



## Evaluation of pharmacokinetics and blood–brain barrier permeability of mitragynine using *in vivo* microdialysis technique



Wai Mun Kong<sup>a</sup>, Zahurin Mohamed<sup>a,b</sup>, Mohammed A. Alshawsh<sup>a</sup>, Zamri Chik<sup>a,b,\*</sup>

<sup>a</sup> Department of Pharmacology, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia

<sup>b</sup> University of Malaya Bioequivalence and Testing Centre (UBAT), Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia

### ARTICLE INFO

#### Article history:

Received 23 December 2016

Received in revised form 8 May 2017

Accepted 11 May 2017

Available online 13 May 2017

#### Keywords:

UFLC-MS

Mitragynine

Microdialysis

Pharmacokinetics

Bioavailability

BBB

### ABSTRACT

A microdialysis system coupled with a sensitive ultra-fast liquid chromatography–mass spectrometry (UFLC-MS) method was developed for the pharmacokinetic analysis of mitragynine in rat blood and striatum. Mitragynine is an active alkaloid of *Mitragyna speciosa* and has been proposed to be used for opioid withdrawal therapy. In this study, chromatographic separation was performed in a gradient elution mode with 0.1% formic acid and acetonitrile on a Zorbax Eclipse C18 column. The mass spectrometric (MS) analysis was carried out in a positive electrospray mode and mitragynine ion ( $m/z$  399.2) was monitored in extracted ion chromatography. A good linearity range was obtained from 10–1000 ng/mL with acceptable accuracy and precision parameters. The microdialysate was collected simultaneously from the striatum and the right jugular vein using microdialysis probes. After a single intravenous administration of 10 mg/kg mitragynine, mitragynine showed a two-compartmental drug elimination pattern with half-life ( $T_{1/2}$ ) of approximately 13 h. The percent of  $AUC_{\text{brain}}/AUC_{\text{plasma}}$  of mitragynine was calculated and shown to be  $65.8 \pm 4.5\%$ . The results indicated that mitragynine could be a suitable molecule to develop into an opioid replacement drug based on its ideal pharmacokinetic properties, namely, small molecular size, lipophilic in nature and with excellent blood–brain barrier (BBB) permeability.

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### 1. Introduction

Mitragynine, an active indole alkaloid ( $C_{23}H_{30}N_2O_4$ ; MW = 398.50 g/mol), is the most abundant active alkaloid found in leaves of *Mitragyna speciosa* [1]. It is widely used for the purpose of pain relief, as anti-diarrhoea, and to increase endurance against fatigue [2]. As with other opioids, mitragynine showed prominent antinociceptive effect by acting on  $\mu$ - and  $\delta$ -opioid receptor in the supraspinal region of the central nervous system [3–5]. However, its anaesthetic effect is much lower as compared with morphine and 7-hydroxymitragynine, the latter two compounds reportedly to be 4–46-fold more potent than mitragynine, respectively [6]. Mitragynine possesses the potential to be developed into a drug for opioid withdrawal therapy by reducing the opioid withdrawal induced elevation of cortisol. From the view of pharmacokinetic/pharmacodynamic (PK/PD) and toxicity, mitragynine poses the advantages of milder adverse effects, lower potency and extremely long elimination half-life (23 h) as compared with morphine and opioids and opioid-like drugs such as methadone

and buprenorphine [7,8]. Oral ingestion of mitragynine in the form of tea, powder and capsules are the most common form of administration used nowadays and traditionally [9], but the resulting therapeutic effects are always unsatisfactory, most likely due to its low aqueous solubility and gastro-intestinal permeability [10]. Besides, the average daily oral dose of mitragynine consumed is relatively low (approximate 0.3–5.1 mg/kg) as compared with the reported therapeutic dosage of about 3–100 mg/kg (i.p.) for antinociceptive activity in mice [11].

Microdialysis is a well-established sampling technique for *in vivo* quantification of exogenous and endogenous substances in both plasma and tissue [12]. It is commonly used for investigation of PK/PD relationship since concentration of the unbound drug is more correlated to the pharmacological effects. By coupling with highly selective and sensitive UFLC-MS, microdialysis is able to overcome several limitations of conventional pharmacokinetic techniques which include simultaneous and continuous sampling in different organs in the same animal [12], minimise the number of animals used and also minimising inter-animal variation. Implantation of a probe in a specific area of interest can also improve the accuracy and quality of the data [12]. However, drug quantification in microdialysis technique remains a major challenge due to the low concentration and small volume of the microdialysate. There-

\* Corresponding author.

E-mail address: [zamrichik@ummc.edu.my](mailto:zamrichik@ummc.edu.my) (Z. Chik).

fore, a highly sensitive bio-analytical instrument such as HPLC and UFLC-MS/MS is always recommended [13].

In the present study, we determined the brain and plasma concentration of unbound mitragynine simultaneously using two probes that were implanted into the jugular vein toward the heart and striatum of the rat. The quantitative analysis was performed using UFLC-MS method. The pharmacokinetic parameters and brain-plasma ratio of mitragynine were calculated and analysed using a two-compartmental technique.

## 2. Experimental

### 2.1. Materials and chemicals

Mitragynine was purchased from ChromaDex (Irvin, USA). All other chemicals used such as HPLC grade acetonitrile (ACN), methanol, formic acid, Tween 20 and normal saline (90% NaCl) were purchased from Sigma Aldrich (St. Louis, MO, USA). The microdialysis probes were purchased from CMA Microdialysis (Kista, Sweden) and used throughout the studies. Ringer's solution was prepared in-house by mixing 147 mM NaCl, 2.7 mM KCl, 1.2 mM CaCl<sub>2</sub>, and 0.85 mM MgCl<sub>2</sub> in Milli-Q water (Millipore Corporation, Billerica, MA). Artificial cerebrospinal fluid (ACSF) was prepared by mixing 145 mM NaCl, 2.7 mM KCl, 1.2 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub> and 2 mM Na<sub>2</sub>HPO<sub>4</sub>, 10 mM HEPES and 10 mM glucose. The solution was filtered through a 0.47 μm filter prior to use.

### 2.2. Animals

Adult, female Sprague-Dawley (SD) rats (8 weeks old, 250–300 g) were obtained from the Animal Experimental Unit, Faculty of Medicine, University of Malaya. The animals were allowed to acclimatize to 12 h light/dark cycle for at least 7 days before start of the study. Normal diet and water was given throughout the study. The study was approved by the Animal Ethics Committee of our institute (ethics reference no.: 2014-03-05/PHAR/R/KWM).

### 2.3. Instruments and UFLC-MS method development

The UFLC-MS system consisted of an ultra-fast liquid chromatography (UFLC) equipped with a mass spectrometer (LC-MS-2020, Shimadzu, Japan). The UFLC chromatographic separation was performed on an Agilent Zorbax Eclipse C18 column (4.6 × 150 mm i.d., 5 μm particle, Agilent, USA) which was kept at constant column temperature of 40 °C. Mobile phase A (0.1% formic acid) and mobile phase B (ACN) was perfused in a gradient elution mode (0–2 min, 10% B; 2–8 min, 10–90% B; 8–10 min 90% B; 10–12 min 10% B) with a flow rate of 0.2 mL/min. The injection volume was 10 μL [14]. The MS analysis was operated in a positive electrospray mode and a selected mitragynine precursor ion was monitored at *m/z* 399. Data acquisition and quantitative processing were accomplished using LabSolutions software (version 5.55, Shimadzu, Japan). UFLC-MS method validations such as linearity, lower limit of quantification (LLOQ), inter-day/intra-day precision, stability, matrix effect, selectivity and extraction recovery of mitragynine were established. The mitragynine standard in Ringer and ACSF solution was calibrated and validated from 10 to 1000 ng/mL prior to sample analysis.

### 2.4. Samples processing

Due to the presence of non-volatile buffer salt in Ringer's and ACSF samples which may be harmful to the mass spectrophotometer (MS) ionizer, a liquid-liquid extraction technique was applied to extract the mitragynine. In brief, the dialysate (50 μL) was added into 20 μL of sodium hydroxide and 600 μL hexane: isoamyl alcohol (98:2, v/v) [14]. The mixture was then allowed to shake for 30 min

on a horizontal shaker and centrifuged at 670 × g for 10 min at 4 °C. The organic phase (500 μL) was transferred into a clear glass tube and allowed to evaporate under a vacuum evaporated system. The extracted mitragynine was reconstituted in 200 μL of methanol and sent for UFLC-MS analysis.

### 2.5. In vitro recovery

The *in vitro* recovery of mitragynine was carried out using recovery by-gain method to validate the microdialysis system before the *in vivo* studies [15]. The probe was placed in a quiescent solution containing 0.01, 0.1, 0.5, 1, 5, and 10 μg/mL of mitragynine at room temperature and was perfused with the respective solutions at 2 μL/min. After equilibration for 1 h, triplicates of 60 μL of dialysates for each concentration were collected and analysed using UFLC-MS. The percent *in vitro* recovery by-gain (RG) was calculated from the slope of the plot concentration in the dialysate (*C<sub>d</sub>*) versus concentration in the perfusate medium (*C<sub>p</sub>*).

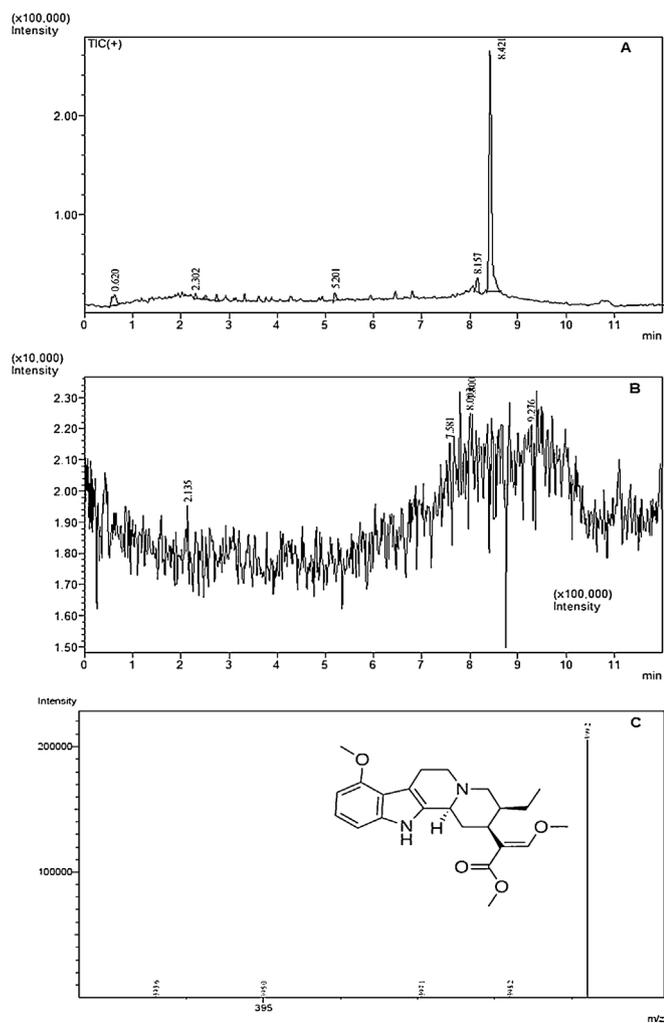
### 2.6. Probe implantation and in vivo probe recovery

The *in vivo* probe recovery was conducted in rats using retrodialysis method (*n* = 4). Firstly, microdialysis probe with cut-off of 20,000 Da (CMA/20 for blood [membrane length 4 mm], CMA/12 for brain [membrane length 3 mm]) were placed into the right jugular vein and striatum according to the stereotaxic coordinates (2.7 mm lateral and 0.8 mm anterior to the bregma and 3.8 mm ventral to the surface of the brain) under ketamine: xylazine (80:20 mg/kg; i.p.) anaesthesia [16]. After 2 h of post-surgical stabilization, microdialysis probes were perfused with Ringer's buffer and ACSF at a constant flow rate of 2 μL/min. Probes were allowed to stabilize for 1 h before sample collection. Subsequently, a solution of Ringer's buffer and ACSF containing 2 μg/mL mitragynine was perfused through the probes at a constant flow rate of 2 μL/min using the microinjection pump. Microdialysates were collected at intervals of 30 min. The probe *in vivo* relative recovery (RR) was determined by comparing the concentration of input and output of mitragynine according to Eq. (1).

$$RR = \frac{C_p - C_d}{C_p} \times 100\% \quad (1)$$

### 2.7. Pharmacokinetic studies

Six adult SD rats were used for the study. After implantation of the probes, the rats were allowed to recover overnight in an isolated chamber with free access to water and food. On the day of the experiment, the rats received a single intravenous dose of mitragynine (10 mg/kg) in 10% Tween 20 aqueous solution. Injection volume of 1 mL/kg was given according to the recommended maximal tolerance volume. The CMA/20 and CMA/12 was connected to the CMA/402 syringe pump through a swivel to prevent the tube from crossing over. Following the setting up of the system, the CMA/20 probe was then perfused with Ringer's buffer at a flow rate of 2 μL/min. On the other hand, the CMA/12 was perfused with ACSF solution. Blood and brain microdialysate samples were collected at intervals of 30 ± 2 min up to 7.5 h. All the microdialysate samples were then extracted and analysed using UFLC-MS within period of 24 h. The actual concentration of mitragynine in the blood and brain was calculated from the *in vivo* recovery. The pharmacokinetic parameters were analysed by two-compartmental method using Phoenix WinNonlin software 6.1 (Pharsight, Mountain View, CA). Lastly, the ratio of the unbound concentration of mitragynine in the brain and plasma (*AUC<sub>brain</sub>*/*AUC<sub>plasma</sub>*) was calculated using the area under the curve (AUC) of mitragynine concentration-time pro-



**Fig. 1.** (A) Total ion chromatogram (TIC) of plasma spiked with mitragynine. (B) Chromatogram of blank plasma. (C) Mass spectrum of mitragynine.

file from time 0 to the last measurable time in the brain ( $AUC_{\text{brain}}$ ) and plasma ( $AUC_{\text{plasma}}$ ) [17,18].

### 3. Results and discussion

#### 3.1. UFLC–MS method validation

In normal circumstances, HPLC will be the most suitable analytical instrument for microdialysis study because of the low concentration quantification and presence of non-volatile buffer salt. However, a more sensitive and reliable UFLC–MS instrument was needed for extremely low concentration quantification. In this chromatographic separation, the mitragynine precursor ion ( $m/z$  399.2) eluted at 8.4 min (Fig. 1). At concentrations between 10 and 1000 ng/mL, mitragynine showed linearity  $Y = 12248X + 12964$  ( $r^2 = 0.999$ ) and  $Y = 10248X - 66119$  ( $r^2 = 0.998$ ) in Ringer's and ACSF solution respectively, where  $Y$  represent the peak area and  $X$  represent the concentration. The LLOQ was set at 10 ng/mL. The UFLC–MS accuracy and precision are summarized in Table 1. Accuracy was defined as percent accuracy (% accuracy) and precision was expressed by the percent relative standard deviation (% RSD).

No significant matrix effect was observed in standards prepared in Ringer's and ACSF solution. The matrix effects observed in biological samples such as plasma and urine are not commonly seen in microdialysis samples due to the filtration process undergone by the samples through use of a probe membrane (cut-off 20,000 Da).

**Table 1**  
Intra- and inter-day accuracy and precision of mitragynine.

Statistical variable	Concentration (ng/mL)		
	10	500	1000
Intra-day			
% Accuracy	81.13	91.90	97.38
Precision (% RSD)	–6.91	2.33	0.81
Inter-day			
% Accuracy	83.71	91.84	93.62
Precision (% RSD)	12.30	2.82	14.71

**Table 2**  
*In vivo* recovery of mitragynine in CMA/20 and CMA/12 using recovery by-loss technique.

Rat number	Percent <i>in vivo</i> recovery (%)	
	CMA 20 probe	CMA 12 probe
1	23.95	16.33
2	20.12	15.34
3	24.86	17.57
4	23.28	15.90
Mean $\pm$ SEM	23.05 $\pm$ 1.03	16.29 $\pm$ 0.47

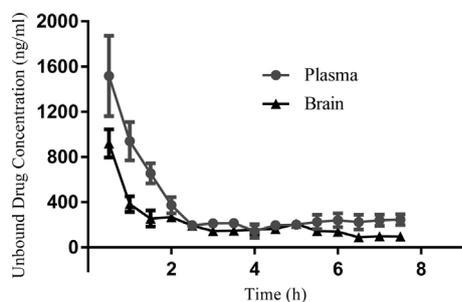
Data are expressed as mean  $\pm$  SEM ( $n = 4$ ).

Ionization interferences of co-eluted matrix components were dramatically improved with liquid–liquid extraction and microdialysis technique [19]. Stability issues (short term 1–4 h in room temperature) was however, not observed. Reports regarding on-bench,  $-20^\circ\text{C}$  freezing and freeze-thaw stability of mitragynine showed that mitragynine is stable in room temperature up to 48 h and more than 1 month in  $-20^\circ\text{C}$  [7].

The selectivity of the method was validated by analysing the blank Ringer's and ACSF samples from four independent rats. A clear chromatogram peak was observed in  $m/z$  399.2 with no other interference peaks exhibited (Fig. 1). The percent of extraction recovery of mitragynine in Ringer's and in ACSF solution were reported to be 77% and 101%, which were consistent over the concentration range 10 to 1000 ng/mL. In summary, this method demonstrated good linearity, accuracy, precision and selectivity. This method was reliable and reproducible and can be used in mitragynine quantification in microdialysates and other pharmacokinetic samples.

#### 3.2. *In vitro* and *in vivo* probe recovery

The *in vitro* recoveries of the CMA/20 and CMA/12 probes were examined in concentration of 10–10,000 ng/mL. The RG for CMA/20 and CMA/12 was determined from the slope and indicated  $50.37 \pm 1.08\%$  and  $21.94 \pm 0.99\%$ , respectively. It demonstrated that the RG of mitragynine was independent of the concentration range explored. Table 2 presents the *in vivo* recovery of mitragynine in four independent rats. The *in vivo* recovery showed  $23.05 \pm 1.03\%$  and  $16.29 \pm 0.47\%$  for CMA/20 and CMA/12 probe without significant variation in this study. Even through *in vitro* recovery is stable and time independent, the use of *in vitro* recovery for *in vivo* drug concentration prediction is very limited because of the substantial difference in diffusion rates for organs and tissues from clean solution [20]. Therefore, back calculations of the actual concentration of the compound in extracellular fluids using *in vivo* probe recovery will be more reliable and accurate to represent the actual physiological concentration [20,21]. Nevertheless, *in vitro* recovery will be useful for method optimization, validation and primary screening of compounds.



**Fig. 2.** Unbound concentration–time profile of mitragynine after an intravenous administration of 10 mg/kg in rats. The data are expressed as mean  $\pm$  SEM ( $n = 6$ ).

**Table 3**

Pharmacokinetic parameters calculated based on unbound concentration of mitragynine in rat blood and brain after an intravenous (10 mg/kg) administration. The data were analysed using two-compartmental pharmacokinetic technique and expressed as mean  $\pm$  SEM ( $n = 6$ ).

Parameters	Unit	IV	
		Plasma	Brain
$K_e$	1/h	0.24 $\pm$ 0.03	0.23 $\pm$ 0.01
$T_{1/2\beta}$	h	13.14 $\pm$ 1.42	13.22 $\pm$ 2.55
$T_{max}$	h	0.5	0.5
$C_{max}$	$\mu$ g/mL	1.52 $\pm$ 0.15	0.92 $\pm$ 0.05
AUC <sub>0-t</sub>	$\mu$ g h/mL	3.47 $\pm$ 0.26	2.24 $\pm$ 0.11
AUC <sub>0-∞</sub>	$\mu$ g h/mL	4.63 $\pm$ 0.44	2.68 $\pm$ 0.14
$V_d$	L/kg	9.84 $\pm$ 0.62	16.94 $\pm$ 1.11
Clearance	L/h/kg	2.26 $\pm$ 0.21	3.78 $\pm$ 0.18

### 3.3. Pharmacokinetics of mitragynine

Fig. 2 shows the concentration–time curve for mitragynine in both blood and brain. The summaries of fundamental PK parameters are listed in Table 3.

Gender-based pharmacokinetic variation is rare for most of the drugs in the market. According to the Food and Drug Administration (FDA), females are apparently more sensitive to drugs as compared with males in terms of drug efficacy and adverse effects. Besides, drugs tend to stay longer and produce greater drug exposure in females due to the physiological differences [22]. Hence, female rats were used throughout the study on determination of the pharmacokinetics of mitragynine. An *in vivo* pilot study was performed to obtain basic information regarding the feasibility of microdialysis technique. Initially, intravenous (5 mg/kg) dose in normal saline was used as reported in conventional PK studies [7,14]. Due to the limitation of microdialysis and unfavourable drug properties such as poor aqueous solubility, high plasma protein binding, the *i.v.* administrated mitragynine was unable to achieve the desired systemic exposure profile in both blood and brain [10,15]. In order to overcome the limitations of the experiment, higher dosage of 10 mg/kg and 10% Tween 20 surfactant was added into the normal saline to improve the solubility of the compound in aqueous solution. Moreover, the dose used in the actual study was closer to the reported antinociceptive dose (*i.v.*, 1–10 mg/kg) in rodents [3,4]. The maximal tolerance dose of surfactant however, should be kept low at 5–10% (*i.v.* and *p.o.*) as recommended for early formulaion study [23].

Mitragynine is a weakly basic drug, this being mainly contributed by the amine groups in its chemical structure. Hence, mitragynine is more predominant in its ionized form and is water soluble in acidic environment such as stomach and upper duodenum. Inversely, it exhibited its non-ionized form and lipophilic properties in basic pH in the lower small intestinal [10,15]. Lipophilic drugs usually express good intestinal permeability, plasma protein binding and BBB penetration in both *in vitro*

and *in vivo* [24]. In this study, unbound mitragynine was able to be detected in both blood and brain within 30 min after the intravenous administration, as demonstrated by rapid and good BBB permeability, indicating  $C_{max}$  value of 1516  $\pm$  145.32 ng/mL and 920  $\pm$  51.16 ng/mL in blood and brain respectively. An earlier time point was not achievable due to the limitation of the sampling method for microdialysis.

Mitragynine elimination parameters were analysed using a two-compartmental drug elimination model which is characterized by a biphasic drug elimination response in log unbound concentration–time profile. In a two compartmental model, a sharp decrease of initial plasma concentration (distribution phase) which was followed by slow drug clearance (elimination phase) was observed, whereby an extraordinary long beta phase half-life ( $T_{1/2\beta}$ , 13 h) was reported. Traceable amount of mitragynine remains detected after 8 h of drug injection. Similar finding was observed in human and rats pharmacokinetic studies using conventional method [8]. High  $V_d$  values (9.8 L/kg in plasma and 16.9 L/kg in brain) that were observed indicated extensive distribution of mitragynine to tissue after the intravenous administration. Since microdialysis is only measuring the unbound fraction of the drug, hence, the high  $V_d$  value could be due to the high plasma protein binding which has been reported in *in vitro* studies [15]. Nevertheless, the high lipophilicity and good membrane permeability of mitragynine was predicted to result in high distribution to the organ or tissues especially the adipose tissue. Together with the low clearance and high metabolic stability, drug clearance from the body was further prolonged [15]. The low bioavailability was explained previously by Ramanathan's study, in which it was stated that mitragynine was unstable and slowly degraded in highly acidic gastric juice [10]. Bioavailability of 3.03% was reported in Parthasarathy's conventional PK study using HPLC–UV method [7]. Bioavailability issues may explain the inconsistency in drug effectiveness, high subject variability and lack of dose proportionality when given orally [25].

The pharmacokinetic studies were carried out in freely moving rats instead of an anaesthetised rat model in order to avoid the anaesthesia induced BBB permeability [26]. Since mitragynine was reported to act on the opioid receptor especially in the supraspinal region, the understanding of BBB permeability is crucial for dose estimation for a therapeutic intention. From the graph in Fig. 2, mitragynine showed good permeability across the blood brain barrier (BBB) with AUC<sub>brain</sub>/AUC<sub>plasma</sub> ratio 65.83  $\pm$  4.54% after an intravenous injection. The result was in contrast with the reported high *in vivo* plasma protein binding of mitragynine [15]. Compounds with high plasma protein binding typically have difficulty to cross the BBB permeability [27,28]. Even through relationship between lipophilicity and permeability has been well established, the fact is, lipophilicity is not always predictive of permeability [24]. Penetration of BBB can be affected by several factors such as the rate of drug–protein dissociation, active transportation, and the adjuvant or surfactant used. In this study, Tween 20 was used to overcome the solubility issue in saline and blood. As a consequence, it may enhance the drug permeability into the CNS [29,30]. Further investigations such as *in vivo* efficacy dose and toxicokinetic studies are necessary to complete the PK/PD profile. Nevertheless, a good oral formulation is needed to improve the gastric stability and better intestinal absorption.

### 4. Conclusion

In order to realise the potential of mitragynine to be developed into an opioid replacement drug, the pharmacokinetics and BBB permeability properties were intensively investigated in this study. Mitragynine demonstrated two compartmental drug elimi-

nation following an intravenous drug administration and showed high effectiveness in BBB penetration. The slow clearance and drug releasing properties of mitragynine in the elimination phase would be an advantage for opioid withdrawal therapy where high frequency of repeated dose can be avoided provided that no side effects occur. *In vivo* correlation of PK/PD, as well as toxicity studies of mitragynine will be further investigated to complete the preclinical profile of this compound.

### Acknowledgement

The research was funded by postgraduate research grant (PPP) University of Malaya (no. PG143-2014B).

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