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Short communication

# Determination of *Z*-3,5,4′-trimethoxystilbene in rat plasma by a simple HPLC method: Application in a pre-clinical pharmacokinetic study

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ABSTRACT

A simple HPLC method had been developed and validated to quantify *Z*-3,5,4′-trimethoxystilbene (*Z*-TMS), a phyto-stilbene with potent anti-cancer activities in rat plasma. Chromatographic separation was achieved on a reversed phase-HPLC column, which was protected by a guard column through a 13.5-min gradient delivery of a mixture of acetonitrile and water at a flow rate of 1.5 ml/min at 50 °C. The UV absorbance at 300 nm was recorded. *Z*-TMS and *E*-stilbene (internal standard) eluted at 8.8 and 9.3 min, respectively. The calibration curve was linear within the range of 33–2500 ng/ml ($R^2 > 0.9995$) and 10 ng/ml was the lower limit of detection. The intra- and inter-day precisions were good and the relative standard deviation was all lower than 10%. The analytical recovery of *Z*-TMS in plasma ranged from 94.6 ± 9.1% to 97.0 ± 2.1%. This HPLC method was successfully applied to assess the pharmacokinetic profile of *Z*-TMS in Sprague–Dawley rats using hydroxypropyl-β-cyclodextrin (HP-β-CyD) as a dosing vehicle. Although *Z*-TMS displayed negligible oral bioavailability, it had a fairly long terminal elimination half-life, abundant plasma drug exposure and limited clearance following intravenous administration. As *Z*-TMS had favorable intravenous pharmacokinetic profile, further investigation on its potential as a cancer chemotherapeutic agent is warranted.



## 1. Introduction

Resveratrol (*E*-3,5,4′-trihydroxystilbene, Fig. 1, I) is a dietary polyphenol that possesses numerous pharmacological activities such as anti-ageing, anti-diabetes, anti-inflammation, anti-obesity, anti-oxidation, cancer chemoprevention, cardio- and neuro-protection [1]. Due to its inferior pharmacokinetic profile including short half-life [2,3], extensive metabolism (glucuronide or sulphate conjugation) [4,5] and low bioavailability [4,6], resveratrol does not appear to be a drug-like molecule. Therefore, it is of interest to identify resveratrol analogs with superior potency and better pharmacokinetic characteristics.

*Z*-3,5,4′-Trimethoxystilbene (*Z*-TMS) (Fig. 1, II) was originally isolated from the bark of *Virola elongate*, a tree whose resin was used as a hallucinogen and arrow poison by the Yanomamö Indians [7,8]. Its presence in a variety of plants had been reported later [9–11]. As a natural occurring analog of resveratrol, *Z*-TMS also exhibited potent anti-proliferative and/or apoptosis-inductive activities in various types of malignant cells [9,12–18]. Its anti-cancer effects may be p53-independent and mediated through the inhibition of tubulin polymerization [9,14,15,18]. Interestingly, the anti-neoplastic activities of *Z*-TMS were better than its *E*-isomer (*E*-3,5,4′-trimethoxystilbene, *E*-TMS, Fig. 1, III) and were about 100 fold more potent than resveratrol [9,12–14,16,17]. As *Z*-TMS usually displayed its anti-cancer effects at sub-micromolar levels [9,12,14–16], it has the potential to be used as a cancer chemotherapeutic agent.

Pharmacokinetics plays an important role in drug discovery and development. The pharmacokinetic profile of *E*-TMS has been reported to be more favorable than resveratrol [19]. We hypothesized that the *Z* isomer (*Z*-TMS) may similarly possess the desirable pharmacokinetics of its *E* isomer. However, the pharmacokinetic profile of *Z*-TMS has not been investigated. In this study, we developed and validated a simple HPLC method to quantify *Z*-TMS in rat plasma. Subsequently, the pharmacokinetics of *Z*-TMS was assessed in Sprague–Dawley rats after the respective intravenous and oral administration. To our knowledge, this is the first report on the pharmacokinetics of *Z*-TMS. A comparison of the pharmacokinetic parameters between *E*- and *Z*-TMS was then carried out.

## 2. Experimental

### 2.1. Special precautions

As stilbenes are light-sensitive, all laboratory procedures involving the manipulation of *E*-TMS, *Z*-TMS and *E*-stilbene were executed in a dimly lit environment.

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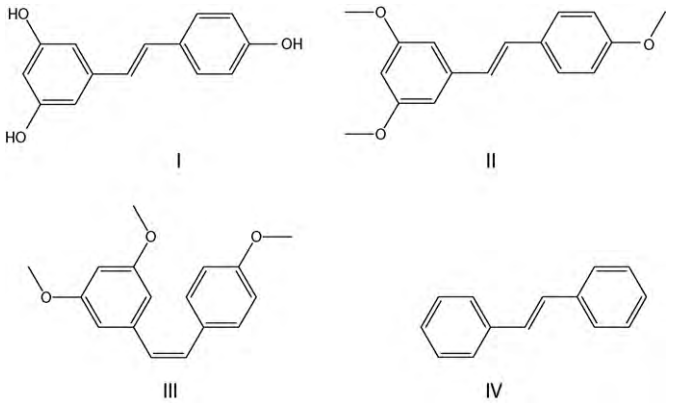


**Fig. 1.** Chemical structures of resveratrol (I), *E*-3,5,4′-trimethoxystilbene (II), *Z*-3,5,4′-trimethoxystilbene (III) and *E*-stilbene (IV, internal standard).

### 2.2. Chemicals and reagents

*Z*-3,5,4′-Trimethoxystilbene (*Z*-TMS, purity > 97%), a colorless oily liquid was synthesized in a previous study [20]. *E*-3,5,4′-Trimethoxystilbene (*E*-TMS, purity ≥ 97%, white solid) and *E*-stilbene (purity 96%, white solid) were purchased from Tokyo Chemical Industry (Tokyo, Japan) and Sigma–Aldrich (St. Louis, MO, USA), respectively. *E*-TMS and *Z*-TMS were stored at −20 °C while *E*-stilbene was kept at room temperature. Hydroxypropyl-β-cyclodextrin (HP-β-CyD) (degree of substitution: ∼0.6) was a generous gift from Roquette Freres S.A. (Lestrem, France). HPLC/Spectro grade acetonitrile and methanol was obtained from Tedia (Fairfield, OH, USA). Analytical grade DMSO was supplied from MP Biomedicals (Solon, OH, USA). Milli-Q water (18.2 MΩ cm at 25 °C) was generated by a Millipore Direct-Q® ultra-pure water system (Billerica, MA, USA) and used throughout the study. Other chemicals used were of at least reagent grade.

### 2.3. HPLC conditions

All chromatographic analyses were carried out with a Shimadzu (Kyoto, Japan) 2010A Liquid Chromatography. The integrated HPLC system comprised of a quaternary gradient low-pressure mixing pump, an online degasser, an auto-sampler, a column oven, a dual-wavelength UV–vis detector and a system controller. The HPLC was controlled by Shimadzu Class-VP Version 6.12 SP1 (Shimadzu, Kyoto, Japan). Chromatographic data analysis was also carried out with the same software.

A 5 μm Zorbax Eclipse Plus C18 column (250 mm × 4.6 mm, Agilent, Santa Clara, CA, USA) coupled with a guard column (12.5 mm × 4.6 mm, Agilent) was used to quantify *Z*-TMS in rat plasma. Chromatographic separation was obtained through a 13.5-min gradient delivery of a mixture of acetonitrile and Milli-Q water at a flow rate of 1.5 ml/ml at 50 °C. The gradient schedule was: (a) 0–5 min, acetonitrile: 57.5%; (b) 5–6.5 min, acetonitrile: 57.5 → 90%; (c) 6.5–10 min, acetonitrile: 90%; (d) 10–13.5 min, acetonitrile: 57.5%. UV absorbance was recorded at 300 and 285 nm but only the data acquired at 300 nm was used in the assay.

### 2.4. Sample preparation

*Z*-TMS (an oily liquid) was dissolved in DMSO and diluted to 1.00 mg/ml. This stock solution was stored at room temperature (24 °C) and protected from light. The calibration standards or quality control samples were prepared from the stock solution with pooled rat blank plasma. The internal standard (*E*-stilbene) was dissolved in acetonitrile and diluted to 125 ng/ml (working solution). During sample preparation, three volumes of *E*-stilbene-acetonitrile working solution were added to one volume of rat plasma. After vigorous vortexing, the samples were centrifuged at 10,000 × *g* for 10 min at 4 °C. Finally, the clear supernatant was placed into a glass insert, which had been pre-installed in a 1.5 ml auto-sampler vial. This simple protein precipitation protocol had been used in the HPLC analyses for *E*-TMS [19], pterostilbene [21] and *E*-3,5,3′,4′-tetramethoxystilbene [22]. During analyses, 50 μl supernatant was injected into the HPLC system. 30 μl plasma was sufficient for a single assay

### 2.5. Assay validation

The validation procedures of this simple HPLC assay involved the assessment of the sensitivity, linearity, precision, accuracy, absolute recovery and the stability profiles of *Z*-TMS under different conditions.

The suitability of the elution conditions was investigated by comparing the chromatograms from 7 individual rat plasma samples and these plasma samples spiked with *Z*-TMS and *E*-stilbene. In the subsequent pharmacokinetic study, pre-dosing plasma samples were also collected from all rats ($n = 11$). The suitability of the elution conditions was further confirmed through a chromatographic comparison between the pre-dosing and post-dosing plasma samples.

The sensitivity of this HPLC assay was represented by lower limit of detection (LLOD) and lower limit of quantification (LLOQ), which were defined as a signal to noise ratio equal to 3 and 10, respectively.

The ratio between the peak area of *Z*-TMS and *E*-stilbene (internal standard) was defined as the analytical response. Linear regression was carried out with GraphPad Prism Version 5.02 (La Jolla, CA, USA), where x was the concentration of *Z*-TMS, y was the analytical response, and $1/x^2$ was used as a weighting factor. The calibration standards of the following concentrations 33, 100, 200, 500, 1000, 1500, 2000 and 2500 ng/ml were used to assess linearity.

Five replicates of calibration standards were analyzed on Day 1 and duplicates of calibration standards were analyzed on Days 2–5. The intra- and inter-day relative standard deviation (RSD) at individual concentration was calculated and used as a precision indicator.

A set of quality control (QC) samples (50, 600 and 2400 ng/ml) were also prepared. The precision, absolute recovery and analytical recovery were measured with such QC samples.

The stability of *Z*-TMS solution was evaluated after storage at room temperature for 7 days. The stability of *Z*-TMS in rat plasma under different conditions was also profiled with the QC samples. The freeze–thaw stability was assessed after three freeze (−80 °C)–thaw (24 °C) cycles. Short-term fridge storage stability (4 °C, 24 h) and long-term deep freezer storage stability (−80 °C, 21 days) was also assessed. The post-preparative stability study was investigated by reanalyzing the samples (kept in auto-sampler vial at room temperature) 24 h later. The stability (% remained) was calculated by comparing the amount of *Z*-TMS detected before storage with the amount of *Z*-TMS detected after storage.

### 2.6. Pharmacokinetic study

The animal handling protocol of this pharmacokinetic study had been reviewed and approved by the Institutional Animal Care and Use Committee of the National University of Singapore (NUS). All animal experiments were carried out in the Comparative Medicine Center of NUS. Sprague–Dawley rats (male, 300–350 g, bred by the Centre for Animal Resources, NUS) were maintained on a 12-h light/dark cycle with free assess to food and water. On the day before pharmacokinetic study, a polyethylene tube (I.D. 0.58 mm,

O.D. 0.965 mm, Becton Dickinson, Sparks, MD, USA) was implanted into the right jugular vein under anesthesia [19,21,22]. Intravenous drug administration and blood sampling were carried out *via* the catheter [19,21,22].

As HP-β-CyD (0.3 M) had been used as a dosing vehicle in the pharmacokinetic study of *E*-TMS [19], it was used again to deliver *Z*-TMS to facilitate the comparison of pharmacokinetic profiles between *E*- and *Z*-TMS.

Eleven rats were divided into two groups. Group 1 ($n=4$) received a single bolus intravenous administration of *Z*-TMS (4 mg/kg), serial blood samples were collected before dosing and at 5, 15, 30, 45, 60, 90, 120, 180, 300, 420, 540 and 720 min post-dosing. Group 2 ($n=7$) received a single oral administration of *Z*-TMS (10 mg/kg) through oral gavage and serial blood samples were collected before dosing and at 15, 30, 45, 60, 90, 120, 180, 300, 420, 540 and 720 min post-administration. To maintain the patency of the cannular, 0.3 ml heparin-saline (10 I.U./ml) was used to flush the cannular after each intravenous injection or blood sampling. The blood samples were centrifuged at 3500 × *g* at 4 °C for 5 min, and the plasma was collected and stored at −80 °C. All plasma samples were analyzed within 1 week after collection.

Pharmacokinetic parameters were calculated by WinNonlin standard version 1.0 (Scientific Consulting Inc., Apex, NC, USA). As the plasma pharmacokinetic profile of *Z*-TMS after intravenous administration displayed a typical bi-exponential decline, the plasma *Z*-TMS concentration-time data was fitted into the classical two-compartment first-order open model ($C = A\,e^{-\alpha t} + B\,e^{-\beta t}$) as described previously [19,21,22]. The plasma exposure (area under the plasma *Z*-TMS concentration-time curve ($AUC_{0\to 12\,h}$)), clearance ($Cl_{0\to 12\,h}$), mean transit time ($MTT_{0\to 12\,h}$) and terminal elimination half-life ($t_{1/2\lambda z}$) were calculated using non-compartmental analysis.

## 3. Results and discussion

### 3.1. Assay validation

Under our gradient chromatographic conditions, *E*-TMS, *Z*-TMS and *E*-stilbene eluted at 8.7, 8.8 and 9.3 min, respectively (Fig. 2B). No notable interference was observed in the chromatograms acquired from either individual blank plasma or pre-dosing plasma samples from the pharmacokinetic study ($n=11$) (a typical chromatogram acquired from a pre-dosing plasma sample is shown in Fig. 2(A)). Furthermore, no notable interference was observed in the chromatograms obtained from the post-dosing samples (Fig. 2C).

Through the assessment of blank plasma from individual rats ($n=7$), the LLOD and LLOQ of *Z*-TMS were determined to be 10 and 33 ng/ml, respectively. The assay sensitivity for *Z*-TMS was about 50% of that for *E*-TMS [19]. This is probably due to the lower UV absorbance of *Z*-TMS in comparison to its *E*-isomer. In the present study, we have tried to maximize the assay sensitivity of *Z*-TMS by reducing its peak width through gradient elution. However, this manipulation resulted in a less than optimal separation of *E*- and *Z*-TMS. Fortunately, *Z*-TMS was not notably converted to its *E*-isomer (Fig. 2C).

The calibration curves of *Z*-TMS were established at the range of 33–2500 ng/ml on 5 continuous days. The calibration curves were all linear with regression correlation coefficients ($R^2$) > 0.9995. The intra-day calibration equation was: $y = 0.0007005x + 0.001613$ while the inter-day calibration was: $y = (0.0007074 \pm 0.0000085)x - (0.0002698 \pm 0.0051917)$. The precision of the calibration standards was good and the intra-day or inter-day RSD was all less than 10%.

Since the sample clean-up procedure only involved protein precipitation by three volumes of *E*-stilbene-acetonitrile, the absolute recovery was excellent (Table 1). According to our experience, such simple clean-up procedure usually provided good recovery [19,21,22]. The accuracy of this assay was also confirmed (Table 1). The analytical recovery ranged from 94.6 ± 9.1% to 97.0 ± 2.1%.

The stability of *Z*-TMS under different storage conditions was also investigated (Table 2). Exposure of *Z*-TMS to plasma even at frozen condition (−80 °C) led to notable degradation. Therefore,

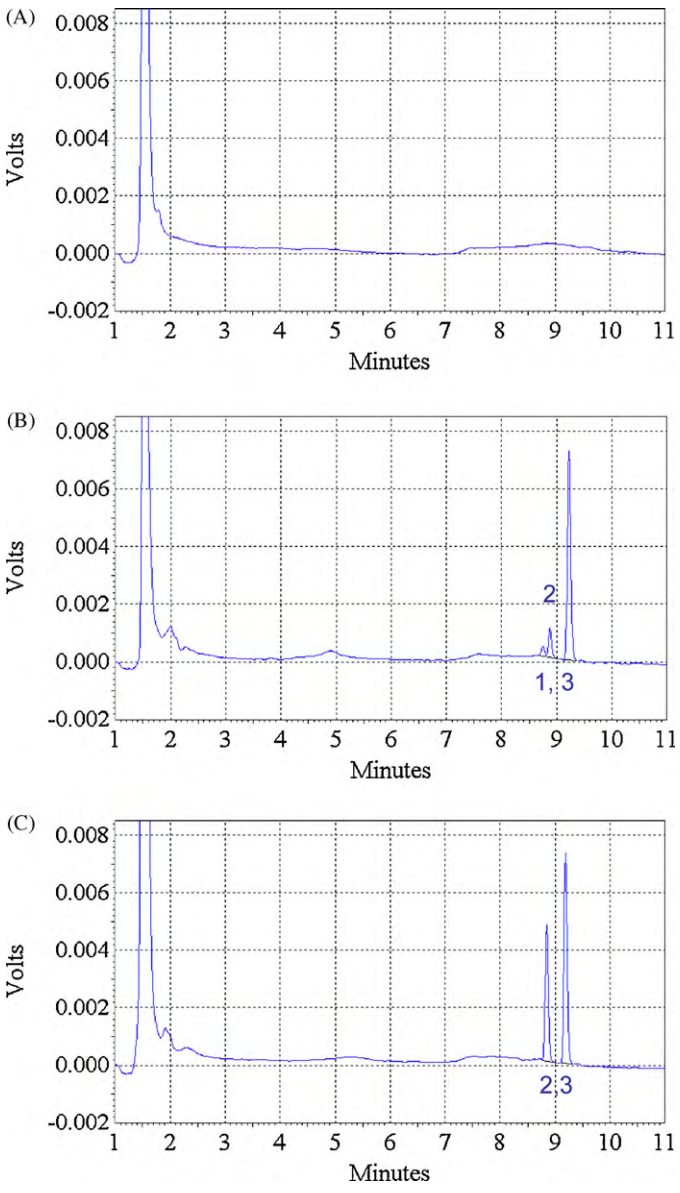


**Fig. 2.** HPLC chromatogram (UV absorbance at 300 nm) of (A) a pre-dosing plasma sample; (B) a blank plasma sample spiked with *E*-TMS (25 ng/ml, peak 1), *Z*-TMS (200 ng/ml, peak 2) and E-stilbene (375 ng/ml, peak 3, internal standard); (C) a plasma sample taken from a rat at 45 min after receiving an intravenous dose of *Z*-TMS (4 mg/kg).

**Table 1**
Absolute recovery and analytical recovery.[a]

| | Spiked concentration (ng/ml) | | |
|---|---|---|---|
| | 50 | 600 | 2400 |
| Absolute recovery (%) | 105.1 ± 10.4 | 106.2 ± 4.7 | 105.7 ± 3.1 |
| Intra-day analytical recovery (%) | 94.6 ± 9.1 | 96.6 ± 1.2 | 96.6 ± 1.0 |
| Inter-day analytical recovery (%) | 95.6 ± 7.9 | 97.0 ± 2.1 | 95.4 ± 1.4 |

[a] Results were presented as mean ± SD ($n=5$).

**Table 2**
Stability of Z-TMS.[a]

| Stability (% remained) | Spiked concentration (ng/ml) | | |
|---|---|---|---|
| | 50 | 600 | 2400 |
| Stock solution stored at 24 °C for 7 days | 100.6 ± 6.8 | 101.1 ± 0.5 | 100.2 ± 1.2 |
| Plasma samples stored at 4 °C for 24 h | 96.7 ± 3.8 | 95.7 ± 0.8 | 97.3 ± 1.3 |
| Plasma samples stored at −80 °C for 21 days | 91.9 ± 6.8 | 93.2 ± 4.1 | 90.3 ± 2.3 |
| Plasma samples after 3 Freeze–thaw cycles | 95.0 ± 5.4 | 96.9 ± 3.2 | 95.1 ± 1.2 |
| Post-preparative samples stored at 24 °C for 24 h | 97.2 ± 2.8 | 99.3 ± 1.4 | 98.5 ± 0.9 |

[a] Results were presented as mean ± SD ($n$ = 5).

Z-TMS should be analyzed as soon as possible after the pharmacokinetic study.

### 3.2. Pharmacokinetics

We subsequently investigated the pharmacokinetic profiles of *Z*-TMS in Sprague–Dawley rats after single intravenous or oral administration. Upon bolus intravenous injection of *Z*-TMS in HP-β-CyD solution (4 mg/kg), the plasma *Z*-TMS levels declined through a classical bi-exponential process (Fig. 3). Therefore, the plasma *Z*-TMS concentration versus time data of individual rats was fitted into the classical two-compartment first-order elimination model (Table 3). *Z*-TMS had a fairly long terminal elimination half-life, abundant plasma drug exposure and limited clearance. Even 12 h after administration, quantifiable levels of *Z*-TMS were detected in plasma (66 ± 23 ng/ml).

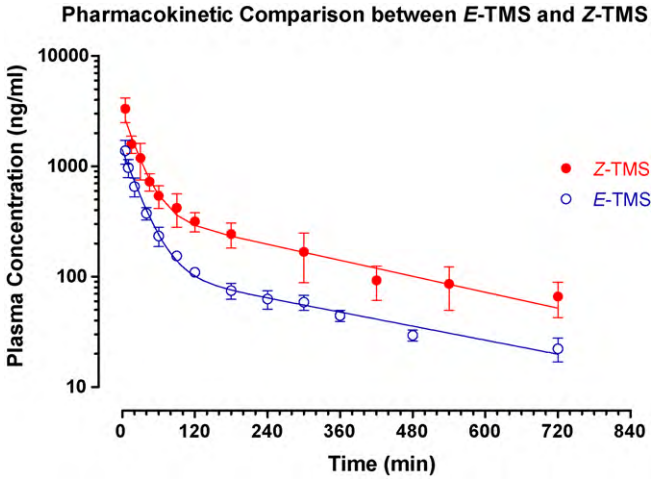


**Fig. 3.** Pharmacokinetic comparison between *E*- and *Z*-TMS after single intravenous administration. Sprague–Dawley rats were received bolus intravenous injection of *E*- or *Z*-TMS (4 mg/kg) solublized in HP-β-CyD. The pharmacokinetic data of *E*-TMS was extracted from a previous report with permission (License No. 2375671003049) [19]. Symbols represent mean values, error bars represent SD and lines represent the predicted data from pharmacokinetic modeling. *E*-TMS: $n$ = 3, *Z*-TMS: $n$ = 4.

**Table 3**
Intravenous pharmacokinetic parameters of *Z*-TMS.[a].

| | |
|---|---|
| Dose (mg/kg) | 4 |
| $A$ (ng/ml) | 2532 ± 1120 |
| $B$ (ng/ml) | 317 ± 163 |
| $\alpha$ (/min) | 0.03550 ± 0.00142 |
| $\beta$ (/min) | 0.00240 ± 0.00082 |
| $V_c$ (ml/kg) | 1633 ± 725 |
| $AUC_{0\rightarrow 12\,h}$ (min ng/ml) | 193526 ± 49783 |
| Cl (ml/min/kg) | 18.5 ± 6.5 |
| $t_{1/2\lambda Z}$ (min) | 395 ± 121 |
| $MTT_{0\rightarrow 12\,h}$ (min) | 158 ± 14 |

[a] Results were presented as mean ± SD ($n$ = 4).

The pharmacokinetic profile of *E*-TMS was assessed in the same animal model using HP-β-CyD as a dosing vehicle [19]. To facilitate the pharmacokinetic comparison between the *E*- and *Z*-isomers of TMS, the intravenous pharmacokinetic data of *E*-TMS was extracted from a recent publication [19] and plotted in Fig. 2. *E*- and *Z*-isomers of TMS shared some similarities in pharmacokinetics. Both of them possessed a typical bi-exponential decline in plasma drug concentration (Fig. 3). They also had similar long terminal elimination half-lives ($t_{1/2\lambda}$: 395 ± 121 min *vs.* 366 ± 104 min) such that their terminal plasma drug concentration-time curves were almost parallel (Fig. 3). Moreover, their mean transit times were similar ($MTT_{0\rightarrow 12\,h}$: 158 ± 14 min *vs.* 144 ± 12 min). However, plasma exposure of *Z*-TMS was about 200% of that of *E*-TMS ($AUC_{0\rightarrow 12\,h}$: 193526 ± 49783 min ng/ml *vs.* 74707 ± 10063 min ng/ml) while its clearance (Cl: 18.5 ± 6.5 ml/min/kg *vs.* 46.5 ± 6.2 ml/min/kg) and apparent volume of distribution of the central compartment ($V_c$: 1633 ± 725 ml/kg *vs.* 2971 ± 667 ml/kg) was about 50% of that of *E*-TMS. As *E*- and *Z*-TMS had similar terminal elimination half-lives, such difference in pharmacokinetic parameters could be attributed to the difference in distribution profile. *E*-TMS may have more tissue distribution and therefore it has less plasma exposure and larger volume of distribution. On the other hand, *Z*-TMS may have less tissue distribution and therefore it has more plasma exposure and smaller volume of distribution. Clearly, the distribution profiles of TMS were dependent of the *E*- and *Z*-isomers.

*Z*-TMS displayed negligible oral bioavailability. After oral dosing (10 mg/kg), *Z*-TMS could be quantified (>33 ng/ml) in only 3 plasma samples from 3 individual rats (69, 40, 34 ng/ml, respectively). These plasma samples were collected within the first hour after oral gavage. Similarly, *Z*-TMS could be detected (>10 ng/ml) in some of the plasma samples collected within the first hour after dosing. Clearly, the oral bioavailability of *Z*-TMS was problematic. The oral pharmacokinetics of *E*-TMS was more favorable than the *Z*-isomer. After oral gavage, *E*-TMS was absorbed rapidly with an absolute bioavailability of 54.9 ± 28.1% [19]. The maximal plasma concentration was fairly high ($C_{max}$ = 721 ± 376 ng/ml) [19]. Our study disclosed the apparent distinction in oral pharmacokinetics between the *E*- and *Z*-isomers of TMS.

It is generally believed that the oral bioavailability of a given drug is dependent on its aqueous solubility, membrane permeability, and metabolic stability [23,24]. When fully solublized by HP-β-CyD, the aqueous barrier is removed. Since *Z*-TMS had a fairly long terminal elimination half-life and limited clearance after intravenous administration, the metabolic rate of *Z*-TMS was not likely to be very high. If physicochemical properties, such as molecular weights, polar surface areas, number of hydrogen acceptors/rotatable bonds are the only determinants of permeability, then with the same physicochemical properties, it would be difficult to link *Z*-TMS with poor membrane permeability as *E*-TMS possessed good oral bioavailability. However, it is unclear whether the isomerism in the respective *E*- and *Z*-forms has a prominent effect on the membrane permeability. The conjecture of impact of isomerism on permeability was supported by the intravenous pharmacokinetic data. The distribution to other

tissues requires penetration through various membranes. *Z*-TMS had less tissue distribution upon intravenous administration, implying lower membrane permeability. Drug efflux transporters, well-known barriers to oral bioavailability [25,26] can be another possible explanation. Although it is unclear whether *Z*-TMS is a substrate of the efflux transporters, some stilbenes such as resveratrol, piceatannol (*E*-3,5,3′,4′-tetrahydroxystilbene) and *E*-3,5,3,4′-tetramethoxystilbene interacted with such transporters [27–29]. Therefore, it is of interest to identify the mechanisms that lead to the poor oral bioavailability of *Z*-TMS in future studies.

## 4. Conclusions

A simple HPLC assay for the quantification of *Z*-TMS in rat plasma had been developed and validated. This rapid method had been successfully applied in the pharmacokinetic study of *Z*-TMS formulated with 0.3 M HP-β-CyD. Although *Z*-TMS displayed negligible oral bioavailability, it had a fairly long terminal elimination half-life, abundant plasma drug exposure and limited clearance following intravenous administration. As *Z*-TMS is a potent anti-cancer agent with favorable intravenous pharmacokinetic profile, this warrants further investigation on its potential as a cancer chemotherapeutic agent.

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