Journal of Pharmaceutical and Biomedical Analysis 51 (2010) 679–684

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

A simple and sensitive HPLC-UV method for the quantification of piceatannol analog *trans*-3,5,3′,4′-tetramethoxystilbene in rat plasma and its application for a pre-clinical pharmacokinetic study

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ARTICLE INFO

Article history:
Received 31 July 2009
Received in revised form 4 September 2009
Accepted 17 September 2009
Available online 25 September 2009

Keywords:
HPLC-UV
Pharmacokinetics
trans-3,5,3′,4′-Tetramethoxystilbene
Piceatannol
Oral bioavailability

ABSTRACT

A simple and sensitive HPLC-UV method was developed and validated for the quantification of piceatannol analog *trans*-3,5,3′,4′-tetramethoxystilbene (M-PIC) in rat plasma. Following protein precipitation with three volumes of acetonitrile, the analytes were separated on a RP-HPLC column, which was protected by a guard column through gradient delivery of a mixture of acetonitrile–water at 40 °C. The UV absorbance at 325 nm was recorded to quantify M-PIC. The retention time of M-PIC and *trans*-3,5-dimethoxystilbene (internal standard) was 7.4 and 8.4 min, respectively. The calibration curves were linear ($R^2 > 0.9989$) with a lower limit of quantification of 15 ng/ml. The intra- and inter-day precisions, in terms of RSD, were all lower than 7.5%. The average analytical recovery ranged from 97.0 to 104.3% while the average absolute recovery ranged from 101.8 to 105.0%. This reliable HPLC method was subsequently applied to assess the pharmacokinetic profile of M-PIC in Sprague–Dawley rats using 2-hydroxypropyl-β-cyclodextrin as a dosing vehicle. The terminal elimination half-life ($t_{1/2\lambda z}$) and clearance (Cl) of M-PIC were 313 ± 20 min and 33.1 ± 3.9 ml/min/kg, respectively; and its absolute oral bioavailability was as high as 50.7 ± 15.0%. M-PIC appeared to have a favorable pharmacokinetic profile and further pharmacological investigation on this phyto-stilbene was warranted.

1. Introduction

Resveratrol (RES, *trans*-3,5,4′-trihydroxystilbene, **1** in Fig. 1), a phytoalexin present in grapes, mulberries and peanuts, has attracted great interests in the past 15 years [1]. Its biological activities, including anti-aging, anti-diabetes, anti-inflammation, anti-obesity, anti-oxidation, cancer chemoprevention, cardio- and neuro-protection have been reported extensively [1,2]. Piceatannol (PIC, *trans*-3,5,3′,4′-tetrahydroxystilbene, **2** in Fig. 1), a well known RES analog, is also present in our diets [3]. Similar to RES, PIC also displayed anti-cancer, anti-inflammatory, anti-oxidant and cardio-protective activities [3–5]. Furthermore, the potency of PIC usually surpasses RES [3,4,6–8]. As RES is converted to PIC by some phase I enzymes in human body [9,10], there lies the possibility for RES to work as a pro-drug for PIC [3].

The pharmacokinetic and metabolic profile of any new drug candidate is one of the key determinants for its success in drug development. In this regard, both RES and PIC lack metabolic stability as they are subjected to extensive phase II metabolism (glucuronide/sulfate conjugation), leading to limited oral bioavailability [1,11,12]. Methylation on the hydroxyl groups of the aromatic ring can avoid the phase II metabolism. Recently, the pharmacokinetic profiles of *trans*-3,5,4′-trimethoxystilbene (fully methylated RES) and pterostilbene (*trans*-3,5-dimethoxy-4′-hydroxystilbene, partially methylated RES) was found to be more favorable than their precursor, RES [13,14]. A fully methylated analog of PIC, *trans*-3,5,3′,4′-tetramethoxystilbene (M-PIC, **3** in Fig. 1), also known as a phytochemical [15], has been recently reported as an anti-allergic, anti-cancer, and anti-inflammatory agent [16–19]. Interestingly, M-PIC also possesses potent inhibitory effects on P-glycoprotein and cyclooxygenases [19,20]. Therefore, M-PIC appears to be an interesting compound for further investigation.

In this study, a simple and sensitive HPLC-UV method was developed and validated for the quantification of M-PIC in rat plasma. This reliable method was subsequently applied to assess the pharmacokinetic profile of M-PIC in Sprague–Dawley rats after single oral or intravenous administration. To our knowledge, this is the first report on the pharmacokinetics of M-PIC. Our study indicated that M-PIC had a superior pharmacokinetic profile over its precursor, PIC.

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doi:10.1016/j.jpba.2009.09.024

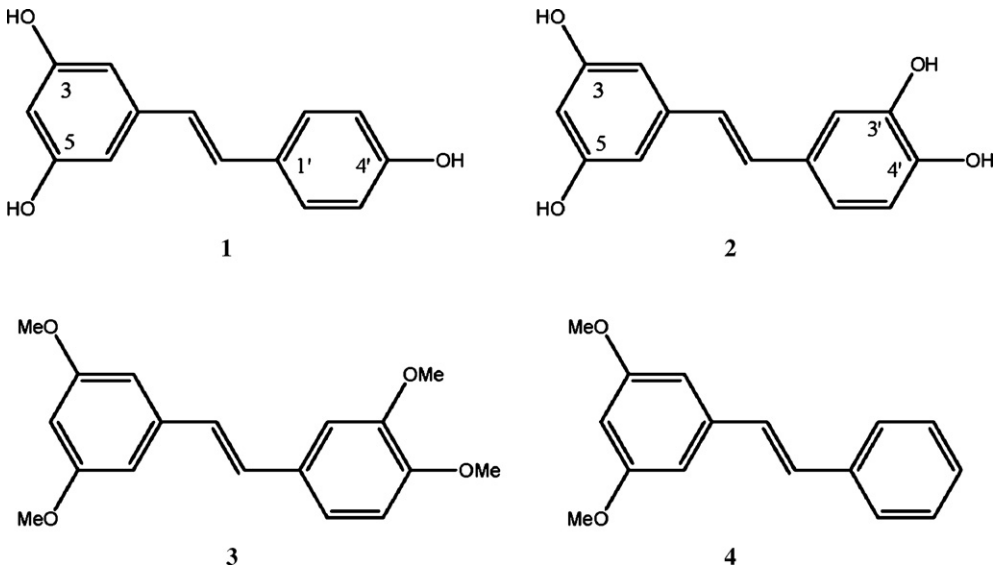

Fig. 1. Chemical structures of resveratrol (**1**), piceatannol (**2**), *trans*-3,5,3′,4′-tetramethoxystilbene (**3**) and *trans*-3,5-dimethoxystilbene (**4**, internal standard).

2. Experimental

2.1. Special precaution

As stilbenes are light sensitive, all laboratory procedures involving the manipulations of M-PIC and *trans*-3,5-dimethoxystilbene were executed in a dimly lit environment.

2.2. Chemicals and reagents

Trans-3,5,3′,4′-tetramethoxy-stilbene (M-PIC, purity > 98%, determined by HPLC) was prepared by some of us employing a previously reported method [21]. *Trans*-3,5-dimethoxystilbene (DMS, **4** in Fig. 1, internal standard, purity > 98%) was purchased from Tokyo Chemical Industry (Tokyo, Japan). 2-Hydroxypropyl-β-cyclodextrin (HP-β-CyD) (degree of substitution: ∼0.6) was a generous gift from Roquette Freres S.A. (Lestrem, France). Gradient grade acetonitrile (Merck, Darmstadt, Germany) and Milli-Q water (18.2 MΩ cm at 25 °C) was used to prepare mobile phase. Other solvents or reagents were at least of analytical grade.

2.3. Chromatographic conditions

HPLC analyses were carried out on a Shimadzu (Kyoto, Japan) 2010A liquid chromatography system, which 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 software Class-VP Version 6.12 SP1 (Shimadzu, Kyoto, Japan) was used for system control and data processing.

A RP-HPLC column (Agilent ZORBAX Eclipse Plus C18: 250 mm × 4.6 mm i.d., 5 μm), which was protected by a guard column (Agilent ZORBAX Eclipse Plus C18: 12.5 mm × 4.6 mm i.d., 5 μm) was selected to quantify M-PIC in plasma. Chromatographic separation was obtained through a 15-min gradient delivery of a mixture of acetonitrile and Milli-Q water at a flow rate of 1.2 ml/ml at 40 °C. The gradient schedule was: (a) 0–1.5 min, acetonitrile: 55%; (b) 1.5–5.5 min, acetonitrile: 55 → 90%; (c) 5.5–10 min, acetonitrile: 90%; (d) 10–15 min, acetonitrile: 55%. UV absorbance at both 325 and 300 nm was recorded but only the data acquired at 325 nm was applied to set up the assay.

2.4. Sample preparation

Stock solution of M-PIC was prepared in DMSO to obtain a final concentration of 1 mg/ml weekly. This stock solution was stored at room temperature (24 °C) and protected from light. The calibration standards for rat plasma were prepared through serial dilution of the M-PIC stock with pooled blank rat plasma. The internal standard (DMS) was dissolved in acetonitrile and diluted to 150 ng/ml (working solution). During sample preparation, three volumes of DMS–acetonitrile working solution were added to one volume of rat plasma. After vigorous vortexing for 20 s, the samples were centrifuged at 10,000 × *g* for 10 min at 4 °C. Finally, the supernatant was transferred into a glass insert that was pre-installed in a 1.5 ml auto-sampler vial. This simple protocol for protein precipitation had been used in the HPLC analyses for *trans*-3,5,4′-trimethoxystilbene and pterostilbene [13,14]. During each assay, 50 μl supernatant was injected into the HPLC system. The minimal volume of plasma required for an analysis was 30 μl.

2.5. Method validation

This HPLC method was validated with regards its selectivity, linearity, precision (intra- and inter-day), sensitivity, accuracy, absolute recovery and stability.

The selectivity was initially investigated by comparing the chromatogram from the pooled blank rat plasma and the chromatogram from the same sample but spiked with M-PIC and DMS. In the subsequent pharmacokinetic study, pre-dosing plasma sample was also collected from each individual rat ($n = 7$). The selectivity of this assay was further documented through a chromatographic comparison between the pre-dosing and post-dosing plasma samples.

The ratio between the peak area of M-PIC and that of DMS (internal standard) was defined as the analytical response. Linear regression was carried out with GraphPad Prism Version 5.01 (La Jolla, CA 92037 USA) via least sum-of-squares method, where x was the concentration of M-PIC, y was the analytical response, and $1/x^2$ was used as a weighting factor [13,14]. The calibration standards of the following concentrations 15, 50, 100, 250, 500, 1000, 1500, 2000 and 2500 ng/ml were used to assess linearity. The plasma M-PIC levels in the subsequent pharmacokinetic study were all covered by this calibration range.

The sensitivity of this HPLC method was examined by the measurement of lower LOD and LOQ. A signal to noise ratio equal to 3 and 10 was defined as lower LOD and LOQ, respectively.

To assess the intra- and inter-day precision, five replicates of calibration standards were analyzed on the same day and on five different days. The RSD at individual concentration was used to indicate the precision.

To evaluate the accuracy of this assay, the analytical recovery of the calibration standards (both intra- and inter-day) were calculated by comparing the amounts of M-PIC detected with the amounts spiked. To further contest the reliability of this assay, the accuracy of another set of quality control (QC) samples (40, 400 and 2400 ng/ml) were also studied in 5 replicates.

The absolute recovery was calculated with the QC samples by comparing the peak areas of M-PIC in the spiked plasma samples with plasma-free samples containing the same amount of M-PIC.

The stabilities of M-PIC in stock solution and DMS in working solution were evaluated after storage at room temperature for 8 days. The stability of M-PIC 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 room temperature stability (24 °C) of M-PIC in rat plasma was investigated after storage of such samples at room temperature for 16 h. Similarly, long-term storage stability of M-PIC in rat plasma was studied after storage at −80 °C for 2 weeks. The post-preparative stability study was conducted by reanalyzing the samples (kept in auto-sampler vial at room temperature) 24 h later.

2.6. Dosing solution

Since HP-β-CyD was able to increase the aqueous solubility of some stilbenes and has been used as intravenous dosing vehicle for these stilbenes [13,14,22], it was also applied to deliver M-PIC in this study. The M-PIC-HP-β-CyD inclusion complex solution was prepared in the following manner: 40 mg of M-PIC was suspended in 10 ml of 0.3 M HP-β-CyD solution prepared with isotonic saline; the suspension was incubated at 65 °C for 2 h and then shaken on a horizontal rotary shaker at room temperature overnight. After filtration through a 0.22 μm syringe driven filter (Millipore, Billerica, MA 01821, USA), the M-PIC-HP-β-CyD inclusion complex solution was collected for pharmacokinetic study. This solution was diluted for 1000 times by methanol and the concentration of M-PIC was measured by HPLC. The M-PIC-HP-β-CyD solution used in the pharmacokinetic study was 3 mg/ml.

2.7. Animals

This pharmacokinetic study was carried out according to the "Guidelines on the Care and Use of Animals for Scientific Purposes" (National Advisory Committee for Laboratory Animal Research, Singapore, 2004). The animal handling procedures of this pharmacokinetic study were reviewed and approved by the Institutional Animal Care and Use Committee of the National University of Singapore (NUS).

Adult male Sprague–Dawley rats (317–359 g) were provided by the Laboratory Animal Center of NUS and maintained on a 12-h light/dark cycle with free assess to food and water. On the day before the pharmacokinetic study, a polyethylene tube (i.d. 0.58 mm, O.D. 0.965 mm, Becton Dickinson, Sparks, MD 21152, USA) was inserted into the right jugular vein through surgical implant. This cannular was used for intravenous dosing as well as for blood sampling. 7 rats were randomly divided into two groups. Group 1 ($n = 3$) received a single bolus intravenous injection of M-PIC at the dose of 4 mg/kg, serial blood samples were collected before dosing and at 5, 15, 30, 45, 60, 90, 120, 180, 300, 480 and 720 min after intravenous administration. Group 2 ($n = 4$) received a single oral administration of M-PIC at a dose of 10 mg/kg through gavage. Serial blood samples were collected before dosing and at 15, 30, 45, 60, 90, 120, 180, 300, 480 and 720 min after oral dosing. 0.2 ml heparin–saline (10 I.U./ml) was used to flush the catheter after each intravenous dosing or blood sampling. After centrifuging at $2000 \times g$ (4 °C) for 10 min, the plasma was collected and stored at −80 °C. All samples were analyzed within 1 week after the pharmacokinetic study.

2.8. Pharmacokinetics

All pharmacokinetic parameters were analyzed by WinNonlin standard version 1.0 (Scientific Consulting Inc., Apex, NC 27502, USA). Since a distribution phase followed by a prolonged terminal elimination phase was observed after intravenous administration in the plasma concentration–time curves, the plasma M-PIC concentration-time data was fitted into the classical two-compartment first-order open model ($C = A \cdot e^{-\alpha \cdot t} + B \cdot e^{-\beta \cdot t}$) using nonlinear least squares method with a weighting factor of $1/y^2$ as described previously [13,14]. The apparent volume of distribution of the central compartment (V_c) was calculated as:

$$V_c = \frac{\text{Dose}}{A + B}.$$

The plasma exposure (the area under the plasma concentration–time curve (AUC)), clearance (Cl), mean transit time (MTT) and terminal elimination half-life ($t_{1/2\lambda z}$) were calculated through non-compartmental method. $AUC_{0\rightarrow 720\,min}$ in rats received oral dose was calculated by standard trapezoidal rule while $AUC_{0\rightarrow 720\,min}$ in rats received intravenous dose was calculated by logarithmic-scale trapezoidal rule. Mean absorption time (MAT) was calculated as: $MAT = MTT_{oral} - MTT_{iv}$. The absolute oral bioavailability (F) was calculated as:

$$F(\%) = \frac{AUC_{0\rightarrow 720\ min}(\text{Group 2})/10\,\text{mg/kg}}{AUC_{0\rightarrow 720\ min}(\text{Group 1})/4\,\text{mg/kg}} \times 100\%.$$

3. Results and discussion

3.1. Selection of chromatographic condition

The objective of this study was to establish a simple and sensitive method to quantify M-PIC in rat plasma. Since M-PIC does not have any ionizable group, the pH value of mobile phase should not have an impact on its elution. Hence, we only used acetonitrile and Milli-Q water as mobile phase. The maximal UV absorbance of M-PIC is at about 325 nm, which was used to quantify M-PIC. DMS was selected as the internal standard for the assay because of its structural similarity with M-PIC. Isocratic elution was attempted at the beginning. However, due to the broad chromatographic peak of M-PIC after isocratic elution, gradient elution was subsequently adopted to overcome this problem. Gradient elution could also improve the sensitivity of the assay. The overall run-time was 15 min for each sample.

3.2. Method validation

The specificity of this simple HPLC-UV method was documented. Under our chromatographic conditions, M-PIC and DMS eluted at 7.4 and 8.4 min, respectively (Fig. 2B). No interference peak was observed at 7.4 and 8.4 min in the chromatograms acquired from either pooled blank rat plasma sample or pre-dosing plasma samples from the respective rats ($n = 7$) used in the study (the chromatogram of a pre-dosing sample is shown in Fig. 2A). Moreover, no notable interferences for M-PIC or DMS were observed in the chro-

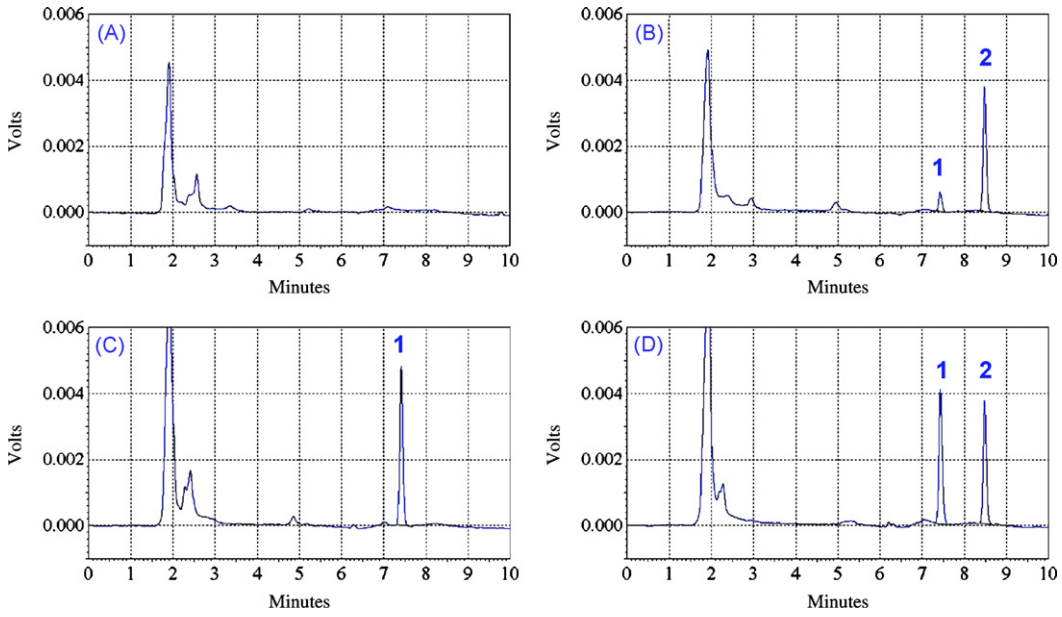

Fig. 2. HPLC-UV chromatogram of (A) a pre-dosing plasma sample, (B) a blank plasma sample spiked with M-PIC (50 ng/ml) and DMS (internal standard) (450 ng/ml), (C) a plasma sample taken from a rat at 90 min after receiving an intravenous dose of M-PIC (4 mg/kg) (without internal standard), (D) a plasma sample taken from a rat at 15 min after receiving an oral dose of M-PIC (10 mg/kg) (with internal standard). Peak 1, M-PIC; peak 2, internal standard.

Table 1
Intra- and inter-day calibration[a].

M-PIC (ng/ml)	Intra-day calibration			Inter-day calibration		
	Analytical response	RSD (%)	Analytical recovery (%)	Analytical response	RSD (%)	Analytical recovery (%)
15	0.0472 ± 0.0033	6.9	101.0 ± 6.6	0.0441 ± 0.0032	7.3	100.7 ± 6.4
50	0.1583 ± 0.0091	5.8	97.0 ± 5.5	0.1569 ± 0.0109	7.0	97.8 ± 6.5
100	0.3279 ± 0.0097	2.9	99.4 ± 2.9	0.3247 ± 0.0124	3.8	99.1 ± 3.7
250	0.8266 ± 0.0097	1.2	99.7 ± 1.2	0.8273 ± 0.0121	1.5	99.9 ± 1.5
500	1.6627 ± 0.0131	0.8	100.1 ± 0.8	1.6665 ± 0.0115	0.7	100.2 ± 0.7
1000	3.3313 ± 0.0255	0.8	100.1 ± 0.8	3.3250 ± 0.0230	0.7	99.8 ± 0.7
1500	4.9844 ± 0.0205	0.4	99.9 ± 0.4	5.0107 ± 0.0542	1.1	100.2 ± 1.1
2000	6.9464 ± 0.1372	2.0	104.3 ± 2.1	6.8324 ± 0.1211	1.8	102.4 ± 1.8
2500	8.1959 ± 0.1888	2.3	98.5 ± 2.3	8.3326 ± 0.1011	1.2	99.9 ± 1.2

[a] Results were presented as mean ± SD ($n = 5$). Intra-day calibration equation: $y = 0.003330x - 0.003188$ ($R^2 = 0.9995$); inter-day calibration equation: $y = 0.003339x - 0.006377$ ($R^2 = 0.9998$).

matograms obtained in the actual pharmacokinetic study (Fig. 2C and D).

The calibration curves were established at the range of 15–2500 ng/ml on 5 continuous days. The calibration curves were all linear with regression correlation coefficients (R^2) > 0.9989. The lower LOD and LOQ were found to be 5 and 15 ng/ml in the plasma from 6 individual rats, respectively. The precision and reproducibility of this simple HPLC-UV method were also confirmed. The intra- or inter-day variations of the analytical responses, in term of RSD, were less than 7.5% (Table 1). Similarly, the accuracy was also documented and the average analytical recovery ranged from 97.0 to 104.3% in both calibration standard samples and QC samples. Furthermore, as the sample clean-up procedure only involved protein precipitation by three volumes of DMS–acetonitrile, the average absolute recovery of M-PIC in QC samples was as high as 101.8–105.0%. The stability profile of M-PIC was also investigated and M-PIC appeared to be stable (Table 2).

This simple HPLC-UV method appeared to be quite sensitive. The good sensitivity could be attributed to the strong UV absorbance of M-PIC as each M-PIC molecule has two aromatic rings conjugated through a double bond. However, the detection sensitivity might be further increased if mass spectrometer detector is applied. In previously studies, the assay sensitivities of LC–MS were found to be higher than that of HPLC-UV in the quantification of RES

Table 2
Stability of M-PIC[a].

Stability (% remained)	Spiked concentration (ng/ml)		
	40	400	2400
Stock solution stored at 24 °C for 8 days	101.6 ± 2.5	101.6 ± 0.5	101.1 ± 0.5
Plasma samples stored at 24 °C for 16 h	96.1 ± 6.5	95.8 ± 4.3	96.6 ± 2.0
Plasma samples stored at −80 °C for 14 days	97.3 ± 6.7	96.9 ± 1.5	98.7 ± 1.1
Plasma samples after 3 Free-thaw cycles	95.5 ± 3.4	96.3 ± 0.9	97.1 ± 0.7
Post-preparative samples stored at 24 °C for 24 h	98.5 ± 2.0	99.7 ± 4.2	100.0 ± 1.0

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

Table 3
Pharmacokinetic parameters of M-PIC[a].

Parameters	Intravenous ($n=3$)	Oral ($n=4$)
Dose (mg/kg)	4	10
A (ng/ml)	1131 ± 179	–
B (ng/ml)	66 ± 18	–
α (/min)	0.01387 ± 0.00106	–
β (/min)	0.00172 ± 0.00021	–
V_c (ml/kg)	3396 ± 507	–
$AUC_{0\rightarrow 12\,h}$ (min ng/ml)	113,508 ± 14,425	143,883 ± 42,625
Cl (ml/min/kg)	33.1 ± 3.9	–
$t_{1/2\lambda Z}$ (min)	313 ± 20	–
$MTT_{0\rightarrow 12\,h}$ (min)	125 ± 3	195 ± 33
MAT (min)	–	70 ± 33
C_{max} (ng/ml)	–	710 ± 219
T_{max} (min)	–	45, 60 or 120
F (%)	–	50.7 ± 15.0

[a] Results were presented as mean ± SD.

and pterostilbene (two stilbenes with structures similar to M-PIC) [14,22–24].

In summary, a simple and sensitive HPLC-UV method had been developed and validated to quantify M-PIC in rat plasma. To our knowledge, this is the first method to determine M-PIC in biological samples. As this HPLC protocol had good linearity, sensitivity, precision, reproducibility and accuracy, it was subsequently used to investigate the pharmacokinetics of M-PIC.

3.3. Application to pre-clinical pharmacokinetic study

Due to the lack of an appropriate analytical method, there was no information regarding the pharmacokinetics of M-PIC before this study. In light of the favorable pharmacological activities such as anti-cancer and anti-inflammation, we assessed its pharmacokinetic profiles in Sprague–Dawley rats after single intravenous or oral administration. The plasma profiles of M-PIC are shown in Fig. 3 while the major pharmacokinetic parameters are listed in Table 3.

After intravenous administration of M-PIC in HP-β-CyD solution, the plasma M-PIC levels declined through a bi-exponential process (Fig. 3). Therefore, the plasma M-PIC concentration versus time data of individual rat was fitted into the classical two-compartment first-order elimination model. The correlation of the fitting of individual rat was good ($R=0.9686$, 0.9742 and 0.9747, respectively), supporting the validity of the modeling. M-PIC had a fairly long terminal elimination half-life ($t_{1/2\lambda z}=313\pm20$ min) and it was still detectable in plasma (19 ± 2 ng/ml) 12 h after intravenous injection. The clearance was found to be 33.1 ± 3.9 ml/min/kg.

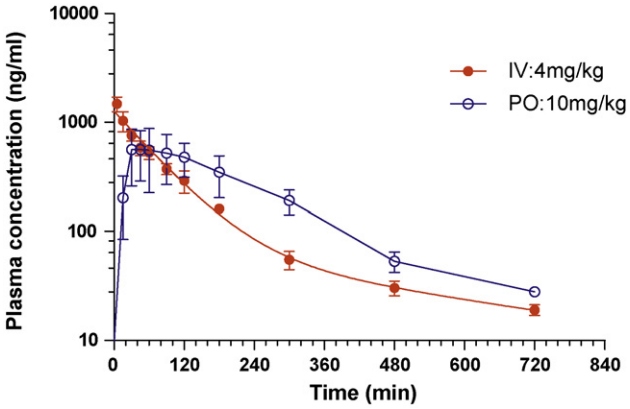

Fig. 3. Pharmacokinetic profiles of M-PIC in Sprague–Dawley rats. Symbols represent mean values and error bars represent SD. Intravenous administration: 4 mg/kg, $n=3$; oral administration: 10 mg/kg, $n=4$.

After oral gavage of M-PIC dissolved in 0.3 M HP-β-CyD, M-PIC was rapidly absorbed and the time to maximal plasma concentration (t_{max}) ranged from 45 to 120 min post-dosing. The mean absorption time (MAT) was 70 ± 33 min, in good accord with rapid absorption phenomenon. After reaching maximal plasma concentration (C_{max}), plasma M-PIC concentration declined gradually and it still remained in the plasma (28 ± 3 ng/ml) 12 h after oral administration. The absolute oral bioavailability (F) was 50.7 ± 15.0%, indicating M-PIC was an orally available when given in a solution form.

The pharmacokinetics of PIC had also been reported in Sprague–Dawley rats [12]. Its apparent volume of distribution was about twofold larger than M-PIC. Interestingly, PIC might undergo entero-hepatic circulation, which led to a secondary peak in the plasma concentration versus time curve [12]. Similar phenomenon was also observed with RES [22]. However, we did not see any secondary peak with M-PIC after oral or intravenous administration. Both RES and PIC are subjected to extensive phase II metabolism such as glucuronidation and sulphation [1,11,12]. Therefore, first-pass effect significantly reduced their oral bioavailability [1,12]. In a preliminary study carried out with rats, the oral bioavailability of PIC was reported to be only ~10% [25]. In the current study, the oral bioavailability of M-PIC was found to be 50.7 ± 15.0%. The improved bioavailability of M-PIC with respect to PIC is clearly attributable to the methylated structure of M-PIC, which made it metabolically stable, due to the chemical stability of the methoxy group with respect to the hydroxyl group. In addition, the presence of methoxy groups instead of hydroxyl group in the resveratrol-related stilbenoid structures has been reported as a structural requirement useful to modulate or to enhance some biological activities, such as antiproliferative or antiangiogenic activity [21,26,27].

4. Conclusions

A simple and sensitive HPLC-UV method was developed and validated for the quantification of M-PIC in rat plasma. This reliable method had been successfully applied in the pharmacokinetic study of M-PIC in rats. As M-PIC possessed good oral bioavailability and potent pharmacological activities, future studies on stilbenes as preventive/therapeutic agents could be extended to M-PIC.

Acknowledgements

The work of Hai-Shu Lin, Chun Wu and Paul C. Ho was supported a research grant from the Agency for Science, Technology and Research (BMRC 06/1/21/19/441), Republic of Singapore. The work of C. Spatafora and C. Tringali was supported by a grant of the Università degli Studi di Catania (Progetti di Ricerca di Ateneo, Catania, Italy) and by MIUR, Ministero dell'Università e della Ricerca (PRIN 2007, Rome, Italy).

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