Formulation and characterization of mometasone furoate and formoterol fumarate containing dry powder inhaler by spray drying and homogenization methods

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ABSTRACT: This study aimed to design and characterize an inhalable dry powder of mometasone furoate monohydrate (MFM) combined with the formoterol fumarate dihydrate (FFD). Homogenization in water and spray drying processes were used to prepare the dry powders for inhalation. The physicochemical characteristics of the dry powders were evaluated by differential scanning calorimetry (DSC), Fourier transform infrared spectroscopy (FT-IR), X-ray diffractometry (XRD) and scanning electron microscopy (SEM). The particle size distribution, content uniformity, solid behavior and aerodynamic properties of DPI were also determined. After the micronization process, the particle sizes of the raw materials significantly decreased. SEM images implied that FFD covered the MFM surface during the spray drying step, and uniform particles were produced. X-ray and DSC results demonstrated that MFM did not show any change in crystal structure after homogenization and spray drying processes. DSC analysis of DPI formulation exhibited that the same melting point of MFM was observed when the drugs were spray dried. FT-IR spectra exhibited the characteristic peaks for MFM in DPI formulation. Content uniformity results showed that the developed production method is suitable for manufacturing uniform formulations. According to angle of repose, Hausner ratio and Carr’s index results, DPI formulation has preferable flow properties. In addition, emitted dose, mass median aerodynamic diameter, fine particle fraction showed that the DPI formulation could ensure drug delivery to the alveoli. This study showed that the combination of homogenization and spray drying methods is suitable to obtain a DPI formulation containing MFM and FFD with a particle size less than 5 µm to reach alveoli.

KEYWORDS: Mometasone furoate monohydrate; formoterol fumarate dihydrate; dry powder inhaler; asthma; combined drug therapy

1. INTRODUCTION

Asthma, which is a chronic inflammatory lung disease, characterized by recurrent episodes of airway obstruction, wheezing coughing, shortness of breath, and chest tightness. The first patient to die of asthma was recorded in 1908, and then the number of asthma patients increased day by day (1). Although asthma is seen in all ages, race and, ethnicity, the severe asthma prevalence was reported as 1.8%-38% of the population (2). It affects approximately 334 million people worldwide. However, only 250,000 patients die annually from asthma (3). Therefore, the treatment of asthma is very important, and formulations taken by oral or pulmonary routes are commercially available to achieve sufficient pulmonary function without any symptoms. In addition, there is no treatment that completely eliminates asthma, but symptomatic treatment of asthma can be achieved by single or multiple use of inhalers, tablets, injections, or surgical treatment.

Among these treatment options, inhalers have many advantages such as rapid effect due to large surface area and highly permeable membrane structure of the lung, minimizing systemic side effects, increasing the treatment efficacy, regular clinical response, overcoming the first-pass effect, and low enzymatic activity (4). Drug administration routes by inhalation are nebulizers, metered dose inhalers (MDIs) and dry powder inhalers (DPIs). Among these, DPI is a dosage form that has been studied frequently in recent years and is superior in the treatment of many lung diseases due to its use for both local and systemic purposes. DPIs are systems in which the active substance is in solid form that provides advantages in terms of drug stability and
ease of use. Since the active substance is contained in a powder formulation, the risks of disintegration, decomposition and microbiological contamination are less than in liquid formulations (5-7). DPIs have important advantages compared to nebulizer and MDIs. For instance, they are portable since they do not require any intermediate device, and because they allow higher dose inhalation with a single breath, the duration of treatment is shorter (8-10). In addition, DPIs do not need hand-mouth coordination and do not contain propellant gas (11, 12). Moreover, when the same dose of drug administered with DPIs or MDIs, the amount of drug reaching the lungs is approximately twice as high with DPIs, and the amount accumulated in the oropharynx is less than that of MDIs.

Formoterol fumarate dihydrate (FFD; Figure 1), a long-acting beta2-adrenoceptor agonist with bronchodilator effect and rapid onset of action, is used in the treatment of asthma, chronic obstructive pulmonary diseases includes chronic bronchitis and emphysema (13). Mometasone furoate monohydrate (MFM; Figure 1) is a corticosteroid which improves lung function and asthma symptom scores (14).

![Figure 1. Chemical structures of a) MFM and b) FFD.](image)

DPPC is one of the primary phospholipids that is naturally found in the lung and essential for proper lung surfactant activity. For this reason, DPPC was used as a surfactant for MFM suspensions in this study (15, 16).

In the treatment of asthma, combination drug therapy with a bronchodilator and a glucocorticosteroid is a common approach. In this study, it was aimed to produce DPI formulation consisting of MFM particles coated by FFD. To achieve this goal, the developed formulation was prepared by spray-drying the mixture containing suspended MFM and dissolved FFD. When this formulation reaches the lung surface, it is expected to exert its FFD effect first to provide bronchodilation, and then to exert its corticosteroid effect by releasing MFM. In this way, it was intended to increase the anti-inflammatory effect of MFM.

In addition, combining the two drugs in a single inhaler not only simplifies the administration regimen but also increase patient compliance. To our knowledge, there is no commercially available DPIs containing MFM and FFD. Two active substances (one water-soluble and the other water-insoluble) at different doses were homogeneously formulated as a carrier-free DPI formulation using combination of high-pressure homogenization and spray drying methods that is simple, cost-effective, and suitable for industrial scale production.

For all these purposes, MFM and FFD containing DPI formulation with a particle size of 1-5 µm was prepared and characterized.

2. RESULTS AND DISCUSSION

2.1. Particle size distribution

The particle size distributions of raw MFM, homogenized suspension of MFM, and MFM+FFD containing DPI formulation were measured. Median volume diameter (Dv50) value was 13.39±1.78 µm for raw MFM. This value decreased with increasing homogenization cycle and changed to 1.49±0.01 µm after 20 cycles (Table 1). When the spray drying process was applied to the obtained suspension, a slight increase in
the Dv50 value was observed compared to the homogenized suspension; however, Dv50 value remained in the respirable range (1-5 µm). The average particle size of the DPI formulation was significantly decreased by 6-fold ($p < 0.05$) compared to the raw MFM. These findings were consistent with the SEM images.

Spray drying is considered a powerful technological process since it brings feasibility to the production of free-flowing particles with well-defined particle size. Spray drying process has been successfully implemented in pharmaceutical technology as it is a suitable method for drying heat-sensitive substances, and obtaining free-flowing particles and well-defined particle size (17). The combination of high-pressure homogenization and spray drying is also advantageous since the high-pressure homogenization produces more homogeneous sprays, thus, narrow particle size distribution, a critical feature in DPI production. Pilcer et al. used combination of these techniques to produce DPI formulations containing tobramycin, and obtained a DPI formulation in an inhalable sizes (18). By following these techniques, the targeted particle size with the unimodal distribution was observed in our MFM+FFD containing DPI formulations (Figure 2). This suggests that the developed DPI formulation can be a suitable and advantageous inhaler dosage form for uniform dosing and targeted lung delivery of MFM and FFD (Table 1).

**Table 1.** Particle size distribution of raw MFM, homogenized MFM suspensions and homogenized+spray dried DPI formulation containing MFM+FFD (n=6, mean±SD).

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>d (0.1) (µm)</th>
<th>d (0.5) (µm)</th>
<th>d (0.9) (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw MFM (5% DPPC)</td>
<td>5.180±0.306</td>
<td>13.390±1.777</td>
<td>122.514±34.510</td>
</tr>
<tr>
<td>MFM (5% DPPC) 10 cycle homogenization</td>
<td>1.19±0.020</td>
<td>2.143±0.011</td>
<td>4.027±0.112</td>
</tr>
<tr>
<td>MFM (5% DPPC) 15 cycle homogenization</td>
<td>0.135±0.537</td>
<td>1.755±0.159</td>
<td>3.317±0.150</td>
</tr>
<tr>
<td>MFM (5% DPPC) 20 cycle homogenization</td>
<td>0.103±0.000</td>
<td>1.485±0.006</td>
<td>2.945±0.014</td>
</tr>
<tr>
<td>MFM (5% DPPC) 20 cycle homogenization+FFD solution (spray dried)</td>
<td>1.463±0.002</td>
<td>2.169±0.003</td>
<td>3.341±0.007</td>
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**Figure 2.** Particle size distribution raw MFM, homogenized MFM suspensions and homogenized+spray dried MFM+FFD DPI formulation (n=6).

### 2.2. Scanning Electron Microscopy (SEM)

SEM images of raw MFM and DPI formulation containing MFM+FFD are shown in Figure 3. SEM images revealed that raw MFM was composed of large crystals. On the other hand, particle size of the DPI formulation containing MFM + FFD was much smaller and uniform than the raw MFM. This observation was also consistent with the results of the particle size distribution measurements (d(0.1), d(0.5), d(0.9)). DPI formulation containing MFM+FFD was prepared from a suspension of homogenized MFM particles, thus they
partially maintained the morphology of raw MFM and have a rigid-non-spherical structure. Moreover, it was seen that FFD covers the MFM surface in the spray drying stage, and uniform particles were produced.

When dissolved substances are spray dried, an amorphous structure is usually obtained. Akdag Cayli et al. aimed to produce DPI formulations containing a combination of an antibiotic and a mucolytic agent. For this purpose, homogenized ciprofloxacin particles were spray-dried together with acetylcysteine solution. SEM analysis demonstrated the presence of acetylcysteine particles on the surface of ciprofloxacin (19), similar to the current study.

In our study, an amorphous image was observed on the surface of the DPI particles as the dissolved FFD dried by covering the surface of the dispersed MFM (Figure 3a). When the SEM images were evaluated together with the XRD results considering the preparation method, it was thought that MFM was in crystalline form and FFD was in amorphous form in the DPI formulation (Figure 3a-3b).

Figure 3. SEM images of DPI formulation containing MFM+FFD (a) and raw MFM (b).

2.3. Differential Scanning Calorimetry (DSC)

DSC analysis verifies the thermogravimetric properties of the active pharmaceutical ingredients and their crystalline structure when mixed in raw form or combined in the DPI formulation. The DSC thermograms of the raw MFM, raw FFD and DPI formulation containing MFM+FFD are presented in Figure 4. DSC thermogram of raw MFM confirmed that endothermic peak between 225-240°C is correlated with the literature (20). DSC analysis of raw FFD demonstrated that raw FFD had three endothermic peaks. The peak around 122°C was ascribed to dehydration of raw FFD. The jagged peak at 163°C displayed a thermal decomposition of the FFD. The endothermic peak at 147°C was compatible with melting point of FFD (21). Additionally, DSC results showed that MFM and FFD used to prepare formulation, had crystalline structures. DSC analysis of DPI formulation exhibited the same MFM melting point as the spray dried drugs. However, as the DPI formulation contains a very low amount of FFD relative to MFM, the melting peak of FFD can be suppressed by the MFM peak in the DPI formulation as shown in DSC thermogram (Figure 4). Besides, while preparing the DPI formulation, MFM was suspended and FFD was dissolved in water, then the mixture of FFD and MFM was spray dried. As there is no FFD peak in the thermogram of the DPI formulation, the DSC results confirms that the FFD is present in its amorphous form, while MFM is in its crystalline form in the prepared DPs.
2.4. Fourier Transform Infrared Spectroscopy (FT-IR)

FT-IR results of DPI formulation and raw MFM were similar, with peaks characteristic of MFM (1600 to 1750 cm⁻¹, carbonyl groups; 1710 cm⁻¹, ester carbonyl; 1658 cm⁻¹, hydrogen bonding; 1732 cm⁻¹, keto carbonyl (20)) in the DPI formulation (Figure 5a). FT-IR data indicated that the chemical structures of MFM was preserved in the DPI formulations. Therefore, neither the manufacturing process (high pressure homogenization and spray drying) nor the addition of FFD affected the molecular structure of MFM. Since the amount of FFD in the formulation was very low compared to MFM, the peaks of functional groups of FFD were suppressed (Figure 5a-5b). However, the presence of FFD in the formulation was demonstrated by HPLC analysis.
2.5. X-ray Diffraction (XRD)

X-ray diffractograms of MFM and DPI formulation revealed that the crystalline structure of MFM was preserved in the DPI formulation produced by spray-drying using homogenized MFM and FFD solution (Figure 6a-6b).

As known, crystalline substances are more stable than amorphous substances due to the lower moisture absorption properties of crystalline materials. Moisture absorption is a very important issue in DPI formulations because aerodynamic parameters change as the moisture content of dry powders change. Variability in aerodynamic parameters can also change the amount of substance reaching the target area in the lungs, and thus the effectiveness of the treatment (22, 23). The lower intensity of the DPI formulation compared to raw MFM is thought to be due to the amorphous FFD coating the surface of the crystalline MFM. The reason why FFD does not contribute to the crystal structure of the DPI formulation is that the amount of FFD in the formulation is very low and that FFD is spray dried by from the solution form during the formulation production (Figure 6a-6b).
Content Uniformity

Content uniformity studies showed that the achieved MFM:FFD ratio was 200.11:4.89, very close to the 200:5 ratio (Table 2). In addition, the amounts of MFM and FFD determined from each DPI sample are close to each other and the standard deviation is less than 0.2 indicating that the developed production method is suitable for manufacturing uniform formulations. The difference between the theoretical and experimental values was thought to be due to the moisture content of raw MFM and raw FFD, and DPPC content of DPI.

Table 2. Amounts of MFM and FFD in 10 mg spray dried DPI formulation (SD: standard deviation, n=3).

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Figure 6. X-ray diffractograms of a) raw MFM and MFM+FFD containing DPI, and b) raw FFD.

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2.7. Characterization of solid behavior of DPI

Although angle of repose is a simple test to assess flow properties of DPI formulations, it provides an important understanding of the friction or cohesion forces between the particles. According to the USP 30 (24), a powder with an angle of repose value within the range of 25–30° exhibits excellent flow property. The estimated angle of repose value for developed DPI formulation was 25.49±2.94, indicating that our formulation is considered to have excellent flow properties.

The flow properties were further examined by the bulk and tapped densities, Hausner ratio and Carr’s index. Estimated tapped and bulk densities of DPI formulation powder were 0.56 ± 0.02 g/cm³ and 0.49 ± 0.03 g/cm³, respectively. For DPI formulations to pass the tests, the Hausner ratio must be between 1.26 and 1.34 and the Carr’s index between 21 to 25. Moreover, the Hausner ratio and Carr’s index values for ideal powders with excellent flow properties in DPIs should be 1.0-1.11 and ≤10, respectively. In our study, produced DPIs showed good flow characteristics with a Hausner ratio of 1.13±0.03 and, Carr’s index value of 11.36±2.66. These results suggest that when the powder is administrated to the lungs, active substances can reach the deeper pulmonary regions, as the powder has the potential to escape inertial and gravitational deposition in the upper respiratory tract. Therefore, the produced DPIs can be considered as an ideal dosage form for the pulmonary drug delivery with excellent dispersibility (25).

2.8. Aerodynamic parameters

Dry powder respirability, expressed by aerodynamic parameters, is given in Table 3. Emitted doses were approximately 53% for MFM, and 75% for FFD. The RS01 device aerosolized DPI formulation: MMADs ranged from 1.7 to 2.2 μm for both MFM and FFD, which represents an appropriate aerodynamic size to administer particles that can reach deep into the lungs. Although FFD had a higher emitted %, MFM had higher FPF%. The FPFs were approximately 85% for MFM, and 69% for FFD. The high FPF (<5 μm) achieved for MFM was the result of the low emitted dose values (19). As shown in Figure 7, although some of the powder remained in the device, a significant amount reached stage 3 and beyond. These results show that the developed DPI can provide drug delivery to the alveoli.
Figure 7. In vitro deposition profile of MFM+FFD containing DPI in Andersen cascade impactor at a flow rate of 60 L/min via RS01 device (mean ± SD; n = 3).

Table 3. In vitro lung deposition of MFM+FFD containing DPI by Andersen cascade impactor (mean ± SD; n = 3).

<table>
<thead>
<tr>
<th></th>
<th>Emitted Dose (mg)</th>
<th>Emitted %</th>
<th>Fine Particle Dose (mg)</th>
<th>Fine Particle Fraction %</th>
<th>Mass Median Aerodynamic Diameter (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MFM</td>
<td>4.78±0.29</td>
<td>53.15±3.22</td>
<td>4.06±0.24</td>
<td>84.81±0.15</td>
<td>1.71±0.04</td>
</tr>
<tr>
<td>FFD</td>
<td>0.37±0.00</td>
<td>74.49±0.25</td>
<td>0.26±0.02</td>
<td>69.33±6.20</td>
<td>2.20±0.44</td>
</tr>
</tbody>
</table>

2.9. HPLC analysis

Quantitative analysis of MFM and FFD from content uniformity samples was performed by an HPLC method. Determination coefficient ($R^2$) values were 0.9999 for MFM (1-500 µg/mL) and 0.9996 for FFD (1-100 µg/mL) indicating linearity of the method. Also, HPLC method used was considered selective as there was no interfering peak with the retention times of MFM (9.7 min) and FFD (1.9 min).

3. CONCLUSION

This study was designed to formulate the combination of MFM and FFD as the DPI formulation for the treatment of asthma. Dry powder formulations were prepared with the combination of both high-pressure homogenization as size reducing process and spray drying. This production process was carried out in water, which could suspend MFM and dissolve FFD. The results obtained showed that the FFD covered the surface of the MFM in the DPI formulation. Thus, FFD will dissolve rapidly and show the bronchodilator effect, and then an anti-inflammatory effect will be obtained with MFM. The results of this study clearly showed that combination of homogenization and spray drying methods is suitable to obtain DPI formulation containing MFM and FFD with particle size less than 5 µm to reach alveoli.
4. MATERIALS AND METHODS

4.1. Materials

Mometasone furoate monohydrate (MFM) and formoterol fumarate dihydrate (FFD) were kindly provided by Drogsan (Turkey) and Abdi Ibrahim (Turkey). RS01 device was donated by Plastiape SpA (Italy). Dipalmitoylphosphatidylcholine (DPPC) was purchased from Sigma Aldrich (Germany). All other chemicals were of analytical grade.

4.2. Methods

4.2.1. Formulation design and development

The preparation method was developed based on our previous study (19). The DPI formulation containing MFM and FFD as model drugs, and DPPC as a surfactant was prepared by combination of high-pressure homogenization and spray drying methods. Surfactants are excipients mostly used in the suspension preparations by high pressure homogenization, therefore, in this study DPPC was used in the DPI formulation and its concentration was determined based on the studies in the literature (26, 27).

A suspension of MFM (0.25% w/v) in 200 mL water (with 5% DPPC) was homogenized for 10, 15, and 20 times at 20000 PSI using a Microfluidizer M-110 P homogenizator (Microfluidics International Corp., MA, USA). After 20 homogenization cycles, suspensions with a mean particle size \( d (0.5) \) of <3 μm were obtained. As the desired particle size was achieved, the number of homogenization cycles was kept at 20. For combination of MFM+FFD formulation, the FFD solution in 550 mL water was added to the MFM suspension prior to the spray drying process (Buchi B-290, Switzerland). FFD concentration was adjusted to obtain 200:5 MFM:FFD ratio. The spray drying process was carried out at the following conditions: 150 °C inlet temperature, 5% pump rate, 85% aspiration, and 667 L/h airflow rate.

4.2.2. Particle size distribution

To investigate the effects of high-pressure homogenization and spray drying on particle size distributions, raw MFM and micronized powders were analyzed by laser diffraction. Samples were dispersed in water. DPPC was added to the dispersion medium to homogeneously disperse the raw MFM. The obtained suspensions were vortexed for 30 s and analyzed using a Malvern Master Sizer 2000 (Malvern Instruments, Westborough, MA), which derives the volume distribution from a laser diffraction intensity distribution. The size of each sample was measured in triplicate under stirring at 1750 rpm without ultrasonication.

4.2.3. Scanning Electron Microscopy (SEM)

The surface morphologies of MFM+FFD containing DPI and the raw MFM were evaluated by scanning electron microscopy (SEM; JSM5600; JEOL Ltd., Japan), with magnifications of 2500×, at 3.00 kV and 10.00 kV, respectively.

4.2.4. Differential Scanning Calorimetry (DSC)

The thermal properties of raw MFM and MFM+FFD containing DPI were investigated using differential scanning calorimetry (DSC; Q100 (TA Instruments, DE, USA)). All samples (5–10 mg) were sealed in aluminum pans and then heated at a 10 °C/min rate under a nitrogen purge at 25–300 °C range.

4.2.5. Fourier Transform Infrared Spectroscopy (FT-IR)

Infrared spectra of raw MFM and MFM+FFD containing DPI formulation were evaluated between 4000 and 600 cm\(^{-1}\) with a Perkin-Elmer FT-IR (MA, USA).

4.2.6. X-ray Diffraction (XRD)

X-ray analyses were performed using a Rigaku Ultima-IV (Japan) for raw MFM and MFM+FFD containing DPI formulation, and Rigaku Miniflex (Japan) for raw FFD at a 40 kV voltage, with a 2°/min scan rate over a 2θ range of 2–40° conditions.
4.2.7. Content Uniformity

To evaluate the content uniformity of DPI formulation containing MFM and FFD, 10 mg powder was accurately weighed, and transferred into a 10 mL volumetric flask. The volume was made up with mobile phase of the HPLC method (acetonitrile:orthophosphoric acid:methanol (60:30:10) mixture), and then magnetically stirred (500 rpm) at room temperature. After 1 h, the samples taken from the medium were filtered using a 0.45 μm cellulose acetate membrane filter. The MFM and FFD content in the samples were then analyzed by an HPLC method.

4.2.8. Characterization of solid behavior of DPI

Flow properties of MFM+FFD containing DPI formulation were characterized determining the angle of repose (α), Hausner ratio, and Carr’s index. The angle of repose was determined using fixed funnel method (n=3). In this method, the DPI formulation was poured through a funnel to form a cone. The height and the base diameter of the powder cone (2r) were measured to calculate the angle of repose using the following equation (28):

\[ \alpha = \tan^{-1}\left(\frac{h}{r}\right) \]  
\( \alpha \) = the angle of repose
\( h \) = the height (cm)
\( r \) = the radius (cm)

Bulk and tapped densities, Carr’s index, and Hausner ratio were also calculated to assess the aerosolization characteristics of the powder. To measure the bulk and tapped volumes, the powder (2 g) was poured into a 10 mL measuring cylinder and the level was uniformed by 10 gentle taps on the hard surface from a fixed distance. After recording the initial volume (\( V_0 \)) of powder, the cylinder was then adjusted and uniformly tapped 90 times more in the same manner. The tapped volume (\( V_{100} \)) of the powder in the cylinder was recorded. Bulk and tapped densities, Carr’s index and Hausner ratio were calculated using following equations (28, 29).

Bulk density = \( \frac{m}{V_{100}} \)  
Tapped density = \( \frac{m}{V_{100}} \)  
Carr’s Index (%) = \( \frac{V_{100} - V_0}{V_{100}} \times 100 \)  
Hausner Ratio = \( \frac{V_0}{V_{100}} \)

4.2.9. Aerodynamic parameters

The aerodynamic parameters of MFM+FFD containing DPI formulation were evaluated according to the United States Pharmacopeia using an Andersen cascade impactor (ACI; Copley Scientific, UK). Size 3 HPMC capsules in an RS01 device (Plastiape SpA, Italy), which was performed at a flow rate of 60 L/min for 4 seconds, was utilized to measure aerodynamic parameters. The cut-off levels for the ACI for 60 L/min flow rate were 8.6, 6.5, 4.4, 3.2, 1.9, 1.2, 0.55 and 0.26 μm. ACI plates were coated with Tween-20 solution in ethanol (1%, w/v) to avoid particle bouncing. The DPI formulations in three capsules were discharged separately for ACI test. After this, the device, capsule, rubber adaptor, induction port (IP), all ACI stages and filter (F) were washed separately with known amount of HPLC mobile phase. The mass median aerodynamic diameter (MMAD) was determined by plotting the cumulative percentage undersize (probability scale) against the aerodynamic diameter (log scale). The Emitted Dose (ED) was the amount of drug ex device measured as the sum of the amount of drug collected from IP to F. The fine particle dose (FPD) was the amount of microparticles with a diameter <5 μm, while the fine particle fraction (FPF) was expressed as the ratio between the FPD and ED. Aerodynamic parameters were calculated for both MFM and FFD.
4.2.10. **HPLC method**

The HPLC method reported by Gujarati et al. was used for the analysis of MFM and FFD in samples. The calibration curves for the HPLC method were obtained for both MFM (1-500 μg/mL) and FFD (1-100 μg/mL). An HPLC system (Shimadzu LC-20 A/Prominence Alliance; Japan) and a Waters Spherisorb ODS2 C18 (250 × 4.6 mm 5μm; USA) column were used for quantitative analyses of MFM and FFD. An acetonitrile : orthophosphoric acid (0.05 M) : methanol (60:30:10, v/v/v) mixture was used as the mobile phase delivered at a flow rate of 1 mL/min, with a 10-min total run time at room temperature. The injection volume was 20 μL and the detection was performed with a DAD detector at 248 nm (30).

4.2.11. **Statistical analysis**

The results were compared with Mann-Whitney U test using GraphPad Prism 6. The difference between the results was considered significant, when p value was less than 0.05.

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**REFERENCES**


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