1.00
3	RT26.911	26.911	124053	0.26	91179	1.09	21.77	1.47
4	RT31.763	31.763	95723	0.20	10772	0.97	6.14	1.73
5	RT45.220	45.220	120784	0.25	53963	1.25	13.44	2.47
Total			47528627	100.00				

Figure–6: Chromatogram–III of Blank (Diluent) & Chromatogram–IV of CMQ.

Observation: R_t of CMQ=18.39 min.

(1) One peak is obtained in the tailing side of the main peak which is not separated in this chromatographic condition. (2) Resolution is very less. (3) Blank peak is observed at same Retention Time, thus blank peak and the main peak are merged. (4) Peak tailing is observed due to lack of sharpness. May be there is an impurity interference in tailing.

Conclusion: To overcome these problems need to change the buffer composition. Next trial is being taken.

Trial No–3

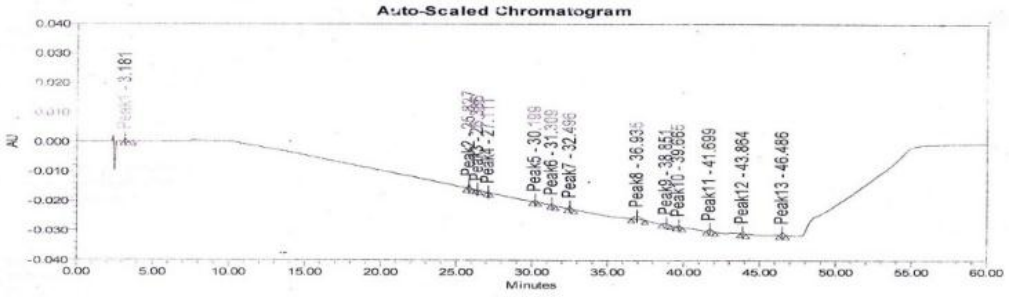
Aim: To change the gradient program as well as 0.01M Ammonium Acetate pH 6.5 (as such) is used.

Table–7: Chromatographic Conditions.

Mobile phase–A	0.01 M Ammonium Acetate in water (Milli Q), pH to 6.5 as such.
Mobile phase–B	Acetonitrile (100%)
Detection wavelength	PDA 210 nm & 225 nm
Flow	1 ml/min
Injection volume	10µl
Column Pressure	1765 psi
Column (Stationary phase)	Inertsil, ODS (C18),3V, (250×4.6mm) 5µ
Column–Temp	30°C
Sample Cooler temperature	25°C
Diluent	0.1% Ammonium acetate in water: ACN = 4:6
Sample Concentration	1000ppm
Time Program	Gradient,

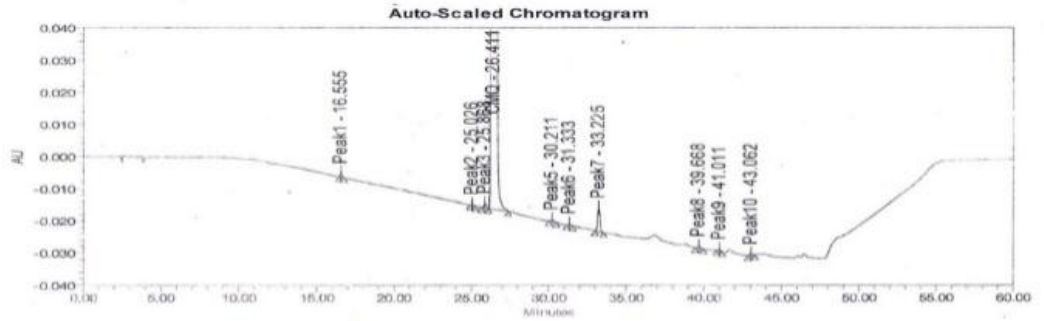
Time (min.)	%MP–A	%MP–B
0.01	90	10
05	90	10
40	25	75
45	25	75
50	90	10
60	90	10

Run time	60 min.



Peak Results

	Name	RT	Area	% Area	RT Ratio	Purity1 Angle	Purity1 Threshold	USP Plate Count	USP Resolution
1	Peak1	3.181	7994	6.61		7.748	8.717	3549	
2	Peak2	25.827	11455	9.48		3.817	4.617	170825	94.82
3	Peak3	26.386	1695	1.40		27.051	45.393	210717	2.29
4	Peak4	27.11*	1583	1.31		14.615	20.351	286228	3.31
5	Peak5	30.169	8486	6.37		12.320	9.469	120774	11.54
6	Peak6	31.309	4977	4.12		22.547	28.682	184680	3.36
7	Peak7	32.496	8667	7.17		12.731	14.842	186200	3.96
8	Peak8	36.935	32009	26.47		6.574	4.839	35871	7.83
9	Peak9	38.851	8958	7.42		22.043	11.427	129941	2.93
10	Peak10	39.666	7253	6.00		26.543	15.893	211516	1.92
11	Peak11	41.699	13334	11.03		11.550	12.545	121419	4.99



Peak Results

	Name	RT	Area	% Area	RT Ratio	Purity1 Angle	Purity1 Threshold	USP Plate Count	USP Resolution
1	Peak1	16.555	10604	0.02	0.63	11.666	13.100	92163	
2	Peak2	25.026	3726	0.01	0.95	22.791	33.902	117695	31.43
3	Peak3	25.868	16990	0.04	0.98	3.640	4.465	140683	2.80
4	CMQ	26.411	47134656	99.68	*	12.106	21.077	87051	1.71
5	Peak5	30.211	10629	0.02	1.14	12.269	8.440	123716	10.84
6	Peak6	31.333	6949	0.01	1.19	6.916	22.489	252323	3.76
7	Peak7	33.225	81122	0.17	1.26	1.337	1.764	217594	7.01
8	Peak8	39.668	10810	0.02	1.50	10.633	10.606	188461	19.28
9	Peak9	41.011	6901	0.01	1.55	12.625	17.523	295099	4.01
10	Peak10	43.062	5799	0.01	1.63	34.489	40.556	352503	7.03

Figure–7: Chromatogram–V of Blank (Diluent)&Chromatogram–VI of CMQ.

Observation: R_t of CMQ=26.41 min.
(1) CMQ, and all other unknown impurities ware separated out. (2) Till base line was not good. (3) Peak shape was found to be good. (4) Thus, need to be resolved.
Conclusion: To overcome the base line problem next trial is being taken.

Trial No–4
Aim: A better chromatogram can be obtained by maintain a buffer – ACN concentration in a such way to maintain the U.V range as such as to get a straight chromatogram.
(1) When ammonium acetate as a buffer uses in HPLC, as the chromatogram progresses the base line drift to downward (negative). (2) pH of ammonium acetate is as such 6.7 to 7.3. (3) Molecular weight of ammonium acetate = 77.08g/ml. (4) Thus we are taking 0.01(M) ammonium acetate i.e. 0.77gm of ammonium acetate.

Solution Stability: Diluent is special type, suppose if the diluent will be 1:1, ACN: water, the impurities peak shape will be different in each runtime.0.1% NH_4OH solution is uses to make a basic condition, i.e. ideal to settle a solution stability. Relative impurities give reproducible peak in each runtime.

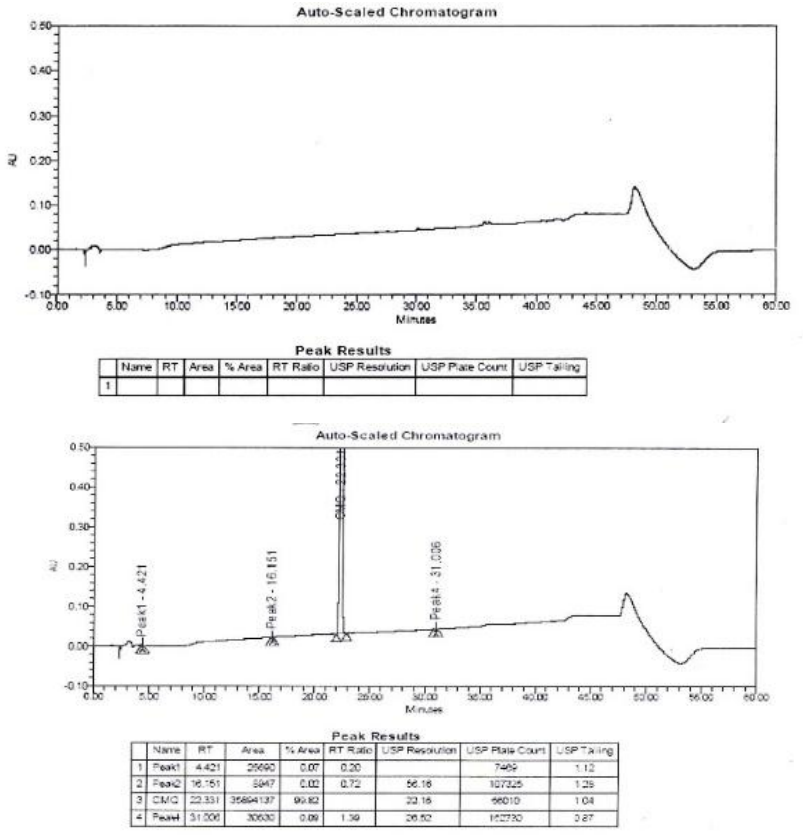
- As per the ICH guideline, the unknown impurities should be <0.1%, and known impurities should be <0.15%.
- In assay the aim is to find out the purity of any substance, whereas in Relative Substances (R.S) the aim is to find out the impurities (known & unknown).
- The pH optimization method is being developed by taking around ±2 of the pKa value of drug.
- Trial and error method is done by taking several pH value, to detect the maximum no of impurities.

Table–8: Chromatographic Conditions.

Mobile phase–A	0.77gm Ammonium Acetate in 900ml water (Milli Q), sonicate to dissolve. Add 100ml of ACN, mix well and filter.
Mobile phase–B	0.77gm Ammonium Acetate in 100ml water (Milli Q), sonicate to dissolve. Add 900ml of ACN, mix well and filter.
Detection wavelength	UV 210 nm
Flow	1 ml/min
Injection volume	10µl
Column Pressure	1765 psi
Column (Stationary phase)	Inertsil, ODS(C18), 3V, (250×4.6mm) 5µ
Column–Temp	30°C
Sample Cooler temperature	25°C
Diluent	0.1% Ammonium solution in water: ACN = 1:1
Sample Concentration	1000ppm
Time Program	Gradient,

Time (min.)	%MP–A	%MP–B
0.01	90	10
05	90	10
40	25	75
45	25	75
50	90	10
60	90	10

Run time	60 min.



Figure–8: Chromatogram–VII of Blank (Diluent)&Chromatogram–VIII of CMQ.

Observation: R_t of CMQ=22.33 min.
(1) Main peak of CMQ is identified and all other unknown impurities ware separated out. (2) Baseline found to be better than all other trials.(3) Peak shape was found to be good.

Conclusion:–The HPLC analytical method is developed of CMQ, % area–99.82% and R_t at 22.33 minute, Peak Area is 3crore58 lakh.

Final method:
Reagent, solvent and Standards: water (Milli Q or equivalent), Acetonitrile (HPLC grade), Ammonium acetate (AR grade), Ammonium solution (AR grade)

Chromatographic condition:
Apparatus: A high performance liquid chromatograph equipped with quaternary gradient pump, variable wavelength UV detector attached with data recorder and integrator software.

Column: Inertsil ODS 3V, 250×4.6mm, 5µ
Column temperature: 30ºC
Sample Cooler temperature: 25ºC
Mobile phase A: 0.77gm of ammonium acetate in 900 ml water, sonicate to dissolve. Add 100 ml of acetonitrile, mix well and filter.
Mobile phase B: 0.77gm of ammonium acetate in 100 ml water, sonicate to dissolve. Add 900 ml of acetonitrile, mix well and filter.

Table–9: Gradient ratio.

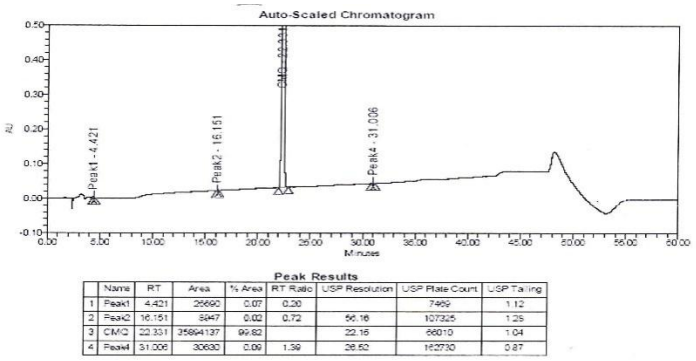
Time (min)	% Mobile Phase A	% Mobile Phase B
0.00	90	10
05	90	10
40	25	75
45	25	75
50	90	10
60	90	10

Diluent: 0.1% ammonium solution in water: Acetonitrile (1:1, v/v)
Flow Rate: 1.0ml/minute
Detection: UV 210nm
Injection volume: 10 µl.

Preparation of Test solution: Weigh accurately about 20.0mg of 2–(chloromethyl)–4–methyl quinazoline and transfer it into 20ml volumetric flask. Add about 10 to 15ml of diluent and sonicate to dissolve. Make up to the mark with diluents and mix.

Procedure: Separately inject equal volumes of blank (diluent), test solution in duplicate and record the chromatogram for all injections eliminating the peaks due to blank. Calculate the chromatographic purity by area normalization method. The retention time of main peak i.e. 2–(chloromethyl)–4–methyl quinazoline is about 23.0 minutes under these conditions.

System suitability test: Tailing factor should not be more than 1.5 of the main peak i.e.2–(chloromethyl)–4–methyl quinazoline from test solution.



Figure–9: Chromatogram–IX.

Calculations:

$$\% \text{ Assay} = \frac{\text{Area of test solution}}{\text{Average area of standard Solution}} \times \frac{\text{Test solution}}{20} \times \frac{\text{Weight of standard in Standard solution}}{50} \times \frac{5}{\text{weight of sample}} \times \frac{20}{5} \times \frac{50}{100} \times \frac{\text{Potency of standard}}{100} \times 100$$

$$\% \text{ Assay (on anhydrous basis)} = \frac{\% \text{ Assay (as such)}}{(100 - \% \text{ Water content})} \times 100$$

P = Potency of in–house reference standard

Method validation: Method validation can be defined as (ICH) "Establishing documented evidence, which provides a high degree of assurance that a specific activity will consistently produce a desired result or product meeting its predetermined specifications and quality characteristics".[1] Method validation is an integral part of the method development; it is the process of demonstrating that analytical procedures are suitable for their intended use and that they support the identity, quality, purity, and potency of the drug substances and drug products. Simply, method validation is the process of proving that an analytical method is acceptable for its intended purpose. All the variables of the method should be considered, including sampling procedure, sample preparation, chromatographic separation, and detection and data evaluation. For chromatographic methods used in analytical applications there is more consistency in validation practice with key analytical parameters. Optimized chromatographic conditions were developed for the separation and quantification of related substances of 2–chloromethyl–4–methyl quinazoline (C.M.Q) according to different individual conditions such as solvent system, pH of the mobile phase, stationary phase, and diluents.[3] This method was validated according to ICH (Q3A, Q2B)[4,5] guidelines, i.e. guidelines for the drug product API and the guidelines for the impurities present in a drug substance.

Table–10: Validation.

Sr. No.	Validation Parameter	Observations	Acceptance Criteria
1	**Specificity**	Method found specific for CMQ No interference observed from any degradation products as well as from unknown impurity	The C.M.Q peak and all other impurity peaks should be well resolved.[8,13]
2	**Stability in solution**	The C.M.Q is stable in the test solution for 48 hours.	The sample preparation to be considered stable as long as there is no significant rise in impurity peaks.
3	**Linearity**	The method is found to be linear from LOQ, 50% to 150% of the test concentration. correlation coefficient greater than 0.99.	Correlation coefficient greater than or equal to 0.99
4	**Limit of Detection**	0.01%w/w of RS concentration for C.M.Q, specified limit 0.20%	Signal to Noise Ratio should be above 3 and %RSD for six injections at this concentration should be less than 33%.[14]
5	**Limit of Quantification**	0.02%w/w of RS concentration for C.M.Q.	Signal to Noise Ratio should be above 10 and %RSD for six injections at this concentration should be less than 10%
6	**Precision** • **System Precision** • **Method Precision** • **Ruggedness**	 %RSD is within desired limits. %RSD is within desired limits. %RSD is within desired limits.	Single maximum impurity should NMT 0.20%.RSD should not be more than 2.0%.
7	**Accuracy**	80µg/ml, 100µg/ml, 120 µg/ml	Mean recovery should lie within 98.0% to 102.0%.
8	**Robustness**	Method is unaffected by small changes in experimental conditions.	%RSD between results obtained with changed condition and that under normal experimental condition should not be more than 2.0%.

Conclusion: Under the conditions described the method is found to be specific, rugged, robust, accurate and linear.

The method is suitable for the **ESTIMATION OF RELATED SUBSTANCES IN 2–CHLOROMETHYL –4–METHYL QUINAZOLINE** as an active pharmaceutical ingredient.

Stability in the Solution: The study reveals that CMQ is found to be stable in the diluent solution for 48 hours at room temperature.

Table–11: Data sheet for solution stability.

Sample	% Any other individual impurity	% Total impurities
0 hr	0.04	0.04
1.5 hr	0.04	0.04
3 hr	0.05	0.05
4.5 hr	0.04	0.04
6 hr	0.05	0.05
12 hr	0.05	0.05
18 hr	0.06	0.06
24 hr	0.05	0.05
36 hr	0.06	0.06
48 hr	0.06	0.06

ND = Not detected

Selectivity: Selectivity is the ability to ensure quantitatively the analyte in the presence of components that may be expected to be present in the sample matrix. Selectivity is done to check interference from diluent and/or degradation products and/or any impurities with main peak. It is observed that Impurity does not interfere with CMQ peak. This shows that the method is selective for estimation of Related substances in CMQ.

Table–12: Sequence for Selectivity.

Sample Name	No. of Injections
Blank	1
2–chloromethyl–4–methylquinazoline sample	2

Table–13: Data Sheet for Reference Solution Injections (Selectivity).

Chromatogram no.	CMQ area
Reference Solution(a)	34492
Reference Solution(a)	34541
Reference Solution(a)	34968
Reference Solution(a)	34095
Reference Solution(a)	34273
Reference Solution(a)	34769
Average	34523
Std Dev	317.97
%RSD	**0.92**

Table–14: Retention time of impurities and main peak.

Selectivity	Retention time
CMQ	24.088 minutes

Specificity of the Method: To check the specificity of the method the compound is subjected to forced degradation under different sets of conditions like temperature, humidity, acid, base, oxidation and photo degradation. After the study, chromatograms are checked for appearance of any extra peak due to degradation of the analyte under stressed conditions and its respective retention time is recorded. Purity of the main peak is also recorded.

Effect of diluents: Diluent is injected in to the column to check the interference from the diluent at the retention time of the main peak. It is observed that diluent {Buffer: ACN (1:1, v/v)} does not interfere with retention time of the main peak or any other impurity.

Sample without Stress Conditions: A sample of CMQ of RS concentration is injected. The CMQ peak is eluted at 24.088 minutes.

Forced Degradation Study: Degradation with Acid: Acid degradation samples are injected at zero hour, after twelve hours, twenty–four hours and after reflux for 4 hours. 1N HCl is used for these studies.[21] No degradation observed at zero & twelve hours. About 60 % degradation observed at 24 hours. The results are reported in Table–17.

Degradation with Base: Base degradation samples are injected at zero hour, after twelve, twenty–four hours. 1N NaOH is used for these studies. 1% degradation observed at zero–hour, twelve hour & 24 hours. The results are reported in Table–18.

Oxidative Degradation: Oxidative degradation samples are injected at zero hour, twelve hours, twenty–four hours. A 30% hydrogen peroxide solution is used for these studies. No degradation observed at zero–hour, twelve hour & 24 hours. The results are reported in Table–19.

Effect of Temperature: It is observed that CMQ undergoes about minor degradation when exposed to temperature (105°C for 24 hours) Table–20.

Effect of Humidity: It is observed that CMQ does not undergoes any degradation when exposed to relative humidity of about 75% for twenty–four–hour Table–21.

Photo degradation: It is observed that CMQ does not undergo any degradation when exposed to the light for a period equivalent to about 1.2 million lux hours (50000lux × 24hrs = 1.2 million lux hours) Table–22.

Table–15: Sequence for Forced Degradation Study.

Sample Name	No. of Injections
Blank	1
Control Sample	1
Blank–1 N HCl	1
Sample– 1(N) HCl–0 hr, 24 hr	1 each
Blank–1(N) NaOH	1
Sample–1(N) NaOH–0 hr, 24 hr	1 each
Blank–3% H_2O_2	1
Sample–3% H_2O_2–0 hr,24 hr	1 each
Sample–Heat–24 hours (105°C)	1
Sample–Humidity–24 hours	1
Sample–Light	1

Table–16: Data sheet for reference solution injections (specificity).

Chromatogram no.	CMQ area
Reference Solution(a)	41795
Reference Solution(a)	42876
Reference Solution(a)	42851
Reference Solution(a)	42336
Reference Solution(a)	42851
Reference Solution(a)	42541
Average	42542
Std Dev	424.96
%RSD	1.00

Purity angle should be less than purity threshold

Table–17: Data sheet for specificity (Acid Degradation).

Sr. No.	Test	Chromatogram Name.	Appearance of Extra Peak	Purity Angle	Purity Threshold	Remarks
1.	1N HCl Zero Hours	Acid sample 0hr	21.63min (0.16%) 23.32min (0.14%)	0.432	1.066	About 1% degradation observed.
2.	Twelve Hours	Acid sample 12hr	27.03min (15.60%) 28.85min (0.37%)	0.249	1.033	About 40% degradation Observed.
3.	Twenty–four Hours	Acid sample 24hr	27.450min (19.0%) 29.175 min (0.55%)	0.605	1.035	About 60% degradation observed.

Table–18: Data sheet for specificity (Base Degradation).

Sr. No.	Test	Chromatogram Name.	Appearance of Extra Peak	Purity Angle	Purity Threshold	Remarks
1.	1N NaOH Zero Hours	Base sample 0hr	21.230 min (18.81%) 26.099min (0.61%)	0.399	1.036	1% degradation observed.
2.	Twelve Hours	Base sample 12hr	21.196 min (23.89%) 26.084 min (0.82%)	0.305	1.026	1% degradation observed
3.	Twenty–four Hours	Base sample 24hr	21.199 min (23.94%) 26.087 min (1.118%).	0.358	1.026	About 5% degradation Observed.

Table–19: Data sheet for specificity (Peroxide Degradation).

Sr. No.	Test	Chromatogram Name.	Appearance of Extra Peak	Purity Angle	Purity Threshold	Remarks
1.	30 %H_2O_2 Zero Hours	Peroxide sample 0hr	15.663min (0.14%) 16.792min (0.15%) 19.062min (0.24%)	0.364	1.025	About 0.5% degradation observed.
2.	Twelve Hours	Peroxide sample 12hr	21.266min (0.20%) 26.057min (0.09%) 27.502min (1.08%) 30.542min (0.14%) 31.280min (0.10%)	0.299	1.028	About 8% degradation Observed

3.	Twenty–four Hours	Peroxide sample 24hr	15.832min (0.06%) 16.925min (0.10%) 19.150min (0.16%) 21.253min (0.23%) 27.480min (1.32%) 30.514min (0.14%)	0.206	1.025	About 18% degradation observed.

Table–20: Data Sheet for specificity (Thermal Degradation).

Sr. No.	Test	Chromatogram No.	Appearance of Extra Peak	Purity Angle	Purity Threshold	Remarks
1.	Effect of Temperature	Thermal sample	——	0.297	1.026 min	Minor degradation observed.

Table–21: Data sheet for specificity (Humidity Degradation).

Sr. No.	Test	Chromatogram No.	Appearance of Extra Peak	Purity Angle	Purity Threshold	Remarks
1.	Effect of Humidity	Humidity sample	21.186min (0.32%)	0.285	1.028	Minor degradation observed.

Table–22: Data sheet for specificity (Photo Degradation).

Sr. No.	Test	Chromatogram No.	Appearance of Extra Peak	Purity Angle	Purity Threshold	Remarks
1.	Effect of Light	Photo sample	——	0.268	1.022	Minor degradation observed.

Limit of Detection (LOD): The limit of detection is determined from the linearity of related substances experiment wherein lower concentrations of each Impurities and CMQ are analysed. The LOD concentration is found to be 0.01 ppm i.e. 0.01%w/w of RS concentration for Unknown Impurities 0.01 ppm i.e for CMQ. The RSD for six replicate injections of drug is evaluated using least square method. Calibration graph will be plotted for the obtained area under the peak of each level against the concentration of 2–(chloromethyl)–4–methylquinazoline. Correlation coefficient, slope, STEYX and intercept will be calculated. Prediction LOD and LOQ values will be calculated using the following formula:

$LOD = (3.3 \times STEYX) \div Slope$

$LOD = (10 \times STEYX) \div Slope$

Preparation of **stock solution A**: Accurately weigh and transfer 20 mg of CMQ standard in 100 ml of volumetric flask. Add about 50–60 ml of diluent and sonicate to dissolve. Make up to the mark with diluent and mix.(200ppm).

Preparation of **stock solution B**: Pipette out 1ml of stock solution A in 100 ml volumetric flask. Dilute and make up to the mark with diluent and mix (2ppm). The results are reported in Table–27.

Limit of Quantification (LOQ): The limit of quantification is determined from the linearity of related substances experiment wherein lower concentrations of Impurities (unknown) and CMQ are analysed. The LOQ concentration is found to be 0.02 ppm i.e. 0.02%w/w of RS concentration for Impurities (unknown) and CMQ. The RSD for six replicate injections of Impurities, CMQ are found to be 2.25% respectively. The signal to noise ratio is above 10. The results are reported in Table No – 28.

Table–23: Dilutions for LOD & LOQ.

Level	Stock Solution	Amount of Stock Solution to be transferred (ml)	Final Volume with diluent (ml)	Concentration
Lin–1.0%	Stock Solution B	1.0	100	0.02
Lin–2.5%	Stock Solution B	2.5	100	0.05
Lin–5.0%	Stock Solution B	5.0	100	0.10
Lin–10%	Stock Solution B	10.0	100	0.20
Lin–15%	Stock Solution B	15.0	100	0.30
Lin–20%	Stock Solution B	20.0	100	0.40

Table–24: Data sheet for prediction LOD & LOQ.

Sr. No	Concentration (%)	CMQ area
1	1	0
2	2.5	705
3	5	1795
4	10	2937
5	15	4604
6	20	5000

Table–25: Data sheet for prediction of LOD & LOQ of related substances.

ITEM	LOD (ppm)	LOD (%)	LOQ (ppm)	LOQ (%)
2–(chloromethyl)–4–methyl quinazoline (CMQ)	0.01	0.01	0.02	0.02

Table–26: Data Sheet for Reference Solution Injections (LOD, LOQ, Linearity).

Chromatogram no.	2–(chloromethyl)–4–methyl quinazoline (CMQ) area
Reference Solution(a)	34785
Reference Solution(a)	34620
Reference Solution(a)	35240
Reference Solution(a)	35438
Reference Solution(a)	35071
Reference Solution(a)	35207
Average	**35060**
Std Dev	305.36
%RSD	**0.87**

Table–27: Data Sheet for LOD.

Chromatogram	2–(chloromethyl)–4–methylquinazoline (CMQ) Area
LOD	8938
LOD	7933
LOD	8285
LOD	8674
LOD	9193
LOD	6869
Average	**8315**
Std Dev	839.718
%RSD	**10.10**

Table–28: Data Sheet for LOQ.

Chromatogram	2–(chloromethyl)–4–methylquinazoline (CMQ) Area
LOD	9216
LOD	9268
LOD	9520
LOD	9194
LOD	9737
LOD	9452
Average	**9398**
Std Dev	211.83
%RSD	**2.25**

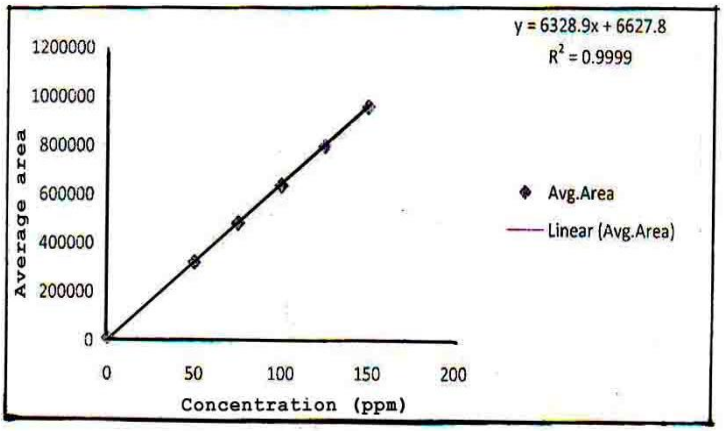
Linearity and Range: Solutions of lower concentrations of CMQ is prepared and each concentration is injected on the same day. The data generated is analysed by linear regression analysis to calculate the slope, intercept and the correlation coefficient. Linearity graphs are plotted. For establishing the linearity for 2–(chloromethyl)–4–methylquinazoline will be prepared to cover a range of 50% to 150% of the test concentration. As the impurity are calculated on area normalization basis, the range proposed for the Linearity determination is 50µg/ml to 150 µg/ml for CMQ with a correlation coefficient greater than 0.99. The results are reported in Table No – 29.

Table–29: Data sheet for linearity of related substances.

Sr. No	Conc. (%)	2–(chloromethyl)–4–methylquinazoline Area	Average area
1	**LOQ**		**9398**
2	50	319626	**319744**
		319808	
		319798	
3	75	479536	**482554.33**
		476852	
		491275	
4	100	638528	**636108.33**
		635539	
		634258	
5	125	797802	**798093**
		798116	
		798361	
6	150	957660	**958399**
		958329	
		959208	

Table–30: Data for calibration of linearity of CMQ.

	2–chloromethyl–4–methylquinazoline (CMQ)
Slope	6328.30
Intercept	6627.82
Coefficient Correlation (R–square)	0.99997



Figure–10: Calibration curve of linearity for CMQ.

Precision: System Precision: System precision is carried out during ruggedness experiment. The RSD for five replicate injections of reference solution is found to be 1.10 % for CMQ. It can be taken part of any experiment or preferably method precision.

Data Evolution: Calculate and report standard deviation and relative standard deviation (%RSD) of the five replicate injections.

Acceptance criterion: The relative standard deviation (%RSD) of the replicate injections of reference solution is NMT 2.0%.

Table–31: Data Sheet for Reference Solution Injections (System Precision).

Chromatogram no.	CMQ Area
Reference Solution	76905
Reference Solution	76544
Reference Solution	77017
Reference Solution	76770
Reference Solution	74956
Average	76438.4
Std Dev	847.218
%RSD	**1.10**

Method Precision: Method precision is performed by preparing six assay preparations of 2–(cholоromethyl)–4–methylquinazoline and injected to HPLC.

Data Evolution: The mean of Assay percentage of 2–(cholоromethyl)–4–methylquinazoline is calculated and report standard deviation and relative standard deviation (%RSD) of the six replicate injections.

Acceptance criterion: The relative standard deviation (%RSD) of the six determinations of assay in 2–(cholоromethyl)–4–methylquinazoline is NMT 2.0%.

Intermediate Precision: Prepare six assay preparations of 2–(cholоromethyl)–4–methylquinazoline using different analyst, a different column on different day and inject in duplicate into a different HPLC.

Data Evolution: The mean of Assay percentage of 2–(cholоromethyl)–4–methylquinazoline is calculated and report standard deviation and relative standard deviation (%RSD) of the twelve replicate injections.

Acceptance criterion: The relative standard deviation (%RSD) of the six determinations of assay in 2–(cholоromethyl)–4–methylquinazoline is NMT 2.0%.

The results are reported in Table No – 34.

Table–32: Sequence for method precision.

Sample Name	No. of injection	Injection Volume (µl)	Run time in min.
Blank	1	10	60
Standard–1	3	10	60
Standard–2	1	10	60
Sample–1	2	10	60
Sample–2	2	10	60
Sample–3	2	10	60
Standard–1	1	10	60
Sample–4	2	10	60
Sample–5	2	10	60
Sample–6	2	10	60
Standard–1	1	10	60

Table–33: Sequence for Intermediate Precision.

Sample Name	No. of injection	Injection Volume (µl)	Run time in min.
Blank	1	10	60
Standard–1	3	10	60
Standard–2	1	10	60
Sample–1	2	10	60
Sample–2	2	10	60
Sample–3	2	10	60
Standard–1	1	10	60
Sample–4	2	10	60
Sample–5	2	10	60
Sample–6	2	10	60
Standard–1	1	10	60

Table–34: Data sheet for precision.

Sample No	Area–1	Area–2	Mean	Sample weight	Assay (as such)	Assay (ODB)
1	5449973	5414052	5432013	21.88	99.39	99.70
2	5582528	5647579	5615054	22.23	101.12	101.43
3	5007954	4955569	4981762	20.12	99.13	99.44
4	4989477	4983617	4986547	20.03	99.67	99.98
5	4876595	4872507	4874551	19.78	98.66	98.97
6	493342	4959871	4946657	19.70	100.53	100.84

Mean	**99.75**	**100.06**
SD	0.916	0.919
RSD	**0.92**	**0.92**

Ruggedness: Ruggedness is the degree of reproducibility of results obtained by the analysis of the same sample under a variety of normal test conditions i.e. different analysts, laboratories, instruments, reagents, assay temperatures, small variations in mobile phase, different days etc. (i.e. from laboratory to laboratory,

from analyst to analyst). This is same as method precision. Six samples are injected by a different analyst on a different day, using a different system. The results are reported in Table–35.

Table–35: Data sheet for ruggedness (Intermediate Precision).

Precision	99.70
	101.43
	99.44
	99.98
	98.97
	100.84
Ruggedness	99.17
	99.89
	98.61
	100.19
	99.60
	101.42
AVG	99.94
STDEV	0.908
%RSD	0.91

Accuracy: Weigh 2–(chloromethyl)–4–methylquinazoline at three different levels:80%,100% and 120% of the specification in triplicate (total nine determinations) and then proceed with sample preparation as per the method for estimation of Assay of CMQ. Injection each of the Sample Preparation in duplicate and then take average area count for calculations. The results are reported in Table–38.

Table–36: Dilutions for accuracy.

Sample Name	Amount of CMQ weight is taken (mg)
Acc–80%/1	16
Acc–80%/2	16
Acc–80%/3	16
Acc–100%/1	20
Acc–100%/2	20
Acc–100%/3	20
Acc–120%/1	24
Acc–120%/2	24
Acc–120%/3	24

Table–37: Sequence for accuracy.

Sample Name	No. of Injections
Blank	1
Standard–1	5
Standard–2	2
Acc–80%/1	2
Acc–80%/2	2
Acc–80%/3	2
Standard–1	1
Acc–100%/1	2
Acc–100%/2	2
Acc–100%/3	2
Standard–1	1
Acc–120%/1	2
Acc–120%/2	2
Acc–120%/3	2
Standard–1	1

Acceptance Criterion:
For each level and each replicate, the following will be calculated:

(i) Amount weighed in mg (Amount actually weighed).
(ii) Amount recovered in mg (quantify against standard response with potency correction).

(iii) Percentage Recovery = Amount recovery/Amount added × 100

The Mean, Standard deviation and RSD will be computed for the nine determinations and reported along with (i), (ii) and (iii).

For the sample the Mean recovery is within 98.0% to 102.0%.

Table–38: Data sheet for accuracy.

Recovery	Wt. taken	Amount added	Area–1	Area–2	Mean	Amount recovered	%Assay	% Mean
80–1	15.88	15.83	3816001	3793737	3804869	15.68	99.05	99.29
80–2	16.19	16.14	3900907	3916894	3908901	16.11	99.82	
80–3	16.23	16.18	3849115	3924160	3886638	16.02	99.01	
100–1	19.88	19.82	4787193	4880139	4833666	19.93	100.56	99.61
100–2	20.02	19.96	4829412	4823780	4826596	19.90	99.71	
100–3	20.12	20.06	4782903	4808165	4795534	19.77	98.57	
120–1	25.66	25.58	6291448	6309802	6300625	25.97	101.52	100.73
120–2	24.12	24.05	5855249	5848734	5851992	24.12	100.31	
120–3	23.88	23.81	5772662	5816507	5794585	23.89	100.35	

Mean	99.88
SD	0.92
RSD	0.92

Robustness of the Method:

a) Change in column temperature (± 5°C).
b) Change in wavelength (±2nm).
c) Change in column Lot (same make, different lot no.).
d) Change in Flow rate (± 0.2 ml/min).
e) Change in Mobile Phase A composition (±10% of nominal concentration)
f) Change in Mobile Phase B composition (±10% of nominal concentration)

Table–39: Sequence for change in experimental conditions.

Sample Name	No. of Injections
Blank	1
Standard–1	3
Standard–2	1
Sample	2

Change in Column Temperature: When the analysis is carried out at a temperature of 25°C the CMQ peak appears at 22.33 minutes and when the analysis is performed at changing condition of column temperature 25°C and 35°C, the results are comparable with that under normal condition. The relative standard deviation determined from reference solution in six replicate injections is below 2.0%.

Change in Wavelength: Normal experimental condition for detection is 210nm. The change in wavelength study is done for actually ±2nm i.e. at two wavelengths, i.e. 208 nm & 212nm. The relative standard deviation determined from the reference solution (a) in six replicate injections is below 2.0%.

Change in Column Lot: Reverse phase HPLC Inertsil ODS 3V, 250 × 4.6mm, 5 µ is the column as described in the method. Two different lots of the column is studied for robustness of the method. This experiment is carried out as a part of ruggedness and it is found that the change in column lot, does not affect the Predetermined HPLC method. The results are comparable with that under normal conditions.

Change in Flow Rate: Normal experimental condition for flow rate is 1.0ml/minute. Change in flow rate is studied for actual ± 0.2ml/minute. The results for the estimation of related substances are comparable with the normal condition in both the flow rates 0.8ml/minute and 1.2ml/minute. The relative standard deviation determined from reference solution in six replicate injections is below 2.0%.

Change in concentration of Mobile phase A: Normal experimental condition for Mobile phase A is Buffer: Acetonitrile (900:100, v/v), Change in Mobile phase A will be studied for Buffer:Acetonitrile (910:90, v/v), and Buffer : Acetonitrile (890:110, v/v). The results for the estimation of related substances are comparable with the normal condition in both the cases.

Change in concentration of Mobile phase B: Normal experimental condition for Mobile phase B is Buffer:

Acetonitrile (100:900, v/v), Change in Mobile phase B will be studied for Buffer:Acetonitrile (90:910, v/v), and Buffer : Acetonitrile (110:890, v/v). The results for the estimation of related substances are comparable with the normal condition in both the cases.

The relative standard deviation determined from reference solution in six replicate injections is below 2.0%. This robustness studies show that method is robust and not affected by any other small changes in the experimental conditions.[28-30]

Table–40: Data Sheet for Robustness: Change in Temperature; (Temperature = 25°C).

Sample	% Any other individual impurity	% Total impurities
Normal Condition	ND	0.08
Temperature=25°C	ND	0.08
Average	**ND**	**0.08**
Std Dev	——	——
%RSD	——	——

Table–41: Data sheet for robustness: Change in Temperature; (Temperature = 35°C).

Sample	% Any other individual impurity	% Total impurities
Normal Condition	ND	0.07
Wavelength=208 nm	ND	0.07
Average	**ND**	**0.07**
Std Dev	——	——
%RSD	——	——

Table–42: Data sheet for robustness: Change in Wavelength (208 nm).

Sample	% Any other individual impurity	% Total impurities
Normal Condition	ND	0.07
Wavelength=208 nm	ND	0.07
Average	**ND**	**0.07**
Std Dev	——	——
%RSD	——	——

Table–43: Data sheet for robustness: Change in wavelength (212nm).

Sample	% Any other individual impurity	% Total impurities
Normal Condition	ND	0.08
Wavelength=222 nm	ND	0.08
Average	**ND**	**0.08**
Std Dev	——	——
% RSD	——	——

Table–44: Data sheet for robustness: Change in Flow rate (Flow rate = 0.8 ml/min).

Sample	% Any other individual impurity	% Total impurities
Normal Condition	ND	0.1
Flow rate=0.8ml/min	ND	0.1
Average	**ND**	**0.1**
Std Dev	——	——
%RSD	——	——

Table–45: Data sheet for robustness: Change in Flow rate (Flow rate = 1.2 ml/min).

Sample	% Any other individual impurity	% Total impurities
Normal Condition	ND	0.1
Flow rate=1.2ml/min	ND	0.1
Average	**ND**	**0.1**
Std Dev	——	——
%RSD	——	——

Table–46: Data sheet for robustness: Change in concentration of Mobile phase A, Buffer: Acetonitrile (910:90, v/v).

Sample	% Any other individual impurity	% Total impurities
Normal Condition	ND	0.09
Buffer:Acetonitrile (910:90,v/v)	ND	0.09
Average	**ND**	**0.09**
Std Dev	——	——
%RSD	——	——

Table–47: Data sheet for robustness: Change in concentration of Mobile phase A, Buffer:Acetonitrile.

Sample	% Any other individual impurity	% Total impurities
Normal Condition	ND	0.09
Buffer:Acetonitrile (890:110, v/v)	ND	0.09
Average	**ND**	**0.09**
Std Dev	——	——
% RSD	——	——

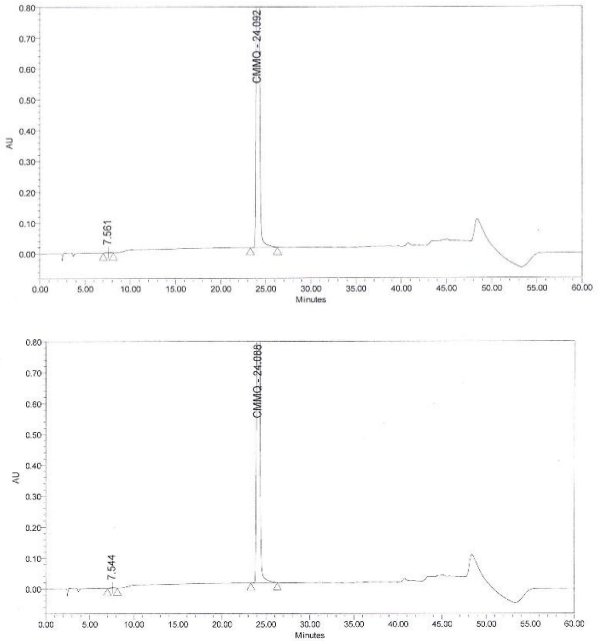
Table–48: Data sheet for robustness: Change in concentration of Mobile phase B, Buffer:Acetonitrile (90:910, v/v)

Sample	% Any other individual impurity	% Total impurities
Normal Condition	ND	0.09
Buffer:Acetonitrile (90:910, v/v)	ND	0.09
Average	**ND**	**0.09**
Std Dev	——	——
% RSD	——	——

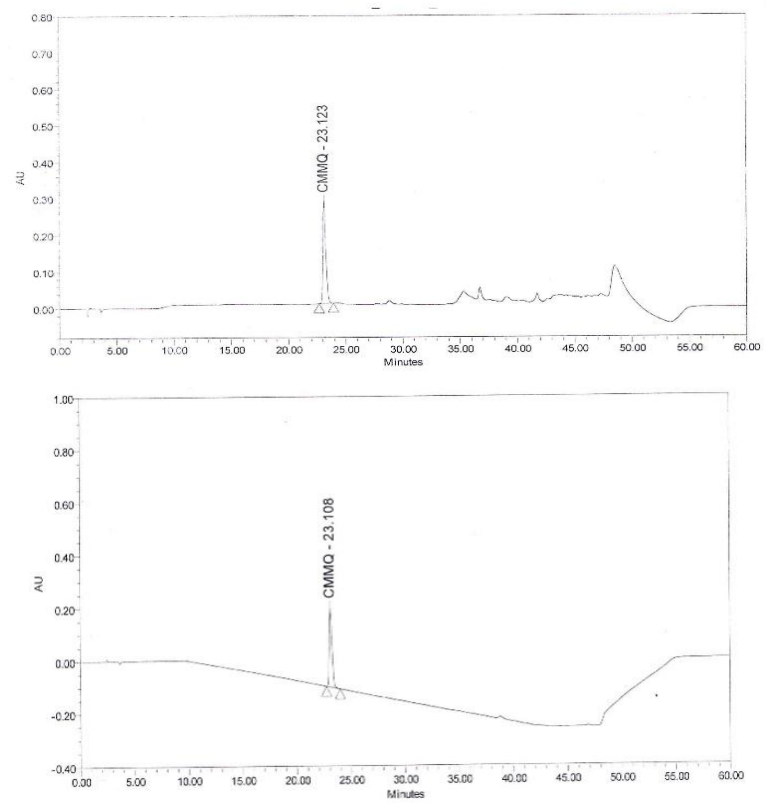
(890:110, v/v)

Table–49: Data sheet for robustness: Change in concentration of Mobile phase B, Buffer : Acetonitrile (110:890, v/v).

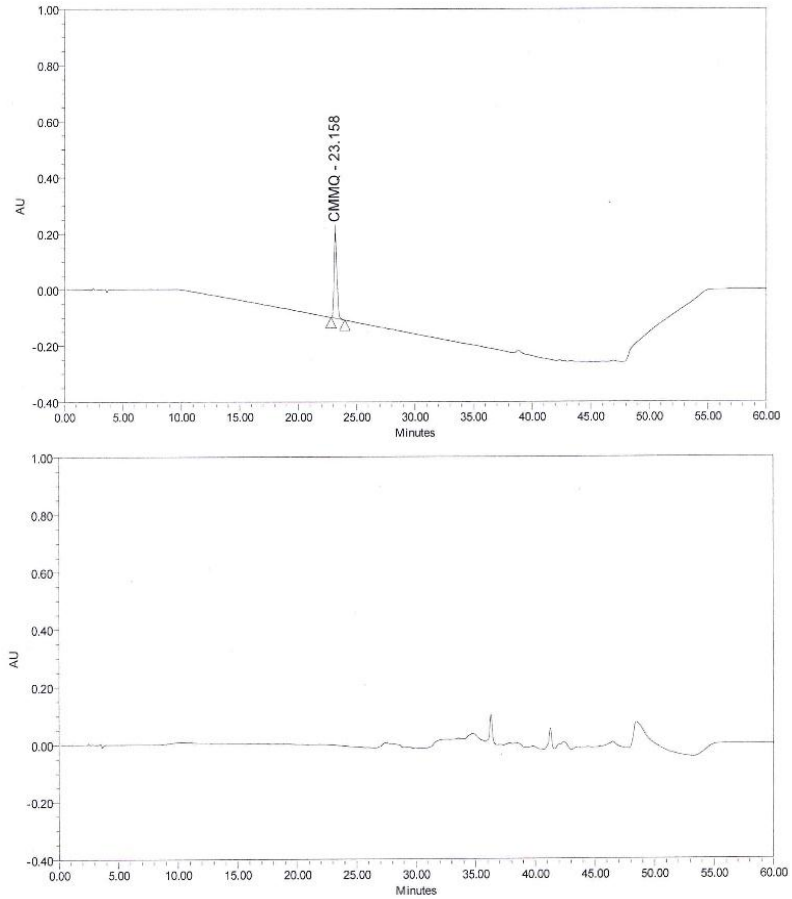
Sample	% Any other individual impurity	% Total impurities
Normal Condition	ND	0.09
Buffer:Acetonitrile (110:890, v/v)	ND	0.09
Average	**ND**	**0.09**
Std Dev	——	——
% RSD	——	——



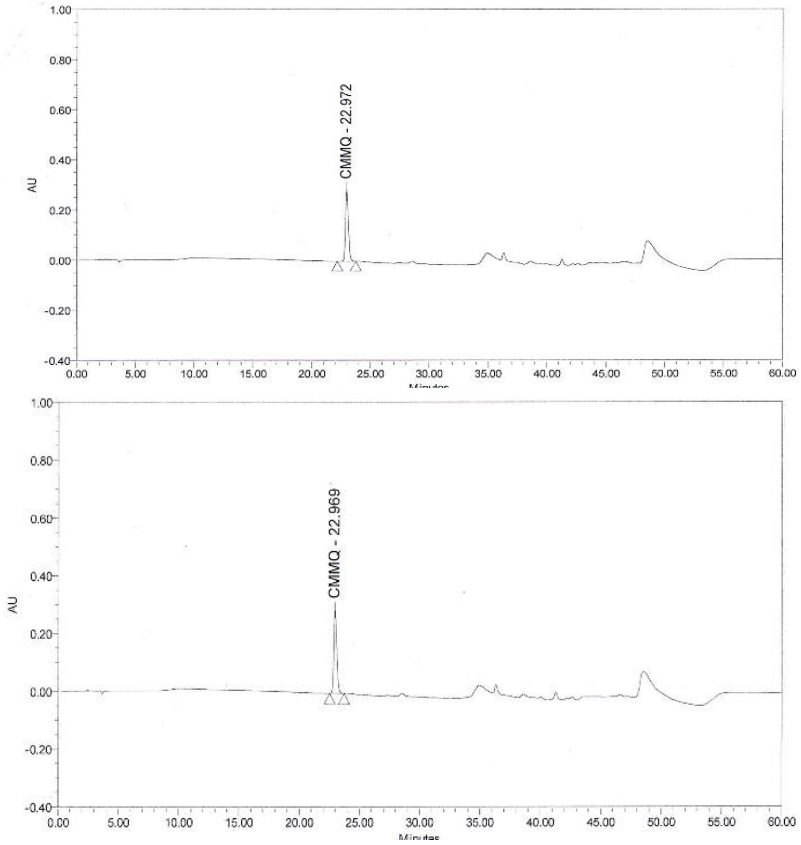
Figure–11: Chromatogram:–X: Solution stability of CMQ after 24 hours; Chromatogram:– XI: Solution stability of CMQ after 48 hours.



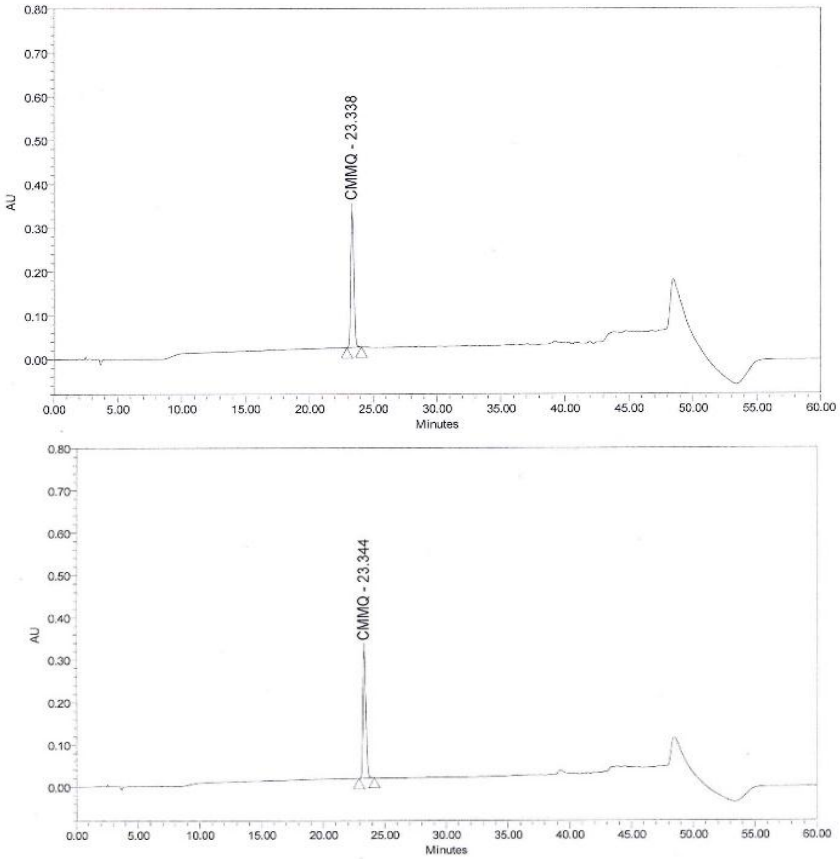
Figure–12: Chromatogram:– XII: Accuracy 100%; Chromatogram:– XIII: Robustness [MP–A–Buffer: CAN, 890:110] Standard.



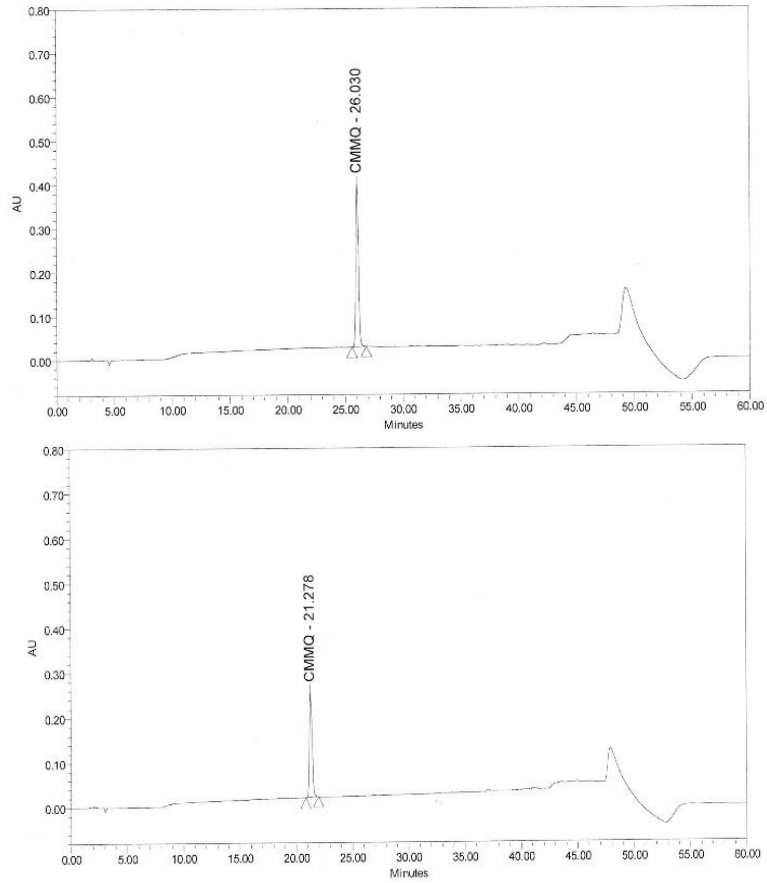
Figure–13: Chromatogram:–XIV: Robustness [MP–A–Buffer:ACN,890:110]Sample (MP–A:+10% ACN)Chromatogram:–XV: Robustness [MP–B–Buffer:ACN,90:910] Blank.



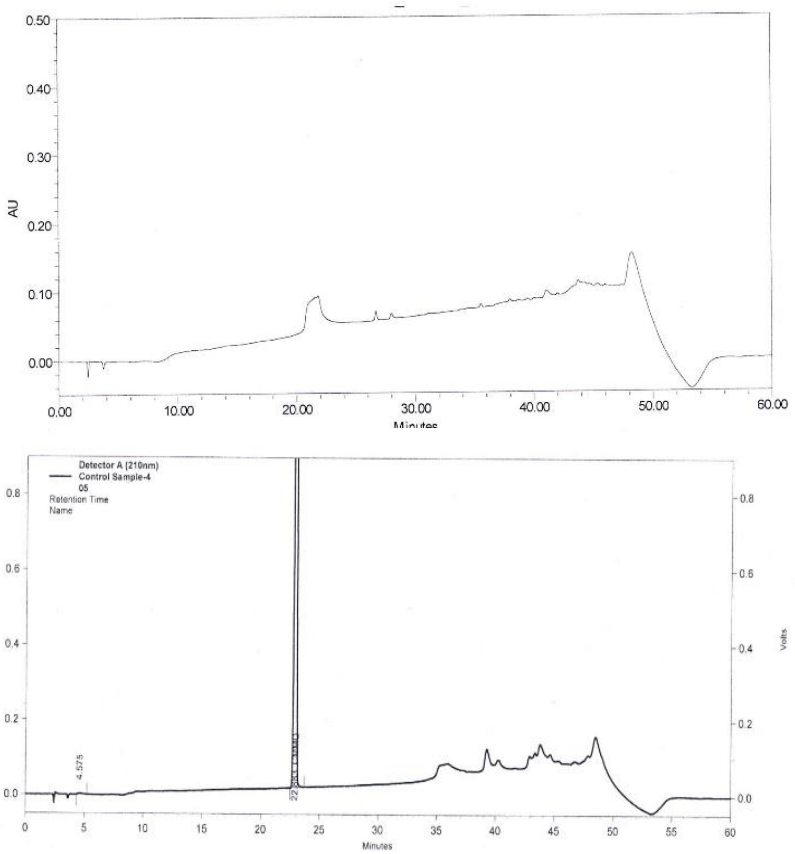
Figure–14: Chromatogram:– XVI: Robustness [MP–B–Buffer:ACN, 090:910] Standard Chromatogram:–XVII: Robustness [MP–B–Buffer:ACN,90:910]Sample (MP–B:+10% ACN).



Figure–15: Chromatogram:–XVIII: Robustness (Low wavelength)–208nmChromatogram:–XIX: Robustness (High wavelength)–212nm.



Figure–16: Chromatogram:–XX: Robustness (Low flow–0.8ml) Sample Chromatogram:–XXI: Robustness (Low flow–1.2ml) Sample.



Figure–17: Chromatogram:–XXII: Ruggednessblank Chromatogram:–XXIII: Ruggednesscontrol sample.

CONCLUSION

We are aware of the fact that quality of the finished pharmaceuticals is mainly based on the quality i.e. purity devoiding of unwanted impurities, safety and efficacy of the active pharmaceutical ingredients (API) used.

A host of impurities in pharmaceutical ingredients do occur that may be partially responsible for toxicity, chemical interference and general instability. In order to ensure that drugs reaching consumers are effective, safe, of good quality regulatory requirement now demands to use standard pure API. Purity of API depends on the synthetic process involving chemical reactions using different reagents under different conditions. Therefore, estimation of purity alongwith impurity profile is necessary to get the pure API. These can only be achieved by thorough analysis of the precursors (Starting Material) used.

Here, the anti–diabetic drug, Linagliptin is synthesized from 2–(chloromethyl)–4–methyl quinazoline (CMQ). Its standardization i.e. purity and impurity profile need to be developed and validated as required by the regulatory authorities. There is no such existing literature reports available for the estimation of CMQ.

The main objective was to isolate, purify the impurities in drug substances as well as to control the actual Impurity or degradation product present in the drug substance at the apparent level of 0.1% (calculated using the **response factor** of drug substance) in case of raw materials, or more (0.15%) in case of intermediate or final API. Considering this fact, an attempt was made to develop a simple, fast, accurate and precise HPLC method, using a mobile phase A [0.77gm Ammonium Acetate in 900ml water (Milli Q), sonicated to dissolve. 100ml of ACN was added, mixed well and filtered.] and mobile phase B (0.77gm Ammonium Acetate in100 ml water, sonicated to dissolve. Then 900ml of ACN was added, mixed well and filtered.) The mobile phase chosen was simple to prepare and economical. The chromatographic condition was set at a flow rate of 1.0 ml/min with the UV detector at 210nm. The developed method was found to be simple, precise, accurate and rapid for the estimation of purity & related substances in the Key Starting Material i.e. CMQ. With the above–mentioned conditions, CMQ gave a good symmetrical peak. In this condition all peaks were well separated. Retention time of main peak was found to be 22.33 minutes, all impurities were in limit, as unknown impurities. This method can be easily and conveniently adopted for routine analysis of CMQ.

The results of the validation and system suitability studies suggested, that the developed RP–HPLC method could be employed successfully for the estimation of CMQ and its related substances.

Conflict of Interest statement

Authors declaredno conflict of interest.

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