ANALYTICAL METHOD VALIDATION, PHARMACOKINETICS AND BIOEQUIVALENCE STUDY OF DIMETHYL FUMARATE IN HEALTHY IRANIAN VOLUNTEERS

GHASEMIE ELHAM¹, SADRAI SIMA², SHOKRI JAVAD³, SAYADI SHAHRAM⁴

¹Department of Pharmaceutics, Faculty of Pharmacy, Islamic Azad University of Damghan, Damghan, Iran, ²Division of Biopharmaceutics and Pharmacokinetics, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran, ³Pharmaceutical department, Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran, ⁴Anesthesiology Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran

Email: ghasemien_elham@yahoo.com

Received: 06 Jun 2021, Revised and Accepted: 31 Jul 2021

INTRODUCTION

Multiple sclerosis (MS) is a chronic autoimmune, inflammatory neurological disease of the central nervous system (CNS) [1] that attacks and destroys the myelinated axons [2]. Most of the MS patients (80–85%) have a Relapsing-Remitting Multiple Sclerosis (RRMS) disease form [3]. Treatment options in RRMS have increased to a dozen different available disease-modifying medicines and a few more are expected to be marketed soon [4].

Dimethyl fumarate (DMF), also known as BG-12, is the first-line oral treatment for RRMS [5] and has immunomodulatory properties [6]. DMF was approved for the treatment of psoriasis in 1959 [7] and got approved under the brand name of Tecfidera® for the treatment of RRMS in 2013 [8]. DMF may cause anti-inflammatory and cytoprotective activities that are mediated by the nuclear factor (erythroid-derived 2)-like 2 (Nrf2) antioxidant response pathway [9].

DMF is rapidly metabolized in the gastrointestinal tract into the primary active metabolite monomethyl fumarate (MMF) [10]. For this reason, DMF is not detectable in plasma after oral administration, and pharmacokinetics measurements are based on MMF concentrations [11]. MMF is dose-proportional over with high inter-subject variability [12]. Protein binding and volume distribution of MMF are 27–45% and 53–73 L, respectively [13]. By attention to this note that DMF microtablets in the capsules have an enteric coating, absorption has a delay leaving the stomach [3]. So, the reported time to peak concentration (T_max) of MMF after oral administration of Tecfidera® capsules is 2–2.5 h and the half-life of MMF is around 1h [14]. Also, the maximum concentration (C_max) of MMF was 1.87 mg/L [14]. An administration of DMF with food delays the time to reach the C_max of MMF up to 5.5h and causes a 40% decrease in C_max but no effect on Area under the curve (AUC) [15].

ABSTRACT

Objective: Pharmacokinetic evaluation of Dimethyl Fumarate (DMF) in the Iranian population wasn’t studied. So, the aim of this research is the validation of the analytical method and evaluation of the pharmacokinetic properties and bioequivalence of the generic form of this drug versus the reference product.

Methods: 2 single-dose, test, and reference DMF products were orally administered to 24 healthy volunteers. The washout period was 28 d between the treatments. Monomethyl fumarate as the metabolite of DMF was analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) and the method was validated. Also, the pharmacokinetic parameters were calculated for bioequivalence evaluation.

Results: The analytical method was validated and linear over the range of 31.25–400 ng/ml (R² = 0.997). In addition, the method was precise and accurate in the low, medium, and high concentrations. The results indicated that the 2 products had similar pharmacokinetics. Further, the 90% CI of the mean ratios of the test versus the reference products of the log-transformed area under the concentration-time curve over 10 h (0.99 to 1.02) and peak concentration (0.98 to 1.03) were within the acceptable range of 0.8 to 1.25 and the generic product of DMF could be similar to that of the reference product.

Conclusion: The applied analytical method is selective, accurate, precise, and repeatable for the analysis of monomethyl fumarate (MMF) in plasma. Also, the bioequivalence study showed no significant difference between the pharmacokinetic parameters of these 2 products. So, the DMF test product can be claimed to be bioequivalent with the reference product.

Keywords: Bioequivalent, Pharmacokinetics, Multiple sclerosis, Dimethyl fumarate, LC-MS/MS

© 2021 The Authors. Published by Innovare Academic Sciences Pvt Ltd. This is an open access article under the CC BY license (https://creativecommons.org/licenses/by/4.0/)

MATERIALS AND METHODS

Subjects

The protocol of this study was approved by the Ethics Committee of Islamic Azad university-Damghan branch, code: IR. IAU. DAMGHAN. REC.1398.004 and was registered in Iranian Registry Clinical Trials (IRCT), IRCT ID: IRCT2020062304792N1. Additionally, written informed consent was obtained from all volunteers before their enrollment. The enrolled volunteers included 14 healthy men and 10 healthy, non-pregnant women with a mean age of 34±5 y (range of 23-43 y), a mean body weight of 75±18 kg (range of 47-110 kg), and a mean height of 173±12 cm (ranging from 151 to 197 cm). Based on the results of the completed clinical assessment, serum biochemistry, hematology, and routine urinalysis, all subjects were found to be healthy.

Drug administration and sample collection

The present single-dose, randomized, 2-treatment and 2-period crossover study was conducted on healthy Iranian male/female volunteers. The test or reference drug was randomly administered in a 1:1 ratio. In addition, all volunteers were fasted at least 10 h before drug administration up to 4 h after that. On the day of the test offered a single oral dose of reference and test formulations with 240 ml of water. The washout period was 4 w. A total of 15 blood samples was collected before 0.5, 0.75, 1, 1.5, 1.75, 2, 2.5, 3, 4, 5, 6, 7,
Chemical
Acetonitrile Pro HPLC (Merck), Zinc sulfate (Sigma), Methanol Pro HPLC (Merck), Formic Acid (Merck).

Sample preparation
To preparation of the standard solution, MMF stock solution (50 µg/ml) and plasma were spiked and mixed at 10: 490 for 2 min. Then, the sample was kept without shaking for 10 min. After that, the prepared content was vortexed for 5 min. Then 50 µl of zinc sulfate solution (1.16 M) and 450 µl Acetonitrile were added to the solution. The sample was vortexed for 5 min and then held for 10 min without shaking. Then the sample was centrifuged at 15 000 rpm for 10 min and the upper phase was separated and injected into LC/MS/MS.

The intended standard plasma concentration range of 31.25-4000 ng/ml was obtained through diluting the MMF standard solution (400 µg/ml).

Chromatographic conditions
Quadrupole mass spectrometer Quattro Micro (Waters-Micromass, UK) equipped with an electrospray source (Z-spray) was applied to conduct mass spectroscopy. Filtered samples were injected in a volume of 20 µl into a Thermo (50×4.6 mm, 5 microns) column at 50 °C and separated by Alliance HT separations module 2795 (Waters, Milford, MA, USA), which consist of a quaternary solvent delivery system, degasser, Autosampler, column heater. Chromatographic separation was performed at a flow rate of 0.5 ml/min using an elution buffer contains 85% of eluent A (0.3% formic acid in water) and 15% eluent B (100% methanol). Mass spectrometry measurements were performed on Mass Lynx software, version 4.1. Samples were introduced to API positive source values as follows: Corona 1 (ua); cone 25 V; extractor, 1 V; RF lens, 1 V; Source temperature: 120 °C; Desolvation temperature: 400 °C; Desolvation gas flow rate: 500 L/h Cone gas (nitrogen 99.99%) purity flow rate: 150 L/h.

Validation procedure
Based on the Food and Drug Administration guidelines, the analytical method was validated in terms of linearity, range, specificity, accuracy, precision, and carryover [16].

Specificity
The Specificity test was conducted by comparing chromatograms of blank plasma, plasma spiked with 1 µg/ml monoethyl fumarate (MEF) as internal standard (IS) and, 31.25 ng /ml MMF as the lower limit of quantification (LLOQ) [17].

Linearity
The spiked standard solutions of MMF (in the range of 31.25-4000 ng/ml) and MEF (1 µg/ml) as an internal standard in plasma were prepared and analyzed by LC-MS/MS system. The final calibration curves included three replicates per calibration concentration, and linearity was assessed by linear regression. The correlation coefficient of Linearity (R²) should be ≥ 0.98.

Accuracy and precision
The precision and accuracy of the assay were determined from the low (62.5 ng/ml), medium (500 ng/ml), and high (3000 ng/ml) Quality Control (QC) plasma samples. The inter-day assay was determined by analyzing QC samples in triplicates and was analyzed on three different days. The intra-day precision and accuracy were determined for each QC sample in plasma, each in triplicate on one day (table 1). The precision determined at each concentration should not exceed 15% of the RSD%. Except for LLOQ (31.25 ng/ml) where it should not exceed 20% of the RSD% [18].

RESULTS AND DISCUSSION
Analytical method validation
Specificity
Under the chromatographic conditions described, MMF and the IS peaks were well resolved. Endogenous plasma components did not have any interfering peaks. Fig. 1 shows typical chromatograms of blank plasma as compare to spiked samples analyzed for a pharmacokinetic study. The average retention times of MMF and MEF were 6.2 and 2.4 min, respectively.

Carryover effect
During the method validation process of MMF, carryover was evaluated by injecting blanks, after previously injected sample with a concentration on Upper Level of Quantification (ULOQ) (3000 ng/ml).

Carryover on the blank should not be more than 20% of LLOQ and 5% for internal standards [19].

Stability
Stability studies were carried out according to EMEA guidelines. The medium concentration (500 ng/ml) of MMF in plasma was prepared in triplicates and kept frozen at 80 °C until analysis. For short-term stability tests one-hour thaw, freeze-thaw cycles were studied. One-hour stability was examined by leaving plasma quality control samples at room temperature on the bench one hour before preparation. Freeze-thaw stability of the samples was obtained after two freeze-thaw cycles, by thawing at room temperature and freezing for 12-24 h for each cycle respectively. The concentration of MMF after each storage period was compared with the initial concentration that was determined for samples that were freshly prepared and immediately processed. The mean area of the stability solution should be±15% of its freshly prepared solution [20].

Pharmacokinetic analysis
The standard non-compartmental procedure was applied to establish or calculate the pharmacokinetic parameters. Maximum plasma concentration (Cmax), time to reach the maximum plasma concentration (Tmax), area under the plasma concentration-time curve from time zero to the last measurable concentration (AUC0-t), and total area under the plasma concentration-time curve (AUC0-inf) were estimated from the plasma concentration-time data [21]. Cmax and Tmax were attained directly from the plasma data, while the AUC0-t was calculated by adding the area from time zero to last sampling time, t (AUC0-t), and the area from time t to infinity (AUCt-inf). AUC0-inf was calculated using the trapezoidal formula, and; AUCt-inf was calculated by dividing the last measurable plasma drug concentration (Ct) with the elimination rate constant (kE) [22-23].

Statistical analysis
The values of Cmax, AUC0-t, AUC0-inf, and Tmax obtained with the two formulations were analyzed using the analysis of variance (ANOVA) procedure which differentiated effects due to subjects, periods, and treatments. Furthermore, AUC0-t, AUC0-inf, and Cmax were used as a base to evaluate the equivalence of the two formulations. The 90% CI of the test/reference mean ratios were determined for Cmax, AUC0-t, and AUC0-inf. The applicable range of 0.8 to 1.25 can lead to the bioequivalence between the 2 formulations. The variations between the 2 compared parameters were statistically significant if the P values were less than 0.05 [24].
Fig. 1: Chromatograms of (A) blank plasma; (B) blank plasma spiked with 1 µg/ml MEF (IS) and (C) blank plasma spiked with 31.25 ng/ml MMF.

Fig. 2: Calibration curve of MMF in plasma.

Accuracy and precision

The precision and accuracy of the assay were determined from the low (62.5 ng/ml), medium (500 ng/ml), and high (3000 ng/ml) Quality Control (QC) plasma samples. The inter-day assay was determined by analyzing QC samples in triplicates and was analyzed on three different days. The intra-day precision was determined for each QC sample in plasma, each in triplicate on one day (table 1). The both precision value (RSD %) determined at each concentration wasn’t more than 8.21%.

Accuracy was expressed as the mean percentage of analyte that recovered in the assay. The results of the accuracy are shown in table 2. As shown, coefficients of variation were less than 10%, which is acceptable for the routine measurement of the accuracy of the Bioanalytical method.

Table 1: Intra-day and inter-day precision of the method for determination of MMF in human plasma

| Concentration of MMF (ng/ml) | Average of drug area/IS area±SD | RSD% |
|-----------------------------|---------------------------------|------|
| Intra-day precision         |                                 |      |
| 62.5                        | 0.08±0.01                       | 8.21 |
| 500                         | 0.73±0.03                       | 4.33 |
| 3000                        | 4.37±0.08                       | 1.92 |
| Inter-day precision         |                                 |      |
| 62.5                        | 0.08±0.00                       | 4.85 |
| 500                         | 0.75±0.00                       | 0.57 |
| 3000                        | 4.42±0.09                       | 1.96 |

Note: Data given in mean±SD, n=3

Table 2: Accuracy of the method for determination of MMF in human plasma

| Concentration of MMF (ng/ml) | Average of drug area/IS area±SD | RSD% | Deviation |
|-----------------------------|---------------------------------|------|-----------|
| Intra-day Accuracy          |                                 |      |           |
| 62.5                        | 0.09±0.00                       | 4.23 | -9.51     |
| 500                         | 0.73±0.02                       | 2.73 | 3.44      |
| 3000                        | 4.37±0.10                       | 2.20 | 5.30      |
| Inter-day Accuracy          |                                 |      |           |
| 62.5                        | 0.09±0.00                       | 0.93 | -8.07     |
| 500                         | 0.77±0.06                       | 7.34 | 1.47      |
| 3000                        | 4.26±0.04                       | 0.92 | 7.50      |

Note: Data given in mean±SD, n=3

Fig. 3: Carryover effect between high concentration sample of MMF and blank.

Carryover effect

Carryover between samples can occur in analytical methods. But in this method development carryover effect was evaluated and no accumulation after a high concentration of MMF was seen (fig. 3). So, it could be concluded no need for a meaningful cleaning procedure between injections.

Stability

The stability of MMF and IS in the short term and freeze and thaw cycles was tested. In all of these stability studies, both MMF and IS did not show any significant degradation (table 4). These results confirmed that MMF was stable in plasma under the storage conditions and during sample preparation.
inter-subject variability in T\(_{\text{max}}\) = 2.1±0.9 h in reference and test formulations (table 5). A higher mean T\(_{\text{max}}\) was observed, which is as a result of variability in gastric emptying time delayed release capsules [25].

As seen in the above table, there is less than a 10% difference between fresh standard and remained sample at room temperature for 1 hour and sample that passed 2 cycles of freezing and thawing.

Pharmacokinetics

35 subjects were screened. 24 subjects were randomized and included in the study. The subjects were divided into two groups according to the randomization table. There was one drop-out (Subject 20, because of fainting before drug administration of the second period). As a result, 23 subjects completed the study and no serious adverse effect was observed in any treatment. The pharmacokinetic parameters (mean±SD) for the test and reference products are summarised in table 5. The logarithmic value of C\(_{\text{max}}\), AUC\(_{0-10}\), and AUC\(_{0-\infty}\) means, ratios, and 90% CIs are summarised in table 6.

Table 4: Stability of MMF and IS in short term and freeze-thaw cycle

| Parameter | Test (Area) | Standard (Area) | Test/STD mean ratio |
|-----------|-------------|----------------|---------------------|
|          | Test        | Reference       |                     |
| C\(_{\text{max}}\) (ng/ml) | 3274.3±204.0 | 3194.3±1308.3 | 1.0 ± 0.05 |
| T\(_{\text{max}}\) (h) | 2.1±0.9 | 2.1±0.9 | 1.0±0.05 |
| AUC\(_{0-\infty}\) (ng·h/ml) | 3200.2±1623.4 | 3225.3±1310.8 | 1.0±0.05 |

Note: data given in mean±SD, n=3

Average plasma concentration-time curves of test and reference products for a single dose of DMF are shown in fig. 4. The reference and test formulations used in the current study have mean AUC\(_{0-\infty}\) values 3200.2±1623.4 ng·h/ml and 3194.3±1308.3 ng·h/ml, respectively. Mean C\(_{\text{max}}\) values for the reference and the test formulations are 1862.7±1191 and 1686.9±716.1 ng/ml, respectively. Further, the mean T\(_{\text{max}}\) values were 2.5±0.9 and 2.1±0.9 h in reference and test formulations (table 5). A higher inter-subject variability in T\(_{\text{max}}\) was observed, which is as a result of variability in gastric emptying time delayed release capsules [25].

The results of the t-test, demonstrate no difference between the average parameters that resulted from sequencing, period, and administering the test and reference products at the significance level of 0.05. The 90% CIs for the mean ratios of the test versus reference formulation of C\(_{\text{max}}\), AUC\(_{0-10}\), and AUC\(_{0-\infty}\) equal to 0.98-1.03, 0.99-1.02, and 0.99-1.01, respectively. Therefore, both are placed in an acceptable range of 0.80 to 1.25 and are found to be bioequivalent.

![Fig. 4: MMF plasma concentration-time in healthy volunteers following consumption of Tecfidera® 240 mg (reference) and Teczifuma® 240 mg (test) (n=23). Concentration presented based on mean±SD](image-url)
CONCLUSION

The optimized LC-MS/MS method is selective, accurate, precise, and repeatable. The method is linear over a wide range and utilizes a mobile phase that can be easily prepared. The run time is short and the protein precipitation technique is very simple. It can be concluded that the method is suitable for the routine quantification of MMF in human plasma.

Overall, in vivo examinations of the test and reference products revealed no significant difference between the pharmacokinetic parameters of these 2 products. Accordingly, the DMF test product can be claimed to be bioequivalent with the reference product and both products were similar in terms of the rate and extent of absorption. Therefore, considering that test product is pharmaceutical equivalent and bioequivalent, indicates that both products are therapeutically equivalent and interchangeable.

ACKNOWLEDGEMENT

We appreciate the Zistdaru Danesh Pharmaceutical Company for the financial support of this study.

AUTHORS CONTRIBUTIONS

All authors contributed to the practical work and writing of the manuscript. Sadrai and Ghasemian planned the study and collected blood samples and analyzed data and prepared manuscript. Shokri analyzed blood samples. Sayadi edited the manuscript. All authors wrote and revised the manuscript.

CONFLICT OF INTERESTS

The authors have declared that they have not any conflict of interest.

REFERENCES

1. Rouini MR, Dibaei M, Ghasemian E. Pharmacokinetics and bioequivalence studies of teriflunomide in healthy Iranian volunteers. Clin Pharmacol Drug Dev 2020;9:341-5.
2. Goldenberg MM. Multiple sclerosis review. PT 2012;37:175-84.
3. Burness CB, Deeks ED. Dimethyl fumarate: a review of its use in patients with relapsing-remitting multiple sclerosis. CNS Drugs 2014;28:373-87.
4. Ingversen J, Aktas O, Hartung HP. Advances in and algorithms for the treatment of relapsing-remitting multiple sclerosis. Neurotherapeutics 2010;6:13:47-57.
5. Carlstrom KE, Ewing E, Granquist M, Gyllenberg A, Ainehband S, Enoksson SL, et al. Therapeutic efficacy of dimethyl fumarate in relapsing-remitting multiple sclerosis associates with ROS pathway in monocytes. Nat Commun 2019;10:3081.
6. Foroughipour M, Gazeran S. Effectiveness and side effects of dimethyl fumarate in multiple sclerosis after 12 mo of follow up: an Iranian clinical trial. Iran J Neurol 2019;18:1-5.
7. Mills EA, Ogrodnik MA, Plave A, Mao Dazzo Y. Emerging understanding of the mechanism of action for dimethyl fumarate in the treatment of multiple sclerosis. Front Neurol 2018;95.
8. Venci JV, Gandhi MA. Dimethyl fumarate (Tecfidera) a new oral agent for multiple sclerosis. Ann Pharmacother 2013;47:1697-702.
9. Fox RJ, Kita M, Cohan SL, Henson LJ, Zambrano J, Scannevin RH, et al. BG-12 (dimethyl fumarate): a review of mechanism of action, efficacy, and safety. Curr Med Res Opin 2014;30:251-62.
10. Cho H, Hartsook MJ, Xu Z, He M, Duh EJ. Monomethyl fumarate promotes NrF2-dependent neuroprotection in retinal ischemia-reperfusion. J Neuroinflammation 2015;12:239.
11. TECFIDERA™ (dimethyl fumarate) delayed-release capsules, for oral use, FDA approved labeling text; 2013.
12. Sheikh SI, Nestorov I, Russell H, O’Gorman J, Huang R, Milne GL, et al. Tolerability and pharmacokinetics of delayed-release dimethyl fumarate administered with and without aspirin in healthy volunteers. Clin Ther 2013;35:1592-94, e9.
13. Dubey D, Kieseier BC, Hartung HP, Hemmer B, Warnke C, Menke T, et al. Dimethyl fumarate in relapsing-remitting multiple sclerosis: rationale, mechanisms of action, pharmacokinetics, efficacy and safety. Expert Rev Neurother 2015;15:339-46.
14. Tecfidera 240 mg gastro-resistant hard capsules: Annex 1: summary of product characteristics. Available from: https://www.ema.europa.eu/en/documents/product-information/tecfidera-epar-product-information_en.pdf. [Last accessed on 02 May 2021].
15. Thomas RH, Wakefield RA. Oral disease-modifying therapies for relapsing-remitting multiple sclerosis. Am J Health Syst Pharm 2015;72:25-8.
16. Bioanalytical Method Validation Guidance for Industry. Food and Drug Administration, Center for Drug Evaluation and Research (CDER), USA; 2018. Available from: https://www.fda.gov/files/drugs/published/Bioanalytical-Method-Validation-Guidance-for-Industry.pdf [Last accessed on 02 May 2021]
17. Guidance for Industry Q2B Validation of Analytical Procedures: Methodology. Geneva; 1996. Available from: https://www.fda.gov/media/71725/download. [Last accessed on 02 May 2021]
18. Tijare LK, Rangari NT, Mahajan UN. A review on bioanalytical method development and validation. Asian J Pharm Clin Res 2016;9 (Suppl 3):6-10.
19. Wenkui L, Ying HL, Austin C, Shaolin Z, Weng N. Simultaneous determination of norethindrone and Ethinyl estradiol in human plasma by high-performance liquid chromatography with tandem mass spectrometry-experience on developing a highly selective method using derivatization reagent for enhancing sensitivity. J Chromatogr B: Anal Technol Biomed Life Sci 2005;825:223-32.
20. Adhuri P, Kumar YS. Development and validation of sensitive LC-ESI-MS/MS method for the simultaneous estimation of dapagliflozin and saxagliptin in human plasma. Int J Pharm Pharm Sci 2019;11:55-9.
21. Harahap V, Malia CD, Sunarish. Pharmacokinetic profile of metformin hydrochloride in dried blood spot of healthy subjects using high performance liquid chromatography-photodiode array. Int J Appl Pharm 2018;10:35-4.
22. Design and Analysis of Bioavailability and Bioequivalence Studies. 2nd ed. Revised and Expanded. Shein-Chung Chow and Jen-Pei Liu; 2000.
23. Guidance for Industry. Bioavailability and Bioequivalence Studies for Orally Administered Drug Products—General Considerations. U. S. Department of Health and Human Services. Food and Drug Administration, Center for Drug Evaluation and Research (CDER); 2003.
24. Drug control division, Food and drug administration, Ministry of Public Health, Thailand. Guideline for the conduct of bioavailability and bioequivalence studies; 2009.
25. Lategan TW, Wang L, Sprague TN, Rousseau FS. Pharmacokinetics and bioavailability of monomethyl fumarate following a single oral dose of bafetam™ (Monomethyl Fumarate) or Tecfidera® (Dimethyl Fumarate). CNS Drugs 2021;35:567-74.