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Pharmacokinetics study of ferulic acid in rats after oral administration of γ-oryzanol under combined use of Tween 80 by LC/MS/MS

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Abstract. – AIM: γ-oryzanol (OZ) is a rich source of commercially-important bioactive phytochemicals, most of them of interest in nutrition, pharmacy and cosmetics. However, the poor solubility of OZ limited the use. In the paper, ultraviolet-visible (UV-Vis) analysis was conducted to analysis the solubilization of OZ under combined use of Tween 80 *in vitro*. In addition, to further confirm the solubilizing effect of Tween 80, a pharmacokinetic study of ferulic acid (FA) in rats after oral administration of OZ 100 mg/kg under combined use of Tween 80 though LCMS/MS was carried out.

MATERIALS AND METHODS AND RESULTS: Solubility enhancement as high as 100-fold is achieved using 1% Tween 80 *in vitro*. Following oral administration of OZ-Tween 80 100 mg/kg, the values of T_{max}, C_{max}, $AUC_{0-\infty}$, $T_{1/2}Ka$ and $MRT_{0-\infty}$ were 46.667 ± 39.328, 129.498 ± 27.025, 63738.28 ± 599, 14.274 ± 7.309 and 859.592 ± 108.780 respectively. The values of $T_{1/2}Ka$, $AUC_{0-\infty}$, MRT_{0-t}, and T_{max} showed up to increase 16%, 58%, 44% and 47% while C_{max} and CL/F decreased 22% and 12%, respectively. The decreased C_{max} value indicated that Tween 80 can hardly enhance the absorption of FA in rats. However, $T_{1/2}Ka$ and T_{max} values showed that the absorption of FA was extended, which resulted the increased values of $AUC_{0-\infty}$ and $MRT_{0-\infty}$.

CONCLUSIONS: Our results reveal that Tween 80 improves solubility of OZ *in vitro* and could enhance the bioavailability of OZ by extending its absorption and elimination.

Key Words:
γ-oryzanol, Solubilization, Tween 80, Pharmacokinetics

Introduction

γ-oryzanol (OZ) is a mixed ester of ferulic acid and phytosterols. It was considered that 24-methylenecycloartanyl ferulate and cycloartenyl ferulate (Figure 1) were the main components among the consisted abundant phytosteryl ferulates, which make up 80% of OZ in rice bran oil[1]. Many researches have been revealed the bioactive activities of OZ, including anti-diabetes mellitus[2], anti-hyperlipidemia[3], anti-oxidant[4], anti-cancer[5] and health properties[6]. In the recent years, it was applied to the industrial use, such as stabilization of fats, frying oils and fried products; a sunscreen in cosmetic formulations because it absorbs UV radiation. And most importantly, in the animal or human studies, it shows the potential to be developed into an effective compound to prevent and cure cardiovascular diseases. However, OZ is insoluble in water[7] and leads to a poor bioavailability for its bad absorption in intestine, which limited its application in food and medical systems. Normally, the solubility limitations could be partially overcome by applying surfactants, cosolvents or new technology. Since OZ is a complex mixture component, there are little relative reports about the pharmacokinetic of OZ *in vivo*. In 2003, Gillespie[8] studied metabolic aspects of oryzanol in rats, but no oryzanol was detected in the serum from rats fed with RO by GCMC analysis or the serum from the human pilot study by HPLC analysis. However, in 1980s, Fujiwara et al took a series studies about OZ, including the metabolism of OZ in rabbit and absorption and metabolism of OZ in rats[9-10]. They found that ferulic acid (FA) might be a good indicator for estimating the absorption of OZ and they successfully determined FA in plasma after oral administration of OZ by a mass fragmentographic (MF)[11] and high-performance liquid chromatography (HPLC)[12], indicating FA, not intact oryzanol, was the main metabolite of OZ. In the present study, we investigate the solubilization behavior of a widely used surfactant, Tween 80 *in vitro*, and then apply it to a pharmacokinetics study in rats with a high-efficient high-performance liquid chromatography coupled with tandem mass spectrometry method (LC/MS/MS).

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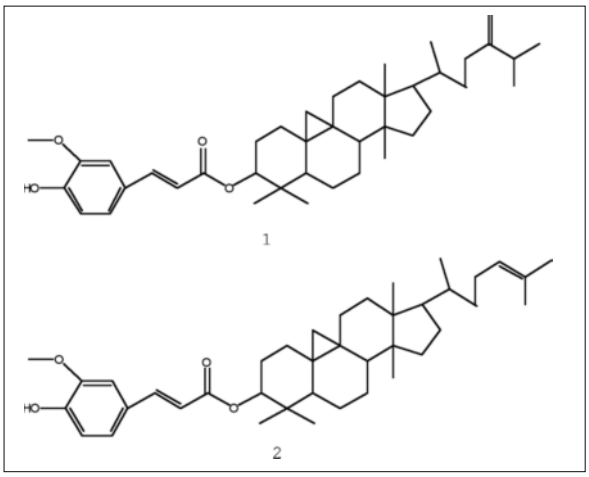

Figure 1. The chemical structures of the major components of oryzanol, compound. **1.** 24-Methylen-cycloartanylferulate and compound. **2.** Cycloartenyl ferulate.

Materials and Methods

Materials

γ-oryzanol (Tokyo, Japan), ferulic acid (FA) was purchased from Beijing Reagent Chemical Company (Guangzhou, China), 3-(4-hydroxyphenyl)-propionic acid (internal standard, I.S.) was obtained from Sigma Alanch. Acetonitrile (HPLC grade) was supplied by Fisher Scientific Products (Fair Lawn, NJ, USA). Annmonium formate was supplied by Agilent Tehcnologies. Others (chemicals, solvents and reagents) were analytical grade.

Instrument and Chromatographic Conditions

The *in vitro* analysis was performed on ultraviolet-visible (UV-Vis) specrophotometer (PE Lambda 35, Perkin-Elmer Corporation, Westbury, NY, USA). The pharmacokinetic experiment was performed on an Agilent Technologies 6460 Triple Quad LC/MS system (Agilent Technologies, Waldbronn, Germany) with an Agilent ZORAX SB-C18 HPLC column (3.5 μm, 150 mm × 2.1 mm). The mobile phase consisted of acetonitrile (A) and 0.1% ammonium acetate-water (B). The column temperature was kept at 30°C with a flow rate of 0.2 ml/min during the whole study. The setting (negative ion mode) of quadrupole mass spectrometer coupled with an electrospray ionization source were as following: 10 L/min for N_2 flow, 30 psi for nebulizer pressure, 330°C for drying gas temperature and 4.5 kV for capillary voltage. 80 V and 110 V were the fragmentor voltage of FA and I.S, respectively. LC/MS/MS was executed in multiple-ion monitoring (MRM, negative) mode applying target ions at m/z 193-> 121 for FA and 165.05-> 121 for the I.S. Figure 3 (A and B) showed the typical product ion spectrum of FA and I.S.

In vitro Study

To evaluate the solubilization behavior of OZ under combined use of Tween 80, we stimulated the *in vivo* situation of stomach and intestines by 0.1 mol/L HCl and phosphate buffer solution (PBS, pH = 6.8), respectively. About 25 mg of OZ was dissolved with 10 ml 0.1 mol/l HCl or PBS that containing 1%, 0.5%, 0.2%, 0.1%, 0.01% and 0% Tween 80. After ultrasonic dissolving for 30 min, the solutions were filtered and tested on UV-Vis. Place cuvette into UV-Vis spectrophotometer and take a background scan (280-400 nm) using 0.1 mol/l HCl or PBS containing different concentrations of Tween 80 as background. Then measure optical absorbance at 328.96 nm. The solubilizing effect of Tween 80 is determined by the absorbance (A).

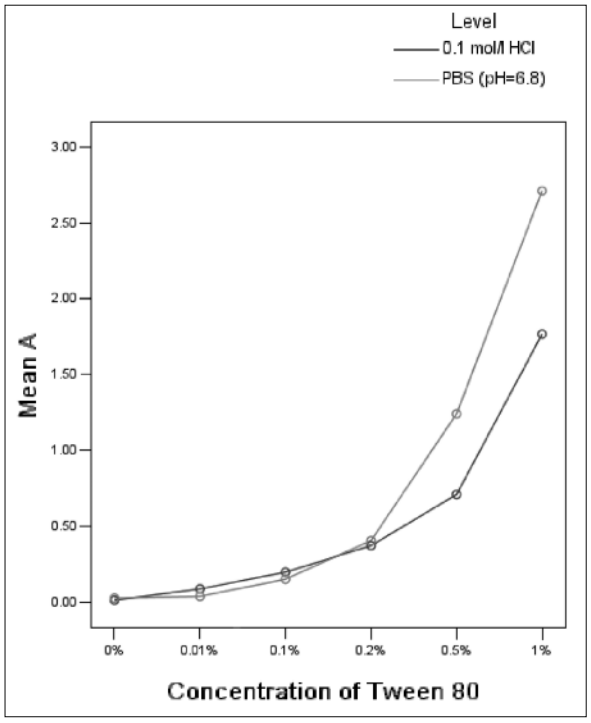

Figure 2. The solubility of OZ in the 0.1 mol/l HCl and PBS (phosphate buffered saline: pH=6.8) with various Tween 80 concentrations. The absorbances of OZ in 328.96 nm were increased with the concentration of Tween 80 increased.

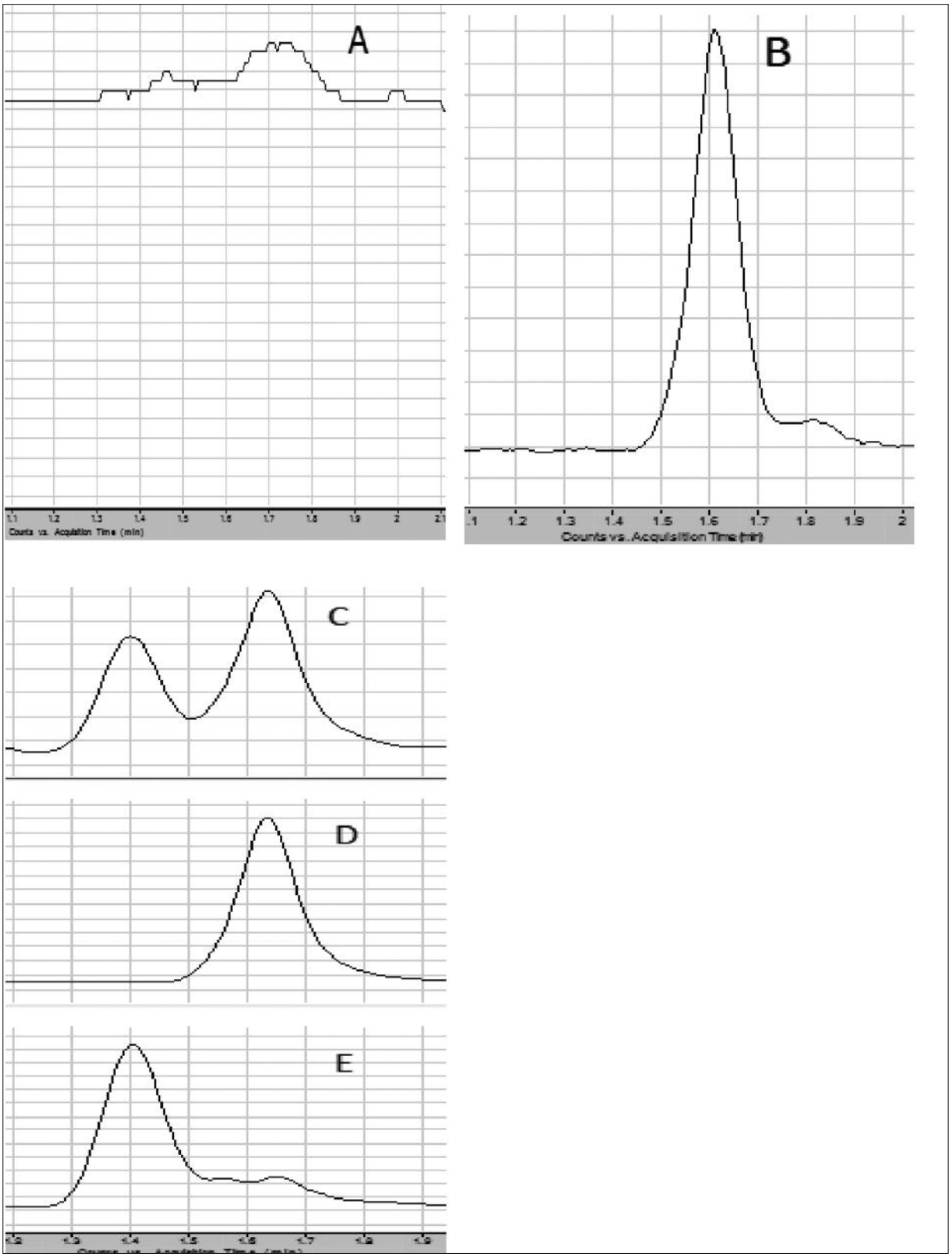

Figure 3. Typical LC/MS/MS chromatograms of blank plasma ***(A)***, FA solution (111.2 ng/ml, ***(B)***, blank plasma spiked with FA (111.2 ng/ml) and I.S. (non-extracted chromatogram, ***(C)***. Extracted chromatogram of FA ***(D)*** and I.S. ***(E)*** from E. I.S. (3-(4-hydroxyphenyl)-propionic acid) was added at the beginning of the sample preparation (41.36 ng/ml).

In vivo Study

To further confirm the solubilizing effect of Tween 80 on OZ, we conducted a LC/MS/MS method to analysis the pharmacokinetics of OZ that suspended in 0.5% CMC-Na solution with or without 1% TW80.

Stock Solutions and Quality Control Sample Preparation

FA (444.8 μg/ml) and I.S. (1034 μg/ml) were prepared with acetonitrile and kept at 4°C as stock solution. The working solution of FA with concentrations of 3.475, 6.95, 13.9, 27.8, 55.6, 111.2, 222.4 and 444.8 ng/ml, were diluted with acetonitrile from the FA stock solution serially. The I.S. solution of 413.6 ng/ml was prepared with acetonitrile as well.

The calibration samples were prepared as follows: 100 μl of blank rat plasma was spiked with 100 μl of the serial working solutions (dried under N_2 stream) to yield the following concentrations: 3.475-444.8 ng/ml. Quality control (QC) samples were prepared as the method mentioned above with concentrations of 8.0064, 80.064 and 400.32 ng/ml.

Sample Preparations

A liquid-liquid extraction technique was employed in the pretreatment of plasma samples. Plasma samples were removed from –80°C and kept in 4°C for 20 min and then immersed at room temperature to thaw. After vortexing, a 100 μl aliquot of rat plasma sample was spiked with 10 μl of the I.S. working solution. Then 50 μl of 1 mol/l HCl and 0.5 ml ethyl acetate was added and then vortex-mixed for 2 min. Following centrifugation at 12000 rpm/min for 10 min, the upper organic phase was moved to another Eppendorf tube and evaporated under a stream of N_2 to dryness (bath water, 40°C). A 2 μl aliquot after resolved the residue with 100 μl of mobile phase (70% A and 30% B) was injected into the LC/MS/MS system for study.

Application to Pharmacokinetic Study

Twelve Sprague-Dawley rats (female and male are half and half), weighing 250-320 g, were supplied by South Medical University (Guangzhou, China). The animals acclimatized to the situation for one week by maintaining them in a conditioned quarter with an air (keep the temperature at 24 ± 2°C), fed with laboratory rodent chow and were allowed to get water freely. All experimental protocols have got clearance from Institutional Animal Ethics Committee of Southern Medical University. OZ suspended in 0.5% CMC-Na solution with or without 1% TW80 was oral administered to the rats (100 mg/kg). The rats were fasted, free access to water for 12 h prior to taking the blood samples. Blood samples (400 μl) were obtained from the rat eye socket vein before dosing and subsequently following oral administration for 2, 5, 10, 20, 30, 60, 120, 180, 240, 360, 480 and 720 min. Bloods were contained in a heparinized Eppendorf tube and centrifuged at 4000 rpm/min for 10 min, the obtained plasma were then transferred to a new Eppendorf tube and frozen at –80°C until use.

Statistical Analysis

To determine the pharmacokinetic action of OZ, the parameters of its metabolite FA were analyzed by Drug Analysis System (DAS, Version 2.1.1, Center of Clinical Pharmacy, Zhujiang Hospital, South Medical University). All results were showed as mean ± standard deviation (SD).

Results

Tween 80 Improve the Solubility of OZ Linearly in vitro

As shown in Figure 2, the solubility of OZ was greatly increased with increasing concentration of Tween 80 from 0%-1%. When containing 1% Tween 80 in 0.1 mol/l HCl solution, the absorbance of OZ at 328.96 nm was 100 times higher than the solution without Tween 80 (A=1.1767 vs A=0.012). And we observed the same result in the group of phosphate buffer solutions. The absorbances were 0.026 and 2.711 when dissolved in phosphate buffer solutions containing 0% and 1% Tween 80. The absorbances were linearly related to the concentration of Tween 80, suggesting that Tween 80 improve the solubility of OZ greatly.

Method Validation of Pharmacokinetic Study

The method was validated according to the USA Food and Drug Administration (FDA) bioanalytical method validation guidance[13].

Selectivity

The selectivity was analyzed by evaluating the blank plasma obtained from six individual rats. As shown in the figure, the chromatograms of FA and IS were overlap with each other (the retention times

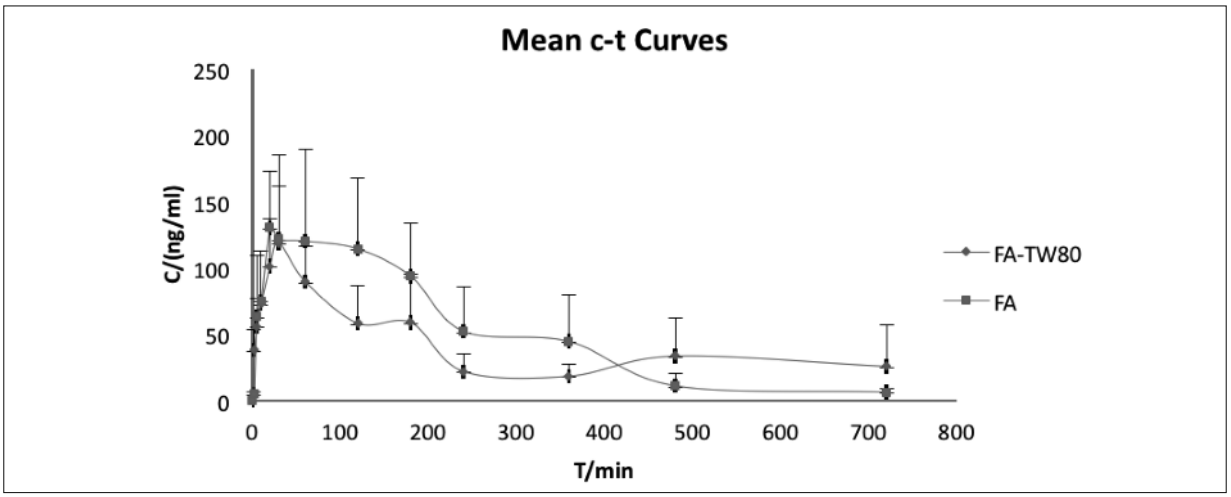

Figure 4. Mean plasma concentration-time profile of FA in six rats after oral administration of 100 mg/kg OZ dissolved in 0.5% CMC-Na solution with or without 1% Tween 80.

of FA and I.S. were 1.616 and 1.638 min, respectively), so an included extraction process was used to eliminate the mutual influence between FA and I.S., and make the results more reliable (Figure 3).

Linearity and Lower Limit of Quantization

The contents of FA in the unknown blood samples were calculated by the accompanying calibration curves in each analytical run. Calibration curves of seven analyses were prepared with fresh diluted standards and were validated after analyzed by the included quantitative analysis software (accuracy range from 85% to 115% and $R^2 > 0.99$). The calibration plasma samples were performed by spiking standard and I.S. solutions with blank rat plasma to get a final concentration of 3.475-444.8 ng/ml for FA, which showed a good linearity ($R^2 > 0.99$). To determine the lower limit of qualification (LLOQ), blank plasma samples were spiked to 3.475 ng/ml FA (n = 5, respectively) and were considered as validated if the signal-to-noise ratio (S/N) = 3.

Precision and Accuracy

QC samples at three different concentrations were analyzed on the same day (the intra-day precision and accuracy) and on three consecutive days (the inter-day precision and accuracy) with five replicate analyses. Correspondingly, the accuracy and precision was expressed as relative standard deviation (R.S.D. %). The results showed that the R.S.D. % was less than 15%, demonstrating the acceptability of the assay (Table I).

Extraction Recovery

The extraction efficiency of the liquid-liquid extraction technique was evaluated as the absolute extraction recovery of QC samples (replicated for five times at each concentration). The recovery was determined by comparing the analytes/I.S. peak area ratio of extracted QC samples to that of samples performed by spiking analyte and I.S. with extracted-blank plasma (n=5 for each level). Recovery of I.S. was also evaluated by the same way at concentration of 41.36 ng/ml

Table I. Precision and accuracy of the determination of FA in rat plasma (inter-day, n = 5; intra-day, n = 5*3)

	Inter-day		Intra-day	
C (ng/ml)	Detected C (ng/ml)	RSD (%)	Detected C (ng/ml)	RSD (%)
8.064	7.80 ± 1.15	14.81	8.16 ± 0.79	9.85
80.064	81.19 ± 8.31	10.24	79.91 ± 2.94	3.69
400.32	341.35 ± 7.63	2.24	402.26 ± 29.65	7.28

Table II. Extraction recovery results for FA and IS.

Sample	C (ng/ml)	Recovery (%)	RSD (%)
FA	8.064	75.57 + 5.76	7.63
	80.064	89.20 ± 16.85	18.88
	400.32	79.86 + 8.45	10.58
I.S.	41.36	75.57 + 5.76	14.26

(n=5). The extraction recovery of the analytes was shown to be consistent and reproducible (Table II).

Matrix Effect

The matrix effect (ME) was examined by comparing the analytes/IS peak area ratios of anayltes that were reconstructed in mobile phase (S1) to analytes that were resolved in the extracted blank plasma samples from five different individual rats (S2): ME (%) = S2/S1 × 100. The ME of the assay was evaluated by QC solutions and five replicates for each concentration were analyzed. The results in Table III showed that the ME values were within the range of 85-115%, indicating that no matrix effect was observed in this assay.

Stability

The stability of FA, such as the freeze and thaw stability, short-tern temperature stability, post preparative stability as well as stock solution stability, was determined by storing QC samples at different conditions by analyzing three different concentrations of QC samples (n = 5). Results were showed in Table IV.

Pharmacokinetics in Rats Following Oral Administration OZ and OZ-Tween 80

OZ is insoluble in water and we applied 0.5% CMC-Na with or without 1% Tween 80 to make it a suspension for oral administration.

The method described above was used to the study the pharmacokinetic of OZ successfully. The concentration of FA in plasma was determined after administration of OZ or OZ couple with TW80 orally. Following oral administration of single OZ, it reached a maximal at 31.667 ± 14.72 min (T_{max}) with similar rapid distribution kinetics for FA ($t_{1/2}\alpha$ = 102.345 ± 92.584 min) and fast clearance (CL/F = 3.15 ± 2.23 L/min/kg). The value of total FA present as the metabolic form was 166.93 ± 54.698 (n = 6) at $AYX_{0-\infty}$ and was 40420.599 ± 15773.439 ng/ml·min. By contrast, when co-administered on rats with 1% TW80, the maximum plasma concentration, T_{max}, prolonged by 47% (T_{max} = 46.667 ± 39.328 min) with corresponding slower distribution kinetics ($t_{1/2}\alpha$ = 239.826 ± 32.167 min), following a slower elimination phases for FA ($t_{1/2}\beta$ = 239.981 ± 32.187 min) and slower clearance (CL/F = 2.777 ± 1.553 L/min/kg). The $AUC_{0-\infty}$ contributed by oral administration of OZ-TW80 was increased by 58%. However, no discernable enhance effect on absorption of FA was observed. The mean FA level present at the peak concentration, C_{max}, was decreased by 22% (Table V, Figure 4).

Discussion

γ-oryzanol (OZ), extracted from rice bran, mainly consisted esters of ferulic acid with sterols and triterpenic alcohols. Other than its remarkable antioxidant activity, recent researches has been revealed that OZ regulated lipid metabolism *in vivo* and *in vitro*. However, to utilize the benefits of OZ as a functional food ingredient and as a stabilizer of lipidic raw materials, we should focus on the insolubility limitation of OZ. Generally, surfactants, co-solvents or new technology are obtained to overcome the limitation. In the present paper, we choose some surfactants and co-solvents, such as sodium dodecyl sulfate (SDS), TW80, poly (ethylene glycol) 400 (PEG 400), glycol to be the appropriate solubilizer for OZ. Finally, we found that TW80 greatly solubilize OZ in a concentration-dependent manner within those

Table III. Matrix effect evaluation of FA in rat plasma (n = 5)

Sample	C (ng/ml)	Mean area (mean ± SD)		Absolute matrix (%)	RSD (%)
		Set 1	Set 2		
FA	8.064	117.8 ± 14.17	117.6 ± 24.60	99.40 ± 15.74	15.83
	80.064	1039.4 ± 47.66	948.6 ± 86.80	91.47 ± 9.74	10.64
	400.32	5333.4 ± 900.50	4872.6 ± 446.48	93.55 ± 18.05	19.29

Table IV. Stability of FA in rat plasma at different conditions (n = 5).

	Accuracy (mean ± SD, %)		
	8.064 ng/ml	80.064 ng/ml	400.32 ng/ml
Short-term stability (room temperature, 6h)	112.51 ± 7.23	107.73 ± 7.07	110.44 ± 2.19
Freeze and thaw stability (–80°C room temperature, 3 cycles)	92.45 ± 11.22	107.03 ± 13.81	88.69 ± 16.02
Long-term stability (–80°C, 5 days)	92.00 ± 6.92	93.22 ± 9.17	103.89 ± 4.45
Post-preparative stability (4°C, 7 h)	89.25 ± 3.09	92.84 ± 7.96	91.70 ± 4.13
Solution stability (4°C, 24 h)	109.89 ± 8.37	82.05 ± 1.34	91.41 ± 4.12

mentioned solubilizer *in vitro*. Therefore, we choose 1% TW80 to be the solubilizer and applied it to pharmacokinetic study in rats to confirm the solubilized effect of TW80 on OZ. And we developed LC/MS/MS, a suitable but sensitive and selective method to determine FA in rat plasma due to the poor absorption of OZ.

Generally, protein precipitation, liquid-liquid extraction and solid phase extraction are common ways applied in the plasma sample preparation. Protein precipitation is simple but we found the recovery is not satisfied and solid phase extraction is expensive. Herein, liquid-liquid could purify and concentrate the sample and ethyl acetate was taken as the extraction reagent. According to our pre-research, we found that the acidified condition is good for yielding the higher recovery for FA, because acid could help to extracted the intact FA from protein and purify the samples.

FA is an acid compound, and a negative ion-monitoring mode was more sensitive than a positive ion-monitoring mode in the LC/MS/MS assay. In selecting target ion of FA and I.S., different fragmentor voltages under scan monitoring were analyzed. Results revealed that, the highest sensitivity was observed under a fragmentor voltage of 80 V at M^- m/z 193 ->134 and 110 V at M^- m/z 165.05 ->121 for FA and I.S. respectively. Therefore, the negative ion m/z 193->134 and m/z 165.05 ->121 were chosen as the target ion in the MRM for FA and I.S., respectively.

Using the developed methods, we first studied the pharmacokinetics of OZ by detecting its metabolism FA by giving a pure OZ with or without TW80 to Sprague-Dawley rats. We found that the disposition process of OZ (FA) in rats could be described by open two-compartment model. Oral administration of OZ and OZ-TW80 to adult SD mice produced plasma of FA that was low (about 167 and 130 ng/ml, respectively) and transitory (about 31 min and 46 min, respectively). The high C_{max} and AUC in the groups demonstrate a high bioavailability of FA. Unfortunately, TW80 disables to interference FA absorption by comparing C_{max}, though it solubilizes OZ greatly *in vitro*. However, from the values of $t_{1/2}\alpha$, $T_{1/2}Ka$, FA in the group without TW80 is a little faster absorption and distribution of FA in rats. In addition, the values of MRT, CL/F and $t_{1/2}\beta$ shows the postpone elimination of FA in rats. From this view of point, TW80 solubilizes OZ without a significant promotion for its absorption, but can prolong its absorption and distribution. This discrepancy should be due to the solubilization of OZ combined use with TW80.

Table V. Main pharmacokinetic parameters FA in rat plasma after oral administration of 100 mg/kg OZ with or without 1% Tween 80 (n=6, mean ± SD).

	FA	FA-Tween 80
T_{max} (min)	31.667 ± 14.72	46.667 ± 39.328
C_{max} (ng/ml)	166.93 ± 54.698	129.498 ± 27.025
AUC_{0-t} (ng/ml*min)	38 941.594 ± 14976.523	30 227.835 ± 8 989.851
$AUC_{0-\infty}$ (ng/ml*min)	40 420.599 ± 15773.439	63 738.28 ± 599
$t_{1/2}\alpha$ (min)	102.345 ± 92.584	239.826 ± 32.167
$t_{1/2}\beta$ (min)	197.739 ± 54.835	239.981 ± 32.187
$T_{1/2}Ka$ (min)	12.357 ± 11.799	14.274 ± 7.309
MRT_{0-t} (min)	190.634 ± 23.543	273.775 ± 95.484
$MRT_{0-\infty}$ (min)	199.398 ± 30.119	859.592 ± 108.780
CL/F (L/min/kg)	3.15 ± 2.23	2.777 ± 1.553

Conclusions

The present study reveals a solubilization of OZ under combined use with Tween 80 *in vitro*. Furthermore, to confirm the effect of Tween 80 on OZ, we carry out a rapid and sensitive LC/MS/MS method to study the pharmacokinetic of OZ (FA) in rats. The findings indicate that Tween 80 may be an ideal solubilizer for OZ for its ability in prolonging OZ (FA) absorption and elimination in rats.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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