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# BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF MONOMETHYL FUMARATE BY LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY AND ITS APPLICATION TO A PHARMACOKINETIC STUDY

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## ABSTRACT
The present research work was to develop a robust, rapid, simple and sensitive liquid chromatography–tandem mass spectrometry (LC-MS/MS) assay method for the quantification of monomethyl fumarate in human plasma. An analytical method employing LC-MS/MS using human plasma was developed and fully validated for the estimation of Monomethyl fumarate. Stable labeled isotopes are used for the quantification of these drugs. The solid phase extraction technique (SPE) was used for the extraction of the drug and Internal standard (IS). The chromatographic separation was achieved on a Zodiac $C_{18}$ column by using a 70:30 (v/v) mixture of acetonitrile and 0.1% formic acid as the mobile phase at a flow rate of 0.5 mL/min. Totally five precision and accuracy batches were performed during the entire validation and intra-day and inter-day precision and accuracy were proved.

**KEYWORDS:** Monomethyl fumarate, Human plasma, LC–MS/MS, Pharmacokinetics, Quantification.

## INTRODUCTION
Monomethyl fumarate is the active metabolite of dimethyl fumarate. Dimethyl fumarate is the methyl ester of fumaric acid and works as hypoxic cell radiosensitizer. Psoriasis can be cured by taking dimethyl fumarate in combination with other fumaric acid esters. Fumaric acid and other esters of it can be used to cure multiple sclerosis.[1,2] Dimethyl fumarate acts as anti-inflammatory and neuro protective agent by activating Nrf2 antioxidant also its active metabolite monomethyl fumarate release transcription factor Nrf2 from cytoplasmic repersion and proteosomal degradation by alkylation of Nrf2 represor keap1(kelch-like erthyroid cell derived protein with CNC homology associated protein).[3] Nrf2 levels increase with this lessening genes of Nrf2 antioxidant pathway. There was an increase in cellular redox and mitochondrial membrane potentials. Adenosine tri phosphate (ATP) levels and increased glutathione levels were observed by activation of Nrf2 antioxidant pathway. Dimethyl fumarate mechanism shows activation of nuclear factor (erythroid derived 2) - related factor 2 (Nrf2) antioxidant response pathway by this mechanism less risk was observed and also high risks such as increase in liver transaminases, lowering of WBC count and Lymphocytes count[4] were seen. HIV progression can be stopped by taking antioxidant dimethyl fumarate and also in treating immune-mediated diseases. Main cause of taking dimethyl fumarate is that its active metabolite, monomethyl fumarate causes adhesion molecule expression, inhibition of pro-inflammatory cytokine signaling, Th1→Th2 lymphocyte shift, suppression of lymphocyte endothelial cell takes place. In vitro macrophages HIV infection can be reduced by dimethyl fumarate and monomethyl fumarate. Neurotoxin production can be suppressed from macrophages infected with HIV driven by CNS neuro degeneration. Because of the oxidative stress and immune activation capability HIV infected with CNS complications can be suppressed with dimethyl fumarate.

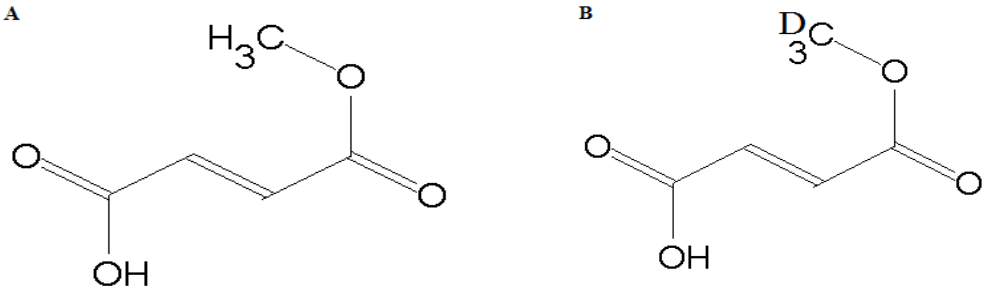


**Fig. 1: Chemical structures of monomethyl fumarate (A) and monomethyl fumarate-$d_3$ (B)**

As per the literature, only two analytical methods had been reported for the determination of monomethyl fumarate. One method reported has employed Protein Precipitation (PP) and was done in human blood over the range of 1-1000 ng/mL which involves the usage of the trapping agents such as tiopronin, sodium fluoride and potassium oxalate. The usage of such trapping agents prevents esterase hydrolysis of dimethyl fumarate and also given that dimethyl fumarate is unstable and has poor ionization. Thus, by the usage of trapping agents makes the method critical.[5] Moreover, intereference in the blank samples, unwanted humps before and after the retention times cannot be removed leading to increase in the run time by employing PP. The method published for simultaneous determination of dimethyl fumarate, teriflunomide and fampridine in human plasma by employing LC/MS-MS with an LLOQ of 5 ng/mL specified a higher run time of 15 minutes.[6] The method mentioned using LC/MS-MS is not suitable for the commercial purposes because of the high run time. Thus, in the present method, without the usage of any of the trapping agents with a less retention time of 2.2 minutes and removing all intereferences in blank and unwanted humps before and after retention times decreases the retention time. The interference and humps were removed by keeping the dip time near the retention time thereby with a less retention time of 2.2 minutes using a triple-quadrupole mass spectrometry (LC–MS/MS) with multiple-reaction monitoring (MRM) mode was used to detect both the precursor ion and fragment ion. Thus, multi step solid-phase extraction with an LLOQ of 10 ng/mL which is sensitive enough and not involving complexity like gradient elution, typical mobile phase just consisting of one or two buffers without the pH adjustment, without longer chromatographic run time (2.2 min) makes the method simple and convenient. This method employs Solid Phase Extraction (SPE) for the sample preparation and does not involve PP and Liquid Liquid Exraction (LLE) which is most likely to cause ion suppression and intereference in blanks and the advantages of SPE are more than that of PP and LLE. The phospholipids, fatty acids, lipids cannot be removed by employing PP and LLE.[7-9] Thus, in the present LC–MS/MS method an aliquot of 300 µL of human plasma was employed for sample preparation and IS used is a stable labeled isotope thereby better recoveries and assays can be achieved.[10] The present method is carried over a range of 10.06 - 4013.83 ng/mL and after elution direct loading into vials without evaporation, drying and reconstitution with mobile phase therby lessening time makes the method more suitable for commercial purposes. The application of this assay method to a clinical pharmacokinetic study in healthy male volunteers following oral administration of dimethyl fumarate is described. The authenticity in the measurement of study data is demonstrated through incurred samples reanalysis.

## MATERIALS AND METHODS

### Chemicals and materials

The reference samples of monomethyl fumarate (99.50%) and monomethyl fumarate-$d_3$ as IS (99.63%) were procured form Vivan Life Sciences Pvt. Ltd., (Mumbai, India). Water used for the LC–MS/MS analysis was prepared from Milli–Q water purification system procured from Millipore (Bangalore, India). Acetonitrile and methanol were of HPLC grade and purchased from J.T. Baker (Phillipsburg, NJ, USA). Analytical grade ammonium acetate and formic acid were purchased from Merck Ltd., (Mumbai, India). The control human plasma sample was procured from Deccan's Pathological Lab's (Hyderabad, India).

### Instrumentation and chromatographic conditions

An HPLC system (Shimadzu, Kyoto, Japan) consisting of a Zodiac C18 column (50 mm × 4.6 mm, 3 µm; Quthbullapur, Hyderabad, India), a binary LC–20 AD prominence pump, an auto sampler (SIL–HTc) and a solvent degasser (DGU–$20A_3$) were used for the study. Aliquots of the processed samples (20 µL) were injected into the column employing an isocratic mobile phase, a 70:30 (v/v) mixture of acetonitrile and 0.1% Formic acid was delivered at 0.5 mL/min into the electrospray ionization chamber of the mass spectrometer. Quantitation was achieved with MS–MS detection in positive ion mode for all the analytes and the internal standard using a MDS Sciex API–3000 mass spectrometer (Foster City, CA, USA) equipped with a Turboionspray ™ interface at 550ºC. The ion spray voltage was set at 4000 V. The source parameters viz. the nebulizer gas, curtain gas, auxiliary gas and collision gas were set at 9, 14, 35 and 10 psi, respectively. The compound parameters viz. the declustering potential (DP), collision energy (CE), entrance potential (EP) and

collision cell exit potential (CXP) were -20, -14, -10, -11 V and -20, -14, -11, -20 V for IS. Detection of the ions was carried out in the multiple reaction monitoring mode (MRM) by monitoring the transition pairs of *m/z* 128.9 precursor ion to the *m/z* 84.7for monomethyl fumarate *m/z* 131.8 precursor ion to the *m/z* 87.9 product ion for the IS. Quadrupoles Q1 and Q3 were set on unit and low resolution. The analysis data obtained were processed by Analyst software™ (version 1.4.2).

**Preparation of stock solutions of analytes and IS**

Initial stock solutions (1 mg/ml) of monomethyl fumarate and monomethyl fumarate-$d_3$ were prepared in methanol. Further dilutions were prepared in a mixture of acetonitrile and water (30:70, v/v; diluent) and also used for preparation of calibration standards and quality control (QC) samples. The stocks were stored at 2-8°C and found to be stable for 6 days. The working IS solution (20 µg/mL) was also prepared in diluent.

**Preparation of calibration curve standards and quality control samples**

Calibration samples were prepared by spiking 950 µL of human plasma with the suitable working standard solution of the analyte (50 µL dilution of monomethyl fumarate). Calibration curve (CC) standards of monomethyl fumarate in blank plasma were prepared by spiking correct volume of the working solutions, giving final concentrations ranging from 10.06 to 4013.83 ng/mL for monomethyl fumarate. The CC samples were analyzed along with the quality control (QC) samples for each batch of plasma samples. The QC samples were prepared at five different concentration levels of 10.14 (lower limit of quantification, LLOQ), 29.83 (low quality control, LQC), 596.66 (middle quality control, MQC–1), 1988.86 (MQC–2) and 3013.42 (high quality control, HQC) ng/mL for monomethyl fumarate in blank plasma. All the prepared plasma samples were stored at −70°C.

**Sample preparation**

All frozen subject samples, calibration standards and quality control samples were thawed and allowed to equilibrate at room temperature prior to analysis. The samples were vortexed to mix for 15 s prior to spiking. Sample was prepared by taking 300 µL of human plasma sample was mixed with 30 µL of the internal standard working solution (20 µg/mL of monomethyl fumarate-$d_3$) followed by addition of 625 µL of the 50mM Ammonium acetate in 0.5% formic acid buffer and vortex mixing for 10 s. The sample mixture was loaded onto an strata X catridge (33 mg/mL) that was pre conditioned with 1.0mL of methanol followed by 1.0mL of water and followed by 1.0mL of 50mM Ammonium acetate in 0.5% formic acid buffer. The extracted catridge was washed with 1.0mL of 50mM Ammonium acetate in 0.5% formic acid buffer followed by 1.0mL of water twice. Analyte and IS were eluted with 0.6mL of mobile phase. Aliquot of 20 µL of the extract was injected into LC–MS/MS system.

**Method validation**

The validation of the method was carried out as per EMEA[11] and US FDA guidelines.[12] The parameters determined were selectivity, specificity, matrix effect, method rugeddness, linearity, precision, accuracy, recovery, stability, run size evaluation and dilution integrity.

**Pharmacokinetic study design**

A pharmacokinetic study was performed in healthy male subjects ($n$ = 6). The ethics committee approved the protocol and the volunteers provided with informed written consent. Blood samples were collected following oral administration of dimethyl fumarate at pre–dose and 0.5, 1.00, 1.33, 1.67, 2.00, 2.33, 2.67, 3.00, 3.33, 3.67, 4.00, 4.33, 4.67, 5.00, 5.33, 5.67, 6.00, 7.00, 8.00, 10.00, 12.00 and 14.00 h, in $K_2$ EDTA vacutainer collection tubes (BD, Franklin, NJ, USA). The tubes were centrifuged at 3200 rpm for 10 min and the plasma was collected. The collected plasma samples were stored at –70°C till their use. Plasma samples were spiked with the IS and processed as per the extraction procedure described earlier. Along with the clinical samples, the QC samples at low, middle 1, middle 2 and high concentration levels were also assayed in triplicate. Plasma concentration–time profile of monomethyl fumarate was analyzed by non-compartmental method using WinNonlin Version 5.1. An incurred sample re–analysis (ISR) was also conducted by selecting the 12 subject samples (2 samples from each subject) near $C_{max}$ and the elimination phase. The percent change in the value should not be more than ±20%.[13,14]

## RESULTS AND DISCUSSION

**Mass spectrometry**

MS parameters were optimized by infusing the standard analyte solution of 100 ng/mL into the mass spectrometer having electrospray as the ionization source and operating in the multiple reaction monitoring (MRM) mode. The signal intensities obtained in negative mode were much higher than those in positive ion mode. Protonated form of each analyte and IS, $[M-H]^+$ ion was the parent ion in the $Q_1$ spectrum and was used as the precursor ion to obtain $Q_3$ product ion spectra. The most sensitive mass transition was monitored from *m/z* 128.9 to 84.7 for monomethyl fumarate *m/z* 131.8 to 87.9 for the IS shown in Fig. 2. As earlier publications have not discussed the details of fragmentation patterns of monomethyl fumarate and IS, we are presenting the data related to this.

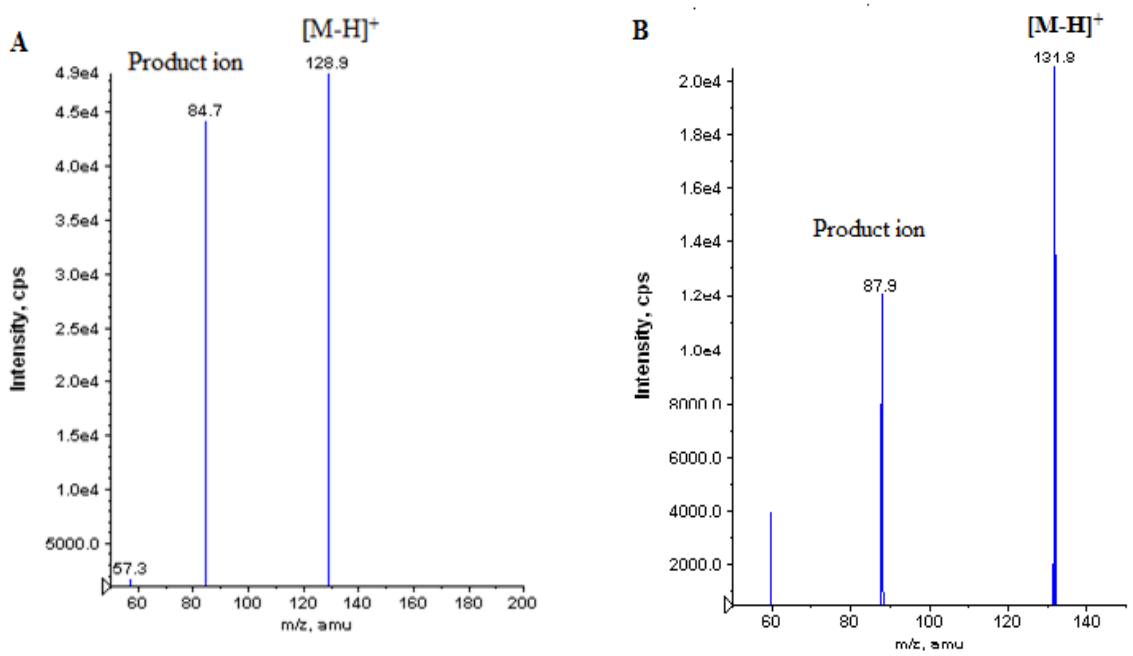


**Fig.2: Product ion mass spectra of $[M-H]^+$ of monomethyl fumarate (A) and monomethyl fumarate-$d_3$ (B)**

**Method development**

To develop a rapid, sensitive and simple assay method for the extraction and quantification of monomethyl fumarate during method development different options were tried to optimize chromatography parameters. The selectivity of MS/MS detection was also expected to be beneficial in developing a selective and sensitive method. The sensitive mass transition was observed from m/z 128.9 to 84.7 for monomethyl fumarate and from m/z 131.8 to 87.9 for the IS. LC–MRM is a very powerful tool for pharmacokinetic studies since it provides sensitivity and selectivity requirements for analytical methods. Thus, the MRM technique was chosen for the assay development. The MRM state file parameters were optimized at a concentration of 100 ng/mL to maximize the response for the analyte.

**Chromatographic conditions**

Chromatographic conditions, especially the composition of the mobile phase, column type, flow rate and column oven temperature were optimized through several trials to achieve good resolution and increased intensity of the signals of the analyte and IS, as well as for the short run time. It was found that a mixture of acetonitrile and 0.1% formic acid (70:30, v/v) could achieve this purpose and was finally adopted as the mobile phase. Zodiac 4.6 X50mm, $C_{18}$ column, 3µm gave good peak shape and response even at lowest concentration level for the analyte and IS. The mobile phase was operated at a flow rate of 0.5 mL/min. The retention time of analyte and the IS were low enough (1.23 min) allowing a small run time of 2.2 min.

**Optimization of extraction procedure**

Protein Precipitation (PP) was tried initially using acetonitrile and methanol as precipitating agents but the response was inconsistent especially at the LLOQ level moreover, moderate drug protein binding of about 27-45% was reported. Thus, the simple SPE technique was employed for the sample preparation in this work and provided high recoveries of the drugs. The use of stable labeled isotopes of the analyte and IS is recommended for bioanalytical assays to increase assay precision and limit variable recovery between analyte and the IS.[15] For an LC-MS/MS analysis, utilization of stable isotope-labeled drugs as IS proves to be helpful when a significant matrix effect is possible. At the initial stages of this work, several compounds were investigated to find a suitable IS and finally monomethyl fumarate stable labeled isotope monomethyl fumarate $d_3$ was found to be best for the present purpose.

**System suitability**

The system suitability was performed daily prior to start of the analysis. A mixture of analyte at medium level concentration and IS at working concentrations constutes the system suitability solution and it is injected. The precision (%CV) for the system suitability test were found to be less than 1% for both retention time and area ratio.

**Selectivity and chromatography**

The degree of interference by endogenous plasma components with the analytes and IS was confirmed by checking chromatograms derived from processed blank plasma sample. As shown in Fig.3 no significant direct interference in the blank plasma traces was observed from endogenous substances in drug free plasma at the retention time of the analytes.

**Sensitivity**

The lowest limit of reliable quantification for the analytes was set at the concentration of the LLOQ. The precision and accuracy at LLOQ concentration were found to be 3.83% and 97.04% for monomethyl fumarate respectively.

**Matrix effect**

SPE technique was employed to get the clear and neat sample thereby endogenous components will be eliminated and clear sample will be obtained there by matrix effect will be reduced or nullified. The results found were well within the acceptable limits as shown in Tab.1. No significant matrix effect was observed in all the lots of human plasma for the analyte at low and high quality control concentrations.

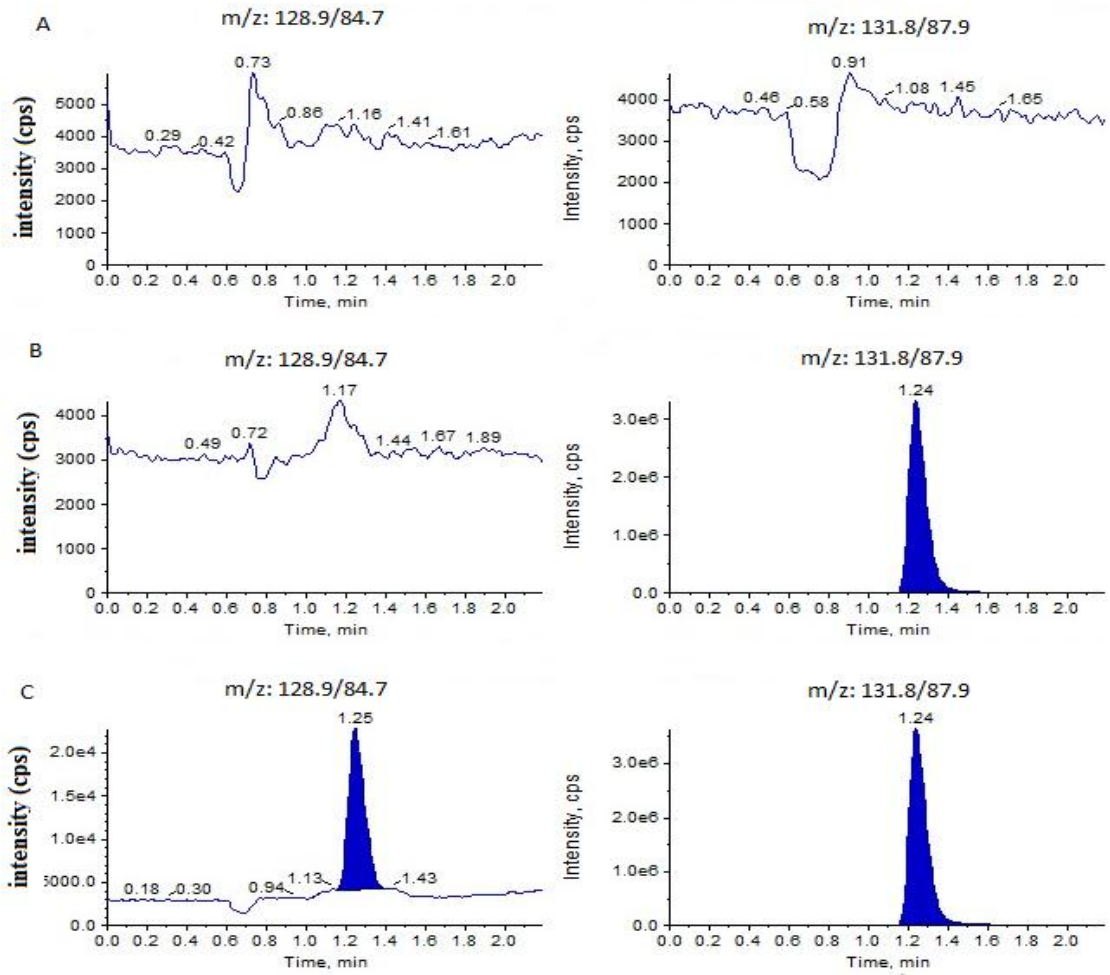


**Fig.3: Typical MRM chromatograms of monomethyl fumarate (left panel) and IS (right panel) in human blank plasma (A) and human plasma spiked with IS (B), a LLOQ sample along with IS (C)**

**Tab. 1: Matrix effect of monomethyl fumarate in human plasma (*n* = 6)**

| Plasma lot | LQC (29.83 ng/mL) | | HQC (3013.42 ng/mL) | |
|---|---|---|---|---|
| | Concentration found (mean ± SD; ng/mL) | % Accuracy | Concentration found (mean ± SD; ng/mL) | % Accuracy |
| Lot 1 | 29.51 ± 0.63 | 98.93 | 2995.14 ± 17.46 | 99.39 |
| Lot 2 | 29.22 ± 1.07 | 97.96 | 2972.04 ± 73.97 | 98.63 |
| Lot 3 | 29.82 ± 0.19 | 99.96 | 3021.51 ± 30.72 | 100.27 |
| Lot 4 | 30.10 ± 0.98 | 100.89 | 2997.12 ± 27.79 | 99.46 |
| Lot 5 | 30.31 ± 1.56 | 101.61 | 2933.47 ± 67.98 | 97.35 |
| Lot 6 | 29.58 ± 1.03 | 99.16 | 3013.69 ± 27.64 | 100.01 |

**Ruggedness**

Ruggedness of the method was proved by analyzing one precision and accuracy batch by a analyst who is not involved in the the particular study on other instrument of the same make. The precision (%CV) and accuracy values obtained were 1.73-6.70% and 96.57-111.54%.

**Linearity**

Ten–point calibration curve was found to be linear over the concentration range of 10.06–4013.83 ng/mL for monomethyl fumarate. After comparing the two weighting models (1/x and $1/x^2$), a regression equation with a weighting factor of $1/x^2$ of the drug to the IS concentration was found to produce the best fit for the concentration–detector response relationship for both the analytes in human plasma. The mean correlation coefficient of the weighted calibration curves generated during the validation was ≥0.99.

**Precision and accuracy**

The results for intra–day and inter–day precision and accuracy in plasma quality control samples are summarized in Table 2. The intra–day and inter day precision deviation values were all within 15% of the relative standard deviation (RSD) at low, middle 1, middle 2 and high quality control level, whereas within 20% at LLOQ QCs level. The intra–day and inter–day accuracy deviation values were all within 100 ± 15% of the actual values at low, middle 1, middle 2 and high quality control level, whereas within 100± 20% at LLOQ QCs level. The results revealed good precision and accuracy.

**Tab. 2: Intra–batch and inter–batch precision and accuracy for monomethyl fumarate**

| QC Levels | QC (spiked concentration ng/mL) | Intra–batch (*n*=12) | | | Inter–batch (*n*=36) | | |
|---|---|---|---|---|---|---|---|
| | | Mean concentration found (ng/mL) | Accuracy | %CV | Mean concentration found (ng/mL) | Accuracy | %CV |
| LLOQ | 10.14 | 9.71 | 95.72 | 4.48 | 10.20 | 100.54 | 8.61 |
| LQC | 29.83 | 29.25 | 98.05 | 1.61 | 29.82 | 99.97 | 3.82 |
| MQC1 | 596.66 | 570.81 | 95.67 | 1.04 | 591.25 | 99.09 | 4.96 |
| MQC2 | 1988.86 | 1850.98 | 93.07 | 0.57 | 1864.89 | 93.77 | 3.90 |
| HQC | 3013.42 | 2734.43 | 90.74 | 0.83 | 2790.07 | 92.59 | 3.71 |

**Recovery**

A simple solid phase extraction with direct elution with mobile phase proved to be robust and provided cleanest samples. The recoveries of the analytes and the IS were good and reproducible. The mean overall recoveries (with the precision range) of monomethyl fumarate were 68.83% with a precision range of 0.55–5.60% respectively. The recovery of the IS was 69.51% with a precision range of 2.98–3.85%.

**Stability studies**

In the different stability experiments carried out viz. bench top stability (12 h), autosampler stability (54 h), repeated freeze–thaw cycles (four cycles cycles), reinjection stability (70 h), wet extract stability (49 h at 2–8°C) and long term stability at –70°C is yet to be proved the mean % nominal values of the analytes were found to be within ±15% of the predicted concentrations for the analytes at their LQC and HQC levels (shown in Tab. 3). Thus, the results were found to be within the acceptable limits during the entire validation.

**Tab. 3: Stability data for Monomethyl fumarate in plasma (n=6)**

| Stability test | QC (spiked Conc., ng/mL) | Mean±SD (ng/mL) | Accuracy/stability (%) | Precision (%) |
|---|---|---|---|---|
| Autosampler stability (at 15ºC for 54 h.) | 29.83 | 31.34 | 105.05 | 3.17 |
| | 3013.42 | 3003.66 | 99.68 | 0.37 |
| Wet extract stability (at 2–8 ºC for 49 h.) | 29.83 | 30.71 | 102.95 | 1.99 |
| | 3013.42 | 3001.75 | 99.61 | 0.43 |
| Bench top stability (11 h. at room temperature) | 29.83 | 30.60 | 102.57 | 4.47 |
| | 3013.42 | 3006.01 | 99.75 | 0.22 |
| Freeze–thaw stability (four cycles) | 29.83 | 31.01 | 103.93 | 2.91 |
| | 3013.42 | 3007.37 | 99.80 | 0.54 |
| Reinjection stability (70 h) | 29.83 | 30.96 | 98.90 | 8.99 |
| | 3013.42 | 2950.09 | 101.37 | 1.79 |

**Run size evaluation**

The total number of samples that can be analyzed in a single run and integrity of the samples demonstrates run size evaluation. The batch size includes 40 sets of each of LQC, MQC1, MQC2 and HQC samples were processed and analyzed for run size evaluation along with freshly spiked calibration curve standards and quality control samples (Low, Middle and High QC samples). 158 QC's out of 160 QC's of run size evaluation were within 15% of their respective nominal (theoretical) values. 24 QC's out of 24 QC's of freshly prepared QCs were within 15% of their respective nominal (theoretical) values.

**Dilution integrity**

The upper concentration limits can be extended to12154.12 ng/mL for monomethyl fumarate by a 1/2 or 1/4 dilution with screened human blank plasma. The mean back calculated concentrations for 1/2 and 1/4 dilution samples were within 85–115% of their nominal. The coefficients of variation (%CV) for 1/2 and 1/4 dilution samples were less than 5% for all the analytes.

**Pharmacokinetic study results**

The sensitivity and selectivity of this method tested for monomethyl fumarate concentrations in human plasma samples collected from healthy male volunteers ($n$ = 6). The mean plasma concentrations against time profile of monomethyl fumarate are depicted in Fig. 4. The pharmacokinetic parameters estimated are shown in Tab.4.

**Tab. 4: Pharmacokinetic Parameter for monomethyl fumarate ($n$=6, Mean ± SD)**

| PK parameter | Monomethylfumarate |
|---|---|
| $t_{max}$ (h) | 4.39±1.02 |
| $C_{max}$ (ng/mL) | 2183.24±489.41 |
| $AUC_{0-t}$ (ng h/mL) | 4481.56±737.04 |
| $AUC_{0-inf}$ (ng h/mL) | 4493.18±740.86 |
| $t_{1/2}$ (h) | 1.21±0.26 |
| Kel ($h^{-1}$) | 0.60±0.13 |

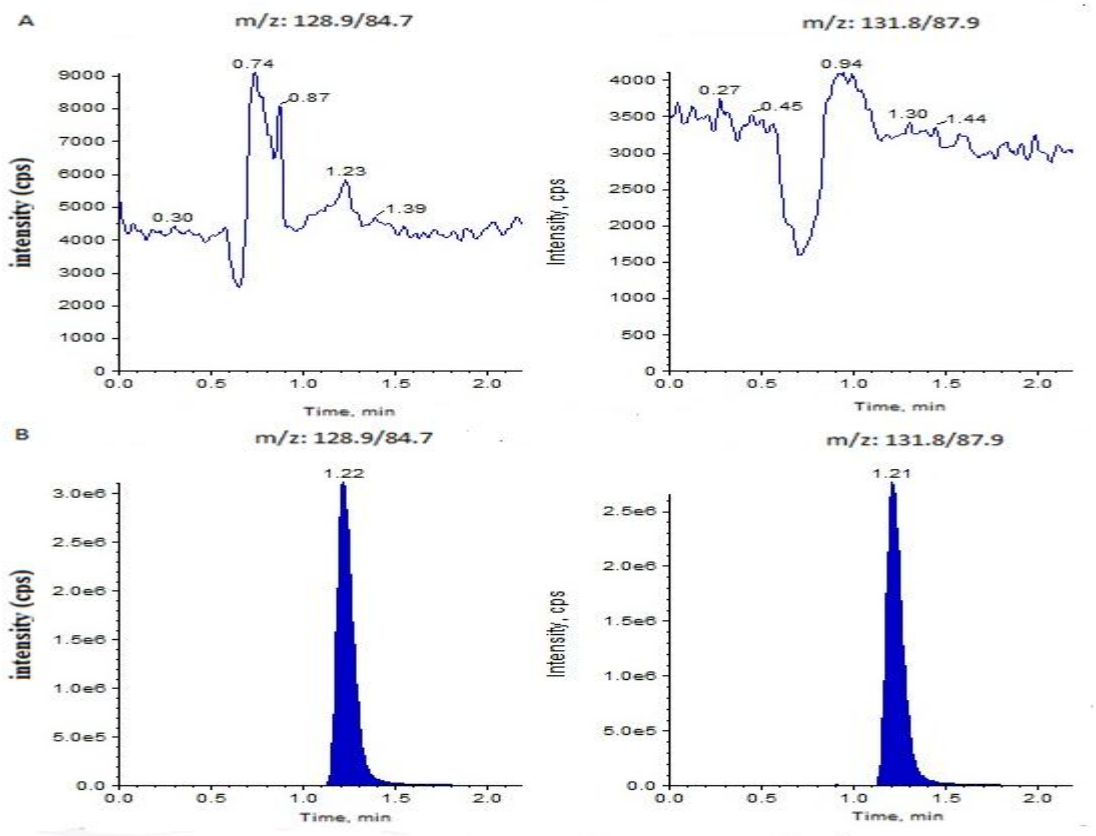


**Fig.4: Typical MRM chromatograms of monomethyl fumarate (left panel) and IS (right panel) in subject blank plasma (A), and 3.30 h of subject plasma sample, after the administration of a 240 mg oral single dose of dimethyl fumarate tablet. The sample concentration was determined to be 2287 ng/mL.**

**Incurred sample reanalysis**

In Crystal City III meeting the FDA has demonstrated the necessity of incurred sample reanalysis evaluation and the importance of assay reproducibility by using dosed subject samples. Incurred sample reanalysis (ISR) was performed using two plasma samples from each subject and re–assayed in a separate batch run. The differences in concentrations between the ISR and the initial values for all the tested samples were less than 20% (shown in Tab. 5), indicating good reproducibility of the present method.

**Tab. 5: Incurred samples re–analysis data of monomethyl fumarate**

| Sample | Initial conc.(ng/mL) | Re-assay conc. (ng/mL) | Difference[a] (%) |
|---|---|---|---|
| 1 | 2506.283 | 2290.458 | 9.00 |
| 2 | 91.039 | 94.568 | 3.80 |
| 3 | 2049.565 | 2041.323 | 0.40 |
| 4 | 53.982 | 56.628 | 4.78 |
| 5 | 1231.503 | 1119.821 | 9.50 |
| 6 | 55.556 | 55.754 | 0.36 |
| 7 | 2371.975 | 2342.389 | 1.26 |
| 8 | 77.307 | 79.728 | 3.08 |
| 9 | 1226.312 | 1289.179 | 5.00 |
| 10 | 76.385 | 83.383 | 8.76 |
| 11 | 1155.776 | 1071.792 | 7.54 |
| 12 | 188.136 | 189.04 | 0.48 |

[a]Expressed as [(initial conc._re-assay conc.)/average] _100%.

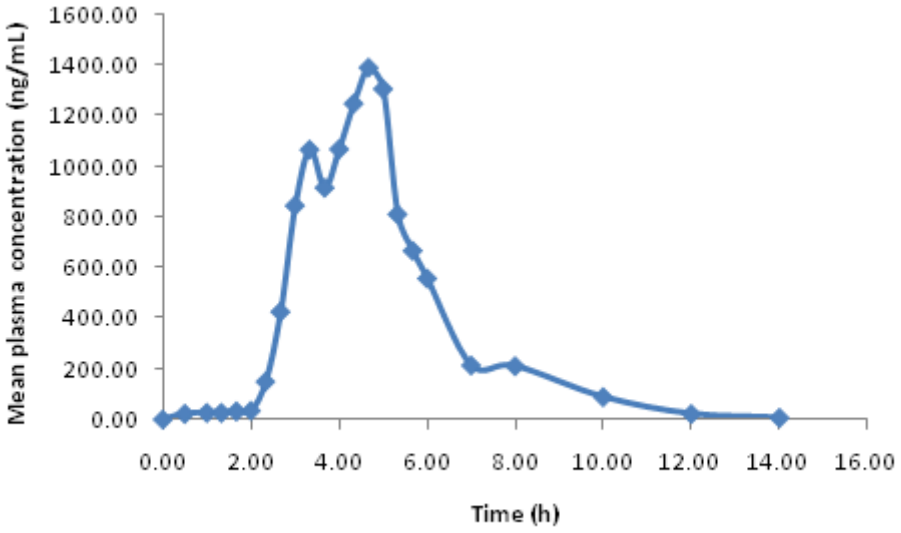


**Fig.5: Mean plasma concentration-time profile of monomethyl fumarate in human plasma following oral dosing of dimethyl fumarate (240 mg) tablet to healthy volunteers (*n*=6)**

## CONCLUSION

The LC–MS/MS method proposed in this paper is rapid, simple, specific and sensitive for quantification of monomethyl fumarate in human plasma and is fully validated according to commonly acceptable FDA guidelines. The method showed suitability for pharmacokinetic studies in humans. The simple SPE method gave consistent and reproducible recoveries for the analytes from plasma and also direct elution without evaporation step considerable reduced the time. The method provided good linearity. The less runtime of 2.2 min makes it an attractive procedure in high-throughput bioanalysis of monomethyl fumarate. From the results of all the validation parameters, we can conclude that the developed method can be useful for bioavailability and bioequivalence (BA/BE) studies and routine therapeutic drug monitoring with the desired precision and accuracy.

## ACKNOWLEDGEMENTS

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## REFERENCES

1. Moharregh-Khiabani D, Linker RA, Gold R, Stangel M. Fumaric acid and its esters: an emerging treatment for multiple sclerosis. Curr Neuropharmacol, 2009; 7: 60-4.

2. Gajofatto A, Benedetti MD. Treatment strategies for multiple sclerosis: When to start, when to change, when to stop. World J Clin Cases, 2015; 3(7): 545–555.
3. Davies TG, Wixted WE, Coyle JE, Griffiths-Jones C, et al." Monoacidic Inhibitors of the Kelch-like ECH-Associated Protein 1: Nuclear Factor Erythroid 2-Related Factor 2 (KEAP1:NRF2) Protein-Protein Interaction with High Cell Potency Identified by Fragment-Based Discovery. J Med Chem, 2016; 59(8): 3991-4006.
4. Gill AJ, Kolson DL. Dimethyl fumarate modulation of immune and antioxidant responses: application to HIV therapy. Crit Rev Immunol, 2013; 33(4): 307–359.
5. Junnotula V, Licea-Perez H. LC-MS/MS quantification of dimethyl fumarate and methyl hydrogen fumarate in rat blood using tiopronin as trapping reagent. Anal. Methods, 2016; 8: 6420-6427.
6. Suneetha A, Raja RK. Comparison of LC-UV and LC-MS methods for simultaneous determination of teriflunomide, dimethyl fumarate and fampridine in human plasma: application to rat pharmacokinetic study. Biomed Chromatogr, 2016; 30(9): 1371-7.
7. Kole PL, Venkatesh G, Kotecha J, Sheshala R. Recent advances in sample preparation techniques for effective bioanalytical methods. Biomed Chromatogr, 2011; 25(1-2): 199–217.
8. Nováková L, Vlcková H. A review of current trends and advances in modern bio–analytical methods: Chromatography and sample preparation. Anal Chim Acta, 2009; 656(1-2): 8–35.
9. Eeckhaut AV, Lanckmans K, Sarre S, Smolders I, Michotte Y. Validation of bioanalytical LC–MS/MS assays: evaluation of matrix effects. J Chromatogr B, 2009; 877: 2198–2207.
10. Katteboina MY, Pilli NR, Inamadugu JK, Satla SR. LC-MS/MS assay for irbesartan in human plasma using solid phase extraction technique: a pharmacokinetic study. Int J Pharm Pharm Sci., 2015; 7: 335-40.
11. Guideline on bioanalytical method validation, Science and Medicinal Health, European Medicines Agency (EMEA), EMEA/CHMP/EWP/192217/2009; 2011.
12. US DHHS FDA CDER Guidance for Industry: Bioanalytical Method Validation US Department of Health and Human Services Food and Drug Administration Center for Drug Evaluation and Research CDER Center for Veterinary Medicine CV 2001. http://www/fdagov/cder/guidance/index.htm.
13. Fast DM, Kelley M, Viswanathan CT, O'Shaughnessy J, et al." Workshop report and follow-up-AAPS workshop on current topics in GLP bioana-lysis: assay reproducibility for incurred samples—implications of crystal city recommendations. AAPS J, 2009; 11(2): 238–241.
14. De Boer T, Wieling J. Incurred sample accuracy assessment: design of experiments based on standard addition. Bioanalysis, 2011; 3(9): 983–992.
15. Ponnuru VS, Challa BR, Nadendla R. Quantitative analysis of eletriptan in human plasma by HPLC-MS/MS and its application to pharmacokinetic study. Anal Bioanal Chem, 2011; 401(8): 2539-48.