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Original Research Article

Effect of Wuling powder on the pharmacokinetics of valproic acid in epileptic rats

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Abstract

Purpose: *To investigate the effect of Wuling powder (WP) on the pharmacokinetics of valproic acid (VPA) in epileptic rats.*

Methods: *A model of epilepsy was established in SD rats by intraperitoneal injection of pentylenetetrazole (PTZ). Twelve epileptic rats were randomly divided into two groups: control group given oral VPA alone at a dose of 180 mg/kg VPA, and drug combination group orally given VPA (180 mg/kg) co-administered with WP at a dose of 200 mg/kg. Blood sample (0.5 mL) was collected at 15, 30, 60, 120, 240 and 720 min after drug administration for measurement of plasma concentrations of VPA using ultraperformance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS).*

Results: *The AUC (0-480min) and maximum plasma concentrations (C_{max}) of VPA in the drug combination group were significantly higher than those in the control group ($p < 0.01$). The half-time ($t_{1/2}$) and time taken to attain maximum plasma VPA concentration (T_{max}) in the combination group were extended, when compared to control group ($p < 0.05$).*

Conclusion: *These results demonstrate that WP increases the plasma concentration of VPA and affects the pharmacokinetic properties of VPA in epileptic rats. Thus, the pharmacodynamic influence of this interaction should be taken into consideration while prescribing WP to epileptic patients already taking VPA.*

Keywords: *Wuling powder, Valproic acid, Pharmacokinetics, Epilepsy*

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INTRODUCTION

Epilepsy is a highly prevalent chronic neurologic disorder which results in social, behavioral, health and economic consequences to the patients and their families [1]. It is estimated that more than 50 million people worldwide are affected by epilepsy [2]. The vast majority of epileptic patients live normal lives after appropriate treatment, but about one- third of the patients have drug-refractory epilepsy which seriously endangers their physical and mental health [3,4].

Valproic acid (VPA) is an anti-epileptic drug used widely for monotherapy or adjunctive therapy of

epilepsy [5]. However, VPA has a narrow therapeutic window, with unstable correlation between serum concentration and dose, which may cause adverse reactions such as teratogenesis, liver injury and hematopoietic injury, thereby endangering the lives of patients [6,7]. The therapeutic dose range of VPA in blood or serum is 50 - 100 μg/mL [8]. Below this concentration range, there is no effective therapeutic effect, while beyond this concentration, the toxicity increases, with no therapeutic effect [9]. Thus, therapeutic drug monitoring (TDM) of the VPA is widely used in the treatment of epilepsy to ensure treatment efficacy and avoid adverse reactions [10].

Traditional Chinese medicine (TCM) is characterized by low side effects, definite curative effects, overall adjustment and low cost, and it has received lots of attention in the treatment of epilepsy [11]. It has been found that some TCMs, bioactive components and their preparations are effective in the control of epilepsy [12]. When combined with western medicines, there are enhanced anti-epileptic effects and reduction of the adverse reactions caused by long-term western medicine. Studies have shown that Chinese and Western medicine interact through pharmacokinetics [13].

Wuling powder is a traditional TCM extracted from the mycelia of *Xylaria Nigripes* ***(KI.)*** [14]. It has been shown that WP exerts anti-epileptic effect and potent anti-depressant property, and it ameliorates memory impairment induced by epilepsy [15]. Moreover, it enhances urate excretion and improves kidney function [16,17]. It has been reported that the combination of WP and traditional western medicine produced therapeutic effect on epilepsy [18,19]. However, there are no published studies on the effect of WP on plasma pharmacokinetics of VPA in epileptic rats. In this study, the effect of WP on VPA pharmacokinetics was investigated.

EXPERIMENTAL

Animals and experimental conditions

Twelve male Sprague-Dawley (SD) rats weighing 200 ± 20 g were kept in colony cages with *ad libitum* access to feed and tap water under standardized conditions (natural light-dark cycle, temperature of 22 ± 3 °C and relative humidity of 40%). The SD male rats were obtained from the Laboratory Animal Service Centre of Lanzhou University (Medical Experimental Animal no. SCXY(G) 2018-0002). All animal experimental procedures were conducted according to the Ethical Procedures and Guidelines of the People's Republic of China. The study was approved by the Ethics Committee of The Second Hospital of Lanzhou University (ref no. 2019A-046).

Study design

Twelve male SD rats were intraperitoneally injected with PTZ at a dose of 35 mg/kg once daily for 28 days. After establishment of the epileptic model, twelve epileptic rats were randomly divided into two groups (n = 6): control group given oral VPA alone at a dose of 180 mg/kg VPA, and drug combination group orally given VPA (180 mg/kg) co-administered with WP at a dose of 200 mg/kg. In the combination group, epileptic rats were administered the drugs via gavage for 7 days. Following fasting for one night, WP was administered before VPA. In the control group, VPA (180 mg/kg) was administered with gavage on the eighth day. The doses of drugs administered to animals were calculated accordingly to body surface area using Meeh-Rubner equation. The VPA and WP were dissolved in normal saline solution before use.

Blood sampling

Approximately 0.5 mL of retro-orbital blood was collected in heparinized tubes before administration and post-dosing at 5, 15, 30, 45, 60, 90, 120, 240, 360, 480 min. The blood samples were centrifuged at 14000 rpm for 25 min, and 200μL of plasma was kept at −80 ℃ until further analysis.

Instrumentation and chromatographic conditions

Liquid chromatography

The chromatographic separation was performed in Thermo Hypersil GOLD C18 column (100 × 2.1 mm, 1.9 μm, Thermo, USA) maintained at 30 °C. The mobile phase consisted of acetonitrile (A) and aqueous solution (B) at a flow rate of 0.25 ml/min, using gradient elution method (0 min: A:B ratio = 65:35, 1.15 min: A:B ratio = 65 :35, 1.2 min : A:B ratio = 90:10, 2.05 min: A:B ratio = 90:10 , 2.1 min: A: B ratio = 65: 35, and 4 min: A:B ratio = 65:35).

Mass spectrometric conditions

Samples were analyzed using a TSQ quantum ultra-mass spectrometric detector with electrospray ionization source (ESI) (Termo, USA) coupled with a Dionex Ultimate 3000 ultra-performance liquid chromatography system. The

operating conditions for mass spectrometry were as follows: electrospray voltage of 3000 V, capillary temperature of 300 ℃, sheath gas pressure of 241.38 kPa, collision gas pressure of 0.13 Pa, and auxiliary gas flow of 2.67 L/min.

Preparation of standard solutions

Standard stock solutions of VPA (1 mg/mL) and benzoic acid (IS; 0.17 mg/mL) were prepared by dissolving the compounds in methanol. The standard stock solutions were stored at 4°C.

Plasma sample preparation

Rat plasma sample (200 µL) was added to IS (10µL) and acetonitrile (400 µL), and vortex-mixed for 1.0 min, followed by centrifugation at 14000 rpm for 5 min. The supernatant was diluted with acetonitrile and treated with 0.22 µm microporous membrane. Then, 5 µL aliquot of supernatant was used for analysis.

Assessment of linearity

The VPA stock solution was diluted with methanol to yield solutions with concentrations of 3, 2.4, 2, 1.6, 0.8, 0.4, 0.2, 0.1 mg/mL. Then, 10 µL of each solution was added to 200µL blank plasma. A series of standard samples of concentrations 150, 120, 100, 80, 40, 20, 10, 5 µg/mL were prepared, and 10 µL IS and 400 µL acetonitrile were added to each standard sample. The mixtures were oscillated for 60 sec and centrifuged at 14000 rpm for 8 min. The supernatants were diluted with acetonitrile and treated with 0.22 µm microporous membrane. Then, 5 µL aliquot was taken for analysis. The calibration curves for VPA were prepared by plotting the peak–area ratio of VPA to IS against the plasma concentrations of VPA. The linear range was subjected to validation procedure.

Determination of specificity of method

Specificity was assessed by assaying blank plasma to investigate potential interferences from endogenous compounds and related metabolites in plasma. Comparison was performed on chromatograms of blank plasma, plasma spiked with analytes and IS, as well as plasma samples after oral administration of VPA.

Blank plasma (200 µL) without IS (replaced by acetonitrile) was treated as indicated *in plasma sample preparation* above. The chromatogram of blank plasma was obtained by system analysis: VPA (10 µL) was added to blank plasma (200 µL) and treated as in *plasma sample preparation*. The chromatogram of blank plasma spiked with VPA was obtained by analysis: IS (10 µL) and VPA (10 µL) were added to the blank plasma (200 µL) and treated as in *plasma sample preparation.* The chromatogram of blank plasma samples spiked with VPA and IS was obtained by analysis. IS (10 µL) was added to rat plasma sample 90min after oral administration of single dosage VPA, treated as in *plasma sample preparation*. Then, 5 µL aliquot was taken for analysis, and the chromatogram was obtained.

Evaluation of precision, accuracy and recovery

Relative standard deviation (RSD) was used to evaluate intra- and inter-day precisions. The intra-day precision was analyzed with quality control samples of low (10 µg/mL), middle (70 µg/mL) and high (130 µg/mL) concentrations in five replicates. The concentrations of quality control samples of low (10 µg/mL), middle (70 µg/mL) and high (130 µg/mL) in the linear range were determined for three continuous days for analysis of inter-day precision. The intra-day and inter-day precisions should be below 15 %. Blank plasma samples of epileptic rats (200-µL portions) were used to prepare quality control samples of VPA at low (10 µg/mL), medium (70 µg/mL) and high (130 µg/mL) concentrations, with 5 replicates per concentration. In line with the method used for assessment of linearity, 5 µL sample was used for calculation of accuracy. The accuracy must be within ± 15 %.

Extraction recovery

Extraction recovery is an indication of the acceptability of the new method used in the study. Blank plasma of epileptic rats (200-µL portions) were used to prepare quality control samples of VPA at low (10 µg/mL), medium (70 µg/mL) and high (130 µg/mL) concentrations (five replicates per concentration). Then, in line with method used in investigation of linear relationship and assessment of linearity, 5 µL of sample was used to calculate the post-extraction concentration (A). The extraction recovery was determined by calculating the ratio of the amount of the extracted compounds from drug-free plasma spiked with low, medium and high concentrations of VPA, to the amount of these compounds added at the same concentrations to methanol (B). The percentage of extraction recovery was calculated as *(A/B) x 100*.

Determination of stability

The stability of VPA in epileptic rat plasma was determined by analyzing replicates (n=3) of plasma samples at low (10 µg/mL), medium (70

μg/mL) and high concentrations (130 μg/mL) under three different storage conditions i.e. storage for 12 h at room temperature, storage at–20 ℃ for 12h, and storage at–20 ℃ for 12h, followed by four freeze–thaw cycles from −20 °C to room temperature.

Long-term stability was determined after storage of the quality control samples at −20 ℃ for 7 days. In line with method used in assessment of linearity, 5 μL sample was taken to determine stability. The results were compared with those obtained for freshly-prepared plasma samples. The stability of IS was determined in a similar way.

Determination of pharmacokinetic parameters

The pharmacokinetic parameters evaluated for VPA were maximal plasma concentration (C_{max}), time after dose at which C_{max} occurred (T_{max}), plasma elimination half-life ($T_{1/2z}$), resident time ($MRT_{(0\text{-}480min)}$), area under the plasma concentration-time curve (AUC), and apparent total body clearance (CLz/F).

Statistical analysis

The DAS3.0 pharmacokinetic analysis software was used to analyze data for plasma concentrations of VPA, and SPSS 22.0 was used for statistical analysis. All data are expressed as mean ± standard deviation (SD). Statistical differences between the two groups were analyzed with unpaired Student's *'t- test.* Statistical significance was assumed at $p < 0.05$ or $p < 0.01$.

RESULTS

Linear relationship

The calibration curves were prepared using a least square linear regression model (Eq 1).

$y = mx + b$ …….. *(1)*

where *y* represents the peak area ratio, *x* represents the concentration, *m* represents the slope of the calibration curve, and *b* represents intercept on the y-axis of the calibration curve.

The equation for the calibration curve of VPA is as shown in Eq 2.

$y = 0.01262x - 0.02101$, $R^2 = 0.9964$ ……. *(2)*

where *y* represents the ratio of peak area to that of IS, *x* is plasma concentration of IS, and R^2 is the regression coefficient. The linear range was 5 - 150 μg/mL, and the lower limit of quantification was 5 μg/mL. These results provided sufficient sensitivity for subsequent pharmacokinetic studies.

Specificity of method

The levels of VPA and IS were determined with electrospray ionization tandem mass spectrometry in the negative ion mode using selective reaction monitoring (SRM). No evident interference was found at the elution times: VPA and IS were eluted at 1.77 and 1.22 min, respectively. The representative chromatograms are shown in Figure 1 and Figure 2.

Precision, accuracy and extraction recovery

The precision of the method was determined by analyzing three different concentrations of VPA (10, 70 and 130 $\mu g \cdot mL^{-1}$) over three validation days. The inter-day precision (RSD) values were 7.21, 4.33 and 3.97 %, respectively, while the inter-day precisions were 8.25, 3.48 and 3.77 %, respectively. The accuracy (RE) values of VPA at three different concentrations were 8.91, 4.63 and 3.83%, respectively. The mean recoveries of VPA were 89.68, 93.70 and 91.62 % at concentrations of 10, 70, 130 μg/mL, respectively. These results are presented on Table 1. The precision and accuracy achieved with this developed method were acceptable.

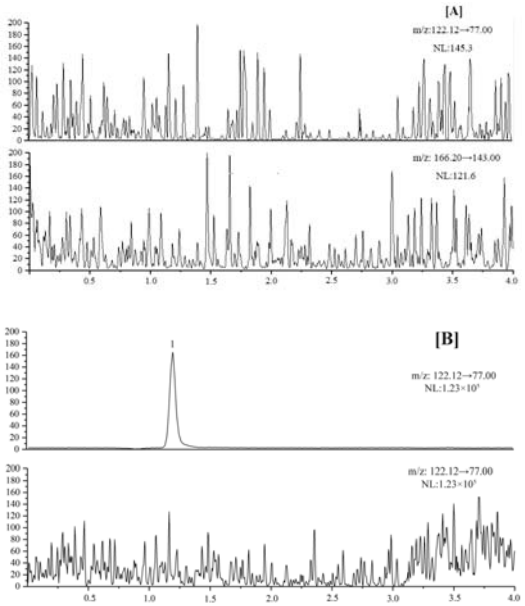

Figure 1: Representative LC-MS chromatograms for VPA (1) and benzoic acid (2, IS) in rat plasma samples. (A) blank plasma sample; (B) blank plasma samples mixed with VPA

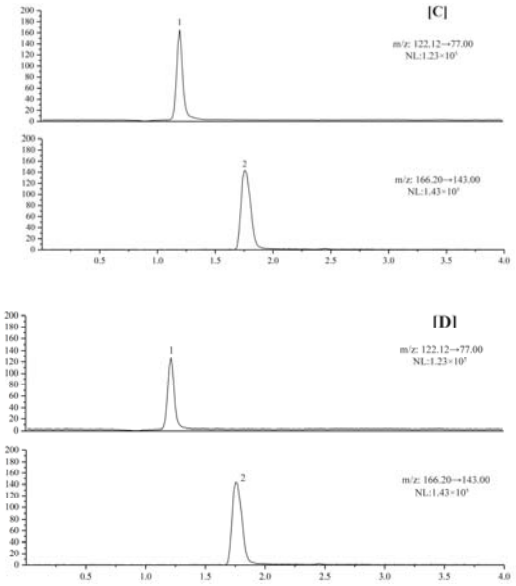

Figure 2: Representative LC-MS chromatograms for VPA (1) and benzoic acid (2, IS) in rat plasma samples. (C) blank plasma samples spiked with VPA and IS; (D) rat plasma sample 90 min after oral administration of VPA spiked with IS

Stability

The results of stability indicated that all analytes in rat plasma were stable at room temperature for 12 h, at -20 ˚C for 12 h, through four freeze–thaw cycles, and at −20 ˚C for 7 days. Stability was evaluated for IS in a similar way. The results of stability experiments are presented in Table 2.

Effect of WP on the pharmacokinetics of VPA in epileptic rats

After single-dose VPA administration and co-administration with WP, blood samples of epileptic rats were collected at indicated time points, and the plasma concentrations of VPA were determined. The mean plasma VPA concentration–time profiles are shown in Figure 2. The data were analyzed with DAS3.0 software, and the results are shown in Table 3.

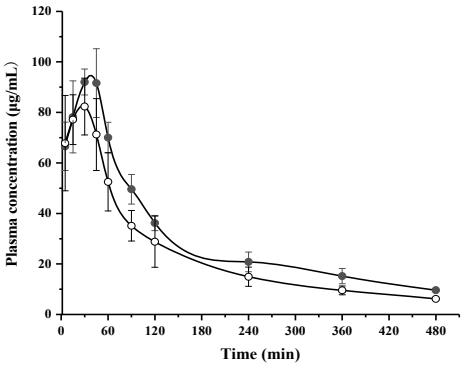

Figure 3: Mean plasma concentration-time curve of the oral administration of VPA without - - or with - - co-administration of WP. Error bars represent standard deviation. Abbreviations: min = minute; VPA = valproic acid; WP = *Wuling* powder

DISCUSSION

Epilepsy is a common neurological disease caused by abnormal discharge from neurons in the brain. Valproic acid (VPA) is a clinically popular anti-epileptic drug which increases the content of gamma-aminobutyric acid (GABA) in the brain, thereby inhibiting epileptic seizures. However, VPA has a narrow therapeutic window. Thus, therapeutic drug monitoring (TDM) of VPA is widely used in the treatment of epilepsy. At present, the commonly used detection methods include fluorescence polarization immunoassay [20], as well as gas chromatography and high-performance liquid chromatography [21].

Table 1: Precision, accuracy and recovery for VPA in rat plasma (n = 5)

Drug	Concentration (μg/mL)	RSD (%)			Recovery	
		Intra-day	*Inter-day*	*Accuracy*	*Mean*	*RSD (%)*
VPA	10	7.21	8.25	8.91	89.68	4.21
	70	4.33	3.48	4.63	93.70	4.38
	130	3.97	3.77	3.83	91.62	5.36

Table 2: Stability of VPA in rat plasma under different conditions (n = 3)

Drug	RSD (%)				
	Concentration (μg/mL)	12 h room temperature	-20 (12 h)	Freeze-thaw	−20 (7days)
VPA	10	8.21	9.02	9.53	11.26
	70	3.86	4.67	4.78	9.25
	130	3.24	3.01	3.21	8.46

Table 3 : Pharmacokinetic parameters and statistical comparisons of VPA with and without WP in pentylenetetrazole-induced epileptic rats

Parameter	VPA	VPA+WP
$AUC_{(0\text{-}480min)}$ (μg/mL·h)	193.42±12.7 9	257±14.62*
$T_{1/2z}$ (h)	2.40±0.25	3.15±0.36#
T_{max} (h)	0.5±0.10	0.75±0.10#
CLz/F(L/h/kg)	0.90±0.12	0.65±0.01
C_{max} (μg/mL)	84.96±3.02	95.57±4.26*
$MRT_{(0\text{-}480min)}$ (h)	2.24±0.89	2.46±0.63

All values are presented as the mean ± SD; #$p < 0.05$, *$p < 0.01$ vs VPA single-dose group; min = minute; VPA, valproic acid; WP, *Wuling* powder

However, due to the symmetry in VPA structure, some detection methods need pre-column derivatization which not only increases the complexity of operation, but also increases measurement error. In contrast, ultra-performance liquid chromatography mass spectrometry (UPLC-MS/MS) does not need pre-column derivatization, which simplifies the operation procedure.

In this study, visual observations revealed that plasma concentrations of VPA increased significantly after VPA co-administration with WP. The area under the plasma concentration–time curve (AUC) and maximal plasma concentration (C_{max}) increased significantly, while the apparent total body clearance (CLz/F) decreased significantly. These results indicate that WP increased the plasma concentrations of VPA in epileptic rats and affected its pharmacokinetic properties.

Previous studies have indicated many possible mechanisms that may be involved in the increase in plasma concentrations of VPA in epileptic rats. Following intestinal absorption, valproic acid is highly bound to plasma albumin, and it undergoes glucuronidation in the liver prior to renal excretion [22]. Valproic acid is eliminated via extensive biotransformation through multiple pathways, including mitochondrion-mediated β-oxidation, glucuronidation and cytochrome P-450 (CYP450)-catalyzed oxidation. The glucuronide derivative is the main metabolic pathway of valproic acid. It is hydrolyzed by β-glucuronidase and undergoes enterohepatic circulation (EHC), and is secreted via the bile into the intestine where it is hydrolyzed by enterobacteria to liberate the parent drug, which is then reabsorbed [23]. It is evident from the mean plasma VPA concentration–time profiles with and without WP, and pharmacokinetic parameters, that WP slowed down the absorption of VPA in epileptic rats, while it increased its peak concentration and slowed down its rate of elimination. Therefore, it can be inferred that WP may increase the plasma concentration of VPA in epileptic rats by inhibiting VPA-glucuronidase and β-oxidation pathways. *In vitro* and *in vivo* studies have shown that CYP2C9 and CYP2C19 are the main metabolic enzymes in the biotransformation of VPA [24]. Therefore, it can be inferred that WP inhibits the activities of these metabolic enzymes, and it very likely involved in raising the plasma concentration of VPA.

Valproic acid (VPA), a first-line anti-epileptic drug, has been found to cause liver damage in patients [25]. In a previous study on the effect of WP on VPA-induced liver injury in epileptic rats, it was shown that inflammatory reaction in hepatocytes was significantly decreased, while the number of hepatocytes was significantly increased. Moreover, edema and steatosis were mitigated, and serum contents of ALP, ALT, AST and r-GT were decreased, indicating that WP mitigated VPA-induced liver injury in the epileptic rats [26]. Thus, the liver plays an important role in the pharmacokinetics of VPA in epileptic rats. Therefore, WP may affect the metabolism of VPA by alleviating the liver damage caused by VPA.

CONCLUSION

A simple and highly sensitive UPLC-MS/MS method was developed and validated for determination of plasma concentration and pharmacokinetic studies of VPA in epileptic rats. The results indicated that WP affected the pharmacokinetic properties of VPA by increasing the plasma concentration of VPA and slowing down its elimination in epileptic rats. These findings provide theoretical support for the clinical application of VPA and promotion of the combination of Chinese and western medicine in the treatment of epilepsy.

DECLARATIONS

Acknowledgement

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Conflict of interest

No conflict of interest is associated with this study.

Contribution of authors

The authors declare that this work was done by the authors named in this manuscript and all liabilities pertaining to claims relating to the content of this article will be borne by them. This study was performed by Yujun Qiao and Yuting Liu. Chen Jia, Rui Zhang and Wen Yin collected the data. Jiali Zhang and Aijia Cao wrote the manuscript. All the experiments were supervised by Haisheng Jiao. Yujun Qiao and Yuting Liu should be considered co-first authors.

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