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A novel HPLC method for the determination of zonisamide in human plasma using microextraction by packed sorbent optimised by experimental design

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A novel high-performance liquid chromatography-diode array detection method based on microextraction by packed sorbent (MEPS) as a sample preparation approach is described for the determination of zonisamide (ZNS) in human plasma. MEPS parameters were optimised using a Plackett–Burman experimental design to achieve the best extraction conditions. The chromatographic separation of ZNS and chloramphenicol [internal standard (IS)] was achieved in less than 5 min on a C18-column, at 35 °C, using a mobile phase composed of acetonitrile/water (35 : 65, v/v) pumped isocratically at 1.0 mL min^{-1}. ZNS and IS were detected at 240 nm. No endogenous and exogenous interference was observed at the retention times of the analyte of interest (ZNS) and IS. A good linearity was obtained for ZNS (r^2 = 0.9960) in the range of 0.2–80 μg mL^{-1} in plasma. The method was shown to be precise (CV ≤ 13.3%) and accurate (bias ±12.3%), and the absolute recovery ranged from 63.8% to 65.2%. The stability of ZNS was demonstrated in plasma samples under all predictable handling and storage conditions. The proposed assay was applied to the analysis of human plasma samples obtained from epilepsy patients under ZNS therapy, and the results supported its usefulness for therapeutic drug monitoring in clinical practice.

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1. Introduction

Zonisamide (ZNS), chemically known as 1,2-benzisoxazole-3-methanesulfonamide,[1,2] is an antiepileptic drug licensed in the European Union for monotherapy or adjunctive therapy of partial seizures with or without generalisation.[1,3] It is characterised by unique structural, mechanistic and pharmacokinetic properties that are distinct from those of other antiepileptic drugs.[4] The mechanism of action described involves the blockage of voltage-dependent sodium and T-type calcium channels interrupting synchronised neuronal firing, thereby preventing the spread of seizure activity and reducing the recurrence of epileptic seizures.[3,5,6] In addition, this drug also has some effects on the synthesis, release and degradation of a number of different neurotransmitters, including glutamate, γ-aminobutyric acid, dopamine, serotonin and acetylcholine, which may lead to an increase in inhibitory synaptic neurotransmission.[3,6–8]

Regarding the pharmacokinetic properties, ZNS is rapidly absorbed from the gastrointestinal tract and presents a high oral bioavailability.[2,3,7] It binds moderately to human serum albumin (about 50%), but tends to accumulate in red blood cells.[3,9] In addition, ZNS is partially metabolised in the liver through cytochrome P450 3A4 (under reductive cleavage) and, subsequently, it is eliminated through the renal route.[2,9] The favourable pharmacokinetic profile of ZNS supports its increasing use. Moreover, it has the advantage of not modifying significantly the plasmatic levels of other antiepileptic drugs administered concomitantly.[3,7]

A therapeutic reference range of 10–40 μg mL^{-1} for ZNS has been proposed for appropriate seizure management.[10–12] In fact, the implementation of therapeutic drug monitoring of ZNS in routine clinical practice requires the availability of suitable bioanalytical methodologies to measure ZNS concentration levels in biological samples in order to adjust the patient's medication regimen and achieve optimal therapeutic outcomes. In this sense, several techniques have been developed and validated to quantify ZNS in human matrices (*e.g.* serum,

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plasma/dried plasma spots, breast milk and urine) through micellar electrokinetic capillary chromatography,[13] enzyme immunoassay,[14] voltammetry,[15] and high-performance liquid chromatography (HPLC) coupled with mass spectrometry (MS)[16,17] and mostly coupled with ultraviolet (UV) light detection or diode array detection (DAD).[18–26] On the other hand, considering the HPLC-UV/DAD methods, the sample preparation/extraction processes employed have been protein precipitation (PP),[20,22,24] liquid–liquid extraction (LLE),[18,19,25] solid-phase extraction (SPE)[21,23] and dispersive liquid–liquid microextraction (DLLME).[26]

As microextraction by packed sorbent (MEPS) is a novel sample preparation approach in the field of bioanalysis, directed towards miniaturisation and automation, and as it has been successfully used for the qualitative and quantitative bioanalysis of drugs and/or metabolites belonging to different pharmacotherapeutic classes[27–31] (nonsteroidal anti-inflammatory drugs,[32] cardiovascular drugs,[33–36] antidepressants,[37,38] and even antiepileptics[39–41]), the application of MEPS was also considered in this work. Briefly, MEPS is a miniaturised format of the conventional SPE technique and consists of enclosing few milligrams (1–4 mg) of a solid sorbent material in a liquid handling gas-tight syringe (100–250 μL) as a plug or placing it between the barrel and the needle as a cartridge.[27,28] Specifically, as far as we know, no methods have been developed and validated for the quantification of ZNS applying MEPS. Therefore, this work was aimed at developing and validating a novel HPLC method to quantify ZNS in human plasma using the innovative MEPS technology optimised by a multifactorial Plackett–Burman experimental design.

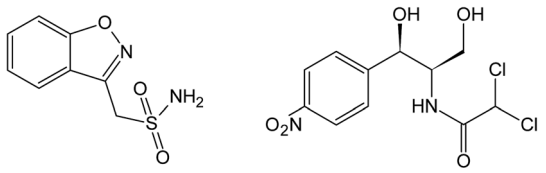

Fig. 1 Chemical structure of zonisamide (ZNS) and chloramphenicol (CAM, used as the internal standard (IS)).

2. Experimental

2.1. Reagents, analytical standards and materials

ZNS (>98% purity) was supplied by Tokyo Chemical Industry (Tokyo, Japan) and chloramphenicol (≥99% purity), used as the internal standard (IS), was purchased from Sigma-Aldrich (St Louis, MO, USA). The chemical structures of these compounds are shown in Fig. 1. Methanol and acetonitrile, both of HPLC gradient grade, were purchased from Chem-Lab (Zedelgem, Belgium) and ultrapure water (HPLC grade, >18 MΩ cm) was prepared by means of a Puranity TU 6 system from VWR (Leuven, Belgium). The MEPS 250 μL syringe and the MEPS BIN (barrel insert and needle) containing ~4 mg of solid-phase silica–C_{18} material (SGE Analytical Science, Australia) were purchased from ILC (Porto, Portugal). Blank human plasma from healthy blood donors was provided by the Portuguese Blood Institute and the plasma samples of the patients under ZNS therapy were obtained after obtaining informed consent from each subject.

2.2. Stock solutions, calibration standards and quality control samples

A stock solution of ZNS (1 mg mL^{-1}) and a working solution (100 μg mL^{-1}) was appropriately prepared in methanol and then adequately diluted in water–methanol (50 : 50, v/v) to afford six spiking solutions with final concentrations of 1, 2, 10, 50, 250 and 400 μg mL^{-1}. Each of these solutions was used daily for spiking aliquots of blank human plasma in order to prepare six calibration standards with concentrations of 0.2, 0.4, 2, 10, 50 and 80 μg mL^{-1}. A stock solution of the IS was also prepared in methanol (1 mg mL^{-1}) and a working solution (500 μg mL^{-1}) was obtained by diluting an appropriate volume of the stock solution in water–methanol (50 : 50, v/v). This IS working solution was used to spike each sample with a final concentration of 100 μg mL^{-1}. All stock, working and spiking solutions were stored at 4 °C and protected from light, except for the IS working solution which was prepared daily. Quality control (QC) samples at four concentration levels, representing the lowest (QC_{LOQ}) and the low (QC_1), medium (QC_2) and high (QC_3) range of the calibration curve, were also independently prepared. For that purpose, aliquots of blank human plasma were similarly spiked to obtain final ZNS concentrations of 0.2, 0.6, 40 and 72 μg mL^{-1}.

2.3. Apparatus and chromatographic conditions

The chromatographic analysis was performed using an HPLC system (Thermo Scientific Dionex Ultimate 3000 UHPLC focused system) that comprised the following modules: a pump (LPG-3400SD), autosampler (WPS-3000SL Analytical), column compartment (TCC-3000SD) and diode array detector (DAD-3000). All instrumental parts were automatically controlled by the Chromeleon 7.2 Chromatography Data System software.

Chromatographic separation of the analyte of interest (ZNS) and IS was carried out at 35 °C on a reversed-phase Acclaim® 120 column (C_{18}, 150 mm × 4.6 mm; 5 μm particle size) purchased from Thermo Scientific (Sunnyvale, USA). An isocratic elution was applied at a flow rate of 1.0 mL min^{-1} with a mobile phase composed of acetonitrile/water (35 : 65, v/v). The mobile phase was filtered through a 0.2 μm filter and degassed ultrasonically for 15 min before use. The injection volume was 20 μL and a wavelength of 240 nm was selected for the detection of both compounds (ZNS and IS).

2.4. Optimisation of the MEPS procedure

For the MEPS optimisation four variables were considered: the pH of the reconstitution buffer of the sample (2, 5 and 8); the number of draw/eject cycles of the sample (2, 5 and 8); the washing volume of water (0, 50 and 100 μL); and the nature of the eluting solvent [methanol, methanol : acetonitrile (50 : 50, v/v) and acetonitrile]. These experiments were carried out using

aliquots of human plasma spiked with ZNS at a concentration of 40 μg mL^{-1} (a concentration value around the middle of the calibration range expected) in order to minimise the interference of other possible confounding variables on the evaluation of the recovery of ZNS (experimental response). The optimisation of the experimental conditions was performed using the Response Surface Methodology and a Plackett–Burman experimental design with 3 central points and 1 replicate in a total of 22 experiments (Table 1). The data retrieved from the experimental design were analysed using the Response Surface Methodology so as to fit a linear equation. To correlate the response variable with the independent variables, multiple linear regression was used. The optimisation was done using the Modde software version 10.1.1 (Umetrics AB, Umeå, Sweden).

2.5. Sample preparation and extraction

The final optimised conditions for sample preparation are described below. Each aliquot (100 μL) of human plasma was spiked with 20 μL of the IS working solution, and then 400 μL of ice-cold acetonitrile was added for PP in order to minimise sample interference in the MEPS step. The mixture was vortex-mixed for 30 seconds and centrifuged at 13 000 rpm (14 000*g*) for 10 min. The resulting supernatant was evaporated under a gentle nitrogen stream at 45 °C and the dry residue was reconstituted with 100 μL of 0.1 M phosphate buffer solution (pH 8.0). This reconstituted sample was then subjected to MEPS.

The MEPS sorbent (C_{18}) inserted into a 250 μL gas-tight syringe was activated with methanol (3 × 200 μL) and passed through ultrapure water (3 × 200 μL). Then, the reconstituted sample (100 μL) was subjected to two draw/eject cycles of the sample, at a flow rate of approximately 10 μL s^{-1}. In the next step, ZNS and IS were eluted with acetonitrile (2 × 30 μL) and diluted with 90 μL of ultrapure water. Then, 20 μL of the final sample extract was injected into the chromatographic system by using an autosampler for further separation and analysis. In order to reuse the MEPS device, the sorbent was reconditioned with 12 × 200 μL of methanol followed by 3 × 200 μL of ultrapure water to avoid transferring ZNS and IS to the next sample (carryover effect). The same MEPS packing bed was reused about 200 times before being discarded.

2.6. Method validation

The international guidelines on bioanalytical method validation include several criteria for specific validation parameters, namely selectivity, linearity, limit of quantification (LOQ), accuracy, precision, recovery and stability, which should be considered in the validation of a quantitative method.[42,43]

The selectivity of the method was evaluated by analysing six blank plasma samples from different subjects to evaluate the existence of matrix endogenous substances at retention times that could interfere with ZNS and IS. Additionally, exogenous interference of other drugs that can be potentially co-administered with ZNS was evaluated by injecting 10 μg mL^{-1} standard drug solutions. The drugs tested in this selectivity assay included other antiepileptic drugs (carbamazepine, lamotrigine, levetiracetam, licarbazepine, phenytoin, primidone and valproic acid), analgesics/antipyretics/anti-inflammatory drugs (acetaminophen, acetylsalicylic acid, diclofenac, ketoprofen, ibuprofen, naproxen and nimesulide), antidepressants (citalopram, duloxetine, fluoxetine, paroxetine,

Table 1 Plackett–Burman experimental design (DoE) with 3 central points and 1 replicate in a total of 22 experiments and the corresponding recovery values obtained for zonisamide (ZNS)

Exp no	pH of the reconstitution buffer (DoE level)	Number of draw/eject cycles (DoE level)	Washing volume (DoE level)	Nature of the eluting solvent (DoE level)	Recovery of ZNS (%)
1	8 (1)	2 (−1)	0 (−1)	Acetonitrile (1)	63.56
2	8 (1)	8 (1)	0 (−1)	Methanol (−1)	63.46
3	8 (1)	8 (1)	100 (1)	Methanol (−1)	51.14
4	2 (−1)	8 (1)	100 (1)	Acetonitrile (1)	57.32
5	8 (1)	2 (−1)	100 (1)	Acetonitrile (1)	54.00
6	2 (−1)	8 (1)	0 (−1)	Acetonitrile (1)	62.70
7	2 (−1)	2 (−1)	100 (1)	Methanol (−1)	46.83
8	2 (−1)	2 (−1)	0 (−1)	Methanol (−1)	55.44
9	5 (0)	5 (0)	50 (0)	Acetonitrile/methanol (0)	57.40
10	5 (0)	5 (0)	50 (0)	Acetonitrile/methanol (0)	61.03
11	5 (0)	5 (0)	50 (0)	Acetonitrile/methanol (0)	57.62
12	8 (1)	2 (−1)	0 (−1)	Acetonitrile (1)	70.21
13	8 (1)	8 (1)	0 (−1)	Methanol (−1)	62.92
14	8 (1)	8 (1)	100 (1)	Methanol (−1)	46.03
15	2 (−1)	8 (1)	100 (1)	Acetonitrile (1)	53.62
16	8 (1)	2 (−1)	100 (1)	Acetonitrile (1)	62.77
17	2 (−1)	8 (1)	0 (−1)	Acetonitrile (1)	66.01
18	2 (−1)	2 (−1)	100 (1)	Methanol (−1)	50.97
19	2 (−1)	2 (−1)	0 (−1)	Methanol (−1)	57.12
20	5 (0)	5 (0)	50 (0)	Acetonitrile/methanol (0)	68.44
21	5 (0)	5 (0)	50 (0)	Acetonitrile/methanol (0)	63.63
22	5 (0)	5 (0)	50 (0)	Acetonitrile/methanol (0)	68.17

trazodone and venlafaxine), antiarrhythmics (amiodarone and flecainide), antifungals (ketoconazole and itraconazole), antihypertensives (amiloride, nifedipine, propranolol and verapamil) and other drugs (cimetidine, dextromethorphan, droperidol, glibenclamide, theophylline and warfarin).

The linearity of the analytical method was evaluated through the construction of three calibration curves on three distinct days ($n = 3$) using six calibration standards in the range of 0.2–80 μg mL^{-1}. The calibration curves were constructed by plotting the ZNS/IS peak area ratio *versus* the corresponding nominal concentrations of ZNS, considering a weighted linear regression analysis.[44]

The LOQ, defined as the lowest concentration of the calibration curve that can be measured with acceptable intra- and inter-day precision and accuracy, was evaluated by analysing plasma samples prepared in five replicates ($n = 5$). The LOQ for ZNS in plasma was assessed considering coefficient of variation (CV) values ≤20% and deviations from nominal concentrations (bias) within ±20%.

The interday precision and accuracy were evaluated after processing four QC samples (QC_{LOQ}, QC_1, QC_2, and QC_3) prepared in plasma, which were tested on three consecutive days ($n = 3$), whereas the intraday precision and accuracy were tested by processing five sets of the corresponding QC samples in a single day ($n = 5$). The acceptance criteria for interday and intraday precision are a CV value lower than or equal to 15% (or 20% in terms of the LOQ) and for accuracy, a bias value lower than or equal to 15% (or ±20% in terms of the LOQ).

The absolute recovery of ZNS and IS from human plasma samples was calculated for the three QC samples (QC_1, QC_2 and QC_3) by comparing the resultant peak areas of the extracted QC plasma samples with those obtained after the direct injection of non-extracted ZNS and IS solutions at the same theoretical concentrations ($n = 5$). The values of absolute recovery for ZNS and the IS were then obtained from the ratio of the peak areas of extracted and non-extracted samples.

The stability of ZNS in human plasma was investigated for QC_1 and QC_3 ($n = 5$) under different experimental conditions. On the one hand, the stability was studied in processed samples (*i.e.*, samples subjected to all the steps described in Section 2.5) kept in the autosampler over a period of 24 h. On the other hand, the stability was also studied in unprocessed samples, simulating short and long-term stability conditions; more specifically, the stability was evaluated at room temperature for 4 h, at 4 °C for 24 h, and at −20 °C for 30 days ($n = 5$). Additionally, the effect of three freeze–thaw cycles on ZNS stability in human plasma samples was also studied at −20 °C. For this purpose, aliquots of pooled plasma samples (QC_1 and QC_3) were stored at −20 °C for 24 h, thawed unassisted at room temperature, and when completely thawed, the samples were frozen again for 24 h under the same conditions until the completion of the three freeze–thaw cycles. Stability was assessed by comparing the data of QC samples analysed before (reference samples) and after being exposed to the conditions for stability assessment (stability samples); a stability/reference sample ratio of 85–115% was accepted as the stability criterion.

2.7. Clinical application

Plasma aliquots from three patient volunteers were analysed to demonstrate the clinical applicability and feasibility of this bioanalytical method. Blood samples were collected in EDTA tubes and centrifuged at 4000 rpm (4 °C) for 10 min. The plasma was transferred to Eppendorf tubes and stored at −20 °C until analysis.

3. Results and discussion

3.1. Optimisation of chromatographic and sample preparation/extraction conditions

A set of preliminary experiments were carried out to optimise the bioanalytical process in order to validate an efficient method for the quantitative analysis of ZNS in human plasma. Initially, the focus was placed on the chromatographic conditions, in which the DAD detection system was considered due to the good absorbance of ZNS in the UV region. Regarding the composition of the mobile phase, and considering the reversed-phase (C_{18}) retention mechanisms of the stationary phase, different percentages of ultrapure water and an organic modifier (acetonitrile or methanol or their mixture) were tested, but the best results were achieved with a mobile phase requiring only water and acetonitrile. The IS selected was chloramphenicol in accordance with a previously reported experiment[18] and due to its good behaviour under the selected chromatographic conditions. In what concerns the detection conditions, although different wavelengths were tested in the range of 215–300 nm, the best compromise between sensitivity and selectivity was achieved at 240 nm (near the ZNS λ_{max}). The final chromatographic conditions established (Section 2.3) enable a symmetric shape and good separation of ZNS and IS in a short running time (only 5 min).

The next step was the development of preparation/extraction conditions for plasma samples using a combination of PP and MEPS. The PP was achieved using cold acetonitrile that has been used as an appropriate protein precipitating agent for plasma samples.[34,39,41] The deproteinization of the plasma samples before sample loading avoids the rapid clogging of the MEPS cartridges.[28,31] Thereafter, the supernatant was collected and evaporated to dryness and the residue was reconstituted in an aqueous buffer before MEPS loading; the evaporation step was required due to the high content of acetonitrile in the sample supernatant, which strongly impairs the retention of the compounds of interest (ZNS and IS) in the MEPS sorbent. In fact, the pH of the aqueous reconstitution solution (0.1 M phosphate buffer solution) was the first factor evaluated during the optimisation of the MEPS protocol. In addition, as mentioned in Section 2.4, other factors that usually affect the MEPS performance were evaluated, namely, the number of draw/eject cycles of the sample, the washing volume of water and the nature of the eluting solvent.[27,28] Then, a Plackett–Burman experimental design was used in order to enable the selection of the best extraction recovery conditions. The Plackett–Burman experimental design is a very efficient two level resolution design. The method requires a small number of

experiments and is very useful for screening the design space to detect the main effects.[45] The conditions tested and the recovery found for ZNS are presented in Table 1. The examination of the model regression coefficients showed that of the four initial factors considered, only two parameters, the washing volume and the nature of the eluting solvent, were significant in the model ($p < 0.05$); however, the other two parameters were maintained in the model. The model for the recovery percentage fitted the experimental data with an R^2 of 0.64 and a Q^2 value of 0.45 demonstrating that a model with a good fit and good predictability was obtained. As can be seen in the contour plot (Fig. 2), the factors that mainly affected the recovery (%) are the washing volume and the nature of the eluting solvent. However, the pH also has some influence, even if it is low. Consequently, the best conditions obtained by this analysis were a washing volume of 0 μL and acetonitrile as the eluting solvent. For the other two parameters, the pH of the reconstitution buffer of the sample and the number of draw/eject cycles of the sample, no significant differences were found under the tested conditions. Thus, pH 8.0 phosphate buffer solution was selected for reconstitution of the sample and the number of draw/eject cycles of the sample was selected to be two in order to increase the speed of the extraction procedure. These conditions correspond to the maximum recovery (%) of ZNS that in Fig. 2 can be seen on the lower right side of the upper left panel.

Finally, the carryover was also investigated: to avoid memory effects, it is necessary to clean the cartridge with 12 × 200 μL of methanol and 3 × 200 μL of ultrapure water after each extraction.

The final sample preparation/extraction conditions established were those previously referred to in Section 2.5. Actually, appropriate sample preparation is a key step and a prerequisite for most bioanalytical procedures. The introduction of MEPS is a very promising technique because it has several advantages, namely, it is a simple, rapid and environmentally friendly technique that enables the reduction of the sample and solvent volumes, and it is a cost-effective tool because the cost per sample is lower than that involving the use of conventional SPE cartridges (the MEPS sorbents are reusable for several samples in comparison with SPE cartridges commonly used only once).[27,28,31]

3.2. Method validation

3.2.1. Selectivity. The chromatograms of blank and spiked human plasma samples are presented in Fig. 3. The analysis of blank human plasma samples from six healthy volunteers

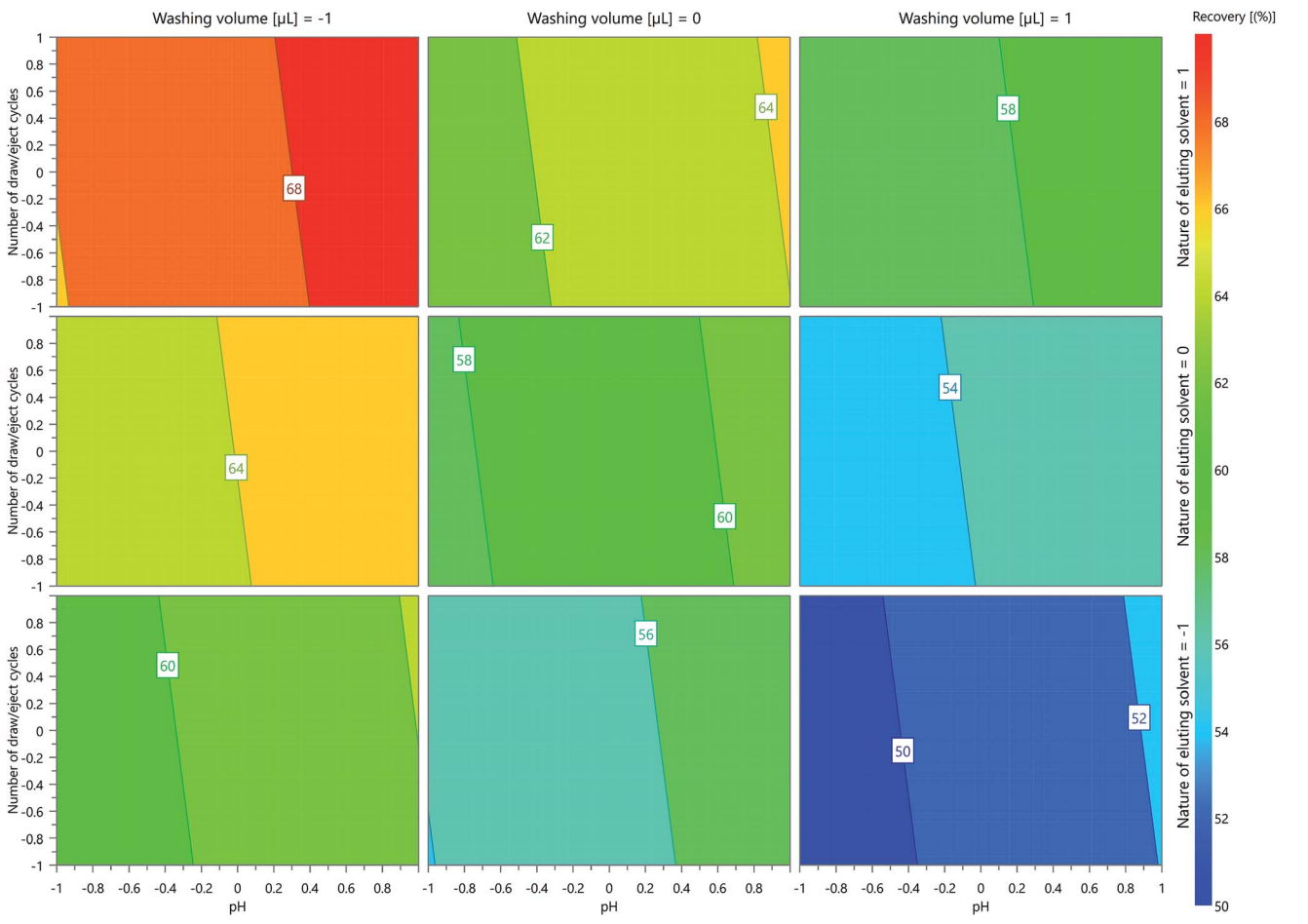

Fig. 2 4D response contour plot obtained for the recovery (%) of zonisamide from the predictive model of the experimental design.

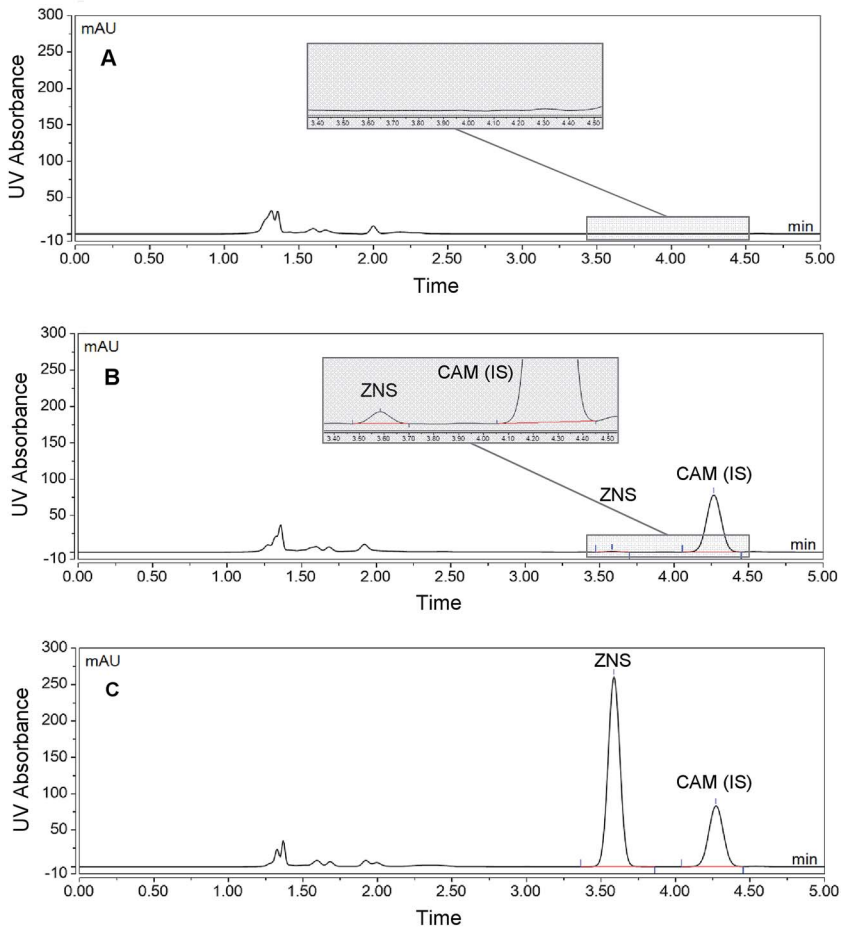

Fig. 3 Typical chromatograms of extracted human plasma samples obtained by the developed MEPS/HPLC-DAD method: (a) blank plasma; (b) plasma spiked with the internal standard (IS) and zonisamide (ZNS) at the limit of quantification (0.2 μg mL^{-1}); and (c) plasma spiked with ZNS and IS at the concentration of the upper limit of the calibration range (80 μg mL^{-1}).

confirmed the absence of endogenous interference in the retention times of ZNS and the IS. The tested drugs potentially co-administered with ZNS were not found to interfere under the established chromatographic and detection conditions.

3.2.2. Linearity and LOQ. The calibration curves obtained for human plasma were linear within the concentration range previously defined and showed a consistent correlation between the ZNS–IS peak area ratios and the corresponding nominal concentrations. A weighted linear regression analysis was performed due to the wide calibration range and to compensate for heteroscedasticity. The calibration curves were subjected to weighted linear regression analysis using $1/x^2$ as the weighting factor. The mean regression equation of the calibration curves and the corresponding determination coefficient (r^2) achieved

Table 2 Inter- and intra-day precision (% CV) and accuracy (% bias) values obtained for zonisamide (ZNS) in human plasma at the limit of quantification (QC_{LOQ}) and at low (QC_1), medium (QC_2) and high (QC_3) concentrations representative of the calibration range[a]

Plasma	$C_{nominal}$ (μg mL^{-1})		$C_{experimental}$ (mean ± SD) (μg mL^{-1})	Precision (% CV)	Accuracy (% bias)
Interday	QC_{LOQ}	0.2	0.181 ± 0.027	12.5	−9.3
	QC_1	0.6	0.530 ± 0.075	13.3	−11.7
	QC_2	40	35.070 ± 3.460	9.9	−12.3
	QC_3	72	65.501 ± 5.866	9.0	−9.0
Intraday	QC_{LOQ}	0.2	0.223 ± 0.013	5.2	11.5
	QC_1	0.6	0.596 ± 0.021	3.3	−0.7
	QC_2	40	37.907 ± 0.809	2.1	−5.2
	QC_3	72	70.990 ± 1.100	1.5	−1.4

[a] $C_{experimental}$, experimental concentration; $C_{nominal}$, nominal concentration.

Table 3 Recovery (values in percentage) of zonisamide (ZNS) from human plasma at low (QC_1), medium (QC_2) and high (QC_3) concentrations of the calibration range[a]

Matrix		$C_{nominal}$ (μg mL^{-1})	Recovery[a] (%) Mean ± SD	CV (%)
Plasma	QC_1	0.6	64.6 ± 1.9	3.0
	QC_2	40	65.2 ± 1.3	2.0
	QC_3	72	63.8 ± 4.6	7.2

[a] $C_{nominal}$, nominal concentration.

for ZNS in human plasma were $y = 0.032774x + 0.00112$ and $r^2 = 0.9960$. The calibration curves were defined within the range of 0.2–80 μg mL^{-1} in order to largely cover the therapeutic range of ZNS (10–40 μg mL^{-1}).[10–12] The LOQ was experimentally determined to be 0.2 μg mL^{-1} for ZNS. It is worthy of note that the LOQ value obtained using our method is lower than those achieved by many other HPLC-UV/DAD and also HPLC-MS(/MS) techniques reported in the literature.[16–18,20,22,24,25] In addition, in comparison with the method described by Behbahani *et al.* (2013) that used DLLME,[26] our method presented a higher LOQ, but it should be taken into consideration that they used a volume of sample 20 to 30 times larger (100 μL *versus* 2000 μL and 3000 μL).

Table 4 Stability (values in percentage) of zonisamide (ZNS) at low (QC_1) and high (QC_3) concentrations of the calibration range in unprocessed and processed human plasma samples[a]

ZNS	QC_1	QC_3
$C_{nominal}$ (μg mL^{-1})	0.6	72
Unprocessed samples		
Room temperature (4 h)	100.9	102.9
4 °C (24 h)	103.4	109.7
Freeze–thaw (3 cycles; −20 °C)	98.8	98.9
−20 °C (30 days)	100.6	101.5
Processed samples		
Autosampler (24 h)	101.0	100.2

[a] $C_{nominal}$, nominal concentration.

3.2.3. Precision and accuracy. The overall intra- and inter-day CV values did not exceed 13.3%, and the intra- and inter-day bias values varied between −12.3% and 11.5% (Table 2). Thus, our method was demonstrated to be accurate, precise and reproducible.

3.2.4. Recovery. Overall, the results for ZNS absolute recovery from human plasma samples, tested at three different concentration levels (QC_1, QC_2 and QC_3), ranged from 63.8% to 65.2% with CV values equal to or lower than 5.5% (Table 3). The absolute recovery of the IS in human plasma was 65.5% with a CV value of 4.9%. Overall, our method enables recovery rates comparable to other ones described in the literature using the MEPS approach.[28,34,38,41]

3.2.5. Stability. The results of ZNS stability in human plasma achieved under the different conditions studied (Table 4) demonstrated that ZNS was stable under the different handling and storage conditions likely to be encountered during the analytical process.

3.3. Clinical application

The plasma samples obtained from three patient volunteers were analysed to demonstrate the clinical usefulness of the method reported herein. Patient ID1 received ZNS (100 mg once every other day; p.o.) and presented a concentration of 3.8 μg

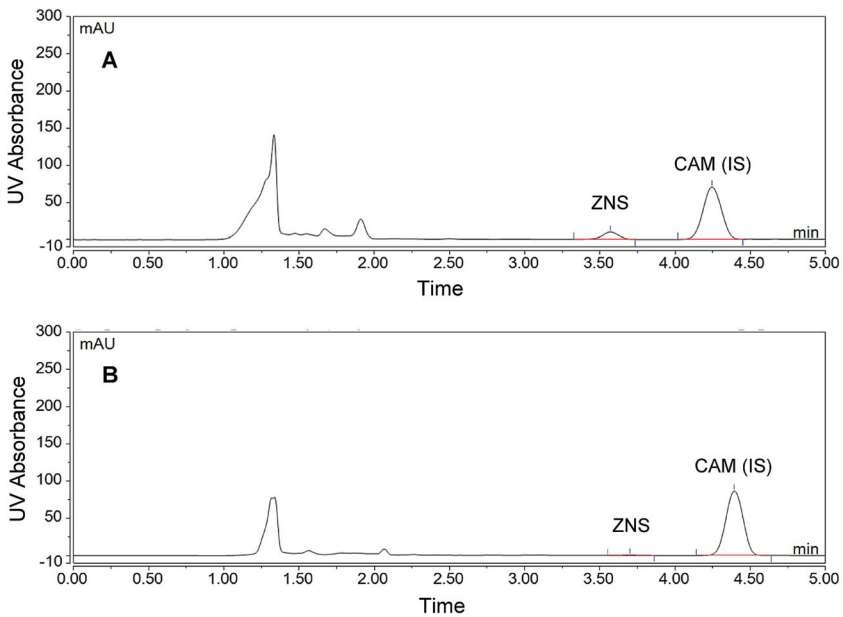

Fig. 4 Representative chromatograms of the analysis of real plasma samples obtained from (a) patient ID1 under treatment with zonisamide (ZNS) at 24 h post-dose and (b) patient ID3 after a period of 30 days of ZNS discontinuation.

mL^{-1} 24 h after the last administration of ZNS. Patient ID1 was also medicated with acetylsalicylic acid (100 mg once-daily, p.o.), ticagrelor (90 mg twice a day; p.o.), pantoprazole (20 mg once-daily; p.o.), allopurinol (100 mg once-daily; p.o.), clonazepam (0.25 mg twice a day; p.o.), quetiapine (25 mg once-daily; p.o.), ramipril (1.25 mg once-daily; p.o.) and levetiracetam (1000 mg three times a day; p.o.). Patient ID2 took ZNS (100 mg once every other day; p.o.) and also carbamazepine (400 mg twice a day; p.o.) and clobazam (400 mg twice a day; p.o.). This patient presented a concentration of 3.3 μg mL^{-1} 36 h after the last administration of ZNS. The concentrations found in the samples of patients ID1 and ID2 are consistent with the literature data, because these patients took only an oral dose of 100 mg of ZNS every 48 h, while the concentrations of the therapeutic window of ZNS are normally achieved with dosages of 300–500 mg per day.[11,24] The last patient, ID3, took levetiracetam (1500 mg twice daily; p.o.) and underwent a gradual discontinuation of ZNS (100 mg once-daily + 200 mg once daily; p.o.) which was substituted by perampanel (2 mg; p.o.). After a period of 30 days of ZNS discontinuation, a plasma sample was collected from this patient. As expected, in this sample, ZNS was only detected at a concentration below the LOQ.

A representative chromatogram of the most polymedicated patient (ID1) is shown in Fig. 4. Indeed, despite the presence of co-medication in all the patients, the peaks obtained from the processed samples of the patients revealed symmetry and good resolution, similar to those obtained in the analysis of spiked human plasma samples. Hence, the simple experimental conditions established could enable the easy implementation of this assay in most hospital settings interested in the therapeutic drug monitoring of ZNS or in other clinical pharmacokinetic-based studies involving this drug.

4. Conclusions

The developed MEPS/HPLC-DAD method for ZNS quantification was successfully validated in plasma samples with high sensitivity, selectivity, precision and accuracy. The small sample volume needed for MEPS processing and the absence of significant chromatographic interference from the biological matrices, as well as from potential concomitant drugs used, together with the short running time in the ZNS analysis and the low LOQ achieved all enhanced the clinical utility of this method. The use of MEPS as a microextraction procedure also has several important advantages, which are usually associated with the miniaturisation and automation of bioanalytical procedures, besides its optimisation through a multivariate approach. Additionally, this analytical method was shown to be appropriate to support the therapeutic drug monitoring of patients under ZNS therapy, and it can also be a valuable tool to support other pharmacokinetic studies.

Conflicts of interest

There are no conflicts to declare.

Abbreviations

CAM	Chloramphenicol
CV	Coefficient of variation
DAD	Diode array detection
DLLME	Dispersive liquid–liquid microextraction
DoE	Experimental design
HPLC	High-performance liquid chromatography
IS	Internal standard
LLE	Liquid–liquid extraction
LOQ	Limit of quantification
MEPS	Microextraction by packed sorbent
MS	Mass spectrometry
PP	Protein precipitation
QC	Quality control
SPE	Solid-phase extraction
ZNS	Zonisamide

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