

**METHOXYAMINE-BASED DERIVATIZATION FOR TRACE-LEVEL CARBONYL
COMPOUND ANALYSIS USING LC-MS/MS**

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ABSTRACT

Carbonyl compounds, including aldehydes and ketones, are ubiquitous environmental pollutants and potential genotoxic impurities in pharmaceutical formulations. Their volatility, instability, and reactivity necessitate derivatization for reliable detection and quantification. In this study, methoxyamine hydrochloride was employed as a derivatizing agent to convert carbonyl compounds into stable oxime derivatives, which were subsequently analyzed using LC-MS/MS and GC-MS techniques. The method was optimized with Chromatopak Peerless C18 column under APCI ionization conditions, achieving high sensitivity with detection limits as low as 0.1 ng/mL for acetophenone and 1 ng/mL for benzophenone. Validation parameters including linearity, accuracy, precision, and recovery demonstrated compliance with accepted analytical criteria ($R^2 > 0.99$, %RSD $< 5\%$). The developed method enables robust identification and quantification of trace-level carbonyl impurities in pharmaceutical formulations, thereby supporting impurity profiling and ensuring drug safety. This approach highlights the importance of sensitive analytical techniques in monitoring genotoxic impurities and mitigating drug–excipient incompatibilities.

KEYWORDS: Carbonyl, Acetophenone, Benzophenone, Derivatization and Oxime.

INTRODUCTION

Carbonyl compounds (CCs), such as aldehydes and ketones, are ubiquitous pollutants that are among the most widespread in the environment.^[1,2] Airborne CCs are primarily emitted by anthropogenic sources, such as the incomplete combustion of fossil fuels or biomass burning activities.^[3] When released into the environment, CCs play an important role in the atmospheric chemistry as the precursors to several species are involved in photochemical smog (e.g., free radicals, ozone and peroxyacyl nitrates).^[4]

The common tests used to identify carbonyl compounds include Tollen's test, Fehling's test and 2,4 Dinitro phenyl hydrazine (2,4-DNPH) test. These tests use different reagents, that react with the carbonyl group and produce characteristic results. The most commonly used test is 2,4-DNPH test, where the carbonyl group reacts

with the amine group of 2,4-DNPH and form imine (Nucleophilic addition reaction).^[5,8]

The principle of nucleophilic addition reaction of carbonyl compounds with amines can be used for their quantification in various matrices. Since the carbonyl group is relatively unstable due to volatility, thermal instability, and sensitivity to acidic environments, it is necessary to derivatize the carbonyl group. Therefore, 2,4-DNPH can react with carbonyl compounds and form a product that could be used as a derivatizing agent for the carbonyl group. The formed hydrazone product can then be identified and quantified.^[9,14]

The same principle can also be applied with another reagent called methoxy amine hydrochloride, where the carbonyl group react with amine group of this reagent and forms omethoximes. So, methoxy amine hydrochloride can also be used as derivatizing agent. The

derivatized products can be identified and quantified using HPLC, GC-MS and LC-MS techniques. The carbonyl group is found to be genotoxic in nature. In the present study, the principal reaction of an amine with a carbonyl group is used, assuming that if any carbonyl residue is said to be present in the formulation (whatsoever the source), it can react with the amine group present in the API and form a product (oxime in the present case). This product can be identified and quantified using GC-MS and LC-MS techniques.^[15,21]

MATERIALS AND METHODS

Chemicals and Reagents: Aldehydes (Formaldehyde, Acetaldehyde, Propanal, Butanal, Benzaldehyde), Ketones (Acetone, Acetophenone, Benzophenone) and internal standard (Triethyl Phosphate) were bought from Sigma Aldrich. Solvents for mass spectrometry analysis were purchased from Fischer chemicals. MilliQ water was collected from (ultrapure direct q®). Ammonium formate was purchased from Sigma Aldrich.

CHROMATOGRAPHIC CONDITIONS

The LC-MS analysis was carried out on Thermo scientific TSQ ALTIS using Chromatopak Peerless C18 column (4.6 mm x 250 mm; 5 µm). The mobile phase used was solvent A: 10 mM Ammonium formate in water and solvent B: 100 % Acetonitrile. The ion source used was APCI. The flow rate was 0.8 mL/min and the run time was 22 min. The data analysis was carried out on Thermo Xcalibur Qual Browser.

GC-MS ANALYSIS

The GC-MS analysis was carried out Agilent 6890N GC with 5973 MSD using DB-624 column. The injector was maintained at 240 °C with a split ratio of 1:10. The column oven temperature program involved an initial temperature of 35 °C for 5 min and increased at 10° C/min to 240 °C and held for 5 min. The carrier gas was helium and was set at constant flow rate of 1 mL/min with a flow pressure of 7.42 psi. EI source was used as ionization source which was held at 230° C and detection was carried out at 150 °C with full scan mode and SIM mode.

PREPARATION OF STOCK SOLUTIONS

The standard stock solutions of 1 mg/mL were prepared by dissolving 1 mg of standard aldehydes and ketones in 1 mL of methanol. The working stock solution of 10 mg/mL of Methoxy amine hydrochloride reagent was prepared by dissolving 100 mg of reagent in 10 mL of water. A solution of 1M HCl was prepared by transferring 4.4 mL of 36 % HCl in 50 mL of water. The stock solution of Triethyl phosphate was prepared by dissolving 1 mg in 1mL of methanol.

DERIVATIZATION

A general reaction of carbonyl compounds with amines forms oximes. The principle of this reaction can be used for the determination of trace levels of carbonyl impurities in formulation as these impurities present in formulation (from whatsoever source) can react with the amine group present in API and can form oximes. Many reagents are being used for the derivatization of carbonyl compounds. A few of the derivatizing reagents that are employed are: O-2,3,4,5,6-(Pentafluorobenzyl) hydroxylamine hydrochloride (PFBHA), hydroxyl amine hydrochloride, Methoxy amine hydrochloride and 2,4-dinitrophenylhydrazine (DNPH).

PROCEDURE

The Stock solution of 10 mg/mL solution of methoxy amine hydrochloride was prepared by dissolving 100 mg of it in 10 mL of methanol. The working stock solutions of aldehydes and ketones were prepared by dissolving 1 mg of each of them in 1 mL of methanol to get a solution 34 of 1 mg/mL separately. The derivatization reaction was carried out by mixing carbonyl compound with methoxy amine hydrochloride. This reaction occurs at a 1:10 ratio of carbonyl compound and methoxy amine hydrochloride reagent in the presence of acid. Hence, 1M HCl was used as acid. The reaction was carried out by mixingThe reaction mixture was heated at 60 °C for 30 minutes.

MS/MS ANALYSIS

The product ion scan (MS/MS) was done at various collision energies by taking the m/z of precursor ion obtained in the HRMS confirmation of o-methyl oximes. Nitrogen gas was used as collision gas. The corresponding product ions at the specific collision energy were noted.

MRM SCAN

The MRM scan was done to quantitate the carbonyl compounds by taking the m/z of precursor ions, their corresponding product ions and collision energies. The specific RT's of the compounds can also be kept (instead of full RT window), in order to increase the sensitivity.

METHOD VALIDATION

Method was validated keeping special emphasis to selectivity and specificity, precision.

RESULTS AND DISCUSSIONS

DERIVATIZATION REACTION

A nucleophilic addition reaction occurs between carbonyl compound and methoxy amine hydrochloride in the presence of acid, where a molecule of water is removed to form a product called oxime.

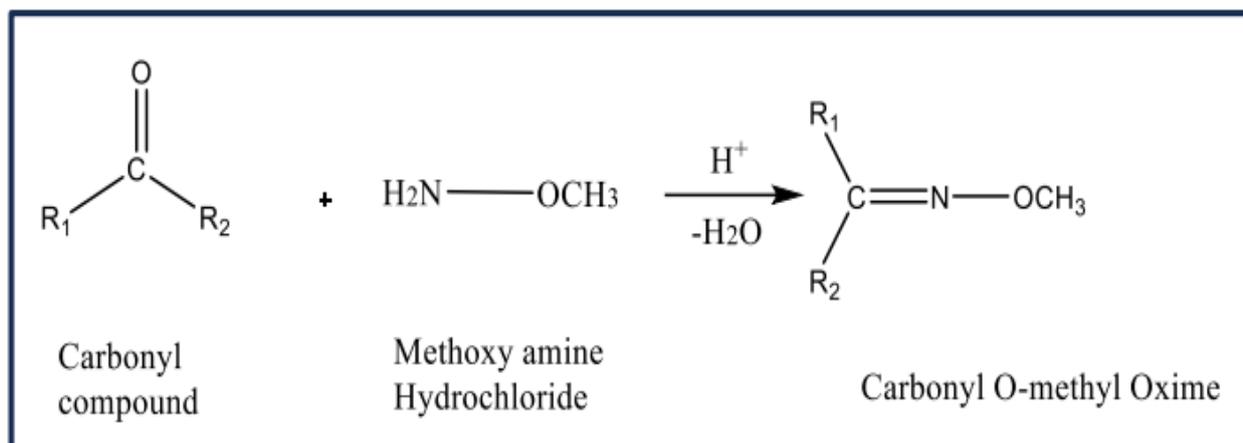


Fig 1: Derivatization reaction.

From above reaction various carbonyl derivatives are produced. They are Formaldehyde-o-methyl oxime, Acetaldehyde-o-methyl oxime, Propanal-o-methyl

oxime, Benzaldehyde-o-methyl oxime, Propane-2-one -o-methyl oxime, 1-Phenylethan1-one -o-methyl oxime & Diphenylmethanone-o-methyl oxime.

Table 1: M/Z of carbonyl-0-methyloximes.

Compound	M/Z
Formaldehyde O-methyloxime	60.04439
Acetaldehyde O-methyloxime	74.06004
Propionaldehyde O-methyloxime	88.07563
Butyraldehyde O-methyloxime	102.09124
Benzaldehyde O-methyloxime	136.07561
Propane-2-one O-methyloxime	88.07569
1-Phenylethan1-one O-methyloxime	150.09134
Diphenylmethanone O-methyloxime	212.1070

Fig 2: EI Spectra of carbonyl compounds (1. Propan-2-one-o-methyloxime, 2. Propanal-o-methyloxime, 3. Butanal-o-methyloxime, 4. Benzaldehyde-o-methyloxime & 5. Acetophenone-o-methyloxime)

VALIDATION

Linearity

Table 2: Linearity data of 1-Phenylethan-1-one-o-methyloxime.

Concentration (ng/mL)	Analyte Area(AA)	Internal standard Area (IA)	AA/IA
1	3514.73	2446783.40	0.001436
2	2046.29	2350179.43	0.000871
5	5727.82	2433218.37	0.002354
10	11406.65	2260319.12	0.005046
20	21186.07	2457114.74	0.008622
50	55624.25	2470188.18	0.022518
100	97014.25	2406991.38	0.040305
200	152301.42	2427320.18	0.062745
500	334635.12	2370812.95	0.141148
1000	644940.53	2345734.47	0.274942
2000	1267695.34	2292735.67	0.552918
5000	4114375.70	1886187.96	2.181318

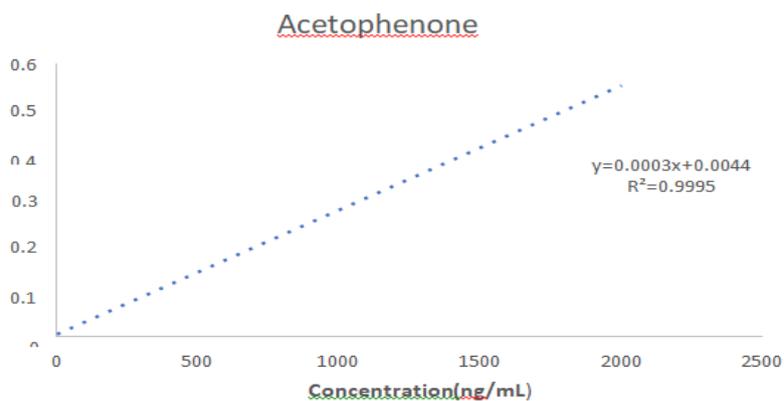


Figure: Calibration curve of 1-Phenylethan-1-one-o-methyl oxime.

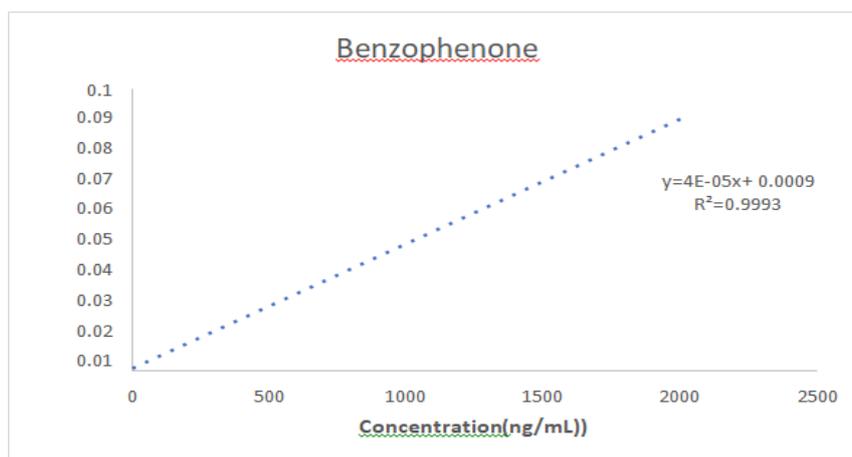


Figure: Calibration curve of Diphenylmethanone-o-methyl oxime.

Table: Linearity data of Diphenylmethanone-o-methyloxime.

Concentration (ng/mL)	Analyte Area (AA)	Internal standard Area(IA)	AA/IA
5	998.52	2433218.37	0.00041
10	2070.73	2260319.12	0.000916
20	3736.36	2457114.74	0.001521
50	8928.12	2470188.18	0.003614
100	16604.98	2406991.38	0.006899
200	30396.46	2527320.18	0.012027
500	54078.36	2570812.95	0.021036
1000	107032.58	2345734.47	0.045629
2000	204180.76	2292735.67	0.089056
5000	615745.75	1886187.96	0.32645

It was observed that the formation of the product increased with the increase in the concentration of the reagent (methoxy amine hydrochloride).

Accuracy

Table: % Recovery of 1-Phenylethan-1-one-o-methyloxime.

CONC (ng/mL)	AA	IS	AA/IS	FC	% Recovery
2	591.65	2350179.43	0.000252	2.167787	108.3893
5	998.52	2433218.37	0.00041	4.433831	88.67662
10	2070.73	2260319.12	0.000916	11.65888	116.5888
20	3736.36	2457114.74	0.001521	20.29468	101.4734
50	8928.12	2470188.18	0.003614	50.20497	100.4099

100	16604.98	2406991.38	0.006899	94.96613	94.96613
200	26396.46	2527320.18	0.010444	191.1112	95.55559
500	59078.36	2527812.95	0.023371	519.2834	103.8567
1000	107032.58	2345734.47	0.045629	1075.715	107.5715
2000	204180.76	2292735.67	0.089056	2161.388	108.0694

Table: % Recovery of Diphenylmethanone-o-methyloxime.

CONC (ng/mL)	AA	IS	AA/IS	FC	% RECOVERY
2	591.65	2350179.43	0.000252	2.167787	108.3893
5	998.52	2433218.37	0.00041	4.433831	88.67662
10	2070.73	2260319.12	0.000916	11.65888	116.5888
20	3736.36	2457114.74	0.001521	20.29468	101.4734
50	8928.12	2470188.18	0.003614	50.20497	100.4099
100	16604.98	2406991.38	0.006899	94.96613	94.96613
200	26396.46	2527320.18	0.010444	191.1112	95.55559
500	59078.36	2527812.95	0.023371	519.2834	103.8567
1000	107032.58	2345734.47	0.045629	1075.715	107.5715
2000	204180.76	2292735.67	0.089056	2161.388	108.0694

Precision

Table: Precision data of 1-Phenylethan-1-one-o-methyloxime.

CONC (ng/mL)	AREA	IS	AA/IS	% RSD
5PPB-1	3801.52	2333668.00	0.001629	4.87
5PPB-2	3672.65	2382182.97	0.001542	
5PPB-3	3814.85	2271740.12	0.001679	
5PPB-4	3943.59	2239930.64	0.001761	
5PPB-5	3653.63	2090123.18	0.001748	
5PPB-6	3917.79	2299491.64	0.001704	
100PPB-1	63981.42	2210534.17	0.028944	2.23
100PPB-2	63046.79	2281239.96	0.027637	
100PPB-3	63073.91	2146339.70	0.029387	
100PPB-4	59362.60	2093919.43	0.02835	
100PPB-5	58098.26	1988110.09	0.029223	
100PPB-6	58457.27	2031071.28	0.028781	
2000PPB-1	1423578.82	2052317.15	0.693645	3.37
2000PPB-2	1302206.96	1985602.87	0.655824	
2000PPB-3	1306037.52	1910170.02	0.683728	
2000PPB-4	1328289.47	1839115.54	0.722244	
2000PPB-5	1309068.32	1901955.21	0.688275	
2000PPB-6	1304068.79	1829900.61	0.712645	

Table: Precision data of Diphenylmethanone-o-methyl oxime.

CONC (ng/mL)	AREA	IS	AA/IS	% RSD
5PPB-1	1864.78	2333668.00	0.0007991	3.7
5PPB-2	1753.53	2382182.97	0.0007361	
5PPB-3	1983.28	2471740.12	0.0008024	
5PPB-4	1782.54	2239930.64	0.0007958	
5PPB-5	1700.16	2090123.18	0.0008134	
5PPB-6	1758.05	2299491.64	0.0007645	
100PPB-1	16160.33	2210534.17	0.0073106	2.93
100PPB-2	16241.04	2281239.96	0.0071194	
100PPB-3	16228.46	2146339.70	0.007561	
100PPB-4	14528.37	2093919.43	0.0069384	
100PPB-5	14644.82	1988110.09	0.0073662	
100PPB-6	14773.65	2031071.28	0.0072738	
2000PPB-1	304961.59	2052317.15	0.1485938	4.7

2000PPB-2	286124.26	1985602.87	0.1440994	
2000PPB-3	286296.44	1910170.02	0.1498801	
2000PPB-4	299158.92	1839115.54	0.1626646	
2000PPB-5	297608.28	1901955.21	0.1564749	
2000PPB-6	293484.74	1829900.61	0.1603829	

LC-APCI-MS/MS Analysis

Among the eight analytes, only 2 analytes were quantified using LC-MS/MS (ThermoScientific TSQALTIMS). The compounds like acetone and propanal are having same molecular weight. So, their derivatized products will also have same molecular weight. This

aspect became difficulty in analysing those two compounds, where they were having different MS/MS spectra but eluting at the same RT. The compounds like formaldehyde and acetaldehyde are more volatile, so this reason made their analysis difficult in LC-MS.

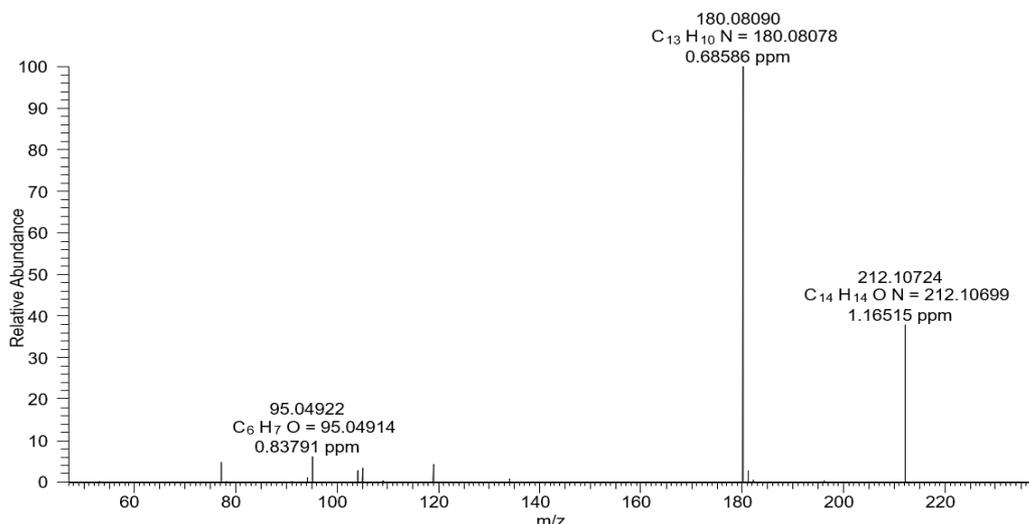


Fig: MS/MS spectrum of Diphenylmethanone-o-methyloxime.

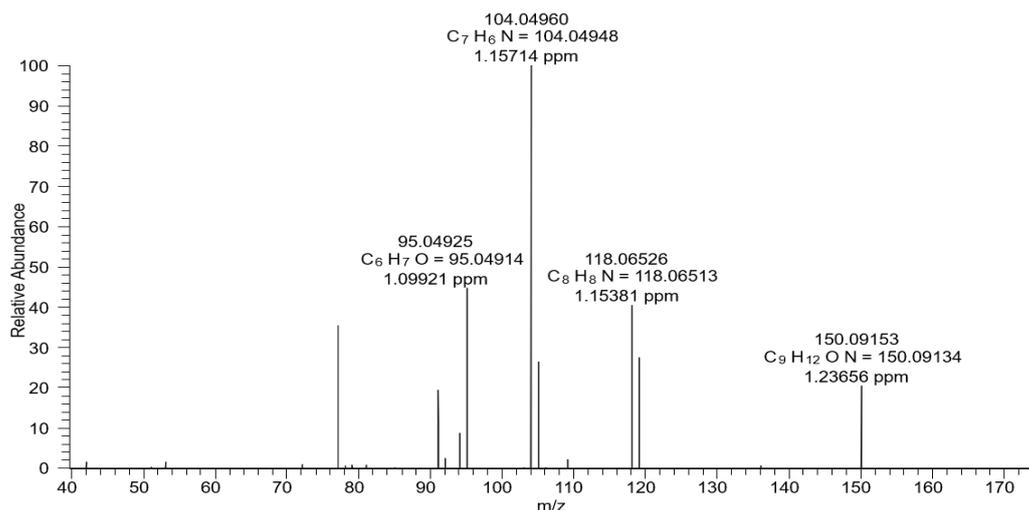


Fig: MS/MS spectrum of 1-Phenylethanone-o-methyloxime.

The linearity range was found to 1 ng/mL to 2000 ng/mL and R² values for acetophenone and benzophenone were 57 found to be 0.9995 and 0.993 respectively. Detection limit (LOD) for acetophenone was found to be 0.1 ng/mL and 1 ng/mL for benzophenone. The LOQ values for acetophenone and benzophenone were 2 ng/mL and 5 ng/mL respectively. The % Recovery or % Accuracy and % RSD were in the range of accepted criteria.

CONCLUSION

Generally, impurities present at trace quantities. Even at these trace levels, they will have impact on safety and efficacy of the formulation. So, this reason necessitates the development of the sensitive methods to identify and quantify these impurities. In addition, these carbonyl compounds will also have genotoxicity. Hence these impurities must be identified and quantified using sensitive techniques. The developed method has a detection limit of 0.1 ng/mL for acetophenone and 1

ng/mL for benzophenone, which were very sensitive. In the present study an attempt has been made to quantify carbonyl residues using LC-MS/MS.

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