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Development and Validation of HPLC-FLD Analysis of Perampanel in MEPS-Processed Rat Plasma Sample

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Abstract: Perampanel, a novel α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor antagonist, is registered for the adjunctive treatment of patients (aged ≥ 12 years) with refractory partial-onset seizures. A simple high-performance liquid chromatographic method fluorescence detection (HPLC-FLD) was developed to analyze perampanel in rats' plasma and validated for bioanalytical purposes. Rats' plasma (50 µL) was processed by microextraction packed sorbent (MEPS). The analytes were separated using a Hypersil Gold octadecyl silane column (250 × 4.6 mm internal diameter, 5 µm particle size) with isocratic elution. A mobile phase consisting of acetonitrile–methanol–water (275:275:450, *v*/*v*/*v*; containing 50 µL triethylamine and pH adjusted to 3.25 with orthophosphoric acid) was used in this analysis. The flow rate was 1.25 mL/min. Analytes were monitored at an excitation wavelength of 285 nm and an emission wavelength of 430 nm. The linearity range for this validated method was from 3.75 to 300 ng/mL. No endogenous peaks were found in the elution of analytes in drug-free rats' plasma. Intra- and inter-batch reproducibility studies demonstrated accuracy and precision within the acceptance criteria. The results indicate that the present method is simple, selective, reproducible, and suitable for the analysis of perampanel in small volume samples. The robustness of the method was accessed using MODDE® design of experiments software version 12.5.

Keywords: MEPS; HPLC; perampanel; fluorescence; rat plasma; development; validation; ruggedness; DoE; MODDE 12.1

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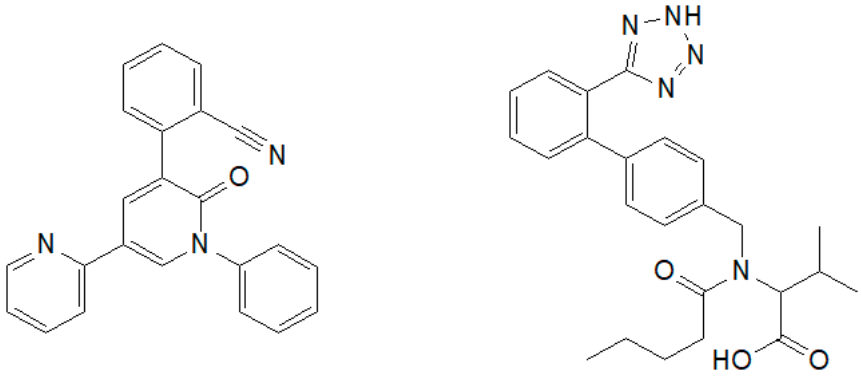
1. Introduction

The European Medicines Agency and the US Food and Drug Administration have approved perampanel (Figure 1a), a highly selective non-competitive allosteric α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid receptor antagonist, for use as an adjuvant treatment for focal seizures and primary generalized tonic–clonic seizures linked to idiopathic generalized epilepsy in patients 12 years of age and older. Chemically, it is 2-(2-oxo-1-phenyl-5-pyridine-2-yl-1,2-dihydropyridine-3-yl) benzonitrile [1]. The mean plasma concentration of perampanel (C_{max}) is recorded between 0.5 h and 4 h after orally administering 1, 2, 4, and 12 mg of a coated tablet in fasting adults. It is rapidly and completely absorbed. C_{max} ranged from 36.8 to 335.7 µg/L following the administration of 1–12 mg tablets orally. The mean oral bioavailability is more than 95%. The mean half-life ($t_{½}$) of perampanel varied from 21.1 to 835.6 h (102.7 ± 74.2 h). Following 1 mg tablets in fed conditions, T_{max} is delayed, and C_{max} ranged from 6.4 to 33.8 µg/L (19.3 ± 6.0 µg/L, n = 24) [2].

The literature describes a number of analytical techniques developed for the estimation of perampanel in human plasma, serum, and rat plasma. These techniques include HPLC-UV/PDA [3–7], HPLC-FLD [3,8–10], LC-MS/MS [8,11–18], UHPLC-QTOF-MS [19], capillary electrophoresis equipped with a fluorescence detector (CE-FLD) [20], HPTLC, and surface-enhanced Raman scattering (SERS) [21].



(**a**) (**b**)

Figure 1. Chemical structure of (**a**) perampanel and (**b**) valsartan (IS).

There is variation in the limit of quantitation in the works showed by different authors using HPLC-UV (2.5–50 ng/mL) [3–7], HPLC-FLD (1–25 ng/mL) [3,8–10], or LC-MS/MS (0.5–7.4 ng/mL) [8,11–18], utilizing 10 to 1000 µL of plasma for sample processing and varied analytical conditions.

There is a need for the development of an analytical method that is sensitive, rapid, and economical for the quantization of perampanel plasma concentration to support animal studies and BA/BE/TDM studies where the sample volume is limited.

It was essential to establish a simple and precise assay capable of quantifying drug concentrations down to 5% of C_{max} (~5 ng/mL) in small sample volumes following the oral/intraperitoneal administration of perampanel for animal studies. The present research describes a simple, rapid, sensitive HPLC-FLD method to determine perampanel in rat plasma utilizing the commercially available internal standard (Valsartan, Figure 1b).

2. Materials and Methods

2.1. Chemicals

Perampanel was obtained from Biosynth USA, through local supplier and valsartan (IS, 99.6%) was procured from Sigma-Aldrich, USA. Figure 1 represents the chemical structures of the drugs. Chemicals like acetonitrile, methanol, and water (Lichrosolv, Merck, Darmstadt, Germany) for the analysis were of HPLC grade. In this experiment, analytical-grade chemicals were used.

2.2. Drug Solutions

All the samples prepared were stored in an amber-colored flask until analysis due to the possible photosensitivity of the drug. The stock solution of perampanel and valsartan (250 µg/mL) was prepared by dissolving a known quantity of the drug in methanol and water (70:30, *v*/*v*). The stock solution was used for the preparation of a working solution and other standard solutions for the analysis. A working solution was prepared by further diluting the stock solution with methanol and water in a ratio of 70:30 (*v*/*v*).

2.3. Working Solutions

Using appropriate aliquots of a stock or working solution of perampanel, various concentrations of perampanel (75, 150, 300, 600, 1200, 1500, 2400, 3000, 4500, and 6000 ng/mL) were prepared, which were later used for the preparation of spiked plasma samples. An internal standard solution (5 µg/mL) was prepared by diluting the stock solution with acetonitrile. All stock solutions were stored between 4 and 8 °C.

2.4. Chromatographic Conditions

Shimadzu Prominence Liquid Chromatographic Instrument consisting of a reciprocating quaternary gradient pump (Shimadzu-20-AD UFLC Degasser–DGU-20A3 Prominence

Degasser), a fluorescence detector (RF-20), an autosampler and injector (SIL-20A Prominence autosampler), a communication bus module (CBM-20A), and Hypersil-Gold column (C-18, 250 mm × 4.6 mm, particle size 5 µm, Thermo-Fisher, Waltham, MA, USA) were used. The generated data were collected with the help of Microsoft Windows 7 based LC-Solution chromatographic software version 1.25. The fluorescence detector was operated at excitation and emission wavelengths of 285 nm and 390 nm, respectively. All analyses were isocratically carried out. The flow rate was 1.25 mL/min.

Mobile Phase

Selection of the appropriate mobile phase is essential in method development. Various factors were considered for method development, including volume fraction of organic solvents (methanol and acetonitrile) in mobile phase and buffer. The optimized mobile phase was methanol 275 mL, acetonitrile 275 mL, and water 450 mL (containing 0.25 mL triethyl amine, pH adjusted to 3.25 using OPA), which was the most suitable mobile phase.

Using a dilute solution of orthophosphoric acid, the mobile phase pH was adjusted to 3.25 ± 0.05. The pH was measured using an Orion Research Model 611 pH meter. The mobile phase was filtered through 0.45 µm Sartorius filter using vacuum and degassed using ELMA ultrasonic bath.

2.5. Plasma Samples

The present study was approved by the Deanship of Graduate Studies Al-Ahliyya Amman University, Amman, Jordan (Number 320 – ك د ع صك ص, 7 April 2019). Rat blood samples (~1.5 mL each) were collected from different adult albino rats (weighing 150–200 g) as per requirement. The plasma was separated, screened, and then pooled together. Pooled blank plasma (950 µL) was spiked with a working solution of perampanel (50 µL) to obtain perampanel concentrations of 3.75, 7.5, 15, 30, 60, 75,120, 225, and 300 ng/mL. Similarly, the quality control samples of perampanel as a single batch (of concentrations 7.5 (low), 150 (medium), and 225 ng/mL (high)) were prepared by spiking 50 µL of appropriate working (150, 3000, and 4500 ng/mL) solution to 950 µL of pooled blank plasma. The quality control samples were divided into aliquots in Eppendorf tubes and were stored at −20 ± 5 °C until analysis.

Sample Processing

Plasma samples (50 microliters) were transferred to Eppendorf tubes. The internal standard (50 µL) was added to all the plasma samples except the blank. Thereafter, samples were diluted with 150 µL of phosphate buffer (pH 7.4), vortexed and processed using MEPS (C-8 cartridge). In brief, MEPS was conditioned twice with ethanol (100 µL) and thereafter with phosphate buffer (100 µL). The sample was loaded to MEPS by a pull-and-push mechanism using a MEPS syringe (75 µL, three times); thereafter, the MEPS cartridge was washed with 100 µL of phosphate buffer, and the sample was eluted with ethanol (50 µL) twice. Samples (2 µL) were injected into an HPLC column, and AUCs were recorded and reported.

2.6. Bioanalytical Method Validation

Validation of the developed method was carried out for selectivity, sensitivity, linearity, precision, accuracy, recovery, and stability. Validation was performed on three different days, with each validation consisting of spiked standard samples with different concentrations: three sets of quality control samples consisting of low, medium, and high QC samples along with blank and blank spiked with internal standard. At the beginning of each validation run, the analysis of the calibration samples was carried out from low concentration to high. The other samples were randomly analyzed between runs with respect to the blank sample, and they were analyzed after the high concentration sample. Evaluation of carry-over effect was done in order to make sure that the rinsing solution (10% aqueous solution of ethanol) used in cleaning the needles, in fact, avoided the carry-over of the injected samples in the succeeding runs. On day 3, along with the validation

sample, the freeze–thaw and stability samples were analyzed. Evaluation of the linearity was done using weighted (1/x) least-squares regression analysis. The correlation coefficient (r) for the calibration curve must be 0.98 or better. A deviation of 15% from the normal value was set as an acceptance criterion for each of the back-calculated standard concentration, and 20% was set for the LLOQ. According to bioanalytical method validation guideline [22], at least 67% of non-zero standard should meet the criteria that are set above including the LLOQ and the upper limit quantitation.

2.6.1. Specificity and Selectivity

Drug-free plasma was used in the testing process to evaluate specificity. The retention time of endogenous compounds from a different matrix (rat plasma) was compared with those of perampanel and valsartan.

2.6.2. Recovery

Recovery of perampanel from the MEPS-processed samples was evaluated by a comparison of the peak area of drugs in processed spiked plasma samples (LQC, MQC, and HQC) with the peak area of drugs in unprocessed samples prepared by spiking supernatant drug-free plasma samples with the same amount of perampanel at the step immediately prior to chromatography.

2.6.3. Linearity

The peak area ratio method was used to evaluate the linearity of the developed method. The calibration samples (3.75, 7.5, 15, 30, 60, 75,120, 225, and 300 ng/mL) along with blank and blank spiked with IS were processed and analyzed. The best-fit regressed curve was constructed with the linear equation $y = mx + c$ using different weighting factors. LOD and LOQ were also calculated from the replicate analyses of different samples.

2.6.4. Accuracy and Precision

Between-batch precision and accuracy were determined by analyzing different sets of quality control samples on three consecutive days, and within-batch accuracy and precision were evaluated by analyzing the QC samples. All the QC samples were daily processed and immediately analyzed after the standard curve, or either in between or at the end. The within-batch and between-batch precision criteria were set for 15% as per the guideline. The standard samples were processed and injected on three different consecutive days and analyzed. The area ratio of perampanel and internal standard was calculated. To define the correlation between the concentration and area response of perampanel and IS, the area ratio was plotted against the concentration of perampanel with a weighting factor 1/x. The method was evaluated by determining intercept values and the correlation coefficient.

2.6.5. Stability

The evaluation of bench-top stability was carried out by keeping the replicates of low, mid, and high QC samples at room temperature for 8 h. The stability of the freeze–thaw sample was achieved by exposing the samples (sets of LQC and HQC) to three freeze–thaw cycles by thawing it for 2–3 h at room temperature and again refreezing for 12–24 h. Auto sampler stability of perampanel was performed by analyzing the processed and reconstituted QC sample that was stored for 24 h in an autosampler tray. Stability test was performed after storing it at −20 °C for 7 days. The analysis was carried out in one batch for each concentration and storage conditions and replicates. Perampanel concentrations after every storage time were correlated with the samples' original concentrations.

2.6.6. Robustness

Robustness is a measure of the reproducibility of test results under normal, expected, operational conditions from one analyst to another [23,24]. Robustness is determined based on precision (% CV or RSD); as determined, the concentration was not expected to go

beyond 15% in bioanalytical studies. To study the robustness in the present test, design of experiment (DOE) was used [22,23]. In this study, to test the robustness, a factorial design was used. Software tools like DOE play a vital role in such studies as they give flexibility in altering the parameters by reducing the number of experiments. In the present research, variables like excitation wavelength (285 or 290 nm), pH (3.00 or 3.50), and flow rate (1.15 or 1.35) were evaluated. Tests were randomly run; the responses selected were retention time (Rt) for perampanel and IS, tailing factor (Tf-P), area count (AUC), and area ratios of perampanel and internal standard [25,26].

3. Results and Discussion

A new HPLC-FLD method for the estimation of perampanel in rat plasma utilizing valsartan (IS) has been developed. To deal with the sample matrix effect, stable analyte must be used as the internal standard. Due to the unavailability of commercially available internal standards, an alternate method has been employed. The internal standard used must match the chromatograph properties, fluorescence properties, and recovery of the analyte. The result shows that the performance of this HPLC-FLD method was neither affected nor worsened by valsartan (IS).

3.1. Separation and Specificity

The developed new HPLC-FID method exhibits high selectivity without any interference at the time of the analyte signal. Appropriate base-to-base resolution was observed for perampanel and valsartan. A symmetric peak shape was achieved for the analyte and the internal standard by optimizing chromatographic conditions several times through method development trials. Excellent separation was achieved by using the mobile phase consisting of a mixture of acetonitrile: methanol: water (27.5:27.5:45.0, $v/v/v$, 0.050 mL TEA, pH adjusted to 3.25). The retention times of perampanel and IS were ~7.0 and 8.5 min, respectively. The specificity of the method was examined by analyzing six different blank rats' plasma. No significant interfering peaks from the plasma were found at the retention time of perampanel or IS (Figure 2).

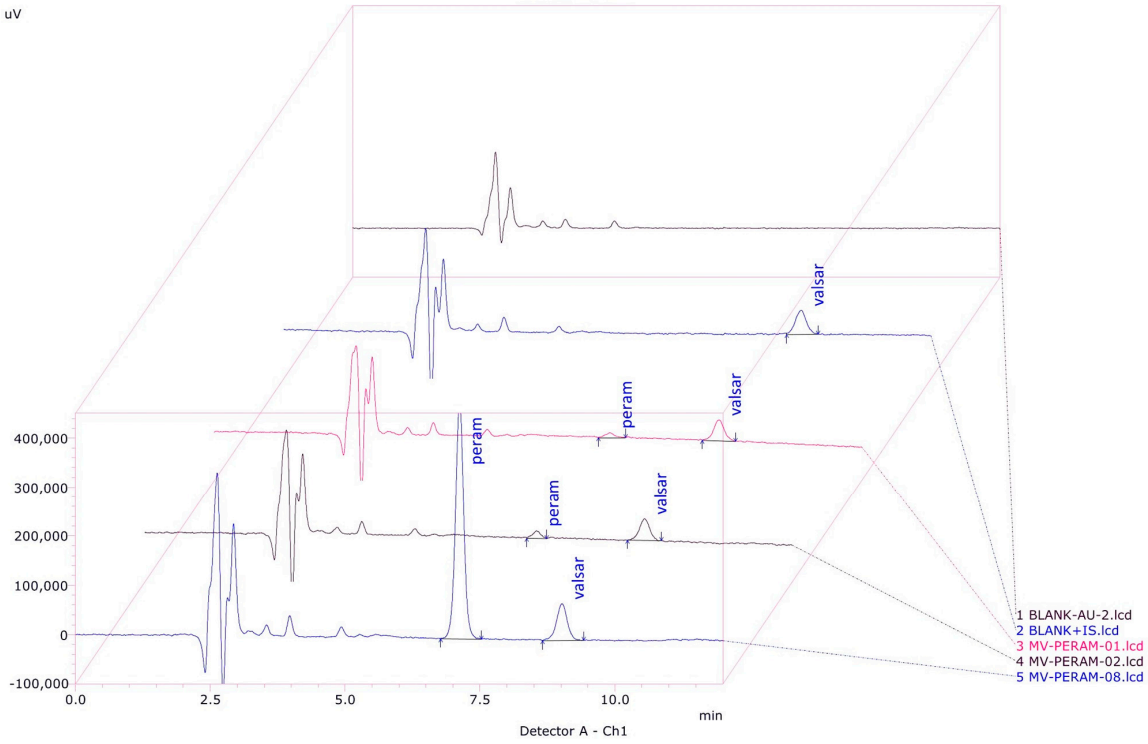


Figure 2. Representative chromatogram of (1) blank plasma, (2) blank + IS, (3) perampanel (3.75 ng/mL) + IS, (4) perampanel (7.5 ng/mL) + IS, and (5) perampanel (150 ng/mL) + IS.

3.2. Linearity

The peak area ratios of perampanel to IS in plasma were linear with respect to the analyte concentration over the calibration range (3.75 to 300 ng/mL). Calibration curves were constructed using both unweighted (x) and weighted (1/x) methods, and |%RE| was calculated. Weighted least-squares linear regression analysis (1/x) of the analytes versus the area ratio of the target drug to that of the internal standard concentration was performed to calculate the calibration equation. The mean linear regression equation ($y = mx + c$) of the calibration curve for perampanel was $y = 0.043\ x - 0.03377$ (r = 0.997367). The correlation coefficient (r) was above 0.999 for perampanel over the concentration range used. The precision, characterized by the relative standard deviation, was less than 15% for perampanel and valsartan (Table 1 and Figure 3).

Table 1. Statistical evaluation of the analysis results for perampanel in standard curves for three days.

SN.	Nominal Concentration (ng/mL)	Area Count of Perampanel	Area Count of IS	Area Ratio	Concentration Found (ng/mL)		Bias	Precision
					Mean	SD	%	%
1	3.75	118,008	1,184,678	0.1078	3.30	0.30	−12.27	9.09
2	7.5	340,613	1,222,874	0.2794	7.30	0.60	−2.57	8.22
3	15	693,368	1,104,327	0.6273	15.33	0.81	2.33	5.27
4	30	1,577,831	1,260,301	1.2592	29.97	1.96	−0.03	6.53
5	60	2,906,440	1,129,887	2.5783	60.50	2.65	0.83	4.38
7	120	6,043,954	1,159,437	5.2098	121.37	2.79	1.10	2.30
8	225	10,786,796	1,145,986	9.3763	217.63	10.78	−3.27	4.95
9	300	15,973,405	1,227,733	13.0217	302.13	9.77	0.73	3.23

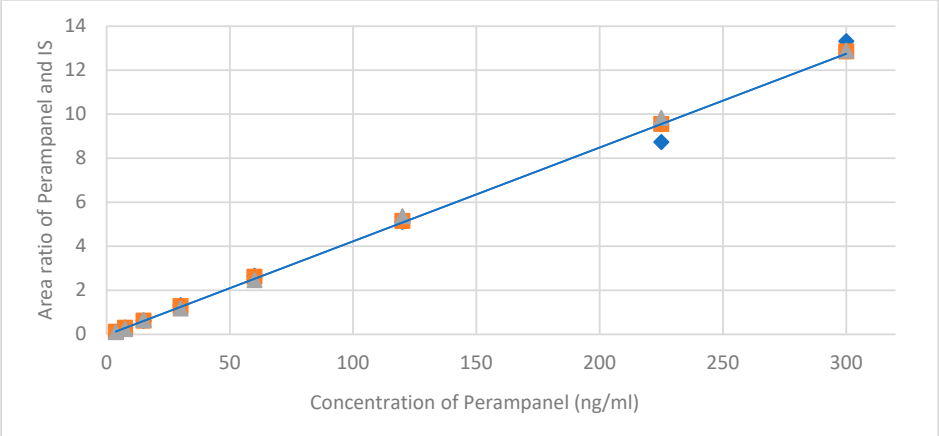


Figure 3. Calibration curve for the analysis of perampanel showing relationship between concentration versus area count ratio.

Mano et al. [9] reported the analysis of perampanel using HPLC-RF utilizing liquid–liquid extraction process, and the calibration curve was found linear over the concentration range from 1 to 500 ng/mL. The slope ranged from 0.0253 to 0.260 with 1.7% RSD, and the intercept was from 0.00355 to 0.00372 with 2.3% RSD. The correlation coefficient was >0.999. In another research, Mano et al. [14] reported the analysis of perampanel using LC-MS/MS; the calibration curve was linear over 0.25 to 200 ng/mL utilizing 100 µL of plasma. In another research, Franco et al. (2016) [4] illustrated the HPLC method with ultraviolet detection for the analysis of perampanel in plasma utilizing 200 µL of a sample processed using acetonitrile as the protein precipitant. Ten microliters of a processed sample was used for analysis. The linearity range of the present method was 25–1000 ng/mL. De Grazia et al. (2018) [15]

reported the analysis and comparison between the developed LC-MS/MS method and the HPLC-FLD method. The linearity range was 50 to 2000 ng/mL utilizing a 50 µL sample.

3.3. Precision and Accuracy

The precision and accuracy of the method can be assured if the precision is less than 15%, indicating that the repeated sample analysis is precise. The precision values were between 5.16 and 10.71%, and for accuracy, they varied from 99.40 to 107.89%; the values of both precision and accuracy were within the accepted range (Table 2).

Table 2. Precision and accuracy of the method of six sets of individually prepared and extracted plasma samples of perampanel (LQC, MQC, and HQC) over 3 different days.

Sample (ng/mL)	Concentration Found		Precision	Accuracy	Bias (%)
	Mean	SD	%	%	%
		Day1			
LQC (7.5)	7.46	0.41	5.51	99.40	−0.60
MQC (150)	153.67	16.45	10.71	102.45	2.45
HQC (225)	240.86	25.66	10.65	107.05	7.05
		Day2			
LQC (7.5)	7.47	0.4	5.34	99.62	−0.38
MQC (150)	155.63	13.56	8.71	103.75	3.75
HQC (225)	236.17	12.19	5.16	104.96	4.96
		Day3			
LQC (7.5)	7.29	0.39	5.39	97.24	−2.76
MQC (150)	161.83	13.8	8.53	107.89	7.89
HQC (225)	241.25	17.54	7.27	107.22	7.22

N = 6, each concentration.

3.4. Stability of Spiked Plasma Samples

The stability of perampanel in the plasma matrix at room temperature and under prolonged storage conditions (−20 °C) during the study period was investigated. The samples LQC (7.5 ng/mL), MQC (150 ng/mL), and HQC (225 ng/mL) were processed and analyzed in triplicate, and the back-calculated values were studied. The samples were stable at room temperature without significant loss up to 8 h (Table 3). The results indicate that the samples were stable for 7 days during the study. The mean percent stability of the samples ranged from 102.84 ± 6.29% to 103.22 ± 6.77%. The % coefficient of variation was less than 6.56% (Table 4).

Table 3. Short-term stability of perampanel and valsartan at room temperature.

SN	(Time, h)	Mean (Area)	SD	RSD %	% Recovery *	Bias (%)
			Perampanel (LQC, 7.5 ng/mL)			
1	0	272,814.7	5747.3	2.11	100.0	0.0
2	4	269,135.3	5239.0	1.95	98.7	−1.3
3	8	271,203.3	7383.4	2.72	99.4	−0.6
			Perampanel (HQC, 225 ng/mL)			
1	0	14,002,961	504,544.5	3.60	100.0	0.0
2	4	13,918,127.7	327,454.7	2.35	99.4	−0.6
3	8	13,860,836.3	506,346.8	3.65	99.0	−1.0
			Valsartan (IS, 250 ng/mL)			
1	0	1,373,928.0	77,441.4	5.64	100.0	0.0
2	4	1,367,948.3	48,899.9	3.57	99.6	−0.4
3	8	1,341,327.0	57,672.8	4.30	97.6	−2.4

* Mean area values at time zero were considered as 100%. Similarly, the following results were compared with zero time (100%).

Table 4. Long-term stability of perampanel in spiked plasma samples stored at −20 °C.

Day	Nominal Concentration (LQC, 7.5 ng/mL)		Nominal Concentration (HQC, 225 ng/mL)	
	Average Concentration Found (ng/mL)	% Recovery	Average Concentration Found (ng/mL)	% Recovery
1	7.72	102.91	221.12	98.28
2	7.98	106.43	215.27	95.68
3	8.05	107.32	239.45	106.42
4	8.29	110.53	214.13	95.17
5	7.75	103.33	251.67	111.85
6	6.91	92.19	238.17	105.85
7	7.29	97.2	245.98	109.32
Mean	7.71	102.84	232.26	103.22
SD (±)	6.29	6.29	6.77	6.77
RSD	6.12	6.12	6.56	6.56

3.5. Freeze–Thaw Stability

The stability of perampanel was determined after three freeze and thaw cycles. Three aliquots of each of the QC samples were stored at −20 °C for 12–24 h and thawed unassisted at room temperature. When completely thawed, the samples were refrozen for 24 h under the same conditions; three freeze and thaw cycles were repeated. The following results were obtained, which were calculated using the calibration curve. The mean percent freeze–thaw stability ranged from 93.2 to 110.3%, which was calculated using the calibration curve. The samples were stable during the three freeze–thaw studies (Table 5).

Table 5. Freeze–thaw stability of perampanel in spiked plasma samples over three freeze-thaw cycles.

Sample LQC	Concentration Found		Precision	Accuracy	Bias (%)
FT Cycle	Mean	SD	%	%	%
Zero time	7.41	0.27	3.6	98.8	−1.2
FT Cycle-I	7.24	0.40	5.5	96.5	−3.5
FT Cycle-II	7.05	0.89	12.7	94.0	−6.0
FT Cycle-III	6.99	0.31	4.4	93.2	−6.8
Sample HQC	**Mean**	**SD**	**Precision**	**Accuracy**	**Bias (%)**
Zero time	248.12	15.74	6.3	110.3	10.3
FT Cycle-I	228.91	18.65	8.1	101.7	1.7
FT Cycle-II	231.28	15.20	6.6	102.8	2.8
FT Cycle-III	233.11	1.69	0.7	103.6	3.6

3.6. Recovery Studies

Recovery studies of perampanel were conducted; the area count of the processed samples and aqueous samples was compared, and the percent recovery was calculated. The percent recovery ranged from 75.1 to 88.2% (Table 7).

3.7. Relative Recovery

Different concentrations (sets of QC samples) of perampanel were spiked into plasma and extracted. Analysis was repeated to determine the recovered concentrations of perampanel (n = 3). Relative recoveries ranged from 96.1 to 105.1%. The mean recoveries ranged from 99.5 to 101.9% (Table 6).

Table 6. Relative recovery studies of perampanel from spiked sample.

Nominal Concentration (ng/mL)	Mean Conc. Recovered (ng/mL)	SD	Precision (%)	Relative Recovery (%)
		Day 1		
7.50	7.42	0.21	2.81	98.89
150.00	154.21	13.96	9.05	102.81
225.00	229.66	11.49	5.00	102.07
Mean recovery				101.26
		Day 2		
7.50	7.33	0.21	2.80	97.78
150.00	154.37	5.35	3.47	102.92
225.00	236.48	20.51	8.67	105.10
Mean recovery				101.93
		Day 3		
7.50	7.42	0.33	4.48	98.89
150.00	144.08	13.48	9.36	96.06
225.00	232.95	22.93	9.84	103.53
Mean recovery				99.49

Table 7. Absolute recovery of perampanel from spiked sample compared with aqueous samples.

Nominal Conc. (ng/mL)	AUC of Perampanel in Spiked Sample	Mean Peak Area	AUC of Perampanel in Aqueous Sample	Mean Peak Area	% Recovery
7.5	254,154 278,421 215,415	249,330	292,275 334,108 290,808	305,730	81.6
150	7,125,415 7,251,489 6,781,524	7,052,809	9,263,035 9,064,365 9,833,209	9,386,870	75.1
225	13,541,549 12,426,515 12,104,554	12,690,873	14,512,457 14,789,545 13,845,125	14,382,376	88.2

3.8. Robustness of Method

As mentioned in an earlier paragraph, robustness was studied using DOE software MOODE GO version 12.1. The parameters used for evaluating robustness were the excitation wavelength (285 or 290 nm), pH (3.00 or 3.50), and flow rate (1.15 or 1.35 mL/min). The deliberate changes in the method do not significantly affect the retention time, tailing factor, and area count for drug (% CV less than 15%). The minor changes in the method had no significant impact on either retention time, tailing factor, or AUC for drug (%CV less than 15%). The scaled and the centered coefficient plot showed that the parameters had no effect on the response. Hence, it can be stated that the method developed is rugged and robust. The flow rate of the mobile phase affected the AUC and the RT of the drug in the investigation (Figures 4 and 5 and Table 8).

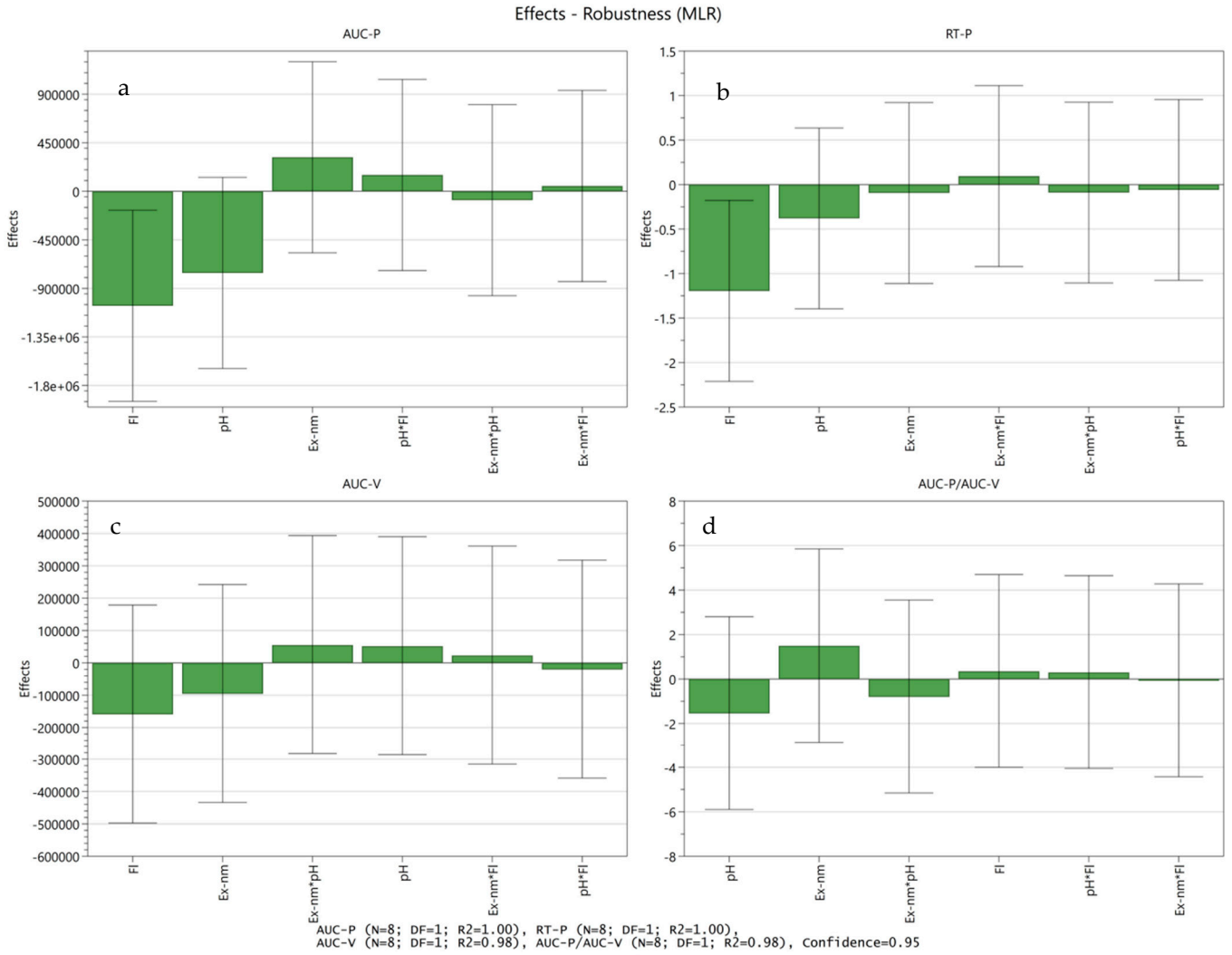


Figure 4. Effects of flow rate, excitation wavelength, and pH on (**a**) area of perampanel (Area-P), (**b**) retention time (RT-p), (**c**) AUC of valsartan (AUC-V), and (**d**) area ratio (AUC-P/AUC-V).

Table 8. Robustness studies of the HPLC-FLD method utilizing design of experiment.

SN.	Factors			Response						
	Ex λ (nm)	pH	Flow Rate (mL/min.)	AUC-P	AUC-V	RT-P	RT-V	TF-P	TF-V	$\frac{AUC-P}{AUC-V}$
1	285	3.0	1.15	7,970,856	965592	7.70	9.77	1.05	1.07	8.25
2	290	3.0	1.15	8,389,145	764,744	7.68	9.76	1.05	1.03	10.97
3	285	3.5	1.15	7,214,936	956,058	7.55	9.97	1.05	1.04	7.55
4	290	3.5	1.15	7,329,742	919,054	7.19	9.87	1.06	1.05	7.98
5	285	3.0	1.35	6,778,935	777,202	6.55	8.31	1.06	0.97	8.72
6	290	3.0	1.35	7,156,018	675,402	6.56	8.32	1.05	1.01	10.6
7	285	3.5	1.35	6,186,015	779,715	6.12	8.4	1.06	1.04	7.93
8	290	3.5	1.35	6,538,541	735,322	6.11	8.38	1.06	0.95	8.89
			Mean	7,195,524	821,636	6.93	9.1	1.05	1.02	8.86
			SD	724,086	109,600	0.68	0.8	0.01	0.04	1.27
			% CV	10.06	13.34	9.78	8.78	0.55	3.94	14.28

3.9. Advantages over Other Reported Methods

Although the LC-MS/MS methods are selective and precise, these high throughput analytical methods are costly and require a skilled operator. The advantages of the present method in comparison with other methods reported (Table 9) are as follows:

(1) In processing of samples, a small volume of plasma is required.
(2) In MEPS, the processed samples are clean with negligible interfering components, which enhance the column life and exhibit better chromatography.
(3) The sensitivity of the method is enhanced as compared with the other reported HPLC-UV and HPLC-FLD methods.
(4) The present method can be successfully utilized for the analysis of perampanel (as low as 5% of C_{max}) following the administration of 2 mg or higher, which provides C_{max} in the range of 60.7 ng/mL to 335.7 ng/mL.
(5) The intrinsic fluorescence of drugs requires less injection volume (2 µL).
(6) Lastly, the method can be explored for the analysis of plasma samples for animal studies.

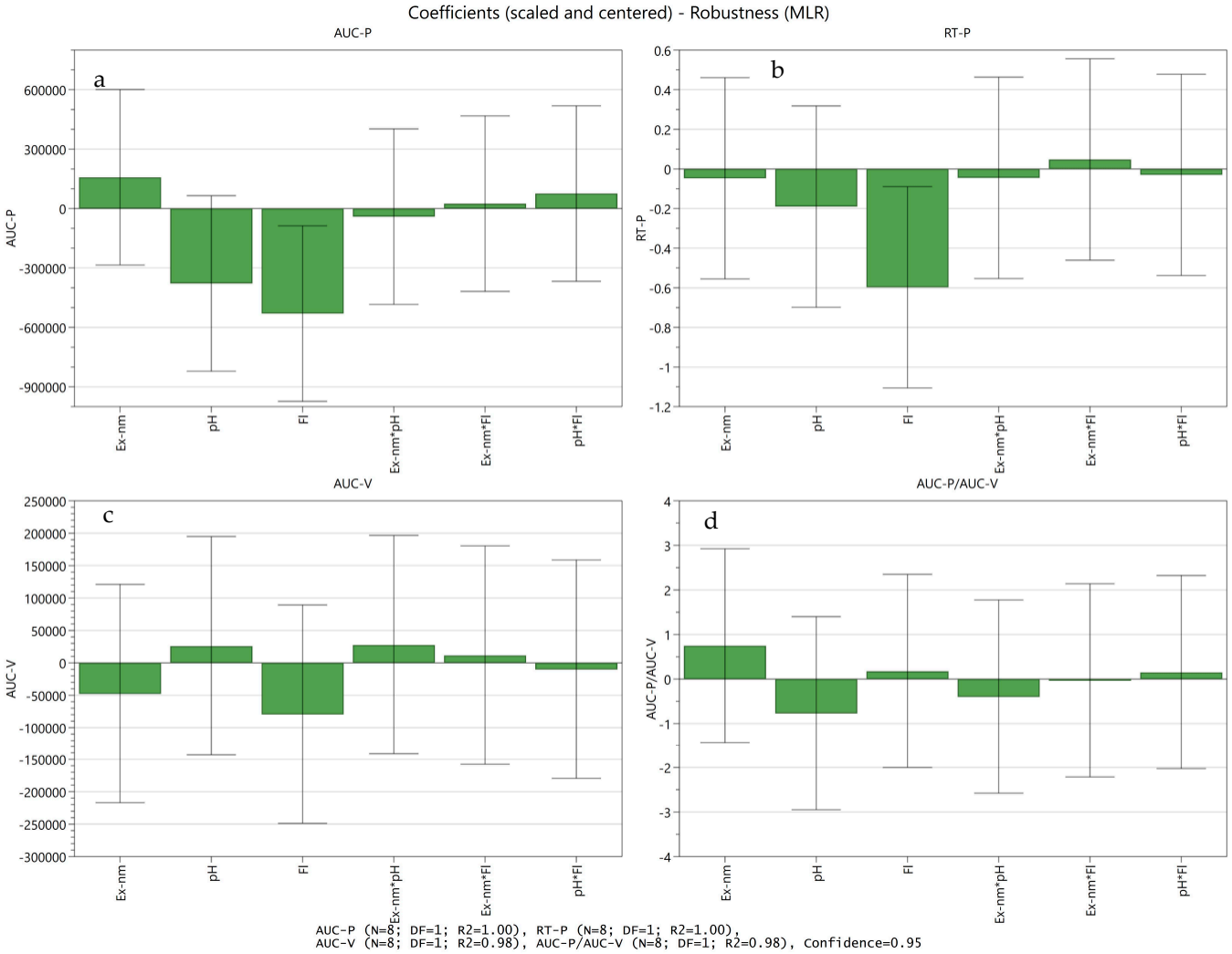


Figure 5. Scaled and centered coefficient of variation (%) of (**a**) area of perampanel (Area-P), (**b**) retention time (RT-p), (**c**) AUC of valsartan (AUC-V), and (**d**) area ratio (AUC-P/AUC-V) for different factors.

As compared with animal studies, which have limited availability of blood volume, this study enables us to collect a greater number of samples at different time points, which is not part of other studies mentioned in Table 9. Since the drug is having a long half-life,

i.e., ~100% bioavailability with no evidence of marked first-pass metabolism, the present method can be utilized in the estimation of perampanel rats' plasma [2].

Table 9. Summary of different analytical methods used for the estimation of perampanel in human and rat plasma.

Method	Chromatographic Conditions/Type of Matrix (Volume)	LOD/LOQ (ng/mL)	Linearity (ng/mL)	Weakness of Method	Strength of Method/Application	Ref.
Present method (HPLC-FLD, Ex/Em λ; 285/430 nm)	Rat plasma (50 µL)	1/3.75	3.75–300	-	The LOQ of 3.75 ng/mL was achieved using low sample volume (only 50 µL), which increases the possibility of collecting a greater number of samples in DMPK/BA studies of PER in a span of 24 h. This LOQ is achieved using expensive LC-MS/MS method [15–19]. Diluted plasma samples processed using a MEPS-equipped syringe result in clean samples. Low sample volume of 2 µL was injected, which increases the column life. These are advantages over a conventional HPLC-UV and HPLC-FLD detector	-
HPLC-UV (300 nm) HPLC-/FLD (Ex/Em λ; 290/430 nm)	Gradient elution using Waters symmetry C18 (75 × 4.6 mm, 3.5 µm column)/human plasma (1 mL)	25/50 1/5	75–1500 5–1500	Sample volume 1 mL required to process	TDM; clinical applications	[3]
HPLC-UV (320 nm)	Two reverse-phase Chromolith performance column, 100 × 4.6 mm internal diameter, RP-18e column/human plasma (200 µL)	10/25	25–1000	Requires two reverse-phase columns together	TDM	[4]
HPLC-PDA (perampanel at 320 nm/Entacapone at 305 nm)	Gradient elution using a LiChroCART® Purospher Star-C18 column (55 × 4 mm; 3 µm particle size)/human plasma (200 µL)	-/30	30–4500	LLE processing time	Clinical applications	[5]
HPLC-UV (254 nm)	C18 XR ODS Shim pack analytical column (4.6 mm I.D. × 50 mm, particle size: 2.2 µm)/human plasma (200 µL)	1.25/2.5	2.5–1000	Extraction cartridges and evaporation at 70 °C, long processing time	Pharmacokinetics studies; clinical applications	[6]
HPLC-UV (320 nm)	Two reverse-phase Chromolith performance column, 100 × 4.6 mm internal diameter, RP-18e column/human plasma (200 µL), glass paper filter discs	10/25	25–1000	Use of serially connected two reverse-phase Chromolith column	Pharmacokinetics studies; clinical applications	[7]
HPLC-FLD (Ex/Em λ; 290/430 nm)	Chromatographic instrumentation not mentioned in text/human plasma (1 mL)	0.25/25	0.25–1000	Minor analytical details not available	Pharmacokinetics studies; clinical applications	[8]
HPLC-FLD (Ex/Em λ; 290/430 nm)	YMC Pack Pro C18 column (150 × 4.6 mm i.d., 5 µm)/human plasma (1 mL)	-/1	1.0–500	Sample volume 1 mL required to process/LLE, long processing time	Short run time (8 min.), pharmacokinetics studies; clinical applications	[9]
HPLC-FLD (Ex/Em λ; 290/430 nm)	Kinetex PFP (100 × 2.6 mm, 4.6 µm) column/human plasma (250 µL)	10/20	20–1000	-	0.8 mL/min flow rate, clinical applications.	[10]
LC-MS/MS	YMC-Pack Pro C8 column (50 mm × 3.0 mm i.d.)/human plasma (250 µL)	0.25/1	0.25–200	Requires a synthetic internal standard (ER-167615)	Flowrate 0.2 mL/min, short run time (5 min), clinical applications	[14]
LC-MS/MS	Poroshell 120 EC-C18 column (50 × 4.6 mm, 2.7 µm particle)/human plasma (50 µL)	-/2.5	2.5–2800	Protein precipitation	Flowrate 0.25 mL/min, clinical applications	[15]
LC-MS/MS	Agela Venusil ASB C8 column (3 µm, 150 Å, 50 × 2.1 mm)/human plasma (50 µL) Saliva (50 µL)	-/0.5 -/1	-	Requires Amicon ultra 30 K, centrifugal filters for clean-up process	Flowrate 0.2 mL/min, short run time (5 min), clinical applications	[16]
VAMS-LC-MS/MS	Phenomenex C18 column (Onyx, 100 × 3 mm i.d.)/human plasma or saliva (30 µL)	0.05/0.5	0.5–300	VAMS Technique, long sample processing	Flowrate 0.9 mL/min, clinical applications	[18]
LC-MS/MS	Serum (10 µL)	-/7.4	7.4–1881	-	Short run time (4 min), clinical applications	[17]
UHPLC-QTOF-MS	Acquity UPLC HSS Cyano column (Waters, USA)(100 mm × 2.1 mm, 1.8 µm)/rat plasma	-/0.4	0.4–400	-	Short run time (4 min), clinical applications	[19]
Capillary electrophoresis (Ex240-400 nm, Em 495 nm)	CE method compared with LC-MS/MS method using Kinetex Biphenyl HPLC column (2.6 µm, 50 × 2.1 mm) human serum (25 µL)	2.9/9.5	10–1000	-	Short run time (4 min), clinical applications	[20]

4. Conclusions

The HPLC-FLD method we developed for the quantification of perampanel in rat samples shows sensitivity, precision, linearity, and accuracy within the acceptance criteria. The method can be utilized for animal research purposes using laboratory settings. The results are within the acceptance criteria for bio-analytical method, and the present method can be applied for the analysis of plasma samples. The linearity range of the method was from 3.75 to 300 ng/mL for perampanel. The LOD was 1 ng/mL, whereas the LOQ was 3.75 ng/mL. The precision of the method was between 5.16 and 10.71, which indicates a highly precise result during the study. The accuracy of the method ranged from 99.62 to 107.89%. The samples were stable during the course of this study. In conclusion, the method presented here is sensitive, rapid, accurate, precise, economic, robust, and selective for the analysis of the drug in plasma and can be utilized for advanced research utilizing animals.

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Abbreviations

CC	Calibration curve
FLD	Fluorescence detector
HQC	High-quality control sample
IS	Internal standard
LLOQ	Lower limit of quantification
LOD	Limit of detection
LOQ	Limit of quantification
LQC	Low quality control sample
MQC	Medium quality control sample
%RE	Percent relative error
RSD	Relative standard deviation
TEA	Triethyl amine
UV	Ultraviolet

References

1. Lee, S.A.; Jeon, J.Y.; Kim, H.W. Effect of perampanel on aggression in patients with refractory focal epilepsy: A 6-month longitudinal study. *Epilepsy Behav.* **2020**, *102*, 106658. [CrossRef] [PubMed]
2. Patsalos, P.N. The clinical pharmacology profile of the new antiepileptic drug perampanel: A novel noncompetitive AMPA receptor antagonist. *Epilepsia* **2015**, *56*, 12–27. [CrossRef] [PubMed]
3. Charlier, B.; Coglianese, A.; Operto, F.F.; De Rosa, F.; Mensitieri, F.; Coppola, G.; Filippelli, A.; Dal Piaz, F.; Izzo, V. Perampanel dosage in plasma samples: Development and validation of a novel HPLC method with combined UV-Fluorescence detection. *J. Pharm. Biomed. Anal.* **2021**, *204*, 114252. [CrossRef] [PubMed]
4. Franco, V.; Marchiselli, R.; Fattore, C.; Tartara, E.; De Sarro, G.; Russo, E.; Perucca, E. Development and Validation of an HPLC-UV Assay for the Therapeutic Monitoring of the New Antiepileptic Drug Perampanel in Human Plasma. *Ther. Drug Monit.* **2016**, *38*, 744–750. [CrossRef] [PubMed]
5. Sabenca, R.; Bicker, J.; Silva, R.; Carona, A.; Silva, A.; Santana, I.; Sales, F.; Falcao, A.; Fortuna, A. Development and application of an HPLC-DAD technique for human plasma concentration monitoring of perampanel and lamotrigine in drug-resistant epileptic patients. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **2021**, *1162*, 122491. [CrossRef]
6. Ohkubo, S.; Akamine, Y.; Ohkubo, T.; Kikuchi, Y.; Miura, M. Quantification of the Plasma Concentrations of Perampanel Using High-Performance Liquid Chromatography and Effects of the CYP3A4*1G Polymorphism in Japanese Patients. *J. Chromatogr. Sci.* **2020**, *58*, 915–921. [CrossRef] [PubMed]
7. Franco, V.; Baruffi, K.; Marchiselli, R.; Crema, F.; Fattore, C.; Romigi, A.; De Giorgis, V.; Tartara, E.; Elia, M.; D'Avolio, A.; et al. Determination of Perampanel in Dried Plasma Spots: Applicability to Therapeutic Drug Monitoring. *Ther. Drug Monit.* **2020**, *42*, 309–314. [CrossRef] [PubMed]
8. Gidal, B.E.; Ferry, J.; Majid, O.; Hussein, Z. Concentration-effect relationships with perampanel in patients with pharmacoresistant partial-onset seizures. *Epilepsia* **2013**, *54*, 1490–1497. [CrossRef]
9. Mano, Y.; Takenaka, O.; Kusano, K. HPLC with fluorescence detection assay of perampanel, a novel AMPA receptor antagonist, in human plasma for clinical pharmacokinetic studies. *Biomed. Chromatogr. BMC* **2015**, *29*, 1589–1593. [CrossRef]
10. Mohamed, S.; Candela, C.; Riva, R.; Contin, M. Simple and rapid validated HPLC-fluorescence determination of perampanel in the plasma of patients with epilepsy. *Pract. Lab. Med.* **2018**, *10*, 15–20. [CrossRef]
11. Liu, P.; An, J.; Wu, H. Evaluation of the Effect of Eslicarbazepine Acetate on the Pharmacokinetics of Perampanel in Rats by Isotope-Dilution-UHPLC-MS/MS. *Drug Des. Dev. Ther.* **2022**, *16*, 4091–4099. [CrossRef] [PubMed]
12. Zhao, T.; Yu, L.H.; Zhang, H.L.; Yu, J.; Feng, J.; Li, H.J.; Sun, Y. Development and application of a novel LC-MS/MS method for human plasma concentration monitoring of perampanel in pediatric epilepsy patients. *Biomed. Chromatogr. BMC* **2022**, *36*, e5446. [CrossRef] [PubMed]

13. Zhang, Y.F.; Yu, Y.; Zhong, M.K.; Wu, X.Y.; Ma, C.L. A high-performance liquid chromatography-tandem mass spectrometry method for quantification of perampanel in human plasma: Effect of concomitant anti-seizure medications on perampanel concentration in patients with epilepsy. *J. Pharm. Biomed. Anal.* **2023**, *223*, 115155. [CrossRef] [PubMed]
14. Mano, Y.; Takenaka, O.; Kusano, K. High-performance liquid chromatography-tandem mass spectrometry method for the determination of perampanel, a novel alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor antagonist in human plasma. *J. Pharm. Biomed. Anal.* **2015**, *107*, 56–62. [CrossRef] [PubMed]
15. de Grazia, U.; D'Urso, A.; Ranzato, F.; De Riva, V.; Contarato, G.; Billo, G.; Perini, F.; Galloni, E. A Liquid Chromatography-Mass Spectrometry Assay for Determination of Perampanel and Concomitant Antiepileptic Drugs in the Plasma of Patients With Epilepsy Compared With a Fluorescent HPLC Assay. *Ther. Drug Monit.* **2018**, *40*, 477–485. [CrossRef] [PubMed]
16. Kim, D.Y.; Moon, J.; Shin, Y.W.; Lee, S.T.; Jung, K.H.; Park, K.I.; Jung, K.Y.; Kim, M.; Lee, S.; Yu, K.S.; et al. Usefulness of saliva for perampanel therapeutic drug monitoring. *Epilepsia* **2020**, *61*, 1120–1128. [CrossRef] [PubMed]
17. Chong, E.; Oh, H.; Kim, D.J.; Kim, S.M.; Lee, S.Y. Quantification of Gabapentin, Lacosamide, Perampanel, Pregabalin, Rufinamide, and Vigabatrin in Serum Using Liquid Chromatography-Tandem Mass Spectrometry. *Methods Mol. Biol.* **2024**, *2737*, 25–32. [CrossRef]
18. Palmisani, M.; Tartara, E.; Johannessen Landmark, C.; Crema, F.; De Giorgis, V.; Varesio, C.; Fattore, C.; Rota, P.; Russo, E.; Franco, V. Therapeutic Salivary Monitoring of Perampanel in Patients with Epilepsy Using a Volumetric Absorptive Microsampling Technique. *Pharmaceutics* **2023**, *15*, 2030. [CrossRef]
19. Paul, D.; Allakonda, L.; Sahu, A.; Surendran, S.; Satheeshkumar, N. Pharmacokinetics and brain uptake study of novel AMPA receptor antagonist perampanel in SD rats using a validated UHPLC-QTOF-MS method. *J. Pharm. Biomed. Anal.* **2018**, *149*, 234–241. [CrossRef]
20. Tuma, P.; Bursova, M.; Sommerova, B.; Horsley, R.; Cabala, R.; Hlozek, T. Novel electrophoretic acetonitrile-based stacking for sensitive monitoring of the antiepileptic drug perampanel in human serum. *J. Pharm. Biomed. Anal.* **2018**, *160*, 368–373. [CrossRef]
21. Tommasini, M.; Lucotti, A.; Stefani, L.; Trusso, S.; Ossi, P.M. SERS Detection of the Anti-Epileptic Drug Perampanel in Human Saliva. *Molecules* **2023**, *28*, 4309. [CrossRef]
22. USFDA. Guidance for Industry: Bioanalytical Method Validation, Centre for Drug Evaluation and Research. 2018. Available online: https://www.fda.gov/files/drugs/published/Bioanalytical-Method-Validation-Guidance-for-Industry.pdf (accessed on 1 June 2019).
23. Dejaegher, B.; Heyden, Y.V. Ruggedness and robustness testing. *J. Chromatogr. A* **2007**, *1158*, 138–157. [CrossRef]
24. Dawud, E.R.; Shakya, A.K. HPLC-PDA analysis of ACE-inhibitors, hydrochlorothiazide and indapamide utilizing design of experiments. *Arab. J. Chem.* **2019**, *12*, 718–728. [CrossRef]
25. Fricke, J.; Pohlmann, K.; Jonescheit, N.A.; Ellert, A.; Joksch, B.; Luttmann, R. Designing a fully automated multi-bioreactor plant for fast DoE optimization of pharmaceutical protein production. *Biotechnol. J.* **2013**, *8*, 738–747. [CrossRef]
26. Eriksson, L. *Design of Experiments: Principles and Applications*; Umetrics Academy: Umeå, Sweden, 2008.