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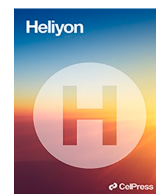

Research article

Therapeutic drug monitoring of free perampanel concentrations in practice: A practical analytical technique based on centrifugal ultrafiltration sample separation

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ABSTRACT

Objectives: The centrifugal ultrafiltration–high-performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) method was established to determine the free perampanel (PER) concentration in children with epilepsy.

Methods: Free PER concentration was obtained using centrifugal ultrafiltration devices. The internal standard was PER-D5. The method was investigated for selectivity, carryover, lower limit of quantification, calibration curve, accuracy, precision, matrix effects, recovery, and stability. The Spearman's correlation coefficient was used to evaluate the correlation between the free and total PER concentrations. A nonparametric test was used to estimate the effects of PER along with other antiepileptic drugs on the total and free PER concentrations.

Results: The free PER concentration was positively correlated with the total PER concentration in the 57 plasma samples ($r = 0.793 > 0$, $P < 0.001$). Additionally, the free PER concentrations were significantly ($P < 0.05$) increased in valproic acid (VPA) co-therapy (9.87 ± 5.83) compared with non-VPA co-therapy (5.03 ± 4.57).

Conclusions: The proposed method is efficient, sensitive, and suitable for detecting free PER concentrations in children with epilepsy. Simultaneously, the free PER concentration response to clinical outcomes in children with epilepsy was more clinically significant, particularly when combined with VPA.

1. Introduction

Epilepsy is a chronic nervous system disease characterized by temporary brain dysfunction involving dysplastic neurons, which manifests as epileptic seizures caused by excessive synchronous discharge of brain neurons [1,2]. Children are particularly vulnerable to developing epilepsy, with those under 18 years of age accounting for over 60 % of all epilepsy patients. Most epileptic seizures are

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relatively sudden and occur frequently, significantly impacting the growth, development, and overall well-being of children and posing serious risks to their physical and mental health and safety [3,4]. Antiepileptic drugs (AEDs) are the primary therapeutic regimen for epilepsy [5,6]. There is a great need to develop AEDs with novel therapeutic targets, better pharmacokinetic characteristics, and reduced adverse reactions to address poorly controlled seizures, particularly in children [7].

Perampanel (PER) is a third-generation novel AED and is considered the first α-amino-3-hydroxy-5-methyl-4-isooxazolpropionic acid (AMPA) glutamate receptor antagonist approved for the treatment of epilepsy. It functions by binding to the AMPA receptor at the postsynaptic level, blocking the connection between the receptor and glutamate. As a result, seizures in epilepsy are highly suppressed, their spread is limited, and the excitability of neurons is significantly inhibited [8]. As of 2018, the Food and Drug Administration has approved PER as an adjunctive therapy for primary generalized tonic–clonic seizures in patients aged 12 years and older and as a monotherapy or adjunctive therapy for partial seizures in patients aged 4 years and older [9]. In July 2021, PER was approved in China as a single agent and an additional therapy for the treatment of focal epilepsy (with or without secondary generalized seizures) in adults and children aged >4 years [10]. Much evidence has suggested that therapeutic drug monitoring (TDM) is useful for personalized dose adjustment of AEDs [11,12].The pharmacokinetics of PER follows a first-order kinetic process, and its PER plasma concentration increases with increasing dose. PER is readily and completely absorbed after oral administration, with 95 % of it bound to plasma proteins. Its plasma half-life is approximately 105 h [13]. Drug–drug interaction studies have revealed that taking enzyme-induced AEDs (such as oxcarbazepine (OXC), carbamazepine (CBZ), and phenobarbital (PHB)) could shorten the half-life of PER by 50 %–70 %, accompanied by a 2–3-fold decrease in PER plasma concentrations [14,15]. A previous study observed that the shortened half-life of PER in children aged 4–11 years indirectly affected plasma concentration, resulting in slightly reduced clearance and plasma concentration in patients with hepatic and renal insufficiency [16,17]. Based on this features, plasma PER concentration is easily affected by albumin level, concomitant medication, age, or hepatic and renal function. Furthermore, excessive plasma concentrations may lead to a higher incidence of drug reactions. Therefore, it is necessary to conduct therapeutic drug monitoring (TDM) for PER, optimize individual therapeutic regimens, monitor toxic adverse reactions, verify medication compliance, and achieve safe and effective therapeutic effects in children.

Some previous reports have established and validated the methods for the determination of PER plasma concentration in patients with epilepsy [18,19]. However, the plasma concentration studied in these reports were total PER concentration consisting of the protein-bound form and free forms. Free PER concentration is a part of the drug in the plasma that is not bound to the protein, that is, the drug concentration of its main pharmacological effect. As mentioned above, PER has a high protein binding rate (95 %∼96 %). Changes in drug–protein binding conditions can cause the free drug concentration to change while the total concentration remains unchanged, leading to potential toxic side effects, particularly in pathological conditions such as hypoproteinemia and changes in renal function [20–23]. Based on this reason, the free PER concentration is easily affected by albumin level, concomitant medication and patient age, that is, PER has high variability between individuals. Hence, monitoring the free concentrations, not the total PER concentration with high plasma protein binding rates, is of great clinical significance. It may be more important to make appropriate dose adjustments for the clinic, and this can also better monitor the therapeutic effectiveness and safety of the drug, which is of great significance for the development of clinical guidelines and personalized treatment for children with epilepsy.

In light of these challenges, this study aims to focus on the development and application of an efficient ultrafiltration–high-performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) approach that combines centrifugal ultrafiltration (CF-UF) with quantitative detection using isotope internal standards (SIL-IS) to determine unbound perampanel in the plasma of children with epilepsy. The relationship between the free and total PER concentrations is analyzed, providing more accurate detection methods and medication guidance for TDM of PER in clinics.

2. Materials and methods

2.1. Chemicals and materials

PER tablets (211194, 2 mg/tablet) were purchased from Eisai Co., Ltd. (Kawashima Factory). PER standards (215906P-WA-01, purity >99 %, CAS: 380917-97-5) and PER-D5 standard (195906PD5-WA-01, purity >98 %, CAS: 2012598-62-6) were purchased from Standard Pharm Co., Ltd. (USA). HPLC-grade methanol was obtained from Thermo Fisher Scientific (Waltham, MA, USA). HPLC-grade formic acid was obtained from Mreda Technology Inc., Ltd. (USA). Ammonium acetate (HPLC–MS-grade) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Purified water was purchased from WaïGroup Co., Ltd. (Hangzhou, China). CF-UF devices (Microcon-10 Ultracel PL-10, REF: MRCPRT010, LOT: R2AB93462, 0.5 mL, cutoff 10 kDa) were purchased from Millipore Corp.

2.2. Instruments

HPLC–MS/MS analysis was conducted using a Shimadzu HPLC system (Kyoto, Japan) coupled with a 4500 MD triple quadrupole mass spectrometer equipped with a Turbo V electrospray ionization source (ESI) (AB Sciex, CA, USA). Analysis software (Analyst 1.6.3 MD, AB Sciex, CA, USA) and quantitative analysis software (version B. 07) were used to collect and analyze the data, respectively. Purified water was prepared using a Milli-Q water purification system (Millipore, ELIX100, USA). An Explorer R quasi-microbalance (Ohaus Corporation, Changzhou, China) was used to weigh the samples. A high-speed freezing centrifuge of BY-R20 type (Beijing Baiyang Medical Instrument Co., Ltd., China) was used to centrifuge the biological samples.

2.3. Chromatographic and mass Spectrometric conditions

Chromatographic separation was performed using a Hypersil GOLD C_{18} (2.1 mm × 100 mm, 3.0 μm) column, with the column oven maintained at 50 °C. The mobile phase consisted of (A) 1 mmoL·L^{-1} of ammonium acetate–water and (B) methanol at a flow rate of 0.4mL·min^{-1}. The gradient elution program was as follows: 0–0.5 min, 90 % A; 0.5–1.2 min, 90 %–5 % A; 1.2–2.0 min, 5 % A; 2.0–2.1 min, 5 %–90 % A, and the remaining time of the gradient elution was 2.1–4.0 min. The injection volume of the biological samples was 5 μL. The mass analyzer equipped with a Turbo V source was then operated in positive mode using multiple reaction monitoring. The parameters of the ion source detector were as follows: ion spray voltage, 5 500 V; ion source temperature, 550 °C; curtain gas, 20 psi; nebulizer gas, 45 psi; and turbo gas, 40 psi. The mass spectrometer was operated under a nitrogen atmosphere using a nitrogen generator. PER produced the predominant quasi-molecular ion [M+H]+ at *m/z* 350.1, and product fragments were observed at *m/z* 219.0. The declustering potential (DP) and collision energy (CE) of the PER were 105 V and 45 V, respectively. Similarly, PER-D5 produced [M+H]+at *m/z* 355.1, and product fragments were observed at *m/z* 220.1. The DP and CE of the PER were 93 V and 47 V, respectively. The chemical structures and mass spectra of PER and PER-D5 are shown in Fig. 1.

2.4. Preparation of blank ultrafiltrate with CF-UF

A blank plasma sample (200 μL), provided by the Department of Transfusion, Children's Hospital of Hebei Province, was taken and added to the CF-UF device. Before this, the CF-UF device was rinsed with 100 μL of 1 mmoL·L^{-1} NaOH solution, followed by a second rinse with 100 μL of distilled water, and kept moist. The blank ultrafiltration solution was obtained by centrifuging the solution at 13 680×*g* for 20 min at 4 °C.

2.5. Preparation of calibrators, quality control samples, and internal samples

The stock solution of PER was prepared by individually weighing it in methanol solution and was then stored in a refrigerator at −80 °C for later use. The stock solution was further diluted with methanol to create a series of concentration standard curve working stock solutions with mass concentrations of 2.5, 10, 25, 125, 250, 500, and 750 ng·$mL^{-1}$and a quality control (QC) working stock solution with mass concentrations of 5, 50, 200, and 600 ng·mL^{-1}, respectively. For calibrators and QC samples, blank ultrafiltrates from blank plasma samples were spiked with appropriate volumes of working stock solutions. In this process, only one volume of the working stock solution was mixed with three volumes of blank ultrafiltration and one volume of IS working solution. Similarly, a stock solution of internal samples with 2.00 mg·mL^{-1} concentration was prepared by diluting PER-D5 in methanol. An appropriate amount of the IS stock solution was diluted with methanol to prepare a solution with a mass concentration of 100 ng·mL^{-1} as the IS working solution. After preparation, the stock and working stock solutions, calibrator samples, QC samples, and IS working solutions were aliquoted and maintained at −80 °C.

2.6. Sample preparation

A plasma sample (200 μL) was taken and added to the CF-UF device (which also treated the same blank ultrafiltrate portion). The ultrafiltration solution was obtained at 13 680 ×*g* for 20 min at 4 °C. For sample preparation, 40 μL of ultrafiltration solution and 10 μL of IS working stock were mixed with 200 μL of methanol mixture in a 1.5 mL polypropylene EP tube for 3 min using a vortex shaker, followed by centrifugation at 13 680 ×*g* for 10 min at 4 °C. The supernatant solution was then transferred to a glass vial with a liner pipe and loaded into an autosampler (maintained at 4 °C), ready for sampling analysis. The details of sample preparation for determination of the total PER concentration were described in our previous study [24].

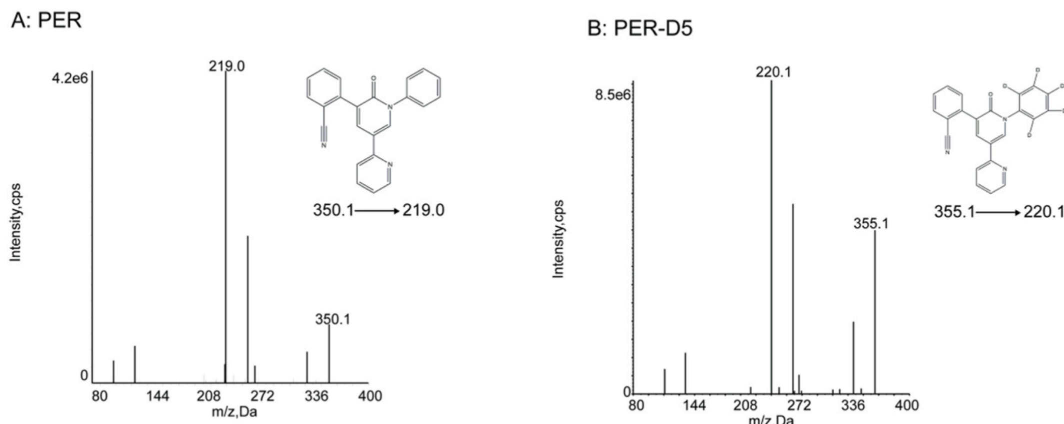

Fig. 1. Positive MS_2-spectras of PER and PER-D5.

2.7. Method validation

Method validation was conducted according to the guidelines for bioanalytical method validation from the Chinese Pharmacopoeia (2020 edition) and the European Medicines Agency (EMA) (July 21, 2011) [25,26]. The evaluation of quantitative processes usually includes the selectivity, carryover, lower limit of quantification (LLOQ), calibration curves, accuracy and precision, matrix effects, recovery, and stability.

2.7.1. Selectivity

Following the EMA guidelines, the selectivity must be demonstrated by analyzing at least six individual sources of the appropriate blank matrix and evaluating them for any interference. Typically, the absence of interfering components is considered acceptable when the response is below 20 % of the LLOQ for the analyte and 5 % for the IS. Hence, six blank plasma samples from different sources and blank plasma spiked with the PER working stock solution were prepared and analyzed, and the corresponding chromatograms were obtained according to the method described in “2.6.”.

2.7.2. Carryover

According to the EMA guidelines, carryover should be evaluated in blank samples after a high-concentration sample or calibration. After injection of the upper limit sample, the carryover in the blank sample should not exceed 20 % of the LLOQ and should not exceed 5 % of the IS.

2.7.3. Calibration curve and LLOQ

The calibration curve was constructed using a linear regression formula relating the peak area ratios of the analytes to the IS. The calibration curve parameters were fitted linearly using the least-squares method, and the concentrations of the corrected standard samples were calculated. According to the EMA guidelines, the back-calculated concentrations of the calibration standards should fall within ±15 % of the nominal value, except for the LLOQ, which should be within ±20 %. To meet this criterion, at least 75 % of the calibration standards, with a minimum of six calibration standard levels, should be within the specified range. In this experiment, a series of calibration curve samples and QC samples were prepared as follows: 30 μL of blank ultrafiltration solution was mixed with 10 μL of the series standard curve working stock solution and 10 μL of IS working solution (100 ng·mL^{-1}). The following process was conducted in the same manner as “mixed with 200 μL of methanol” under “2.6.”. The calibration curve mass concentrations were set to 0.5, 2, 5, 25, 50, 100, and 150 ng·mL^{-1}, while the QC sample mass concentrations were set to 1, 10, 40, and 120 ng·mL^{-1}, respectively. The curve displayed a linear relationship, and the weighting factor was set to $1/x^2$.

2.7.4. Accuracy and precision

The analytical method accuracy describes the proximity of the determined value obtained by the method to the nominal analyte concentration (expressed as a percentage). For accuracy and precision, the relative standard deviation (RSD) should generally not exceed 15 % within and between batches, while for the LLOQ, the RSD should not exceed 20 %. Five QC samples of LLOQ, low, medium 1, medium 2, and high concentration were prepared, and each concentration was tested in parallel. The within-run accuracy was investigated using the same method described for “mixed with 200 μL of methanol” under “2.6.”. Furthermore, the between-run accuracy was evaluated by conducting three batches of repeated tests (within three days) for each batch, following the aforementioned operation methods.

2.7.5. Matrix effect and recovery

Matrix effects should be evaluated using at least six blank matrices from different donors to assess the peak area of the analyte at the QC level along with its IS. The matrix factor (MF) is generally calculated as the ratio of the peak area with the matrix (blank ultrafiltration solution plus QC working solution) to that without the matrix (pure QC working solution). The IS-normalized MF was also calculated by dividing the MF of the QC sample by that of the IS. According to the EMA and Chinese Pharmacopoeia guidelines, the coefficient of variation (CV) of the IS-normalized MF for matrix effect calculations should not exceed 15 %.

Recovery is defined as the ratio of the peak area of the analyte after extraction to that of the unextracted analyte (blank ultrafiltration solution substitution is extracted). One set of QC samples was prepared and analyzed as described in the normal processing flow according to the method in “2.6.” (peak area A). Another set of blank ultrafiltration solutions was obtained and subsequently spiked with the QC working solution to obtain the final QC-level samples (peak area B). The extraction recovery was calculated as the ratio of the peak area of A to that of B using the MS/MS method. Similarly, the extraction recovery should conform to a CV value of less than 15 %.

2.7.6. Stability

Stability should be ensured under different conditions during biological quantitative analysis. We assessed the stability of the samples under the following conditions: storage at room temperature (About 25 °C) for 4 h, storage in the autosampler at 5 °C for 8 h, freeze–thaw stability (subjected to three freeze–thaw cycles), frozen at −20 °C for 15 and 30 days, and frozen at −80 °C for 45 and 60 days. For freeze–thaw stability, QC samples were frozen for at least 12 h before thawing in each cycle. The freeze–thaw cycles were conducted at −20 °C for congelation and at room temperature for complete thawing. The stability under different conditions was evaluated by analyzing five individual samples at each QC level. For stability experiments, the RSD under different conditions should not exceed 15 %.

2.8. Sample collection

The study protocol was approved by the Ethics Committee of the Children's Hospital of Hebei Province. A total of 57 plasma samples were obtained from 57 children with epilepsy between March 15, 2022, and November 2, 2022. The inclusion criteria were as follows: (1) consistency with the latest seizure type and epilepsy diagnosis of the International League Against Epilepsy; (2) age 0–16 years; (3) regular treatment with PER and regular monitoring of PER blood concentration; and (4) stable medication state, with the same dose of PER taken for at least 4 consecutive weeks. Only if the above four criteria are met at the same time can they be included. The exclusion criteria were as follows: (1) blood collection time point did not meet the requirements; (2) medication time did not reach steady state; (3) children with incomplete or missing general information; (4) those not considered suitable for inclusion by the researchers of the research group. If one of these items is met, it can be excluded. All children received an oral PER maintenance dose in the evening, and 2 mL of blood was collected between 8:00 and 10:00 a.m. the following day. The plasma samples were collected in anticoagulant centrifuge tubes containing EDTA and centrifuged at 1 610 $\times g$ for 5 min, and the resulting plasma was transferred to clean centrifuge tubes. The obtained plasma samples were immediately analyzed or stored at -80 °C for testing.

2.9. Statistical analysis

Statistical analysis was performed using SPSS software (version 21.0; IBM SPSS, USA). Spearman's correlation coefficient was used to evaluate the correlation between free and total PER plasma concentrations. A nonparametric test was used to estimate the effects of combined PER and other AEDs on total and free PER concentrations. Data assessments were double-tailed, and statistical significance was set at $P < 0.05$. Data were expressed as mean standard deviation.

3. Results

3.1. Method validation

3.1.1. Selectivity

There was no interference from endogenous substances at the retention time of the tested substances. The retention times of PER and PER-D5 were 2.38 and 2.40 min, respectively (Fig. 2).

3.1.2. Carryover

Carryover was evaluated by injecting blank samples after reaching the upper limit of quantification. The results showed that carryover met the requirements of the Chinese Pharmacopoeia and the EMA.

3.1.3. Calibration curve and LLOQ

The calibration curve parameters were fitted linearly using the least-squares method, and the concentration of the corrected standard sample was calculated. The standard curve regression formula for unbound PER was $Y = 0.02\,828X + 0.0\,817$ (correlation

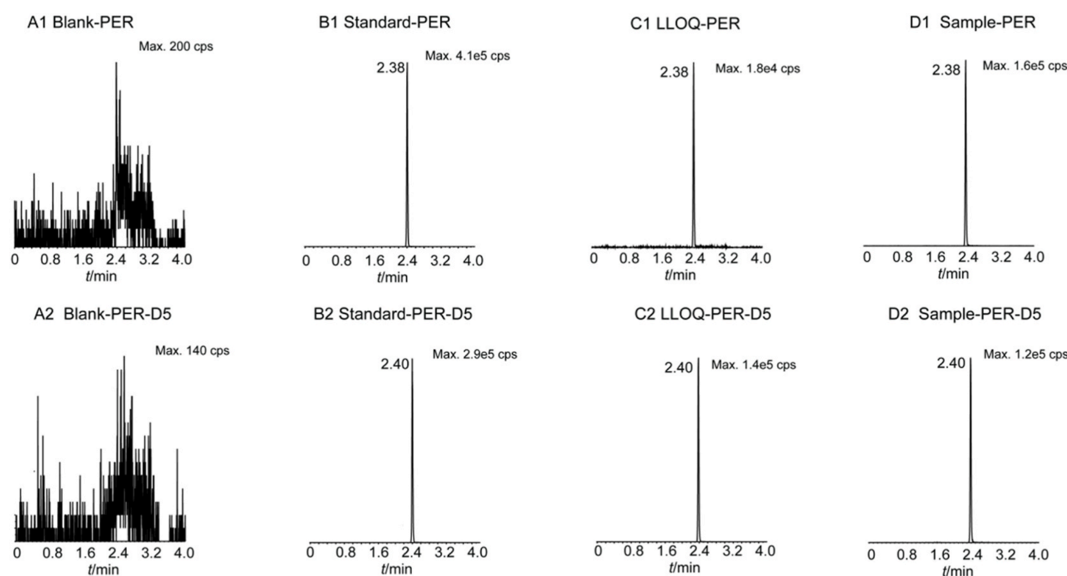

Fig. 2. Representative HPLC-MS/MS chromatograms of PER and PER-D5 A blank plasma; B standard solution; C blank plasma spiked with the analytes at LLOQ; D patient's sample.

coefficient $r = 0.9\ 986$), indicating a strong linear relationship within the range of 0.5–150 ng·mL^{-1}. This linear range complies with the requirements of the biological sample chromatography guidelines of the Chinese Pharmacopoeia and EMA. The LLOQ was 0.5 ng·mL^{-1}, and the signal-to-noise ratio (S/N) was much greater than 5.

3.1.4. Accuracy and precision

The precision RSD of PER within and between batches was ≤9.43 %, and the accuracy was 93.25 %–98.34 %, which met the basic requirements for quantitative analysis of biological samples according to the guidelines of the Chinese Pharmacopoeia and EMA. The data for within- and between-run accuracy, precision, and RSD are summarized in Table 1.

3.1.5. Matrix effect and recovery

The MF is generally calculated as the ratio of the peak area with the matrix (blank ultrafiltration solution plus QC working solution) to the peak area without the matrix (pure QC working solution). The IS-normalized MF was also calculated by dividing the MF of the QC sample by that of the IS. Upon calculation, the IS-normalized MF of PER ranged from 93.96 % to 103.72 %, and the RSDs were all ≤9.59 % (Table 1), indicating that different matrices of the blank plasma ultrafiltrate had no effect on the detection results of PER.

The extraction recovery was calculated as the ratio of the peak areas of A to the peak areas of B, which ranged from 92.58 % to 111.74 %, and the RSDs were all ≤6.49 % (Table 1). The results showed that the extraction recovery satisfied the requirements for quantitative analysis of biological samples.

3.1.6. Stability

The results showed that the stability of the RSDs under different conditions (the samples stored at room temperature for 4 h, in an autosampler at 5 °C for 8 h, freeze–thaw stability, frozen at −20 °C for 15 and 30 days, and frozen at −80 °C for 45 and 60 days) were all less than 9.20 %, indicating good stability under these conditions. The PER stability data under different conditions are summarized in Table 2.

3.2. Application to clinical samples

Sample information and the corresponding results are presented in Table 3. According to a previous research method, the total plasma PER concentration (n = 57) ranged from 90.17 to 1867.73 ng·mL^{-1}, and the mean concentration was 620.15 ± 396.53 ng·mL^{-1}; the free plasma PER concentration (n = 57) ranged from 0.42 to 23.14 ng·mL^{-1}, and the mean concentration was 8.09 ± 5.85 ng·mL^{-1}. Owing to the skewed distribution of both free drug concentrations ($P < 0.001$) and total drug concentrations ($P = 0.002 < 0.05$), the correlations between the total and free PER plasma concentrations for the 57 plasma samples were assessed using Spearman's product moment coefficient. The free PER concentration was positively correlated with the total PER concentration ($r = 0.793 > 0$, $P < 0.001$). We plotted scatter plots based on the total and free PER concentrations, while curve fitting was performed based on the goodness of fit and complexity of the equation (Fig. 3).

This study analyzed the effects of PER along with other AEDs on the total and free PER concentrations. We classified the AEDs as strong enzyme inducers (OXC, PHB, and CBZ), weak enzyme inducers/no inhibitors (levetiracetam, topiramate, lamotrigine, lacosamide, and clobazam), and enzyme inhibitors (VPA). Eleven samples were treated with enzyme inducers (group A), 10 samples were treated with no strong enzyme inducers/no inhibitors (group B), 31 samples were treated with VPA (group C), and five samples with enzyme inducers + VPA (group D). Total and free PER concentrations were compared among the patient groups treated with different AEDs using the Kruskal–Wallis H nonparametric test. The results showed that the total PER concentrations (Chi-Square = 13.358, $P = 0.004 < 0.05$) and free PER concentrations (Chi-Square = 11.452, $P = 0.010 < 0.05$) of the four groups were significantly different, as shown in Table 4 and Fig. 4. Pairwise comparisons were performed using the Mann–Whitney U method when the rank sum test indicated a significant difference among the subgroups. The total PER concentration was significantly ($P < 0.05$) lower in group A (311.32 ± 190.14) than that in group C (745.60 ± 415.53) and group D (780.14 ± 459.18). However, free PER concentrations were significantly ($P < 0.05$) increased in group C (9.84 ± 5.66) versus group A (4.80 ± 3.70) and group B (5.28 ± 5.57). Because VPA is not only an enzyme inhibitor but also a high-affinity, highly protein-bound drug, it significantly interferes with the total and free PER concentrations. We further examined the effect of VPA co-therapy on total and free PER concentrations in patients with epilepsy (57 samples). The result displayed that the total PER concentration was significantly ($P < 0.05$) increased in VPA co-therapy (750.40 ± 415.02) compared with non-VPA co-therapy (396.87 ± 236.55). Similarly, free PER concentrations were significantly ($P < 0.05$) increased in VPA co-therapy (9.87 ± 5.83) compared with non-VPA co-therapy (5.03 ± 4.57) (see Table 5).

4. Discussion

4.1. Optimization of ultrafiltration conditions

Various assays have been used to determine free drug plasma concentrations, including equilibrium dialysis, ultrafast centrifugal gel filtration, and CF-UF. CF-UF involves the selective separation of ultrafiltration membranes with different molecular weight limits [27]. Small molecules are filtered, whereas high-molecular-weight substances larger than the membrane aperture are trapped in the ultrafiltration membrane. Compared with the equilibrium dialysis method, CF-UF avoids dilution of the analyte by the dialysate and degradation of the analyte during prolonged equilibrium dialysis. *In vitro* analysis showed that CF-UF can rapidly separate bound and free drugs, is fast and simple, has a short sample preparation time, and can be used for the simultaneous processing of multiple samples

Table 1
Accuracy, precision, recovery, and matrix effects for the analysis of unbound PER.

Nominal concentration/ng·mL^{-1}	Within-run			Between-run			Recovery		Matrix effect	
	Calculated concentration (mean ± SD) /ng·mL^{-1}	Accuracy/ (%)[a]	Precision (RSD, %)[a]	Calculated concentration (mean ± SD) /ng·mL^{-1}	Accuracy/ (%)[b]	Precision (RSD, %)[b]	Recovery/ %)[c]	RSD/ %	Matrix effect(%)[c]	RSD/ %
0.5	0.47 ± 0.04	94.54	9.43	0.47 ± 0.03	94.86	6.51	–	–	–	–
1	0.94 ± 0.03	94.23	3.37	0.94 ± 0.04	93.82	4.51	95.57 ± 2.99	3.12	99.38 ± 4.54	4.56
10	9.72 ± 0.41	97.17	4.20	9.83 ± 0.36	98.34	3.69	108.40 ± 3.34	3.08	96.55 ± 2.59	2.68
40	37.36 ± 1.05	93.39	2.80	37.30 ± 1.01	93.25	2.72	99.17 ± 4.25	4.29	101.14 ± 2.58	2.55
120	112.46 ± 1.78	93.72	1.58	113.47 ± 5.23	94.56	4.61	102.25 ± 1.86	1.82	100.48 ± 2.09	2.08

Abbreviations: RSD, relative standard deviation.
[a] n = 5 for accuracy and precision validation in within-run.
[b] n = 15 for accuracy and precision validation in between-run.
[c] n = 6 for recovery and absolute matrix effect validation.

Table 2
The stability results for the analysis of unbound PER ($n = 5$).

Nominal concentration/ ng·mL^{-1}	Short-term 4 h ($\bar{x}$ $\pm s$) / ng·mL^{-1}	RSD/ %	Autosampler 5°C-8 h ($\bar{x}$ $\pm s$) / ng·mL^{-1}	RSD/ %	Freeze-thaw Stability ($\bar{x}$ $\pm s$) / ng·mL^{-1}	RSD/ %	frozen at −20 °C for 15 days ($\bar{x}$ $\pm s$) / ng·mL^{-1}	RSD/ %	frozen at −20 °C for 30 days ($\bar{x}$ $\pm s$) / ng·mL^{-1}	RSD/ %	frozen at −80 °C for 45 days ($\bar{x}$ $\pm s$) / ng·mL^{-1}	RSD/ %	frozen at −80 °C for 60 days ($\bar{x}$ $\pm s$) / ng·mL^{-1}	RSD/ %
1	1.01 ± 0.09	8.49	1.03 ± 0.05	4.73	1.02 ± 0.05	5.32	0.96 ± 0.09	9.20	0.96 ± 0.08	7.89	0.97 ± 0.02	2.27	0.95 ± 0.07	7.83
10	9.94 ± 0.42	4.22	9.99 ± 0.37	3.13	9.99 ± 0.08	0.78	9.99 ± 0.22	2.17	9.79 ± 0.21	2.16	10.03 ± 0.38	3.75	10.85 ± 0.38	3.49
40	38.09 ± 1.40	3.66	37.53 ± 1.46	3.89	37.39 ± 0.53	1.43	39.06 ± 1.06	2.72	38.87 ± 1.52	3.92	43.90 ± 1.23	2.81	43.64 ± 1.27	2.90
120	111.97 ± 2.47	2.20	113.16 ± 0.76	0.68	115.95 ± 6.88	5.93	121.27 ± 3.40	2.80	119.85 ± 2.22	1.85	130.97 ± 2.18	1.66	132.67 ± 1.39	1.05

Table 3
The TDM results of PER in 57 samples and its basic clinical data.

Characteristic	mean ± SD (range) (n = 57)
Gender	male (29, 50.88 %) Female (28, 49.12 %)
Age (years)	7.35 ± 3.10 (0.83–15.00)
Body weight (kg)	29.69 ± 12.19 (10.00–70.00)
Dose (mg·d^{-1})	4.56 ± 1.80 (2.00–8.00)
Total concentration (ng·mL^{-1})	620.15 ± 396.53 (90.17–1867.73)
Free concentration (ng·mL^{-1})	8.09 ± 5.85 (0.42–23.14)
Plasma protein binding rate (%)	98.64 ± 0.63 (96.66–98.58)
combined with other AEDs	
Group A	11 (19.30 %)
Group B	10 (17.54 %)
Group C	31 (54.39 %)
Group D	5 (8.77 %)

Interpretation: A, enzyme inducers; B：not strong enzyme inducers/not inhibitors; C: enzyme inhibitors (VPA); D: enzyme inducers + VPA.

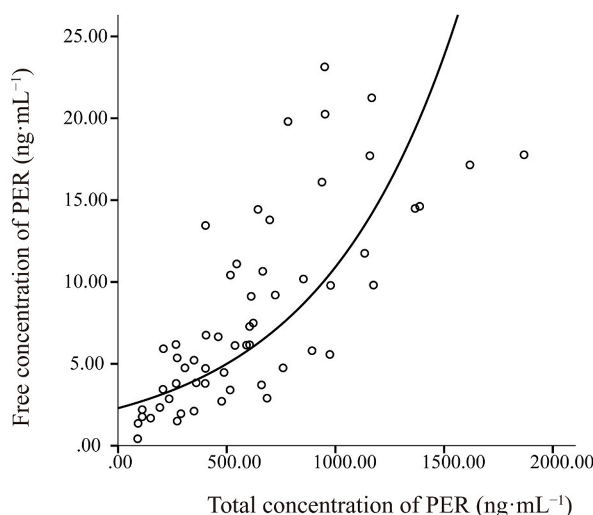

Fig. 3. The correlation of total and free PER concentration.

Table 4
Total and Free concentration of PER in groups of cotherapy other AEDs.

Variable	Group A(n = 11)		Group B(n = 10)		Group C(n = 31)		Group D(n = 5)		Chi-Square	P
	mean ± SD	Mean Rank	mean ± SD	Mean Rank	mean ± SD	Mean Rank	mean ± SD	Mean Rank		
Total PER concentration/ ng·mL^{-1}	311.32 ± 190.14	14.27	490.97 ± 255.71	25.00	745.60 ± 415.53	34.55	780.14 ± 459.18	35.00	13.358	0.004
Free PER concentration/ ng·mL^{-1}	4.80 ± 3.70	19.50	5.28 ± 5.57	19.00	9.84 ± 5.66	34.73	10.09 ± 7.54	34.40	11.452	0.01

Interpretation: A, enzyme inducers; B：not strong enzyme inducers/not inhibitors; C: enzyme inhibitors (VPA); D: enzyme inducers + VPA.

Table 5
Total and Free concentration of PER in groups of cotherapy VPA.

Variable	VPA cotherapy(n = 36)		not VPA cotherapy(n = 21)		Mann-Whitney U	P
	mean ± SD	Mean Rank	mean ± SD	Mean Rank		
Total PER concentration/ng·mL^{-1}	750.40 ± 415.02	34.61	396.87 ± 236.55	19.38	176.00	0.001
Free PER concentration/ng·mL^{-1}	9.87 ± 5.83	34.68	5.03 ± 4.57	19.26	173.50	0.001

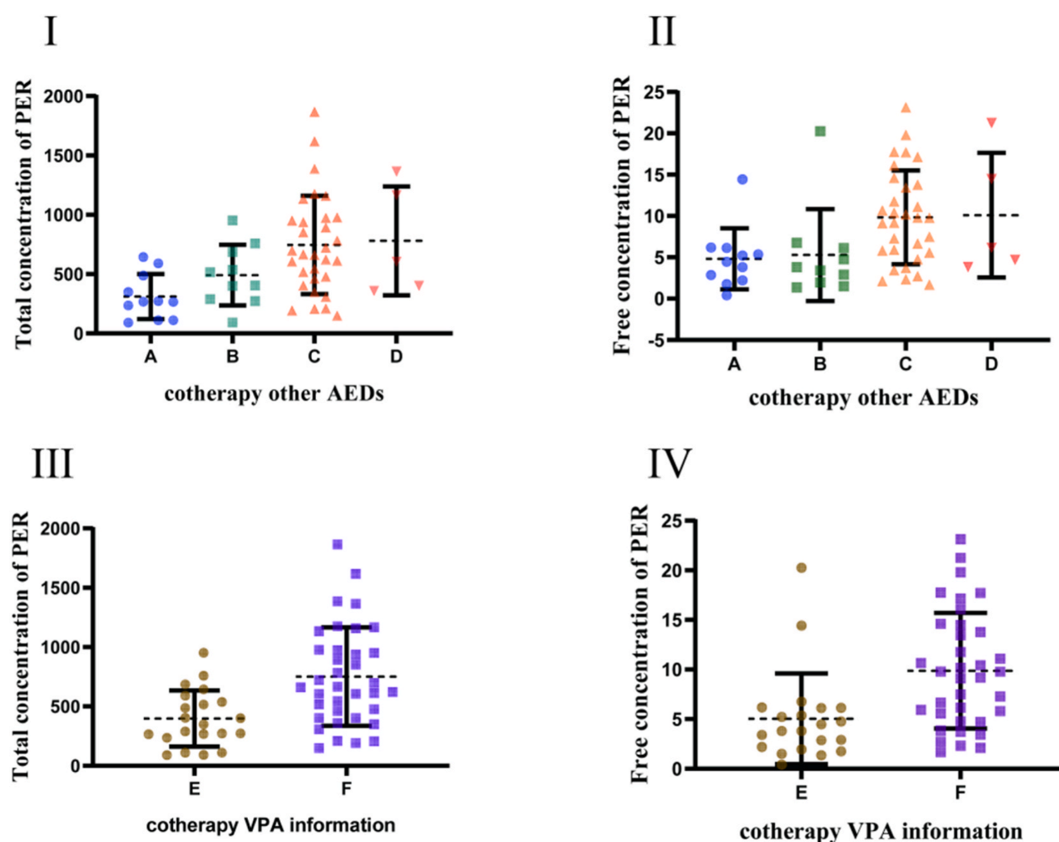

Fig. 4. The distribution of total and free PER concentration in groups of cotherapy other AEDs (A:cotherapy enzyme inducers; B:cotherapy no strong enzyme).

[28]. CF-UF devices are readily available and are relatively inexpensive. In this experiment, the ultrafiltration conditions were optimized by first selecting the ultrafiltration tube varieties. Among the main Millipore filtration products, there are two series of ultrafiltration tubes, namely, the Microcon centrifugal and Amicon Ultra, both of which are centrifugal membranes of reclaimed cellulose. The Amicon Ultra centrifugal ultrafiltration tube is mainly used for the concentration, desalination, and percolation of proteins and antibodies due to its groove-designed built-in filter membrane. In contrast, the built-in filter membrane of the Microcon centrifugal tube is designed with a plane that has a larger filter surface and a smaller dead volume, which is more beneficial for the filtration of microsolutes. Therefore, a Microcon centrifugal ultrafiltration tube was used to separate unbound and bound PER. Subsequently, the ultrafiltration membrane aperture was selected. The pore sizes of ultrafiltration membranes generally correspond to 10, 30, 50, and 100 kDa. According to an EMA report, PER is mainly bound to albumin *in vivo* and a small part to α-acid glycoprotein. The molecular weight of albumin is 66 kDa, while that of α-acid glycoprotein is 40 kDa [29]. Therefore, these two binding proteins can be completely intercepted using a 10 kDa ultrafiltration membrane aperture. Then, because the ultrafiltration membrane of the ultrafiltration tube contained trace amounts of glycerin at the factory, we cleaned it with NaOH solution and distilled water before using the CF-UF device to avoid subsequent interference. Finally, the ultrafiltration conditions were investigated, which are centrifugation speed (6 080, 9 500, and 13 680×*g*) and centrifugation time (10, 15, and 20 min). Too little ultrafiltrate was collected to meet the requirements for analysis and determination when the rotational speed was too low and the centrifugal time was too short. However, an excessively high rotation speed can result in the rupture of the ultrafiltration membrane [30]. Consequently, centrifugal conditions of 13 680 ×*g* and a centrifugation time of 20 min were determined through investigation to ensure the integrity of the ultrafiltration membrane and obtain a sufficient volume of the ultrafiltration liquid.

4.2. Correlation analysis of total and free drug PER concentrations

PER is a high-affinity, highly protein-bound drug (binding rate is approximately 95 %–96 %), with only 5 % of the free PER concentration available to exert pharmacological effects [31]. TDM is usually performed by measuring the total drug concentration [32]. Under normal circumstances, the free drug and total drug concentrations maintain a certain balance, and the ratio of the free and total drug concentrations is constant. However, changes in the binding conditions of the drug and protein can alter the free drug concentration, even if the total drug concentration remains unchanged. This variation in free concentration may have mental side effects, particularly under pathological conditions (hypoproteinemia or renal function changes) or during drug co-therapy [20–23].

Therefore, it is necessary to monitor the free drug concentrations. Fig. 3 shows a positive correlation between free and total PER concentrations in 57 plasma samples. It was observed that the free concentration increased exponentially as total concentration increased, as shown in Fig. 3. The reference range of blood PER concentration was 86–1000 ng·mL^{-1} [15,33], and the total PER concentration was positively correlated with free PER concentration. However, when the blood PER concentration exceeded 1000 ng·mL^{-1}, the free PER concentration rapidly increased. This indicates that in clinical practice, the total PER concentration does not fully represent the free PER concentration, and when the total PER concentration reaches saturation, the free PER concentration increases sharply, causing neurological toxicity or other adverse effects.

The free drug concentration can be calculated from the total drug concentration and plasma protein binding rate in normal patients. Referring to relevant references [15,33], the reference range for the total PER concentration was 86–1000 ng·mL^{-1}. According to the theoretical plasma protein binding rate (95 %), the reference range of free PER concentration was 4.47–52.63 ng·mL^{-1}. In actual clinical samples, seizures in child No. 38 were not effectively controlled, with an average frequency of 2–3 per month. The daily dose of PER was 2 mg·d^{-1}, the total drug PER concentration was 289.24 ng·mL^{-1}, and the free drug PER concentration was 1.95 ng·mL^{-1}. Although the dosage and total drug PER concentration were within the reference range, the free drug concentration was lower than the theoretical reference range, that is, the actual active PER did not reach the effective concentration. After communication with the clinician, the child did not exhibit drowsiness or abnormal mental behavior; therefore, it was recommended to increase the PER dose to 4 mg·d^{-1}, continue to closely observe the changes in the condition, and review the plasma PER concentration when necessary. Meanwhile, similar clinical samples, such as No. 3, No. 5, No. 8, No. 20, No. 26, No. 45, and No. 46, were recommended by the clinician. On the contrary, after changing the previous OXC treatment to VPA and PER, no convulsive symptoms appeared in patient No. 25 and epilepsy was effectively controlled. The daily dose of PER was 5 mg·d^{-1}, the total drug PER concentration was 1158.38 ng·mL^{-1}, and the free drug PER concentration was 17.71 ng·mL^{-1}. Although the total drug concentration was outside the reference range, the free drug concentration was within the reference range, and the child experienced no mental or psychiatric adverse reactions. Therefore, clinicians were advised to continue treatment at this dose. Meanwhile, similar clinical samples were recommended by the clinician such as No. 6. In summary, practice has proven that monitoring free PER concentration is crucial for clinical patients.

4.3. Effects of PER combined with other AEDs on total and free PER concentrations

As a third-generation AED, PER is generally used in combination with other AEDs to treat epilepsy in clinical practice. The main metabolic pathway of PER was in the liver involves oxidative metabolism, followed by glucuronidation, which is mediated by cytochrome P450 (CYP) 3A4/5, CYP1A2, and CYP2B6 enzymes [34]. Drugs that induce or inhibit these isoenzymes may affect PER clearance and plasma levels [5]. For instance, drugs that induce liver CYP3A4/5 can reduce PER's half-life by 50 %–70 %, indirectly affecting the blood PER concentration, resulting in a 2–3-fold decrease in blood concentration [12]. OXC can increase the clearance rate of PER and thus decrease the mean PER AUC values by 50 % [35]. Other AEDs, such as PHB and CBZ, which also induce the same enzyme as OXC, can reduce PER clearance [5,36]. In contrast, the broad-spectrum enzyme inhibitor VPA may increase the plasma PER concentration in patients with epilepsy [37,38]. Our results showed that the total PER concentration was significantly reduced in the group treated with enzyme inducers compared with the group treated with enzyme inhibitors, which is consistent with the results reported in the aforementioned literature. However, free PER concentrations were significantly increased in the VPA group compared with those in the group treated with enzyme inducers and the group not treated with strong enzyme inducers/noninhibitors. The results showed that the effect of VPA on free PER concentration was more pronounced than that on total PER concentration. This may be attributed to VPA's potent inhibition of CYP2C9 activity in human liver microsomes, with only minor inhibitory effects on the CYP2C19 and CYP3A4 subtypes [39]. Hence, VPA does not significantly affect the total PER concentration. As PER is highly protein-bound in plasma, other highly protein-bound drugs, such as VPA, can potentially displace PER, increase free PER concentrations, and cause neurological toxicity or other adverse effects. We recommend closely monitoring the free PER concentration in patients using VPA along with PER to ensure medication safety.

4.4. Limitations

When using the CF-UF method to determine the free drug concentrations, the ultrafiltration membrane exhibits similar nonspecific adsorption properties to those of the cellulose membrane. This can lead to a concentration polarization effect, where a thin and dense protein layer is formed on the ultrafiltration membrane, accompanied by filtered plasma. Consequently, some free drugs may fail to enter the ultrafiltration membrane, resulting in lower concentrations of free drugs and higher plasma protein binding rates. Furthermore, *in vivo*, PER is mainly bound to albumin and α-1-acid-glycoprotein, and a decrease in protein content due to pathological conditions results in a poor correlation between free and total PER concentrations. However, whether children with epilepsy have hypoalbuminemia and whether an unusually high free PER concentration can be induced in patients with hypoalbuminemia are not investigated in this study. Therefore, in the future, the difference in free PER concentrations between normal and low protein levels needs to be studied and more clinical samples from patients with hypoalbuminemia are needed for evaluation.

5. Conclusion

Herein, we established a method combining CF-UF with HPLC–MS/MS for determining the free PER drug concentration in plasma samples. This method offers the advantages of being simple, rapid, highly stable, highly sensitive, and accurate, making it suitable for the detection of plasma PER-free concentrations in children with epilepsy. In addition, the response of free PER concentration to

clinical outcomes in children with epilepsy is clinically significant, particularly when combined with VPA. Future research should investigate the impact of protein levels on free PER concentrations in children with epilepsy, particularly in cases of hypoalbuminemia.

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Ethics and consent statement

This study was reviewed and approved by [Medical Research Ethics Committee of Hebei Children's Hospital] with the approval number: [No. 202206-15], dated [20220307]. The biological samples we analyzed were derived from the secondary use of blood from the therapeutic drug monitoring program, so we inform the patients verbally.We have obtained verbal consent from each participant, and consent was obtained from minors and the parents/legal guardian.

Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Ying-Hua Ma: Writing – review & editing, Writing – original draft, Methodology, Funding acquisition, Formal analysis. **Lei Dong:** Writing – review & editing, Supervision, Funding acquisition. **Jia-Xuan Wu:** Software. **Shi-Yuan Hu:** Investigation. **Xiang-Fei Meng:** Validation, Data curation. **Yi-Le Zhao:** Visualization, Supervision. **Kang Liu:** Resources. **Dan-Ni Yan:** Investigation. **Su-Zhen Sun:** Supervision, Project administration.

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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