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Pharmacokinetics and tissue distribution of 5,7-dimethoxyflavone in mice following single dose oral administration

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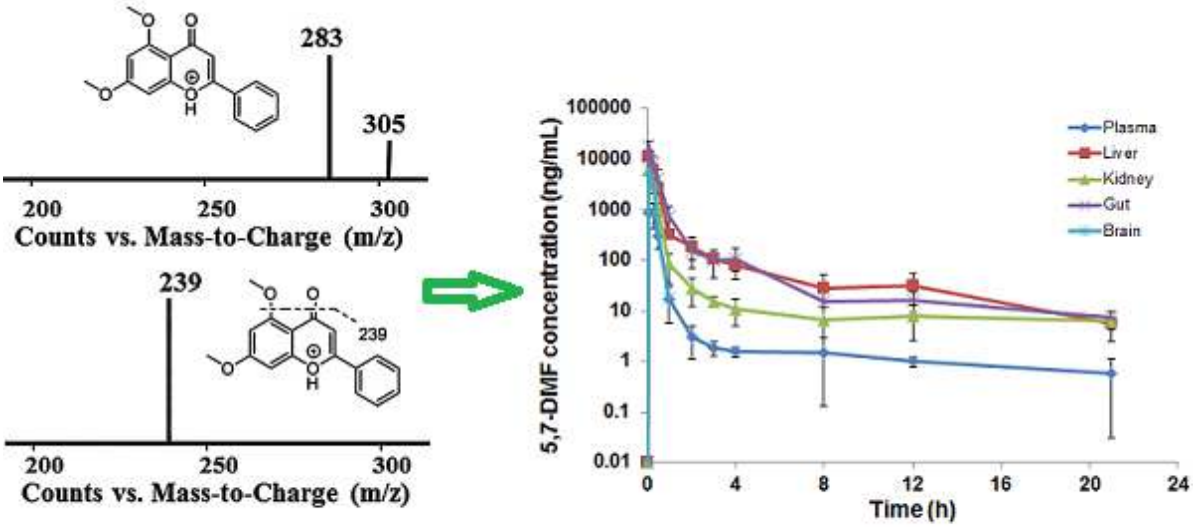
Graphical Abstract



In vivo study in mice and sample analyses with LC-MS/MS

5,7-DMF PK and tissue distribution profile

Highlights

- The PK and tissue distribution of 5,7-DMF was evaluated for the first time in mice
- Peak 5,7-DMF concentrations in mouse plasma and tissues were reached within 30 min.
- 5,7-DMF oral dose led to extensive tissue distribution.
- 5,7-DMF was abundant in gut> liver>kidney>brain>spleen>heart>lung>adipose>muscle.
- The partition coefficient (Kp) of these tissues were 0.65 to 12.9.

Abstract

5,7-dimethoxyflavone (5,7-DMF) is a major active constituent of many herbal plants, such as *Kaempferia paviflora*, *Piper caninum*, and *Leptospermum scoparium*. 5,7-DMF has demonstrated many beneficial pharmacological effects in vitro, including anti-infammatory, anti-oxidant, cardioprotection effects, as well as chemopreventive and chemosensitizing properties . In contrast to the extensive in vitro investigations, the information of the pharmacokinetic (PK) profile of 5,7-DMF in vivo is very limited. In this study we investigated the PK and tissue distribution of 5,7-DMF in mice following single oral dose of 10 mg/kg 5,7-DMF. Mouse plasma, heart, lung, liver, kidney, intestine, brain, spleen, muscle and fat tissues were collected and analyzed using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Maximal 5,7-DMF concentrations in plasma and tissues were reached within 30 min. The peak plasma concentration (C_{max}) was 1870 ± 1190 ng/mL, and area under the curve (AUC_t) was 532 ± 165 hr*ng/mL and terminal half-life was 3.40 ± 2.80 hr. The volume of distribution was 90.1 ± 62.0 L/kg. Clearance was 20.2 ± 7.49 L/hr/kg. Except for muscle and adipose, other tissues had higher C_{max} than plasma, ranging from 1.75- to 9.96-fold. After oral administration, 5,7-DMF was most abundant in gut, followed by liver, kidney, brain, spleen, heart, lung, adipose and muscle. The partition coefficient (Kp) of these tissues were 0.65 to 12.9. In conclusion, we reported for the first time the PK and tissue distribution of 5,7-DMF in mice. These results will be critical in evaluating if those beneficial in vitro effects can be translated in vivo.

Abbreviations

5,7-DMF: 5,7-dimethoxyflavone

PK: pharmacokinetic

LC-MS/MS: Liquid Chromatography-Tandem Mass Spectrometry

CYP: Cytochrome P450

P-gp: P-glycoprotein

MRP: multidrug resistance associated-proteins

BCRP: Breast Cancer Resistance Protein

MDR: multidrug resistance

LLOQ: lower limit of quantification

PBS: phosphate-buffered saline

I.S: internal standard

QC: quality control

HPLC: high performance liquid chromatogram

NCA: non-compartmental analysis

XIC: extracted ion chromatogram

BBB: blood-brain barrier.

Keywords: 5,7-dimethoxyflavone; pharmacokinetics; tissue distribution; natural products.

1. Introduction

5,7-Dimethoxyflavone (5,7-DMF) is a pharmacologically active compound abundant in plant sources such as *Kaempferial paviflora*, *Piper caninum* and *Leptospermum scoparium* [1-3], all of which have been used as folk medicine to treat gastrointestinal disorder, infections and hypertension [1-2]. 5,7-DMF has been evaluated extensively in vitro and the results showed that 5,7-DMF has many beneficial pharmacological activities. For example, it has anti-inflammatory activity [4], vasorelaxation and cardioprotection effect by increasing potassium efflux and inhibiting calcium influx [5], and selectively inhibitory effect against butyrylcholinesterase versus acetylcholinesterase [6]. Moreover, Walle's group conducted a series of in vitro studies to evaluate the chemotherapeutic and chemopreventive potential of this compound [7-9]. They found that 5,7-DMF can prevent hepatic carcinogenesis by inhibiting Cytochrome P450 (CYP) 1A1 activity, which subsequently decreased the carcinogenbenzo[a]pyrene (BaP)-induced DNA adduct formation [7]. The chemopreventive effect of 5,7-DMF was also observed in human esophageal cancer cells and human oral carcinoma cells by regulating CYP1B1 activity [8-9]. 5,7-DMF was also reported to have chemosensitizing effect in hetapocellular carcinoma cells and human leukemic cells through tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-mediated apoptosis [10-11]. In addition, 5,7-DMF was shown to improve the accumulation of rhodamine 123 into the cells with overexpression of P-glycoprotein (P-gp), an efflux transporter which is present in various cancer cells and plays an important role in multi-drug resistance (MDR) [1]. In addition to P-gp inhibition, 5,7-DMF also has inhibitory effect on other two efflux transporter members - multidrug resistance associated-proteins (MRPs) and Breast Cancer Resistance Protein (BCRP). For example, 5,7-DMF can increase the accumulation of doxorubicin in A549 cells as a result of suppression of MRPs [12]. 5,7-DMF demonstrated very potent BCRP inhibition both in vitro and in vivo even at low micro molar concentrations [13-14]. The

plasma and tissue concentrations of mitoxantrone, an anticancer drug with high affinity to BCRP, were significantly increased in vivo with the co-administration of 5,7-DMF [13]. MDR is the major cause for the failure of cancer chemotherapy treatment. An important mechanism for MDR is the decreased intracellular concentration of anticancer drugs due to the overexpression of efflux transporters, including P-gp, BCRP, and MRPs [15]. The broad inhibitory effect of 5,7-DMF on P-gp, BCRP, and MRPs makes it a very promising chemosensiting agent. Although 5,7-DMF has numerous beneficial pharmacological activities as mentioned above, it should be noted that most of the results were obtained in vitro. To evaluate if those in vitro effects can be translated in vivo, it is important to know the PK information of 5,7-DMF, especially the in vivo concentrations of 5,7-DMFat the target site(s) (i.e. tissues). To date the PK information of 5,7-DMF is very limited [16-18]. Thus the aim of our study was to evaluate the PK and tissue distribution of 5,7-DMF in mice.

Previously, the analytical methods available can only quantify 5,7-DMF with the lower limit of quantification (LLOQ) of more than 800 ng/mL [16,19]. In our recent study, we established a fast, accurate and sensitive LC-MS/MS method to quantify 5,7-DMF in mouse plasma with the LLOQ of 2 ng/mL [20]. It was applied to this study to investigate the PK and tissue distribution of 5,7-DMF in mice.

2. Material and methods

2.1. Chemicals and reagents

5,7-DMF (>99%,) and 5,7,4'-trimethoxyflavone (TMF, >99%) were purchased from Indofine Chemical Company, Inc (Hillsborough, New Jersey, US). Ammonium formate (99%) was obtained from ACROS Organics (New Jersey, US). Analytical HPLC grade acetonitrile (ACN), isopropanol, water, ethanol, phosphate-buffered saline (PBS) 10X solution, Triton lysis buffer (pH 8.0) and formic acid were purchased Fisher Scientific (Pittsburg, Pennsylvania, US). Analytical spectrophotometric grade ethyl acetate (99.5%) was obtained

from Alfa Aesar (Ward Hill, Massachusetts, US). Cyclohexane Hexahydrobenzene (99.5%) was purchased in VWR International LLC (Philadelphia, Pennsylvania, US). Heparin-treated mouse plasma was purchased from BioreclamationIVT (East Meadow, New York, US). Poly(ethylene glycol) Mn 400 (PEG400) were purchased through Sigma Aldrich (St. Louis, Missouri, US). Heparin Injectable (1,000 U/mL) was purchased from Patterson Vet Generics (Devens, Massachusetts, US). Isoflurane was provided in Animal Facility surgery room under IACUC protocol.

2.2. Animals and in vivo study

4-6 weeks old Harlan's ND4 male Swiss Webster mice with the average body weight of 26.5 g were obtained from Harlan Laboratories. Before experiment, all mice were settled and housed in the University of Florida (UF) Animal Research Facility for a week following a 12 hr light/dark cycle. All mice had access to normal/standard diet and water and this in vivo study was carried out in accordance with IACUC protocol evaluated and approved by review board of UF Animal Care and National Institutes of Health.

In this study, 30 mice were randomly separated into 10 groups based on pre-determined time points, with 3 mice at each time point (N=3). 5,7-DMF was first dissolved in dimethyl sulfoxide at the concentration of 20 mg/mL and prior to dosing, it was further diluted as solution in the vehicle consist of 35% PEG400, 2% ethanol and 63% deionized water at the final concentration of 2 mg/mL. This solution containing 2 mg/mL 5.7-DMF was stable in the vehicle for at least 24 hr under room temperature. The target dose of 10 mg/kg 5,7-DMF was administered through oral gavage to the mice. The mice had free access to food and water before and after oral administration. Blood, heart, lung, gut, brain, liver, kidney, spleen, muscle and adipose tissues were collected at time points 5, 15 and 30 min and 1, 2, 3, 4, 8, 12 and 21 hr after oral dosing. Mice were euthanized by isoflurane followed by cervical dislocation. The heparinized blood was immediately centrifuged at 2,000 g for 8 min with a

mini-centrifuge. Supernatant layer of the blood was thus collected as plasma and all the plasma and tissue samples were stored at -80ºC until analysis.

2.3. Sample preparations and LC-MS/MS assays

The LC-MS/MS condition was the same as previously reported [20]. Briefly, under positive mode of Agilent triple quadruple 6460, 5,7-DMF (m/z 283 > m/z 239) and I.S (m/z 313 > m/z 298) were simultaneously monitored with isocratic elution at a flow rate of 0.4 mL/min. The mobile phase is 50/50 (v/v) of 20 mM ammonium formate in water and acetonitrile. Plasma sample preparation method was completed following previous reported method [20]. For tissue samples, tissue was weighed and added PBS buffer (pH=7.4) based on 10-fold volume of each tissue weight. They were cut into smaller pieces before homogenization. Then 10 µL of I.S was spiked into 100 µL of tissue homogenate. Other steps were the same as plasma sample preparation. After vortex mixing for 1 min, 600 µL of ethyl acetate was added for liquid-liquid extraction on a mechanical shaker for 5 min. Then the supernatant layer was transferred to clean tubes after the centrifuge at 17,000 rpm under 4 ºC for 4 min, and placed under nitrogen for evaporation. The residue was reconstituted with 100 µL of mobile phase and centrifuged at 17,000 rpm under 4 ºC for 4 min to get rid of precipitation. 5 µL of the supernatant aliquot was injected to the LC-MS/MS. Calibration curve were prepared at concentrations 2, 5, 10, 20, 50, 100, 200, 500 and 1000 ng/mL in blank mouse tissues. Quantification of these samples was carried out based on our previously reported method [20].

2.4. Pharmacokinetic analysis

The non-compartmental analysis (NCA) was employed to calculate the PK parameters using Phoenix 1.3 (Pharsight, Mountain View, CA). Lambda z (λ_z) is the elimination rate constant at the terminal phase, and it was calculated as the slope of the linear regression on the

terminal data points. C_{max} is the maximal plasma concentration based on the experimental data. The area under the plasma concentration time curve from time zero to the last time point (AUC_t) was calculated by linear trapezoidal rule. AUC infinity (AUC_{inf}) was determined by AUC_t plus extrapolated portion C_t/λ_z. Terminal half-life was calculated as $0.693/\lambda_z$. The systemic clearance (CL_F) was determined as Dose/AUC_{inf}. The apparent volume of distribution (V_z_F) was calculated as CL_F/λ_z. The partition coefficient in tissue (Kp) was calculated by AUC_t (area under the curve from 0 to the last time point that the analyte can be measured) of tissue divided by AUC_t of plasma.

3. Results

3.1. LC-MS/MS method development and validation

As reported, our LC-MS/MS quantification method on mouse plasma was proven to be accurate, reliable, fast, sensitive and selective [20]. It was fully validated and the precision and accuracy of various QC samples all passed the criteria based on FDA guideline [21]. In this study, it was applied and validated in tissue matrices. As indicated by the FDA guideline and the newest draft guidance [21, 22], the change in the biological matrices within same species is the typical method modification and only partial validation is needed. Further, it is indicated in these guidance that, the total number of QC samples should be either 5% of the total samples to be analyzed or at least 6 QC samples in total, whichever number is greater [21, 22]. Therefore, in this case, for each tissue analysis, we performed intra-day validations on various concentrations with each concentration in triplicates (N=3) and the method validation results were shown in Table 1. Our results indicated QC samples at LLOQ were within 20% of the nominal concentrations and QC samples at higher concentrations were within 15% of the nominal concentrations. Thus these quantification methods were successfully validated and sample analyses using these assays were reliable. The extracted ion chromatograms (XIC) of 5,7-DMF in tissue samples were shown in Fig 1.

The sample preparation method for tissue samples was optimized based on the comparison of four tissue preparation methods from a pilot study. In method A, 70% ice cold methanol was used as homogenate buffer then ethyl acetate was added prior to the liquid-liquid extraction. In method B, PBS buffer was used as the homogenate buffer. 500 µL of acidified acetonitrile (acetonitrile with 0.2% formic acid) was added for further protein precipitation. The samples were placed in -20ºC freezer for 5 min before centrifugation. The supernatant layer was dried and reconstituted prior to injection. In method C, acidified methanol was used as the protein precipitation agent. Other steps were the same as that in method C. In method D, PBS buffer was used as the homogenate buffer and ethyl acetate was added for extraction. Among these four methods, the measured extraction efficiency was the highest in method D. Due to the simple and efficient procedure of method D, it was applied for tissue preparations in this study. One limitation of our study is that the stability of 5,7-DMF was only evaluated in plasma. The stability of 5,7-DMF in mouse tissues was assumed to follow the same pattern as tested in mouse plasma in the previous report [20]. Further stability tests of 5,7-DMF in mouse tissues are warranted.

3.2. Pharmacokinetic analysis and tissue distribution in mice

The NCA result in Table 2 showed that the maximal plasma concentration was 1870 ± 1190 ng/mL. The terminal half-life $t_{1/2}$ was 3.40 ± 2.80 hr. The volume of distribution by the area method V_z_F was 90.1 ± 62.0 L/kg. Clearance (CL_F) was 20.2 ± 7.49 L/hr/kg. The drug exposure AUC_t and AUC_{inf} was 532 ± 165 and 537 ± 167 hr*ng/mL, respectively.

The concentration-time profiles of plasma, liver, kidney, gut and brain were presented in Fig 2. Through the oral gavage, the C_{max} of 5,7-DMF was quickly reached among plasma and tissues within 0.5 hr after dosing due to fast absorption, with mean T_{max} ranging from 0.14 to 0.36 hr. The semi-log scale plots indicated a bi-exponential disposition pattern of 5,7-DMF. The plasma concentration after T_{max} dropped drastically due to fast and vast distribution into

tissues and then it followed a long terminal phase probably because of the contribution of the 5,7-DMF in the tissue back to plasma. The analyte was detectable up to 21 hr (the last time point) in mouse plasma, gut, liver, kidney, spleen and adipose. The time-course of mouse liver, kidney, gut, spleen and adipose followed the same pattern as that of mouse plasma. As shown in Fig 2, the 5,7-DMF concentration in mouse tissues were much higher than that in mouse plasma. Except for muscle and adipose, other tissues had the C_{max} higher than that in the plasma, ranging from 1.75- to 9.96-fold. The highest tissue C_{max} was in mouse gut, followed by liver, brain, kidney, heart, spleen, lung, muscle and adipose. As indicated by the NCA analysis results listed in Table 3, AUC_t of gut, liver, kidney, brain and spleen were 6850 ± 1360, 4640 ± 518, 2100 ± 667, 1520 ± 554 and 1250 ± 82 hr*ng/g, respectively. Thus, 5,7-DMF exposure in these tissues were 2.35- to 12.9-fold higher than that of mouse plasma. It is shown that 5,7-DMF through oral dose was most abundant in gut, followed by liver, kidney, brain, spleen, heart, lung, adipose and lastly, muscle..

The calculated Kp values for major tissues were listed in Table 3. Kp values for intestine, liver, kidney, brain, spleen, heart, lung, fat and muscle were 12.9, 8.71, 3.94, 2.86, 2.35, 2.31, 1.47, 0.99 and 0.65, respectively. They will be further evaluated in our future investigation on the physiologically based PK modeling of 5,7-DMF.

4. Discussion

The pharmacokinetic parameters of 5,7-DMF after 10 mg/kg oral dose in mice were determined in this study. Based on the previous literature, 10 and 30 mg /kg equivalent 5,7-DMF oral doses were proven safe in rats [16]. In addition to 10 mg/kg oral dose, we also evaluated 10, 25 and 50 mg/kg intravenous dose in our pilot study (data not shown) and no toxicity was observed at any dose tested, indicating the favorable safety profile of 5,7-DMF. In addition, our pilot study (data not shown) had demonstrated the linear relationship

between dose and concentration on 10, 25 and 50 mg/kg intravenous dose of 5,7-DMF. Therefore the C_{max} and AUC can be reasonably estimated within that dosing range.

It has been reported that the IC_{50} value of 5,7-DMF for BCRP inhibition in vitro is 1.41 μM and at this concentration 5,7-DMF can significantly increase the intracellular accumulation of mitoxantrone in the BCRP-overexpressing cells [13, 14]. Our result revealed that under 10 mg/kg oral dose, 5,7-DMF could easily reach the concentration of 1.41 μM (equivalent to 398 ng/mL) in plasma and various tissues. Based on our results, it is reasonably to speculate that the BCRP inhibitory effect of 5,7-DMF observed in vitro can be extrapolated to in vivo. Similarly, 5,7-DMF was reported to increase the doxorubicin accumulation into P-gp overexpressed cell-line in a concentration-dependent manner within 1-300 μM (282-84600 ng/mL) [1], and this in vitro P-gp inhibitor effect should be able to be translated to in vivo as well since our PK data showed that the C_{max} of 5,7-DMF in plasma and different tissues range from 1870 to 18600 ng/ml (Tables 2 and 3).

Terminal half-life of 5,7-DMF calculated in plasma was 3.40 ± 2.80 hr, so it might take 17 hr (5 half-lives) to clear 5,7-DMF completely (>95%). T_{max} for mouse gut, plasma, liver and kidney were 0.083 ± 0.00, 0.14 ± 0.10, 0.14 ± 0.10 and 0.19 ± 0.10 hr, respectively, indicating rapid distribution of the 5,7-DMF from plasma to tissues [17]. The peak concentration in major tissues such as liver, brain, kidney, heart, and lung were higher than plasma peak concentration, with the C_{max} ratio to plasma of 5.86, 3.88, 3.85, 2.67 and 1.62, respectively. The high tissue accumulation was also in accordance with Walle's paper, in which maximal liver concentration of 5,7-DMF was 7-fold higher than that of plasma level and the peak lung and kidney 5,7-DMF were almost 2-fold higher than plasma concentration [17].

The large value of volume of distribution indicated extensive accumulation in tissues, which was consistent with observations in Mekjaruskul's and Walle's studies [16, 17]. This may indicate tissues as the favorable sites for 5,7-DMF to exert the greatest pharmacological action. Among different studies and different species, 5,7-DMF may have different tissue distribution profile. In our study, we found that in mice, the compound after oral dosing was most concentrated in gut > liver > spleen > kidney > brain > heart > lung> adipose > plasma > muscle. And Walle's paper reported 5,7-DMF most accumulated in rats liver > lung > kidney > plasma [17] whereas Mekjaruskul's paper reported rats concentration liver > plasma > kidney > lung [16]. 5,7-DMF in killifish was reported to have highest concentration in liver > brain > gut > gill > skin [18]. But in general, liver and kidney had higher 5,7-DMF than other tissues and plasma probably because they are well-perfused organs. It is noteworthy that liver was the major tissue for 5,7-DMF accumulation. 2-20 μM (564-5640 ng/mL) of 5,7-DMF was achievable through this 10 mg/kg oral dose which was reported to inhibit benzo[a]pyrene-induced DNA binding in liver [7]. Also, 2.5-10 μM (705-2820 ng/mL) of 5,7-DMF was achievable in vivo to chemosensitize Trail-induced apoptosis in liver carcinoma cells [10]. Interestingly, 5,7-DMF could penetrate blood-brain barrier (BBB) and blood-testicular barrier, which was demonstrated by our study in mouse brains and also Mekjaruskul's study in rat brains and testicles [16]. This may imply promising medical use of 5,7-DMF for brain and testicular cancer and Alzheimer's diseases [6].

5. Conclusions

In summary, this study described the PK of 5,7-DMF in mice following 10 mg/kg oral dose for the first time. It established the tissue distribution profile and PK parameters in vivo and provided further basis for physiologically based PK modeling, drug-drug interaction studies, dosing regimen and prediction in clinical trials in the future.

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Figure Captions

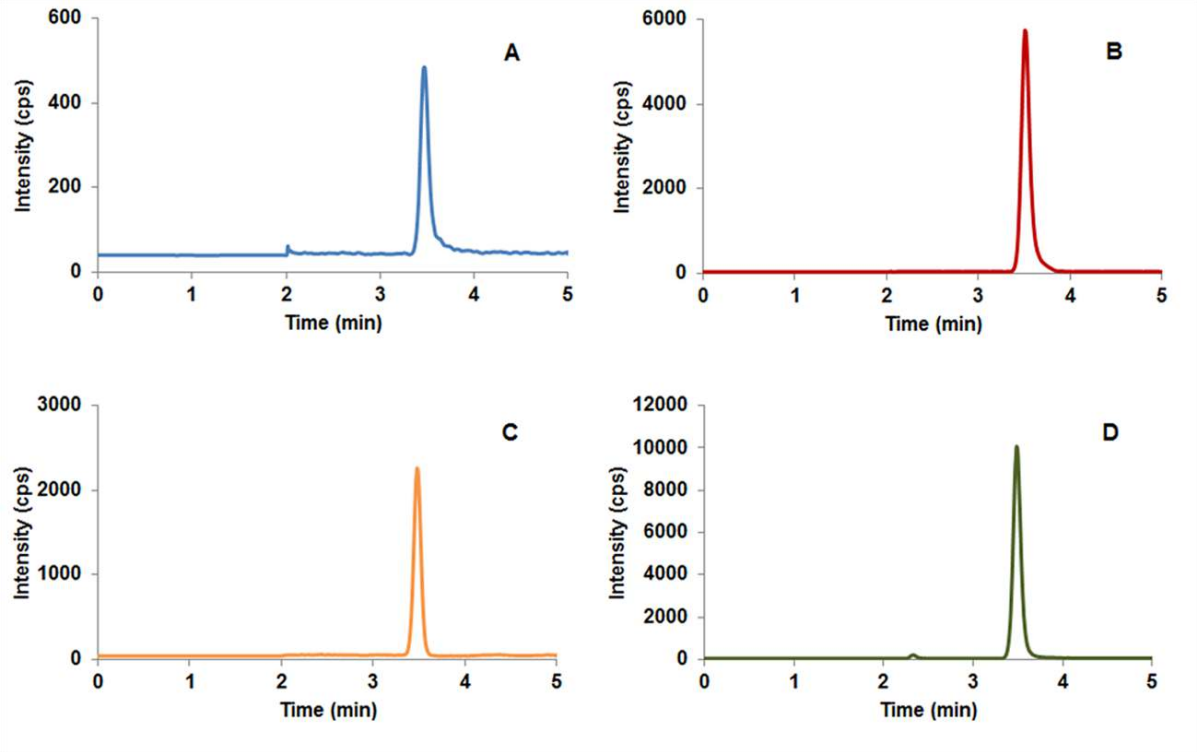

Figure 1. XIC of the 5,7-DMF. A, B, C, D, E, F, G, H, I and J represented the XIC of 2 ng/mL 5,7-DMF in mouse plasma, liver, kidney, intestine, spleen, brain, heart, lung, muscle and fat, respectively. .

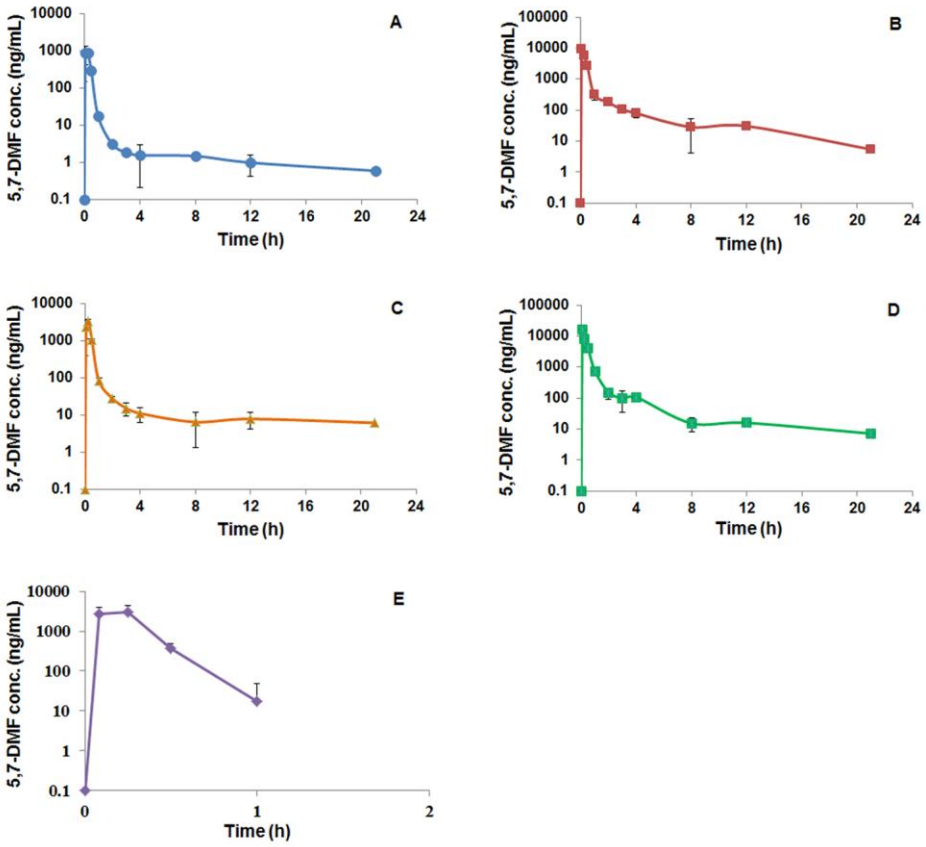

Figure 2. Concentration-time profiles of 5,7-DMF in mice following oral administration of 10 mg/kg 5,7-DMF (N=3). A, B, C, D and E represented the pharmacokinetic profile of 5,7-DMF in mouse plasma, liver, kidney, small intestine and brain.

Tables

Table 1. Method validations of 5,7-DMF in mouse plasma and tissues (N=3).

Tissue	Conc. (ng/mL)	Mean conc. (ng/mL)	Accuracy (%)	CV (%)
Plasma	2	2.18	109	3.54
	500	552	110	0.92
Liver	2	1.84	92.2	6.17
	500	525	105	1.50
Kidney	2	2.18	109	3.30
	500	536	107	1.58
Brain	2	1.96	98.1	1.70
	500	528	106	5.10
Intestin e	2	1.95	97.5	12.3
	500	506	101	2.37
Spleen	2	1.92	95.8	3.10
	500	460	92.0	*
Heart	2	1.97	98.5	6.59
	500	552	111	2.75
Lung	2	2.13	107	19.6
	500	555	111	2.28
Muscle	2	2.29	115	3.05
	500	554	111	2.79
Fat	2	4.93	98.7	1.56
	500	496	99.1	2.06

(*denotes one determination)

Table 2. Non-compartmental parameters of 5,7-DMF in mouse plasma after oral administration of 10 mg/kg 5,7-DMF (N=3). The data were presented as mean and standard deviation.

Parameter	Estimate
λ_z (1/hr)	0.30 ± 0.17
C_{max} (ng/mL)	1870 ± 1190
T_{max} (hr)	0.14 ± 0.10
AUC_t (hr*ng/mL)	532 ± 165
AUC_{inf} (hr*ng/mL)	537 ± 167
$T_{1/2}$ (hr)	3.40 ± 2.80
CL_F (L/hr/kg)	20.2 ± 7.49
V_z_F (L/kg)	90.1 ± 62.0

Table 3. PK parameters of 5,7-DMF in mouse tissues after oral administration of 10 mg/kg 5,7-DMF (N=3). The data were presented as mean and standard deviation.

Parameter	C_{max} (ng/g)	AUC_t (hr*ng/g)	Kp
Intestine	18600 ± 4290	6850 ± 1360	12.9
Liver	10900 ± 3140	4640 ± 518	8.71
Kidney	7200 ± 5820	2100 ± 667	3.94
Brain	7260 ± 5740	1520 ± 554	2.86
Spleen	3260 ± 2200	1250 ± 82	2.35
Heart	4990 ± 4220	1230 ± 355	2.31
Lung	3030 ± 1450	781 ± 148	1.47
Fat	558 ± 290	529 ± 293	0.99
Muscle	1180 ± 739	345 ± 110	0.65