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ABSTRACT

A simple and sensitive static head space gas chromatographic method was developed for the determination of Methanol as a residual solvent in Dimethyl fumarate API. ZB-624 capillary column of 30 meters length, 0.53 mm internal diameter and 3.0 μm film thickness was used as the stationary phase. Helium was used as the carrier gas. The quantitative estimation of Methanol was done using the Flame ionisation detector along with Empower 3 software. The method was optimised to get a good peak shape and resolution between the analyte and the diluent peak. The developed method proved to be specific, precise, accurate, robust and rugged when validated as per the ICH Q2 (R1) guidelines. The LOD and LOQ of the method were found to be 2 ppm and 6 ppm respectively. Linearity of results was obtained in the concentration range of 6 to 4500 ppm. This method would help Dimethyl fumarate API and formulation manufactures to ensure that their products are free from the toxicological levels of Methanol.
INTRODUCTION:
A number of solvents are used or produced in the manufacturing process of active pharmaceutical ingredients (APIs), excipients and drug products. These solvents may be a critical element in the synthesis or the formulation, as they may enhance the yield or determine characteristics like purity, crystal form or solubility. They are mostly not completely removed by the pharmaceutical techniques and remain in the API or excipient or the drug product as residual solvents or the organic volatile impurities (OVIs). Since the residual solvents do not have any therapeutic value, they should be removed as much as possible. There are three classes of solvents. Class 1 solvents are known to cause unacceptable toxicities or deleterious environmental effects; hence their use should be avoided unless their use can be strongly justified by a risk-benefit assessment. Class 2 solvents are associated with less severe toxicity. Their use should be limited in order to protect patients from potential adverse effects. Class 3 solvents include the less toxic solvents and these should be used where practicable.\[1, 2]\n
Dimethyl fumarate (Figure 1) [1, 4-dimethyl (2E)-but-2-enedioate] is from a class of medications called Nrf2 activators. It is known for its anti-inflammatory and immunomodulatory effects. Dimethyl fumarate (DMF) is indicated for patients with relapsing multiple sclerosis. It works by decreasing inflammation and preventing nerve damage that may cause symptoms of multiple sclerosis. DMF is a prodrug which metabolizes to Monomethyl fumarate (MMF) prior to entering the systemic circulation. MMF upregulates the transcription factor (Nuclear factor erythroid-derived 2)-related factor 2 (Nrf2) pathway that is activated in response to oxidative stress. DMF is also used in the treatment of psoriasis.\[3, 4]\n
As per the literature, it was studied that methanol is used in the process of synthesis of DMF. It is used in the reaction involved in the production of DMF and sometimes used for the purification of the synthesized DMF. The DMF produced is freed from methanol by drying it.\[5]\ But, methanol may still remain in the DMF API as an organic volatile impurity. Methanol is classified under Class 2 residual solvent as per International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) Harmonised Tripartite Guideline for Residual Solvents Q3C (R6). The use of Class 2 residual solvents in the manufacture of drug substances, excipients, dietary ingredients, and official products should be limited because of the inherent toxicities. The limits for Class 2 solvents are based on the toxicological permitted daily exposure (PDE). The PDE of Methanol is 30 mg/day and its concentration limit is 3000 ppm.\[1, 2]\n
The literature survey revealed no study been done on the analysis of Methanol as a residual solvent in Dimethyl fumarate API.\[6-20]\ Hence the authors decided to develop a sensitive, precise and accurate method which would help to determine the content of Methanol residual solvent in DMF API with ease. There are various methods used to analyse residual solvents like, Loss on Drying (LOD), Gas chromatography (GC), Thermogravimetry- Mass spectrometry (TGA- MS), Infrared spectroscopy (IR) and Nuclear Magnetic Resonance (NMR). Head space Gas chromatographic (HS-GC) technique was chosen for this research as it is known to be a very sensitive, specific and accurate method. While drawbacks like non-specificity, prolonged time of analysis, less sensitivity are faced by using the other methods for the analysis of residual solvents in pharmaceuticals.\[21]
MATERIALS AND EXPERIMENTAL CONDITIONS:

Chemicals, Reagents and Materials:
Dimethyl fumarate API samples (Batch no. DVm0340516 and Batch no. DVm0450616) were obtained from MSN Organics Private Limited (Hyderabad, India). Methanol (ACS grade) purchased from Sigma Aldrich (Merck KGaA, Darmstadt, Germany) was used as the standard. The diluent N, N-Dimethyl formamide (GC grade) was purchased from Rankem™ (Avantor, Inc.). Helium was used as the carrier gas. Nitrogen and Air were used as the fuel gas and combustion gas respectively.

Instrumentation:
Perkin Elmer gas chromatographic system of Clarus 580 module with Head Space auto sampler Turbo matrix 40 was used for the analysis of the residual solvents. To detect and quantitate the residual solvents in the sample the system was equipped with FID (Flame ionization Detector). The GC system was operated using the Empower-3 software. ZB-624 capillary column of 30 meters length, 0.53 mm internal diameter and 3.0 μm film thickness (Make: Zebron; Part No: 7HK-G005-36) was used as the stationary phase. Semi micro balance of Make: Mettler-Toledo (Model: XSE205DU) was used for weighing during sample and standard preparation. To measure the sample and the standard, micro pipette of Make: Eppendorf was used. Head space Vials (20 mL) (Make: Perkin –Elmer) and aluminium crimp caps with septas (PTFE/ Silicone) manufactured by Cyberlab manufactures were utilised for the research work.

Preparation of the standard and sample solutions:

Preparation of standard stock solution:
Transfer about 76 μL of Methanol (density= 0.791) into a 10 mL volumetric flask containing about 5 mL of diluent and make up the volume up to the mark with diluent.

Preparation of standard solution:
Transfer 200 μL (0.2 mL) of the standard stock solution into a 20 mL volumetric flask containing about 10 mL of diluent and make up the volume up to the mark with the same diluent. This solution contains about 3000ppm of Methanol with respect to 20 mg/mL of test concentration.

Standard preparation:
Pipette 2 mL of standard solution into a 20mL of head space vial fitted with a septum. Crimp the cap immediately.

Blank preparation:
Pipette out 2 mL of diluent into a 20mL of head space vial fitted with a septum. Crimp the cap immediately.

Sample preparation:
Weigh accurately 40 mg of test sample and transfer it into a 20 mL head space vial. Add 2 mL of diluent to it and crimp the cap with the septum immediately.

Gas Chromatographic procedure:
The head space gas chromatographic system (Clarus 580 & Turbomatrix 40) equipped with FID and integrator was used. ZB-624 capillary column of 30 meters length, 0.53 mm internal diameter and 3.0 μm film thickness (Make: Zebron; Part No: 7HK-G005-36) was used as the stationary phase. Helium

Figure 1: Chemical structure of (A) Dimethyl fumarate and (B) Methanol
was used as the carrier gas. The head space auto-
sampler parameters and the gas chromatograph
parameters were set as per the conditions given in
Table 1. The injection volume was fixed to 50 µl. A
Split mode of injection for the analyte was used. The
split ratio of 1:3 was fixed. The carrier gas flow rate
was fixed to 5 mL/min (velocity=30 cm/sec). A flow
rate of 400 mL/min was fixed for Air and 40 mL/min
was fixed for Hydrogen gas.

Table 1: Optimised parameters of the Gas chromatograph instrument equipped with a Head space auto sampler:

<table>
<thead>
<tr>
<th>GC instrument</th>
<th>Perkin Elmer instrument (Clarus 580)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Detector</td>
<td>Flame Ionisation Detector</td>
</tr>
<tr>
<td>Carrier gas</td>
<td>Helium</td>
</tr>
<tr>
<td>GC cycle time</td>
<td>40 minutes</td>
</tr>
<tr>
<td>Detector temperature</td>
<td>260 ºC</td>
</tr>
<tr>
<td>Injector temperature</td>
<td>180 ºC</td>
</tr>
<tr>
<td>Oven temperature programme</td>
<td>Initial Temp.</td>
</tr>
<tr>
<td></td>
<td>(ºC)</td>
</tr>
<tr>
<td></td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>130</td>
</tr>
<tr>
<td>Carrier gas flow rate</td>
<td>5 mL/min</td>
</tr>
<tr>
<td>Carrier gas linear velocity</td>
<td>30 cm/sec</td>
</tr>
<tr>
<td>Flow rate of Air</td>
<td>400 mL/min</td>
</tr>
<tr>
<td>Flow rate of Hydrogen</td>
<td>40 mL/min</td>
</tr>
<tr>
<td>Split ratio</td>
<td>1:3</td>
</tr>
<tr>
<td>Head space parameters</td>
<td>Head space auto sampler Turbo matrix 40</td>
</tr>
<tr>
<td>Oven temperature</td>
<td>80 ºC</td>
</tr>
<tr>
<td>Needle temperature</td>
<td>140 ºC</td>
</tr>
<tr>
<td>Transfer line temperature</td>
<td>180 ºC</td>
</tr>
<tr>
<td>Vial thermostat time</td>
<td>20 minutes</td>
</tr>
<tr>
<td>Shaker</td>
<td>On</td>
</tr>
<tr>
<td>Vial Pressurization time</td>
<td>0.20 minute</td>
</tr>
<tr>
<td>Vial withdrawal time</td>
<td>0.2 minute</td>
</tr>
<tr>
<td>Inject time</td>
<td>0.05 minute</td>
</tr>
</tbody>
</table>

**Method Validation:**

The method was validated as per the ICH Q2
(R1) guidelines. Various validation parameters like
System suitability, Specificity, Limit of Detection,
Limit of Quantitation, Linearity, Range, Precision,
Accuracy, Ruggedness and Robustness were studied.
**System Suitability:**

System suitability was determined by
injecting six replicate injections of the standard
solution. Various test parameters like % RSD of the
Retention time (Rt) of the analyte peak, % RSD of the
peak areas of the analyte, USP Plate count and USP
tailing were measured and calculated.

**Specificity:**

The test for specificity was done to determine
any interference at the Rt of the peak of Methanol.
This was performed by analysing the blank solution,
sample solution and methanol solution as per the
methodology.
Limit of Detection (LOD) and Limit of Quantitation (LOQ):
The LOD and LOQ were established by the signal to noise (S/N) ratio method. These were determined by analysing solvent (Methanol) with concentrations lesser than its standard concentration which would give a S/N ratio of 3 for LOD and S/N ratio of 10 for LOQ.

Linearity and Range:
The Linearity of the method was evaluated at five different concentration levels. Concentrations at LOQ, 50 %, 100 %, 120 % and 150 % of the specification limit with respect to the test sample were prepared. Thus, the range of concentrations from 6-4500 ppm was analysed by the above developed method. A calibration plot of plotted of the obtained peak areas versus the concentrations of Methanol. The coefficient of correlation was determined.

Precision:
Precision of the method was demonstrated by performing the System precision, Method precision and precision at the LOQ level. System precision was evaluated by injecting the standard solution of methanol six times into the system. In Method precision, six test preparations were prepared from a homogeneous sample by spiking it with Methanol of working concentration. These were analysed along with two control samples. Content of Methanol (ppm) in each test preparation was calculated as per equation (1) and % RSD for content (ppm) was determined. Precision at the LOQ level was determined for the solvent by spiking the diluent with Methanol at LOQ level concentration. % RSD of the peak area of Methanol was determined for each type of precision evaluated.

Accuracy:
Accuracy of the method was evaluated by preparing the sample solutions by spiking the Methanol solvent at LOQ to 150 % of specification limits in triplicate as per the test method. The % Recovery, Mean % Recovery and % RSD for each level was calculated.

Robustness:
The Robustness of the method was assessed by analysing the effect of variation in the flow rate (±10 %), injector temperature (±5 %), detector temperature (±5 %) and head space vial’s incubation temperature (±5 %).

Ruggedness:
Ruggedness also known as intermediate precision was evaluated by performing the same test as for method precision by another analyst on a different day.

Content of solvent in ppm=
Solvent area in sample x (µL of solvent x density) x 0.2mL x dil. of the sample (mL) x 10<sup>6</sup>
Solvent avg. area in standard x 10 mL X 20 mL X Weight of the sample (mg)

APPLICATION FOR DIMETHYL FUMARATE API SAMPLE ANALYSIS:
Two batch samples (Batch no. DVm0340516 and Batch no. DVm0450616) obtained from MSN Organics Private Limited, Hyderabad, India were analysed by the developed HS-GC method. Test sample preparations were prepared in duplicate for each batch by the above given procedure. The content of Methanol in Dimethyl fumarate API of the two batches was determined by using the optimized method.

RESULTS AND DISCUSSION:
Method development:
The method development included the selection of column, carrier gas, diluent and the detector. Optimization of the various GC instrument parameters was also done.

Selection of the diluent:
The diluent chosen was based on the solubility of Dimethyl fumarate and the partition coefficient of Methanol. Water was not selected as diluent as the solubility of Dimethyl fumarate is less in water (1.6 mg/mL). Methanol also forms hydrogen bonding with water due to which equilibrium of Methanol in the head space of the vial does not take place. Dimethyl fumarate is very soluble in Dimethyl sulfoxide (DMSO) (29 mg/ mL) and N, N-Dimethyl formamide (12 mg/ mL). The peak shape of Methanol was broader
and the GC cycle was longer when DMSO was used. The sensitivity of the method was improved by using N.N- Dimethyl formamide; hence it was selected as the diluent.

**Selection of the detector:**

The Flame ionization detector (FID) was used for the research work, as it is the most sensitive general detector in GC. It is a mass sensitive detector. The sample undergoes combustion in hot hydrogen- air flame. Ions and free electrons are hence formed. These charged particles produce a measurable current flow in the gap between the electrodes. The current formed is greater than that produced by the carrier gas and the fuel gas flame alone. Hence it provides information about the number of carbons in the sample. The detector gases, hydrogen and synthetic air were used as the fuel gas and the oxidising gas respectively during the combustion process in the FID.

**Selection of column and carrier gas:**

The selection of the column depends on the nature of analyte to be analysed. For the analysis of Methanol, columns equivalent to USP Phase G43 Inertcap 624 columns were tried out. These columns are having medium polarity. They are made up of bonded 94 % Dimethylpolysiloxane and 6 % Cyanopropylphenyl. Various fused silica columns like DB-624, OV1-G43, ZB-624 were tried during the method development. Amongst the above ZB-624 was finalised, as more theoretical plates and sharper peaks were obtained using it.

Helium or nitrogen are normally used as carrier gases for the FID, sometimes hydrogen is also used. Helium was used as the mobile phase as it is inert, non-flammable and has a low molecular weight. The linear velocity of Helium was fixed at 30 cm/ sec. At this linear velocity the more efficiency of the column and good peak shape were observed. Trials were carried out with flow rate of 2- 5 mL/min. The carrier gas flow rate was fixed at 5 mL/min.

**Temperature programming of the column oven:**

Methanol is a highly volatile compound having a boiling point of 64.7 ºC. Linear temperature program was trialled with initial temperature at 50 ºC, final temperature of 240 ºC and ramp rate of 5 ºC/min and 10 ºC/min. As Methanol is a highly volatile compound, its peak was obtained below retention time of 10 minutes. Gradient temperature programs were also trialled with. The optimised column oven temperature program which resulted in a Methanol peak with a good shape, less retention and a good resolution between the Methanol and the diluent peak is given in Table 1.

**Optimisation of the Head space auto-sampler parameters:**

The Head space vial was thermostatted at 80 ºC and shaked to assist the attainment of equilibrium in it. This improved the sensitivity of the method. The needle temperature and transfer line temperature was maintained at 140 ºC and 180 ºC respectively so as to prevent the condensation problems.

**Method Validation:**

After the method is developed it had to be validated to check its efficiency and reproducibility of results. For this the developed GC method was validated as per ICH Q2 (R1) guidelines. The System suitability parameters like USP plate count, USP tailing measures the performance of the entire GC system by analysing its ability to separate components. While parameters like % RSD of peak area and Rt of the peak, measures the system reproducibility by analysing the consistency of separation from injection to injection. The results of system suitability test (given in Table 2) show that the analyte, equipments, analytical operations, chromatographic system, the electronics and the software used for the integration of results form an integral system.

In the test for specificity, no interference from the blank solution or sample solution was observed at the retention time of the Methanol peak. The Methanol peak also did not co-elute with the diluent peak. Hence the method was specific. The chromatograms are shown in Figure 2. The LOD and LOQ were calculated based on the signal to noise ratio. The LOD was found to be 2 ppm with S/N ratio of 3.4. And the LOQ was found to be 6 ppm with S/N ratio of 10.3. The calibration curve of Methanol \((y = 41.634x – 271.87)\) showed good linearity in the concentration range of 6 – 4500 ppm. The coefficient of correlation was found to be 0.9998. Figure 3 depicts the Calibration curve of Methanol.
The system and the method proved to be precise with the results having % RSD of less than 15% (Results are given in Table 3). With a recovery between 80% to 120% and %RSD of less than 15% at each tested concentration level, accuracy of the developed method was observed (Results are given in Table 4). The method was found rugged with a % RSD less than 15% when analysis was done by another analyst on another day.

The results obtained by inducing variations in the flow rate, injector temperature, detector temperature and head space vial’s incubation temperature were similar to those obtained using the optimized method. Hence the method was found to be robust.

**Result of the sample analysis:**

The Dimethyl fumarate sample analysis showed that DMF API (Batch no. DVm0340516) there was no Methanol present. While in DMF API (Batch no. DVm0450616) there was 7 ppm of Methanol content present as residual solvent. Both the DMF samples pass the Residual solvent test as, the Methanol content in both is less than 3000 ppm. The chromatograms of the API sample analysis of Dimethyl fumarate are given in Figure 4.

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**Table 2: System suitability test results:**

<table>
<thead>
<tr>
<th>PARAMETER</th>
<th>RESULT OBTAINED</th>
</tr>
</thead>
<tbody>
<tr>
<td>% RSD of Retention time</td>
<td>0</td>
</tr>
<tr>
<td>% RSD of Peak area</td>
<td>8.9</td>
</tr>
<tr>
<td>USP Plate count</td>
<td>58801</td>
</tr>
<tr>
<td>USP Tailing</td>
<td>1.1</td>
</tr>
</tbody>
</table>

**Table 3: Summary of results of the Precision studies:**

<table>
<thead>
<tr>
<th>LEVEL</th>
<th>MEAN % RECOVERY</th>
<th>SD</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>System Precision (Peak area)</td>
<td>129334.7</td>
<td>2587.0</td>
<td>2.00</td>
</tr>
<tr>
<td>Method Precision (Methanol content in ppm)</td>
<td>2976.5</td>
<td>104.67</td>
<td>3.52</td>
</tr>
<tr>
<td>Intermediate Precision (Methanol content in ppm)</td>
<td>3002.5</td>
<td>95.67</td>
<td>3.19</td>
</tr>
<tr>
<td>Precision at LOQ level (Peak area)</td>
<td>1398.8</td>
<td>56.7</td>
<td>4.05</td>
</tr>
</tbody>
</table>

**Table 4: Recovery study results for Methanol solvent:**

<table>
<thead>
<tr>
<th>LEVEL</th>
<th>MEAN % RECOVERY</th>
<th>SD</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Level-1 (LOQ)</td>
<td>92.5 %</td>
<td>1.25</td>
<td>1.35</td>
</tr>
<tr>
<td>Level-2 (50%)</td>
<td>100.3 %</td>
<td>0.72</td>
<td>0.72</td>
</tr>
<tr>
<td>Level-3 (100%)</td>
<td>99.7 %</td>
<td>1.56</td>
<td>1.56</td>
</tr>
<tr>
<td>Level-4 (120%)</td>
<td>97.9 %</td>
<td>1.17</td>
<td>1.20</td>
</tr>
<tr>
<td>Level-5 (150%)</td>
<td>99.6 %</td>
<td>0.70</td>
<td>0.70</td>
</tr>
</tbody>
</table>

**Table 5: Results of Dimethyl fumarate API sample analysis:**

<table>
<thead>
<tr>
<th>Batch no.</th>
<th>Mean Peak area</th>
<th>Content of Methanol (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DVm0340516</td>
<td>Peak missing</td>
<td>Not detected</td>
</tr>
<tr>
<td>DVm0450616</td>
<td>15769</td>
<td>7</td>
</tr>
</tbody>
</table>
Figure 2: Chromatograms of (A) Blank, (B) Control sample, (C) Standard solution and (D) Methanol at LOQ level

Figure 3: Calibration curve of Methanol
CONCLUSION:

Gas chromatographic technique is a very sensitive technique and is used for the analysis of volatile substances. The significance of this technique was utilized by us in our research for the development of a method to determine the content of Methanol in Dimethyl fumarate API (Active Pharmaceutical Ingredient). Methanol is a Class 2 solvent as per ICH Q3C (R6) guidelines. This solvent has toxicological effects if present above the specification limits as per the guidelines. Hence, analysis of Methanol residual solvent in Dimethyl fumarate is crucial. The developed HS-GC method proved to be simple, sensitive, precise and accurate.

Our research would benefit the Dimethyl fumarate API manufacturers to do a quality check of their product and thereby optimize the process of synthesis accordingly. Dimethyl fumarate formulation manufacturers can approve the API from their vendors by doing the analysis of Methanol residual solvent by our method. This will help them in vendor selection for their API. Also screening of the API batches to be used for their Dimethyl fumarate formulations will be possible. The developed HS-GC method will hence give pharmaceutical industries an assurance that the Dimethyl fumarate formulations produced by them are free from toxicologically significant levels of Methanol.

ACKNOWLEDGEMENT:

The authors are very grateful to MSN Organics Private Limited, Hyderabad, India for the Dimethyl fumarate API samples. The authors are also immensely grateful to Graviti Pharma Pvt. Ltd., Isnapur, Hyderabad, India for providing excellent facilities for carrying out this research work.

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education/10187/gas-chromatography-gc-with-flame-ionization-detection

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Source of Support: Nil
Conflict of Interest: None declared

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