LC-MS/MS assay for the therapeutic drug monitoring of perampanel in children with drug-resistant epilepsy

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ABSTRACT

Perampanel (PER) is the first clinically available selective antagonist of α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptor approved globally for the treatment of epilepsy. Studies have recently underlined the significant association between dose-exposure-effect-adverse events of PER in patients with epilepsy, so the therapeutic drug monitoring (TDM) of PER is critical in clinical practices, especially for pediatric patients with drug-resistant epilepsy. Due to several limits in previous published analytical methods, herein, we describe the development and validation of a novel liquid chromatography tandem mass spectrometry (LC-MS/MS) method for monitoring PER in human plasma samples. Protein precipitation method by acetonitrile containing PER-d5 as internal standard was applied for the sample clean-up. Formic acid (FA, 0.2 mM) in both aqueous water and acetonitrile were used as the mobile phases and the analyte was separated by an isocratic elution. Qualification and quantification were performed under positive electrospray ionization (ESI) mode using the m/z 350.3 → 219.1 and 355.3 → 220.0 ions pairs transitions for PER and PER-d5, respectively. Potential co-medications of anti-seizure medications (ASMs) have no interference to the analysis. Calibration curves were linear in the concentration range of 1.00–2,000 ng mL⁻¹ for PER. The intra- and inter-batch precision, accuracy, recovery, dilution integrity, and stability of the method were all within the acceptable criteria and no matrix effect or carryover was found. This method was then successfully implemented on the TDM of PER in Chinese children with drug-resistant epilepsy. We firstly confirmed the apparent inter- and intra-individual PER concentration variabilities and potential drug-drug interactions between PER and several concomitant ASMs occurred in Chinese pediatric patients, which were also in line with previous studies in patients of other race.

KEYWORDS

perampanel, LC-MS/MS, therapeutic drug monitoring, drug-resistant epilepsy, drug-drug interactions

1. INTRODUCTION

Perampanel (PER), a novel anti-seizure medication (ASM), is the first clinically available selective non-competitive α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptor antagonist [1], which reduces the neuronal excitability through inhibiting glutamatergic postsynaptic transmission by selective inhibition of AMPA receptors [2]. PER was approved by the U.S. Food and Drug Administration (FDA) and the European Medicines
Agency (EMA) for the treatment of partial-onset seizures (POS) with or without secondarily generalized seizures in patients ≥4 years of age and as an adjunctive therapy for primary generalized tonic-clonic seizure (GTCS) in patients with epilepsy ≥12 years of age [3]. Most recently, the National Medical Products Administration (NMPA) of China has approved PER as a monotherapy or an adjunctive treatment for partial-onset seizures (with or without secondarily generalized seizures) in patients with epilepsy aged at 4 years and older [4].

Several studies have confirmed the significant association between dose-exposure (plasma concentration)-effect-adverse events (AEs) of PER in adult and pediatric patients with epilepsy [5–9], but the reference concentration ranges of PER are still controversial [5, 10]. On the other hand, PER is substantially (approximately 95%) bound to plasma proteins [11] and metabolized by cytochrome P450 (CYP450) enzyme, especially for CYP3A4 [9, 12–14], which partly determine the efficacy and AEs related to the plasma concentration of PER [5]. Therefore, precise individual PER therapy regimen is a vital strategy to achieve optimal clinical outcomes, and this would be guided by therapeutic drug monitoring (TDM) [15–17]. Satisfactory TDM is partly dependent on the availability of rapid and convenient biochemical analysis for measuring PER in blood or other biological samples.

Several analytical methods have been proposed for the evaluation of PER concentration in human plasma or serum [1, 13, 18–23]. As shown in Table 1, several shortcomings existed in these assays are worthy of improvement [24], such as the large sample volume required for analysis (e.g. 100–1,000 μL), complex sample pretreatment processes (e.g. liquid-liquid extraction), narrow quantitative range, and long running time. Therefore, it is necessary to develop a novel determination method of PER that suit for clinical application.

Hitherto, because of the excellent intrinsic sensitivity and selectivity, liquid chromatography tandem mass spectrometry (LC-MS/MS) methods are considered as the gold standard for the TDM of various medications such as ASMs [24]. In this study, we developed and validated a sensitive, convenient, and robust LC-MS/MS method for the measurement of PER in human plasma samples. Most importantly, the developed method was successfully applied to quantify PER concentrations in Chinese pediatric patients with drug-resistant epilepsy involved infants within 4 years of age.

2. EXPERIMENTAL

2.1. Chemicals and materials

The reference standard of perampanel (batch No. 21J079-X4; purity: 98%) and perampanel-d5 (batch No. 4-LDO-89-3; purity: 98%), which was used as the internal standard (IS), was purchased from Shanghai ZZbio, Co., Ltd, (Shanghai, China). HPLC-grade acetonitrile and methanol were purchased from the Merck KGaA (Darmstadt, Germany). Isopropanol of HPLC grade was purchased from Fisher Scientific (Fairlawn, U.S.). HPLC-grade formic acid (FA) was supplied by Tedia Company, Inc. (Fairfield, U.S.). Ammonium acetate (NH4Ac, ACS Reagent) was provided by Sigma-Aldrich, Inc. (Burlington, U.S.). Ultrapure water was prepared using an in-house Milli-Q water purification system (Millipore, Bedford, MA, U.S.). All other chemicals used were of analytical grade or better.

Normal blank, hemolyzed and lipemic human plasma were obtained from the Blood Transfusion Center of Children’s Hospital of Nanjing Medical University (Nanjing, China).

2.2. Chromatographic and mass spectrometric conditions

The LC-MS/MS system consisted of a Triple Quad™ 4500MD mass spectrometer (AB Sciex Pte. Ltd, Singapore) interfaced via a Turbo V™ ion source with a Japer™ liquid chromatography system (AB Sciex Pte. Ltd, Singapore), which comprises a binary pump (Sciex Dx™), an online degasser (Sciex Dx™), an autosampler (Sciex Dx™), and a column oven (Sciex Dx™). The AB-SCIEX Analyst software package (version 1.6.3) was used to control the LC-MS/MS system, as well as for data acquisition and processing.

The chromatographic separation was conducted on a Kinetex® C18 column (2.1 × 50 mm, 5 μm, 100 Å, Phenomenex) at 30 °C, before which a security Guard Cartridges Kit C18 column (4 × 2.0 mm, Phenomenex) was installed. Isocratic elution was applied using mobile phases consisting of 0.2 mM FA in both aqueous water (phase A) and acetonitrile (phase B) delivered at a flow rate of 0.35 mL min⁻¹. The autosampler were kept at 4 °C.

Mass spectrometer was operated in the electrospray ionization (ESI) positive ion mode. Pneumatically nebulized ESI spraying was achieved by using inner coaxial nebulizer N2 gas (GS1) of 40 psi through a Turboionspray probe, a voltage of +3000 V was applied to the sprayer tip and heated dry N2 gas (GS2) of 50 psi at 650 °C from two turbo heaters adjacent to the probe. To prevent solvent droplets entering and contaminating the ion optics, a curtain N2 gas (CUR) of 25 psi was applied between the curtain plate and the orifice. The in-source collision gas (CAD) flow was set at level 8. Qualification and quantification were operated using multiple reaction monitoring (MRM) mode with the following transitions: m/z 350.3 → 219.1 for PER (Fig. 1A) and m/z 355.3 → 220.0 for PER-d5 (Fig. 1B). Specific mass spectrometric parameters of RER and IS are shown in Table 2.

2.3. Stock solutions, calibration curve and quality control (QC) samples

Stock solutions (1.00 mg mL⁻¹) of PER and IS were dissolved in acetonitrile and then diluted to working solutions with acetonitrile: water (1:1; v/v). All solutions were stored at −20 °C before use. The stability of the stock solutions was confirmed by comparing measurements of freshly prepared stock solutions with stock solutions stored for at least 1 month.
Table 1. Comparison of this study with the published analytical methods for PER

<table>
<thead>
<tr>
<th>Year</th>
<th>Method</th>
<th>Internal standard</th>
<th>Matrix</th>
<th>Matrix volume (µL)</th>
<th>Sample preparation</th>
<th>Elution</th>
<th>Column</th>
<th>Mobile phase</th>
<th>Linearity range (ng mL⁻¹)</th>
<th>Analytical time (min)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>2015</td>
<td>LC-MS/MS</td>
<td>ER-167615</td>
<td>Plasma</td>
<td>100</td>
<td>LLE by MTBE</td>
<td>Isocratic</td>
<td>YMC-Pack Pro C8 column (50 × 3.0 mm)</td>
<td>0.1% AA and 0.1% 200 mM NH₄Ac in water and acetonitrile</td>
<td>2.50-2,000</td>
<td>5</td>
<td>[21]</td>
</tr>
<tr>
<td>2015</td>
<td>HPLC-FLD</td>
<td>ER-167615</td>
<td>Plasma</td>
<td>1,000</td>
<td>LLE by DEE</td>
<td>Isocratic</td>
<td>YMC-Pack Pro C8 column (150 × 4.6 mm, 5 µm)</td>
<td>Acetonitrile–water–AA–sodium acetate (840:560:3:1.8, v/v/v/w)</td>
<td>1.00-500</td>
<td>8</td>
<td>[1]</td>
</tr>
<tr>
<td>2016</td>
<td>HPLC-UV</td>
<td>Promethazine hydrochloride</td>
<td>Plasma</td>
<td>200</td>
<td>PPT by acetonitrile</td>
<td>Isocratic</td>
<td>Merck RP C18 column (100 × 4.6 mm)</td>
<td>0.1% of 85% PA (pH = 2.3) in water and acetonitrile</td>
<td>25-1,000</td>
<td>6</td>
<td>[20]</td>
</tr>
<tr>
<td>2016</td>
<td>LC-MS/MS</td>
<td>NR</td>
<td>Serum</td>
<td>24</td>
<td>PPT by acetonitrile</td>
<td>NR</td>
<td>HiQ sil C18 column</td>
<td>NR</td>
<td>2.00-800</td>
<td>NR</td>
<td>[23]</td>
</tr>
<tr>
<td>2018</td>
<td>HPLC-FLD</td>
<td>Mirtazapine</td>
<td>Plasma</td>
<td>500</td>
<td>PPT by acetonitrile</td>
<td>Isocratic</td>
<td>Kinex-PFP column (100 × 4.6 mm, 2.6 µm)</td>
<td>30 mM sodium acetate (pH = 3.7) and acetonitrile (40:60, v/v)</td>
<td>20-1,000</td>
<td>5.6</td>
<td>[22]</td>
</tr>
<tr>
<td>2018</td>
<td>LC-MS/MS</td>
<td>Levetiracetam-d₆</td>
<td>Plasma</td>
<td>50</td>
<td>PPT by acetonitrile</td>
<td>Gradient</td>
<td>C18 Hypersil Gold column (50 × 2.1 mm, 1.9 µm)</td>
<td>FA in water and acetonitrile</td>
<td>2.50-2,800</td>
<td>10.3</td>
<td>[19]</td>
</tr>
<tr>
<td>2021</td>
<td>HPLC-UV</td>
<td>Ketoprofen</td>
<td>Plasma</td>
<td>100</td>
<td>PPT by methanol</td>
<td>Gradient</td>
<td>Waters Symmetry C18 column (4.6 × 75 mm, 3.5 µm)</td>
<td>0.1% PA in water and methanol</td>
<td>5.00-1,500</td>
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<td>[18]</td>
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<tr>
<td>2021</td>
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<td>Entacapone</td>
<td>Plasma</td>
<td>200</td>
<td>LLE by EA</td>
<td>Gradient</td>
<td>Purospher Star- C18 column (55 × 4 mm, 3 µm)</td>
<td>0.1% of 85% PA in water (pH = 2.79) and acetonitrile</td>
<td>30-4,500</td>
<td>12</td>
<td>[13]</td>
</tr>
<tr>
<td>This study</td>
<td>LC-MS/MS</td>
<td>Perampanel-d₅</td>
<td>Plasma</td>
<td>50</td>
<td>PPT by acetonitrile</td>
<td>Isocratic</td>
<td>Kinex-C18 column (2.1 × 50 mm, 5 µm)</td>
<td>0.2 mM FA in water and acetonitrile</td>
<td>1.00-2,000</td>
<td>2</td>
<td>/</td>
</tr>
</tbody>
</table>

Abbreviations: AA, acetic acid; AF, ammonium formate; C8, octyl carbon chain; C18, octadecyl carbon chain; DAD, diode-array detector; DEE, diethyl ether; EA, ethyl acetate; FA, formic acid; FLD, fluorescence detector; HPLC, high-performance liquid chromatography; LLE, liquid-liquid extraction; MTBE, methyl tert-butyl ether; LC-MS/MS, liquid chromatography tandem mass spectrometry; NH₄Ac, ammonium acetate; NR, not reported; PA, phosphoric acid; PPT, protein precipitation; UV, ultraviolet.
Calibration curve samples and QC samples were diluted from separate stock solutions. Calibration samples were prepared by spiking the working solutions into drug-free blank plasma at a ratio of 1:20 to obtain serial concentrations of 1.00, 2.00, 5.00, 10, 60.0, 120, 600, 1,200, and 2,000 ng mL\(^{-1}\). QC samples, including the lower limit of quantification (LLOQ), low QC (LQC), geometric medium QC (GMQC), middle QC (MQC), high QC (HQC), and upper limit of quantification (ULOQ) were spiked in the same way at concentration levels of 1.00, 2.50, 50.0, 800, 1,600, 2,000 ng mL\(^{-1}\), separately.

2.4. Sample preparation

Plasma samples were prepared by a protein precipitation (PPT) method at a precipitant-to-plasma volume ratio of 6:1 (v/v). In brief, a 50 μL aliquot of plasma sample was mixed with 300 μL of acetonitrile containing the IS (100 ng mL\(^{-1}\)). The mixture was mixed by vortex-shaking for 5 min and centrifuged at 12,000 rpm for 5 min. 50 μL of the supernatant were transferred to another 1.5 mL Eppendorf tube containing 300 μL of acetonitrile: water (1:1; v/v). Then, the mixture was vortexed for 5 min and a 5 μL volume of the mixture were directly applied for the LC-MS/MS analysis.

2.5. Method validation

Full validation of the proposed method was carried out according to the U.S. FDA guidance on bioanalytical method validation: Bioanalytical Method Validation Guidance for Industry (May 2018) [25] to demonstrate the newly developed bioanalytical method was reliable for the intended applications.

2.5.1. Selectivity and specificity (potential impact of ASMs)

Drug-free blank human plasma from six different sources which including the hemolyzed and lipemic plasma were assayed. The chromatograms were inspected for any endogenous peaks that could interfere with PER or the IS. Furthermore, the interference between PER and IS was also assessed using the ULOQ without IS (PER working solution was spiked at the ULOQ concentration but without IS added.
into blank plasma and blank control (QC₀) samples (only IS working solution was spiked into blank plasma).

For the specificity assessment, potential interference due to the presence of possible co-medicated ASMs on the elution of PER and IS was tested. Seventeen commonly prescribing ASMs were added into PER-free blank plasma at their clinically relevant concentrations [21]. The 17 kinds of ASMs used in this study and their concentrations in plasma were valproic acid and lamotrigine, as well as 10-phenytoin, carbamazepine, and oxcarbazepine for 1.00 μg mL⁻¹.

2.5.2. Linearity and limit of quantitation. Linearity was accessed by plotting the peak area ratio of PER/IS versus the analytes’ nominal concentration of the calibration samples, using linear regression analysis with a weighting factor of 1/x². The LLOQ was defined as the lowest concentration on the calibration curve which should be within acceptable limits of accuracy and precision, and the signal-to-noise ratio (S/N) should be no less than 5, at least.

2.5.3. Precision and accuracy. Intra- and inter-batch precision as well as accuracy were evaluated with the analysis of six repeat spiked samples at five different concentrations in three consecutive batches that analyzed on different days. Precision was expressed as percentage of coefficient of variation (CV%) at each concentration, while accuracy was estimated using percentage of bias (Bias%) of QC samples from the nominal concentration. Acceptance criteria for CV% were set at 15% for QCs and 20% for LLOQ samples. Acceptability limits of accuracy and precision, and the signal-to-noise ratio (S/N) should be no less than 5, at least.

2.5.4. Recovery and matrix effect. The extraction recoveries of PER and IS were determined by comparing peak area ratios of PER/IS of extracted samples with those obtained from the extracted blank matrix fortified with the corresponding concentrations of working solutions at the same nominal concentrations.

Matrix effect was assessed by calculating the ratio of peak areas of the extracted blank plasma matrix, fortified with working solutions to those of the analytes in working solutions diluted to corresponding concentrations. IS-normalized matrix factors served as the index for the assessment of the matrix effect of PER.

These two procedures were repeated six times for the three different concentration levels of QC.

2.5.5. Carryover and dilution effect. To assess the carryover effect, blank plasma samples were injected immediately after the ULOQ of the calibration samples. Acceptability criteria was the carryover in the blank sample following the ULOQ samples should not be greater than 20% for PER and 5% for IS of the analyte response at LLOQ samples.

To test the clinical applicability of the method, a dilution of QC samples above the ULOQ (6,000 ng mL⁻¹) with blank plasma matrix to obtain three different concentrations of PER samples (120, 600 and 1,200 ng mL⁻¹) falling within the calibration interval. This experiment was performed in six replicates.

2.5.6. Stability. Stability of PER in plasma was evaluated by analyzing three replicates per concentration of LQC and HQC samples under different storage temperature and time conditions. The short-term (bench-top) stability was evaluated by maintaining QC samples at room temperature for more than 12 h. Long-term stability was assessed at −20°C for a period of 30 days. Post-preparative (autosampler) stability was measured by reanalyzing extracted QC samples under autosampler conditions (4°C) for more than 6 days. Stability of five freeze–thaw cycles was measured by completely thawed QC samples at room temperature and then refrozen at −20°C for at least 24 h until five cycles had been completed.

2.6. Method application in clinical practice

The validated method was applied in clinical practice for TDM of PER in plasma samples of Chinese pediatric subjects admitted to the Children’s Hospital of Nanjing Medical University. Twenty children were diagnosed with drug-resistant epilepsy and receiving different therapeutic regimens that orally treated with PER (Fycompa®, Eisai Co., Ltd., Tokyo, Japan) in combination with other ASMs. The off-label use of PER was consented by the guardian of patients ≤4 years of age before clinicians prescribing PER. The study was conducted in accordance with the Helsinki Declaration and protocol of this study was approved by the ethics committee of Children’s Hospital of Nanjing Medical University (Protocol number: 202008094-1). This study focused on the LC-MS/MS method development, validation, and clinical implementation for routine monitoring plasma PER levels in pediatric patients and reporting results to ordering clinicians. The informed consent to participate is not applicable.

Blood samples were collected at steady-state concentration of PER which defined as the patient had remained on the same PER dosing schedule for at least 21 days [26]. Twenty hours after the administration of PER in the previous evening, blood samples were collected to K₂-EDTA tubes and centrifuged at 4,000 rpm (20°C) for 8 min to obtain plasma. Plasma was immediately separated and frozen at −20°C prior to analysis. Calibration curve standards and spiked QC samples were routinely thawed and analyzed with patient samples. Patients were anonymized and their demographic characteristics, type of epilepsy and seizure condition, PER dosage as well as concomitant ASMs were gathered.

2.7. Statistical analysis

The weight-adjusted dosage and concentration/dose ratios (C₃/D) of PER were processed using IBM SPSS statistics 22.0 (IBM, New York, U.S.). Correlations were tested by the
Spearman’s correlation coefficient analysis. Comparison between two groups was performed by the Mann-Whitney U test. Difference was defined as significant at a probability level of < 0.05.

3. RESULTS AND DISCUSSIONS

3.1. Method development and optimization

3.1.1. Mass spectrometry optimization. Positive and negative ionization ion modes were investigated and compared to obtain satisfied mass spectrometric conditions by infusing standard stock solution of PER and PER-d5 through a syringe pump. MRM in positive mode was finally adopted due to the absence of product ions under negative mode. As shown in Fig. 1, the mass spectra of PER and PER-d5 revealed peaks at m/z 350.3 and 355.3, respectively, as protonated molecular ions [M+H]+. The dominant product ions of PER and PER-d5 for further qualification and quantification are summarized in Table 2. After ascertaining the precursor and product ions, the mass spectrometry parameters that included compound-dependent parameters (DP, EP, CE, CXP, shown in Table 2) and source-dependent parameters (CAD, CUR, GS1, GS2, IonSpray Voltage, Heated Temperature, as shown in the above Section 2.2) were optimized to achieve a satisfactory mass response of the analyte.

3.1.2. Liquid chromatography and sample preparation optimization. Chromatographic conditions were optimized using several assays to achieve a better resolution and peak shape as well as intensity. Firstly, a Kinetex® C18 column (2.1 × 50 mm, 5 μm, 100 Å, Phenomenex) column was chosen for the chromatographic separation of PER and PER-d5 due to the good performances.

Then we investigated the influence of different kinds and concentrations of additives in mobile phases to the sample clean-up. Three concentration levels of QC samples prepared by methanol containing PER-d5 as described in Section 2.4. After that, different concentrations of FA (0.008, 0.04, 0.10, 0.20, 0.50, 1.00, and 5.00 mM) were added in water and methanol as mobile phases. Highest sensitivity was found at 0.20 mM concentration of FA (Fig. 2A). To further explore whether NH4Ac could improve the peak intensity of the analytes, four concentration levels of NH4Ac (0, 0.50, 1.00, 2.00 mM) were added in mobile phases that containing 0.20 mM FA. Interestingly, peak intensity of the analytes only increased slightly in 0.50 mM NH4Ac but decreased obviously in higher concentration of NH4Ac (Fig. 2B), which may be caused by NH4Ac competitively inhibiting the ionization of the analytes [27]. Considering the simplicity and practicality of method, NH4Ac wasn’t spiked in the mobile phases.

To design a simple but effective preparation method, an ingenious crossover design using methanol or acetonitrile as mobile phase B or PPT agent was applied to investigate the effects on peak intensity of the analytes. Shortly speaking, LQC and HQC samples were prepared triplicate by methanol or acetonitrile containing PER-d5 as described in Section 2.4, six samples were analyzed under the mobile phase B as 0.20 mM FA in acetonitrile or methanol, respectively. The results showed that acetonitrile as the precipitation agent as well as phase B could significantly improve the peak intensity and narrow the peak shape of PER (Fig. 3), which might because it has a higher solubility in acetonitrile [28]. Thus, acetonitrile was determined as the precipitation agent while 0.2 mM FA in aqueous water and acetonitrile as the mobile phases, finally.

![Fig. 2. Effects of additives in mobile phases to the detection of PER (blue) and IS (red) in three QC levels. (A): six concentration levels (0.008, 0.04, 0.10, 0.20, 0.50, 1.00, and 5.00 mM) of formic acid (FA) added in water/methanol; (B): four concentration levels (0, 0.50, 1.00, 2.00 mM) of ammonium acetate (NH4Ac) mixed with 0.2 mM FA added in water/methanol.](https://example.com/figure2.png)
To shorten the sample clean-up time, column temperature, flow rate and the ratio of mobile phase A to B were adjusted to 30 °C, 0.35 mL min⁻¹, and 50:50 (v/v) respectively. Moreover, a complex needle wash solution containing acetonitrile: methanol: isopropanol: water (4:4:1:1, v/v/v/v) was utilized to overcome potential carryover effect that may be caused by isocratic elution [29]. PER and PER-d5 were detected at merely 1.14 min, and the system running time was within 2 min in total at these conditions.

It is well known that matrix effect caused by co-eluting endogenous compounds (lipids, phospholipids, fatty acids, etc.) in samples may affect the precision and accuracy of bioanalytical LC-MS/MS methods through suppressing or enhancing the ionization especially in ESI mode, and the stable isotope labeled internal standard (SIL-IS) is highly recommended to eliminate matrix effect since it shows almost identical behavior to the analyte in sample pretreatment, chromatography, as well as in ionization [30, 31]. Thus, the SIL-IS of PER (PER-d5) was applied in this study to overcome matrix effect as much as possible, although it was rarely used in previous studies [32].

3.2. Method validation

3.2.1. Selectivity and specificity. As is shown in Fig. 4, plasma samples have no interference to the detection of the target analytes PER and PER-d5. There is no interference between PER and PER-d5 through calculate the peak intensity of ULOQ without IS and QC0 samples. Benefit from

Fig. 3. Effects of protein precipitation (PPT) agent and mobile phase B to the detection of PER (blue) and IS (red) in two QC levels. M_M: methanol as PPT agent and mobile phase B; M_A: methanol as PPT agent while acetonitrile as mobile phase B; A_M: acetonitrile as PPT agent while acetonitrile as mobile phase B; A_A: acetonitrile as PPT agent and mobile phase B

Fig. 4. Representative LC-MS/MS chromatograms of PER (blue) and IS (red) in extracted human plasma samples. (A) – (C): double blank samples of normal, hemolyzed, and lipemic plasma; (D): double blank sample spiked with 17 kinds of potential co-medicated ASMs; (E): blank control (QC0) samples; (F): upper limit of quantification (ULOQ) without IS sample; (G): lower limit of quantification (LLOQ) sample; (H): plasma sample of children treated with PER and other ASMs
the high selectivity of LC-MS/MS method [33], 17 kinds of potential co-medicated ASMs were confirmed to produce no discernible impact on the analysis of PER and PER-d5 (Fig. 4D).

3.2.2. Linearity and limit of quantitation. Calibration curves were linear over the concentration range of 1.00–2,000 ng mL\(^{-1}\) with a weighting factor of 1/x\(^2\) for PER in plasma. Equations (mean ± SD, n = 6) of the regression lines were \( Y = 0.00331 \pm 0.00007 \) X - 0.000354 (± 0.00016), with the correlation coefficients \( r \geq 0.9970 \). The LLOQ was established at 1.0 ng mL\(^{-1}\) (S/N > 10), which is suitable for the TDM for pediatric patients.

3.2.3. Precision and accuracy. The intra- and inter-batch precision and accuracy results of plasma PER samples (LLOQ, LQC, GMQC, MQC, and HQC) are summarized in Table 3. Both intra- and inter-assay precision (CV, %) and accuracy (Bias, %) were ≤ ± 10% for the samples of whole 5 concentration levels. Therefore, the precision and accuracy data were within the acceptable criteria (≤ ± 15%, except for ≤ ± 20% at LLOQ) [25] and allowed the accurate analysis of PER in plasma of pediatric patients with epilepsy.

3.2.4. Recovery and matrix effect. The extraction recoveries of PER from human plasma were 99.7 ± 3.5%, 99.2 ± 2.2%, and 98.8 ± 1.4% at three QC levels (2.50, 800, and 1,600 ng mL\(^{-1}\)), respectively, and the recovery of PER-d5 was 101.2 ± 3.7%. A similar percentage recovery was achieved between PER across all concentrations measured and PER-d5.

Matrix factors are shown in Table 4 for three different concentrations of PER in six different lots of independent drug-free plasma from healthy donors. Due to the add-in of SIL-IS (PER-d5), no matrix effect was observed to interfere the analysis of PER. Most importantly, hemolyzed and lipemic plasma were proved to have no influence in this method, which suitable for pediatric patients because of the high incidence of hemolysis in pediatric setting, especially at the time of sample collection or during specimen transport [34].

3.2.5. Carryover and dilution effect. Chromatograms obtained when blank plasma was injected after the ULOQ of the calibration samples did not reveal a signal greater than the acceptability criteria. The carryover effect was consequently considered to be negligible for both the PER and PER-d5, which indicated that the following sample could be analyzed reliably, regardless of the concentration of the sample injected before.

The accuracy and precision of diluted samples are shown in Table 3. These results illustrated that the current analytical method was trustworthy by diluting samples with blank plasma back to dynamic range of the calibration curve if the concentration of PER in clinical samples exceeded the ULOQ of the calibration curves.

3.2.6. Stability. Results of the stability assessment are summarized in Table 6. PER was stable in plasma for up to 30 days at –20°C. Bench-top stability in plasma was observed for 15 h at ambient temperature and PER was

### Table 3. Intra-batch and inter-batch precision (CV, %) and accuracy (Bias, %) of PER in human plasma. (n = 6 × 3)

<table>
<thead>
<tr>
<th>Nominal concentration (ng mL(^{-1}))</th>
<th>Measured concentration (mean ± SD, ng mL(^{-1}))</th>
<th>Precision (CV, %)</th>
<th>Accuracy (Bias, %)</th>
<th>Measured concentration (mean ± SD, ng mL(^{-1}))</th>
<th>Precision (CV, %)</th>
<th>Accuracy (Bias, %)</th>
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</thead>
<tbody>
<tr>
<td>1.00</td>
<td>1.06 ± 0.05</td>
<td>4.7</td>
<td>6.0</td>
<td>1.08 ± 0.04</td>
<td>3.9</td>
<td>8.0</td>
</tr>
<tr>
<td>2.50</td>
<td>2.45 ± 0.06</td>
<td>2.4</td>
<td>–2.0</td>
<td>2.54 ± 0.24</td>
<td>9.6</td>
<td>1.6</td>
</tr>
<tr>
<td>50.0</td>
<td>48.3 ± 0.79</td>
<td>1.6</td>
<td>–3.4</td>
<td>47.2 ± 2.70</td>
<td>5.7</td>
<td>–5.6</td>
</tr>
<tr>
<td>800</td>
<td>828 ± 12.78</td>
<td>1.5</td>
<td>3.5</td>
<td>836 ± 27.59</td>
<td>3.3</td>
<td>4.5</td>
</tr>
<tr>
<td>1,600</td>
<td>1,638 ± 31.89</td>
<td>2.0</td>
<td>2.4</td>
<td>1,677 ± 135.34</td>
<td>8.1</td>
<td>4.8</td>
</tr>
</tbody>
</table>

Abbreviations: Bias, deviation from nominal value; CV, coefficient of variation; n, number of replicates; SD, standard deviation.

### Table 4. Matrix effect of PER in six different lots human plasma at three QC levels (n = 6 × 3)

<table>
<thead>
<tr>
<th>Nominal concentration (ng mL(^{-1}))</th>
<th>Plasma lots ID.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td>1.00</td>
<td>101.60 ± 0.71</td>
</tr>
<tr>
<td>800</td>
<td>97.2 ± 0.71</td>
</tr>
<tr>
<td>1,600</td>
<td>101.60 ± 0.71</td>
</tr>
</tbody>
</table>

| IS-normalized matrix effect (mean ± SD, %) | 1.00  | 0.7 ± 0.07 | 0.2 ± 0.05 | 0.5 ± 0.05 | 1.3 ± 0.16 | 1.6 ± 0.11 | 1.7 ± 0.14 | 1.7 ± 0.14 | 1 ± 0.1 |
| CV (%)                                  | 0.7   | 0.2 ± 0.07 | 0.2 ± 0.05 | 0.5 ± 0.05 | 1.3 ± 0.16 | 1.6 ± 0.11 | 1.7 ± 0.14 | 1.7 ± 0.14 | 1 ± 0.1 |
| Replicates                              | 6     | 6 ± 0.07  | 6 ± 0.05   | 6 ± 0.05   | 3 ± 0.16   | 3 ± 0.11   | 3 ± 0.14   | 3 ± 0.14   | 3 ± 0.1 |
| Mean matrix effect (mean ± SD, %)       | 100.01 ± 1.25 | 3 ± 0.16 | 3 ± 0.11   | 3 ± 0.14   | 3 ± 0.14   | 3 ± 0.14   | 3 ± 0.14   | 3 ± 0.14   |
| Total CV(%)                             | 1.2   | 1 ± 0.1   | 1 ± 0.1    | 1 ± 0.1    | 1 ± 0.1    | 1 ± 0.1    | 1 ± 0.1    | 1 ± 0.1    | 1 ± 0.1 |

Abbreviations: CV, coefficient of variation; n, number of replicates; SD, standard deviation.
stable after five freeze/thaw \((-20^\circ C)\) cycles. PER in processed samples for injection remained stable for 6 days and 3 h at \(4^\circ C\).

### 3.3. Application to children with epilepsy

#### 3.3.1. Patients’ characteristics.

The developed LC-MS/MS method was successfully applied to 20 Chinese pediatric patients with drug-resistant epilepsy. During the study period, 36 plasma samples are routinely submitted to our laboratory for monitoring plasma PER levels (1 sample from 12 individual patients; 2 samples from 4 individual patients; and 3 or more samples from 4 individual patients). The demographic and clinical characteristics of these children are shown in Table 7. The age of onset of epilepsy ranged from 4 months to 9 years and 10 months. The age at which PER treatment was initiated ranged from 10 months to 12 years and 9 months. The epilepsy seizures were classified as focal in 11 cases, generalized in two cases, and combined generalized and focal in 7 cases. The main seizure types were focal to bilateral tonic-clonic seizures \((n = 7)\) and focal clonic seizures \((n = 6)\).

#### 3.3.2. Therapeutic regimens.

These patients had all received at least 2 appropriately chosen and used ASMs schedules after diagnosed with epilepsy. Unfortunately, symptoms of resistant to treatment were observed and finally they were diagnosed with drug-resistant epilepsy [35]. To control seizures, PER as an add-on drug were accede to regiments.

In patients older than 4 years, the initial dose of PER was 2 mg per day at night, increasing 2 mg every 2 weeks. The PER dose could be increased to a maximum of 12 mg day\(^{-1}\) based on the clinician’s judgment [3]. Patients younger than 4 years received a lower initial dose (2 mg per day at night) and the dose was increased in increments of 2 mg every 2 weeks for the first 3 months, followed by monthly increments of 2 mg every 2 weeks if necessary.

### Abbreviations

- ASMs: anti-seizure medications
- SD: standard deviation
- PER: zonisamide
4 years were started on PER at lower doses (0.50 mg day$^{-1}$), adjusted according to age and body weight. As is shown in Table 7, all patients had been treated with more than two concomitant ASMs. Ten patients had received one concomitant enzyme-inducing ASMs (carbamazepine, oxcarbazepine, phenobarbital, or topiramate), while one patient had been co-medicated with two kinds of enzyme-inducing ASMs (oxcarbazepine and topiramate). Of note, oxcarbazepine is not classified as enzyme inducer or inhibitor in most instances; however, in the interaction with PER, it showed a potent inducing effect of the same order of magnitude of strong enzyme inducers, such as carbamazepine and phenytoin [9, 10, 36–38].

3.3.3. Plasma concentrations. As is shown in Fig. 5A, plasma concentrations of PER measured by this method were ranged from 26.70 to 1,020 ng mL$^{-1}$, while the mean ± SD was 288.41 ± 244.08 ng mL$^{-1}$. The weight-adjusted PER dose was 0.146 ± 0.080 mg/kg/d, and the range was 0.035–0.300 mg/kg/d. Mean values and ranges of PER plasma concentrations in this study were similar to those Japanese children with drug-resistant epilepsy [6, 7], at comparable PER dosages.

To evaluate the correlation of PER concentrations with weight-adjusted dose, linear regression analysis was applied in these data. As is shown in Fig. 5B, no significant correlation ($r = 0.430$) was found between PER concentrations and weight-adjusted dose. However, the correlation could be significantly improved if we separate patients into concomitant enzyme-inducing ASMs groups ($r = 0.628$) and concomitant non-enzyme-inducing ASMs groups ($r = 0.624$), which was consistent with previous studies in children and adults [5, 6, 8]. What’s more, the concomitant use of enzyme-inducing ASMs resulted in a marked decrease of the regression equation slope (2254.2 versus 1037.8), which was also in line with previous studies [6, 26].

Interestingly, a prospective observational study in Italian adult epilepsy patients indicated that PER plasma concentration-
dose linearity was found in the subgroups treated with the enzyme inducer and the non-inducer/inhibitor plus valproic acid co-medicated subgroups, whereas no significant correlation was found in the enzyme inducer plus valproic acid and non-inducer/inhibitor subgroups [38]. The small number of plasma specimens in the enzyme inducer plus valproic acid subgroup might have contributed to these results according to the authors. Of note, the authors confirmed in a larger number of patients of the same cohort the evidence of higher mean $C_{0/D}$ of PER in the subgroup treated with valproic acid [39], which illustrate the complex drug-drug interactions (DDIs) between PER and other ASMs. In this study, all patients were co-medicated with valproic acid, so this DDIs were not shown.

To further investigate the influence of concomitant ASMs on PER, the $C_{0/D}$ of PER in patients receiving different ASMs regimens were calculated. As is shown in Fig. 6A, apparent inter- and intra-individual variabilities (mean CV% were 99.15% and 23.01%, respectively) were observed in the $C_{0/D}$ of PER in these Chinese pediatric patients, reflecting the heterogeneous contribution of the patient himself and different concomitant ASMs [5, 26, 38], which indicates the importance of TDM for PER, especially for pediatric patients because the enzyme activity of hepatic cytochrome P450 including CYP3A4 changes greatly during ontogeny [40], particularly when in combination with multiple ASMs [6, 36].

The mean ± SD for the $C_{0/D}$ of PER in patients co-administered with and without enzyme-inducing ASMs were 3026.7 ± 1,647.7 (CV% = 54.43%) and 1,150.9 ± 784.7 (CV% = 68.18%) (ng mL$^{-1}$)/(mg/kg/d), respectively. There were significant differences ($P < 0.001$) between the two groups by the Mann-Whitney $U$ test (Fig. 6B). The $C_{0/D}$ of PER in this study were similar to previous studies in children and adult epilepsy patients and the same DDIs were also found [7, 10, 26]. Recently, a prospective real-life study in China confirmed the satisfactory efficacy and tolerability of adjunctive PER in patients ≥14 years of age with focal-onset seizures. However, plasma concentrations of PER were not measured in the study, and the author concerned that they could not precisely assess the influence of concomitant enzyme inducers on the plasma levels of PER associated with seizure control and AEs [41]. Another retrospective observational study has suggested the effectiveness and safety of PER in Chinese pediatric patients (2–14 years) with refractory epilepsy [42]. But the author did investigate the plasma concentration of PER in these children, too. Therefore, the bioanalytical method and analysis results proposed in this study exactly fill the gaps in the above research.

4. CONCLUSION

This is the first TDM study of PER in China. Due to several limits in previous published methods, a convenient, sensitive, reliable, and fast LC-MS/MS method for the TDM of PER was herein developed and validated. This method has distinctive advantages compared to the previous published methods, because of the simple sample preparation, the quick analytical time, and the minor sample volumes. More importantly, this method was successfully applied to the clinical TDM practices of PER in Chinese pediatric patients with drug-resistant epilepsy. By this method, we identified the apparent inter- and intra-individual PER concentration variabilities and the potential DDIs between PER and several concomitant ASMs in Chinese pediatric patients, which provide valuable information to enable clinicians for the reasonable application of PER in the future.

Declaration of competing interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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