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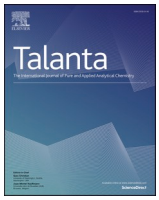

Quantitative dried blood spot microsampling for therapeutic drug monitoring of -antiseizure medications by design of experiment and UHPLC-MS/MS

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A B S T R A C T

Background: Therapeutic drug monitoring (TDM) of Antiseizure Medications (ASMs) is an essential tool for persons with epilepsy (PwE). Compared to traditional venipuncture, microsampling requires lower blood volume through less painful and invasive fingerprick offering a sampling methodology potentially performed at-home. This study aimed to validate the extraction method of ASMs from Capitainer®-qDBS microsampling. Five ASMs were considered. According to EMA guidelines, through technical and clinical validation, ASMs' quantification from qDBS device was performed by UHPLC-MS/MS. Extraction parameters were optimized by Design of Experiment. Clinical validation was performed to compare ASMs concentrations in Capitainer®-qDBS with those in plasma in 30 PwE.

Results: The method used in the chosen extraction procedure was proven to be accurate and precise. Intra and inter-assay reproducibility analyses showed accuracy and precision ≤15 % across the calibration range. Recovery was >75 %, and matrix effect >75 %, for most of the ASMs analyzed. Stability was tested at 7, 15, and 30 days of storage, showing mutual robustness at 30 days at room temperature. No hematocrit effect was observed over a range of 20–70 %. Linear regression and Bland-Altman analysis indicated a good correlation for the ASMs considered.

Significance: A UHPLC-MS/MS assay was developed and validated according to EMA guidelines for quantifying ASMs from qDBS devices. This validation has the advantage of allowing the potential to utilize the new microsampling qDBS device, providing a patient-friendly approach to blood sampling eventually even at-home.

1. Introduction

Therapeutic Drug Monitoring (TDM) of Antiseizure Medications (ASMs) is fundamental for the follow-up of persons with epilepsy (PwE), enabling the optimization of individual drug therapy. Venipuncture is considered the reference-standard method for TDM, but it requires high-volume blood taken by skilled nurses and can be challenging to perform in pediatric or uncooperative PwE [1,2]. Moreover, PwE often have driving restrictions resulting in difficulties in reaching the hospital and/or a diagnostic center for venous blood collection [1,2]. Micro-sampling, increasingly used for TDM, is proposed as a clinically practical and reliable sampling alternative. Indeed, compared with traditional venous blood collection, it requires a lower blood volume through a less painful and invasive fingerprick. Dried Blood Spot (DBS) is a longstanding microsampling technique that allows remote collection without necessitating hospital access. Despite the many advantages of DBS, this method lacks volumetric control and has been associated with the well-described hematocrit (HCT) effect [3–5]. New microsampling devices overcome these limitations by precisely controlling the blood volume collected. Among these, the most widespread microsampling techniques on the market are Volumetric Absorptive Microsampling (VAMS) and quantitative Dried Blood Spot (qDBS). In particular, qDBS devices are proposed to be free from over-sampling and capable of mitigating HCT-induced inaccuracies. Indeed, qDBS offers several advantages, including minimally invasive sampling, which makes it easier and more comfortable for patients, especially in populations that are difficult to sample. It requires a small blood volume that is ideal for pediatric or vulnerable patients or can potentially be used it in an

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emergency setting. Additionally, qDBS samples are stable at room temperature, simplifying storage and transportation without the need for refrigeration. Its versatility allows for the analysis of different analytes, making it particularly useful for TDM and improving patient compliance in regular testing[3.

A few studies have demonstrated extraction methods for various drugs and biomarkers [6–10]. Deprez et al. evaluated the use of the Capitainer®-qDBS for combined TDM of four immunosuppressants and creatinine through LC-MS/MS. This application was based on venous qDBS patient samples and compared with venous whole blood [8]. Carling et al. assessed the monitoring of phenylalanine levels in patients with phenylketonuria using three different dried blood devices and compared them with the conventional DBS cards using flow injection analysis MS/MS. The author concluded that the volumetric collection devices will overcome the significant pre-analytical issues associated with conventional DBS collection [6]. Wikstrom et al. determined lithium concentrations for monitoring the treatment of bipolar disorder. The author used a Atomic Absorption Spectrometer in flame ionization mode, demonstrating the versatility of coupling qDBS with different analytical techniques. They statistically compared venous and capillary concentrations [9]. In addition to drug analysis and TDM, proteins can be determined, as in the case of measuring anti-S antibody in blood by qDBS combined with an automated anti-SARS-CoV-2 S immunoassay. This simplified the evaluation of the immunity status against SARS-CoV-2 in large populations [7]. The advantages of volumetric blood collection in analytical chemistry are accompanied, as described above, by the great comfort for patients. An example is the use of qDBS for pharmacokinetic studies of 8 antibiotics in neonates, with the obvious advantage of the non-invasiveness of the sampling [10].

Despite these evidences studies employing qDBS devices for ASMs TDM remain limited. The present study aimed to validate the extraction method of ASMs from the Capitainer®-qDBS (10 μL) device followed by ultra-high-pressure liquid chromatography combined with tandem mass spectrometry (UHPLC-MS/MS) determination. By the Design of Experiment (DoE) approach, we optimized the extraction method for five ASMs, including carbamazepine (CBZ), lacosamide (LCM), levetiracetam (LEV), lamotrigine (LTG), and valproic acid (VPA). DoE investigated the simultaneous effects of multiple experimental factors at different levels with a specified number of experiments. This approach considers all potential interactions among the factors [11,12]. The qDBS method has been technically and clinically validated according to European Medicine Agency (EMA) guidelines and clinically validated through comparison with conventional plasma samples collected via venipuncture in the ambulatory setting.

This study aims to broaden the range of applications of volumetric microsampling devices, in particular qDBS, by showing comparable or even better validation parameters values to classic venous sampling. In addition, our work confirmed the potential great qualities of minimal invasiveness, comfort, high stability with consequent possible application in telemedicine of this type of sampling.

2. Materials and methods

2.1. Chemicals and reagents

1 mg/mL standard solutions of CBZ, LEV, LTG and VPA in methanol (MeOH) and LCM in acetonitrile (ACN) were sourced from Merck-Lifescience (certified reference material – Cerilliant®). Carbamazepine D_{10} solution (100 μg/mL in MeOH), levetiracetam D_6 solution (1.0 mg/mL in MeOH), lamotrigine ^{13}C, $^{15}N_4$ solution (500 μg/mL in MeOH), lacosamide ^{13}C-D_3 solution (1.0 mg/mL in ACN) and valproic acid D_6 solution (1.0 mg/mL in MeOH), used as internal standards (IS), were provided by Sigma-Aldrich (St. Louis, Missouri, USA) with a purity of≥ 99 %. Organic solvents such as ACN provided by VWR Chemicals (Radnor, PA, USA), LC-MS/MS gradient-grade MeOH provided by MerckMillipore (Darmstadt, Germany), ethanol (EtOH) provided by PanReac AppliChem - ITW Reagents, and formic acid (FA) provided by Carlo Erba Reagents S.r.l. (Milan, Italy) were used. Ultrapure water was obtained from a MilliQ® integral apparatus (Merck Millipore), which guarantees the elimination of impurities that could potentially impact experimental outcomes. Regenerated Cellulose (RC) membrane 0.22 μm 4 mm syringe filters were purchased from Phenomenex (Torrance, CA, USA). The Chromsystems kit (Gräfelfing, Germany) was used to perform cross-validation.

2.2. Stock and working solutions

To ensure accurate and consistent concentration levels, reference stock solutions of each ASM were diluted to produce a series of working solution levels with MeOH and ACN, respectively. Stock and working solutions were stored at −20 °C. These several solutions were used for the in-solution calibration and provided a comprehensive range for qDBS calibration and Quality control (QCs) purposes. They were used to prepare spiked blood samples for QCs and calibration solutions (see Section 2.6). IS working solutions were obtained by diluting the starting standard solution (see Section 2.1) in ACN to a final IS mixture at concentrations of 10 μg/mL for CBZ D_{10}, 50 μg/mL for LEV D_6, 10 g/mL for LTG ^{13}C, $^{15}N_4$, 5 μg/mL for LCM ^{13}C-D_3, and 80 μg/mL for VPA D_6.

2.3. Venous blood collection and plasma sample preparation

Venous blood samples were drawn into BD vacutainer lithium heparin tubes (6 ml capacity) and aliquoted into two parts, (1) and (2). Plasma samples obtained from the blood aliquot (1) were centrifuged at 3000 rpm at 4 °C for 10 min and stored at −20 °C until the analysis. The aliquot (2) was collected for qDBS device preparation (see Section 2.5). The blood samples were obtained from 13 healthy volunteers to prepare calibrators and QC and from 30 PwE for clinical validation. With the aim of cross-validate the original microsampling method, we analyzed plasma from venous blood collected in PwE with a well-established commercial kit and compared the ASM concentrations to those obtained in qDBS samples.

2.4. Patient enrollment

Between July to September 2023, 30 adult PwE (age ≥18 years) attending the Epilepsy Centre of our Institute were enrolled. Eligible participants were defined as PwE in therapy with at least one of the ASMs in the exam. On the day of enrollment, informed consent forms were signed and collected in the ambulatory setting. A 5 mL venous blood sample was drawn from each patient during the morning, before taking the therapy. The study was approved by the Independent Ethics Committee of Area Vasta Emilia Centro (792-2022-DISP-AUSLBO) on 18 January 2023.

2.5. qDBS preparation and extraction

Quantitative dried blood spot (qDBS) (Capitainer®, Stockholm-Sweden) was prepared by carefully pipetting 25 μL of whole blood into the designated inlet port of each device for sample collection. The device is designed to collect 10 μL per spot. In order to ensure this amount was collected and to mimic the blood drop coming from a patient's fingertip, we used a 25 μL pipette for each sample. For each point on the calibrators/QC and PwE samples one qDBS device was used.

Venous blood from healthy volunteers and patients was respectively used for technical and clinical validation.

The prepared devices (blood-qDBS) were allowed to air-dry for approximately 2 h at room temperature (RT) in a zip-closure plastic bag to preserve the blood samples. The pre-perforated paper discs containing the collected samples were delicately separated using tweezers. The discs were then placed individually into 2 mL Eppendorf tubes.

ASMs extraction from the qDBS device was optimized following the

multifactorial DoE (see Section 2.7), and the final conditions are as follows. We add 250 μL of Millipore water (stored at 4 °C) to the 2 mL Eppendorf tube for the hydration step. Then, 250 μL ACN, for protein precipitation, and IS mixture were added. Both steps were followed by 5 min of 50 °C sonication to facilitate the release of the drugs. Samples were then centrifuged at 3000 rpm, 4 °C for 10 min 50 μl of supernatant was mixed with 450 μL of water, providing a 1:10 dilution, filtered, and injected into the UHPLC-MS/MS system.

2.6. Calibration curves and quality control

For calibration curve preparation, we obtained spiked whole blood at five final concentration levels (I-low, II, III-medium-, IV, V-high-) for each drug (CBZ levels were 2 μg/ml, 5 μg/ml, 10 μg/ml, 12 μg/ml, 15 μg/ml; LCM levels were 1 μg/ml, 3 μg/ml, 5 μg/ml, 8 μg/ml, 10 μg/ml; LTG levels were 2 μg/ml, 5 μg/ml, 10 μg/ml, 15 μg/ml, 20 μg/ml; LEV levels were 5 μg/ml, 25 μg/ml, 50 μg/ml, 70 μg/ml, 80 μg/ml; VPA levels were 30 μg/ml, 50 μg/ml, 80 μg/ml, 100 μg/ml, 120 μg/ml). Solutions were incubated at 37 °C for 30 min to mimic physiological conditions. The qDBS calibration curve was prepared by adding 25 μL spiked whole blood onto the devices (spiked blood-qDBS) and following the ASMs extraction procedure described in Section 2.5. QCs were prepared in the same way as the calibration curve at points II (lowQC) and IV (highQC) for each drug. The calibration curve and QCs were freshly prepared for each experiment session.

2.7. Design of experiment

The DoE is a methodology used to investigate the simultaneous effects and interactions of multiple experimental factors at different levels with a specified number of experiments. DoE was applied to optimize the sample extraction procedure. In this study, we applied the D-optimal design to maximize the statistical efficiency [11,13,14]. The experimental design aimed to optimize the recovery of the drugs in exams. The factors analyzed in the experiment were the following.

- Extraction 1 Time (t1): 5 min; 15 min; 30 min
- Solvent (solv): ACN; ACN + 0.1 % FA; MeOH; MeOH + 0.1 % FA; MeOH + EtOH (1:1); MeOH + EtOH (1:1) + 0.1 % FA
- Extraction 2 Instrument (ins): Vortex; Ultrasound sonicator
- Extraction 2 Time (t2): 5 min; 15 min; 30 min

The candidate set for the experimental design was generated considering all possible combinations of the levels for each factor (see Table S1 of the supplementary material). To generate the experimental design, we used the D-optimal design approach using the 'skpr' package in R software, which maximizes the statistical efficiency of the experiments [13,15–17]. The power of the experimental design was evaluated to ensure that the design could reliably detect significant effects for the factors under study.

2.8. UHPLC-MS/MS method

After qDBS extraction, all the solutions (calibration, QCS, and patient samples) were analyzed by UHPLC-MS/MS, modifying the method reported in our previous paper [18]. Instrumentation consists of a triple quadrupole mass spectrometer equipped with a turbo ion spray source (Sciex 4500 QTRAP, Concord, Ontario, Canada) coupled with a UHPLC system (Nexera X2 UHPLC, Shimadzu Corporation, Kyoto, Japan). The control and management of the UHPLC-MS/MS system were executed through the SCIEX Analyst 1.6.3 software. The column was a Kinetex C18, 30 × 4.6 mm I.D., 2.6 μm 100 Å column (Phenomenex, Torrance, CA, USA).

The mobile phases were 0,1 % formic acid water (A) and 0,1 % formic acid acetonitrile (B). The gradient elution was as follows: 0–0.20 min, 0 % mobile phase B; 0.21–3.75 min, 0–100 % mobile phase B; 3.76–4.25 min, 100 % mobile phase B; 4.26–4.27 min, 100-0 % mobile phase B; 4.28–5.40 min, 0 % mobile phase B. Injected volume was 20 μL, at a flow rate of 0,6 mL/min, and the column temperature was maintained at 30 °C. Mass spectrometry parameters were optimized by directly infusing reference standards and IS-ASMs solutions. The ion source gas was set at 45 PSI and 60 PSI. The ion spray probe temperature was set at 500 °C. MRM-optimized parameters, such as precursor ions, fragment ions, and collision energies, are reported in Table S2. Our original method was cross-validated by comparison with the well-established UHPLC-MS/MS plasma analysis [18].

2.9. Method validation

The validation was performed using EMA guidelines for bio-analytical methods, and the following parameters were tested [19].

Linearity: spiked blood-qDBS calibrators and spiked blood samples were prepared at the calibration concentrations (I, II, III, IV, V levels for each ASM) (see Section 2.6). The peak area ratios between the analytes and IS were plotted against the nominal concentrations. The calibration curves were set up using a linear least-square regression method with a weighting factor of 1/x. Linearity was assessed by determining the coefficient of determination (R^2). The acceptance requisite was $R^2 \geq 0.996$. The back-calculated concentrations of the calibration standards have to be within ±15 % of the nominal value.

Selectivity: was evaluated through injections of blank blood matrices. The method was considered selective and accepted if the area of ASMs and IS was less than 20 % of the LLOQ for the analyte and 5 % for the IS.

Precision and accuracy: intra- and inter-day precision and accuracy were assessed by determining the coefficient of variation (CV%) and relative error (RE%), respectively.

Intraday precision and accuracy were evaluated by analyzing six spiked blood-qDBS samples at LLOQ, as well as lowQC, mediumQC, and highQC, compared to nominal standard solutions at the same spiked concentrations. Interday precision and accuracy were evaluated by analyzing lowQC, mediumQC, and highQC samples from three different runs on three different days. The acceptance criteria should not exceed 15 % CV for the spiked samples, except for the LLOQ, which should not exceed 20 %.

Hematocrit impact (HCT): to assess the impact of HCT on assay precision (RE%) and accuracy (CV%), lowQC and highQC samples were prepared at 20 %, 41 %, and 70 % HCT levels [20]To achieve these different HCT levels, an initial blood sample with a known HCT of 41 % was split into three aliquots: one was left untreated (41 % HCT), a second was centrifuged at 1500 rpm for 10 min to remove plasma (70 % HCT), and an aliquot of the removed plasma was then added back to a separate aliquot of the initial blood sample (20 % HCT).

Lower limit of detection (LLOD): LLOD was determined experimentally in triplicate by comparing measured signals from spiked blood-qDBS samples at known lowQC ASMs concentrations (serial dilutions) with those of spiked blank-plasma. LLOD was calculated as 3 times the baseline noise.

Lower limit of quantification (LLOQ): LLOQ was experimentally defined as the lowest quantifiable concentration of analyte, with acceptable accuracy and precision (≤±20 %) and a signal-to-noise ratio of 10:1. LLOQ was estimated by analyzing blood sample spiked solutions at serial dilution concentrations, based on the criteria of accuracy, precision, and signal-to-noise ratio.

Recovery: was assessed by analyzing lowQC, mediumQC, and highQC spiked blood-qDBS samples in triplicate at LLOQ, lowQC, mediumQC, and highQC concentrations. The recovery (R%) was determined as the percent ratio between the mean of the peak areas of the analyte in spiked blood-qDBS samples (pre-spiked samples) and those obtained by spiking the solution after qDBS extraction (post-spiked samples). The acceptance criteria should be ≥ 75 %.

Matrix effect: was investigated in triplicate at medium

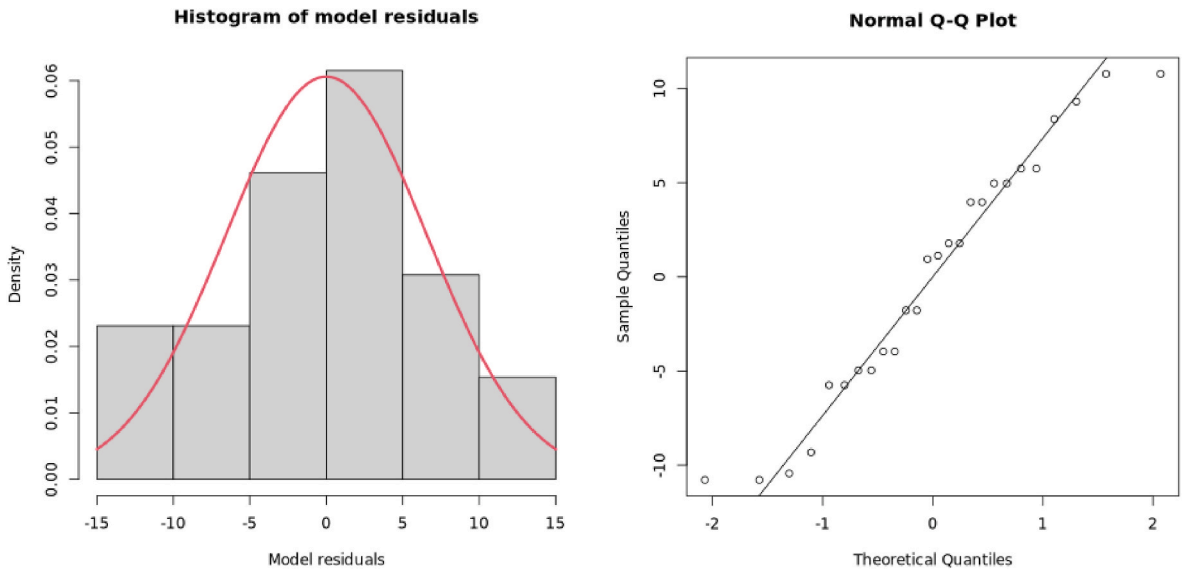

Fig. 1. (a) Histogram of model residuals and fitted normal distribution. (b) Anormal Q-Q plot confirms the normal distribution of residuals and the absence of outliers.

concentrations, using 6 different lots of blood-qDBS samples from healthy volunteers spiked after the extraction procedure. The matrix factor (MF%) was calculated as a ratio between post-spiked samples and standard analyte solutions. The MF%, calculated as the ratio between post-spiked sample and standard solution and should be between 80 and 120.

Carry-over: was performed by injecting a blank sample after the highest calibrator (n = 3). Carry-over was considered negligible if it was ≤20 % than LLOQ and ≤5 % than IS concentrations.

Stability tests: to assess stability, spiked blood-qDBS at lowQC, mediumQC, and highQC were prepared, dried for at least 2 h at RT, and stored in plastic bags with desiccant packages. The stability was tested at 7, 15, and 30 days of storage, at RT, and 7 days at −20 °C. In order to establish the stability of the extracts, we performed the analysis after storing them in autosampler (T = 4 °C) for 24 h and 5 days and at −20 °C for 15 days. Blood-qDBS and extracts were considered stable if their mean concentrations were within ±15 % of the nominal concentration.

2.10. Statistic for cross-validation

Cross-validation was assessed by comparing our original qDBS microsampling method with the well-established plasma UHPLC-MS/MS analysis. Data were collected from analyses of blood-qDBS and plasma samples from 30 PwE, with qDBS results adjusted based on the blood-to-plasma ratio ($R_{b/p}$) calculated for each ASM. To assess agreement and correlation between methods, ASMs concentrations in plasma and blood-qDBS were compared using Bland-Altman analysis and linear correlation respectively with a 95 % confidence interval [21]. Statistical analyses were conducted using MedCalc software (Version 22.030).

3. Results and discussion

3.1. Optimization of qDBS extraction by DoE

DoE is particularly useful for optimizing multivariate systems, such as multi-step sample preparation combined with different analytes. Effective optimization involves adjusting several experimental variables to ensure conditions are suitable for all the analytes to maximize the detection. This optimization aims to achieve maximum recovery as an indicator of method sensitivity and minimal variability. A particularly effective type of DoE is the D-optimal design [11,16], which is employed to maximize the statistical efficiency of the experimental results. D-optimal design selects a subset of experimental runs that provide the most information about the system, minimizing the variance of the estimated effects. The experimental design comprised 25 experiments, as already reported in Table S1, which were carried out and subsequently analyzed using a linear model. This approach allowed for the assessment of the main effects and interactions of the factors on ASMs recovery, ensuring that the optimized conditions were based on statistically sound results.

We fit a linear model with interaction terms:

$$Y = \beta_0 + \beta_{t1}\cdot t_1 + \beta_{solv}\cdot solv + \beta_{ins}\cdot ins + \beta_{t2}\cdot t_2 + \beta_{solv:t1}\cdot solv\cdot t_1 + \beta_{solv:ins}\cdot solv\cdot ins + \epsilon \quad \text{(Eq. 1)}$$

In this linear model, “Y” is the sum of the five drugs’ recoveries, “t1” and “t2” are time intervals for the first and second extraction, respectively, “solv” is the solvent (6 categories), “ins” is the instrument (ultrasound sonicator or vortex), “β_s” are model coefficients and “ϵ'' are model’s residuals. The linear model has been fitted to pilot experimental data using the R’s‘stats’ package [16]. The histogram of the model’s residuals, Fig. 1a, follows a normal distribution (Shapiro-Wilk normality test [22] p-value = 0.23): this means that residuals are independent and identically distributed and the homoscedasticity holds, which are the fundamental assumptions of linear models. Moreover, the normal Q-Q plot, Fig. 1b, shows a linear trend, confirming the normal distribution of residuals and the absence of outliers. The model’s adjusted R squared is 0.905, and the model’s p-value is 7.6×10^{-4}. Estimated coefficients and associated p-values are shown in Table S3. ”ACN" and “Ultrasound sonicator” were used as references to compute Solvent and Instrument coefficients, respectively. The relatively high value of R^2 indicates that the model seems to explain the variability of the dataset quite well. Including remaining interaction terms would have reduced the R^2. Table S3 shows that both “t1” and “t2” do not significantly influence ASMs recovery, and their estimated coefficients are relatively small (<1). The vortex instrument shows no significant difference with respect to the ultrasound sonicator (USS), even if its negative coefficient suggests that using this last one would improve drug recovery. Considering solvent coefficients, the ones with MeOH show relatively small p-values (two of them are below 0.05), indicating a significant difference with respect to ACN, which is the reference solvent. Since estimated coefficients are negative, using solvents with MeOH will reduce the ability to obtain a satisfying recovery. Solvent with ACN +0.1 % FA has a positive coefficient, but its p-value does not show significant differences

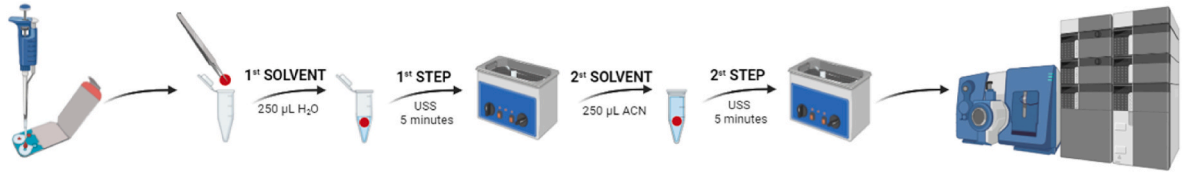

Fig. 2. qDBS extraction conditions (Created in BioRender.com).

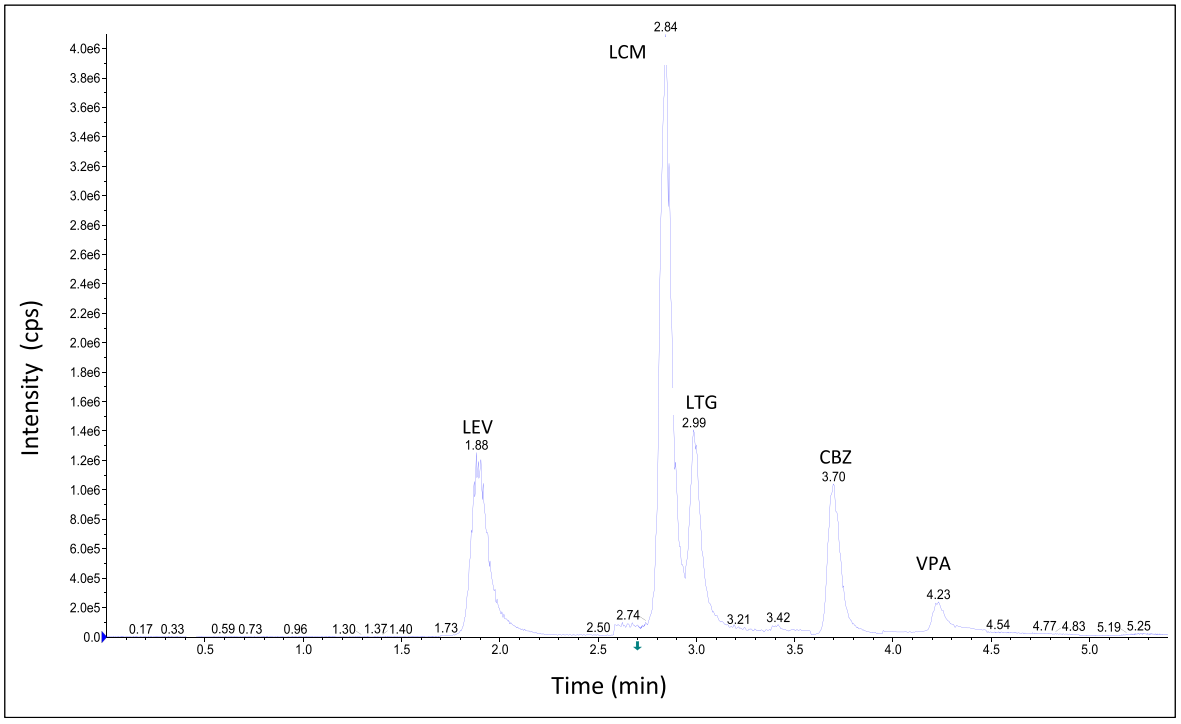

Fig. 3. MRM chromatograms of spiked blood-qDBS at "medium" concentration point. MRM transitions for the analytes are: 238.1 m/z > 193.06 m/z for CBZ, 252.1 m/z > 108 m/z for LCM, 172.1 m/z > 70 m/z for LEV, 256.1 m/z > 210.9 m/z for LTG, and 143.1 m/z > 99.05 m/z for VPA.

Table 1
Linearity, intra-day, and inter-day precision and accuracy were assessed by calculating the coefficient of variation (CV%) for precision (SD/mean x 100) and the relative error (RE%) for accuracy ([(mean concentration found – nominal concentration)/nominal concentration] x 100). The table presents results obtained across multiple concentrations.

Intraday (n = 6; 1 day)							Interday (n = 9; 3 days)					
Linearity							Linearity					
ASMs	**R^2**		**Mean conc. (µg/mL)**	**Std Dev.**	**%CV**	**%RE**	**R^2**		**Mean conc. (µg/mL)**	**Std Dev.**	**%CV**	**%RE**
CBZ	0.998						0.998					
		LowQC	2,18	0,15	6,88	8,26		LowQC	2,14	0,04	1,87	6,54
		HighQC	14,5	0,47	3,24	−3,45		HighQC	13,91	0,32	2,28	−7,84
LCM	0.998						0.997					
		LowQC	0,95	0,02	2,11	−5,26		LowQC	0,92	0,01	1,30	−8,70
		HighQC	9,48	0,45	4,75	−5,49		HighQC	9,50	0,06	0,65	−5,26
LTG	0.999						0.998					
		LowQC	1,91	0,05	2,62	−4,71		LowQC	1,76	0,12	6,70	−13,64
		HighQC	19,06	1	5,25	−4,93		HighQC	20,72	1,38	6,66	3,47
LEV	0.998						0.998					
		LowQC	5,80	0,41	7,07	16,00		LowQC	5,62	0,14	2,42	11,03
		HighQC	91,43	1,53	1,67	14,29		HighQC	85,28	0,32	0,38	6,19
VPA	0.996						0.996					
		LowQC	25,24	3,42	13,55	−18,86		LowQC	22,46	6,00	26,71	−33,57
		HighQC	104,33	7,8	7,48	−15,02		HighQC	110,67	1,27	1,15	−8,43

Table 2
Recovery (R%) and Matrix Effect (MF%), calculated respectively at LowQC and HighQC and MediumQC concentrations for ASMs.

ASMs		Nominal conc. (μg/mL)	R%; n = 3	MF%; n = 3
CBZ				
	LowQC	2	83,2	
	MediumQC	10	74,5	81,47
	HighQC	15	79	
LCM				
	LowQC	1	76	
	MediumQC	5	86	80,52
	HighQC	10	83	
LTG				
	LowQC	2	98	
	MediumQC	10	86	97,97
	HighQC	20	97	
LEV				
	LowQC	5	102	
	MediumQC	50	99	102,27
	HighQC	80	104	
VPA				
	LowQC	30	21	
	MediumQC	80	44	28,20
	HighQC	120	31	

with respect to ACN. Using a solvent with ACN will, therefore, maximize the recovery of ASMs. The only significant interaction term is Solv [MeOH + 0.1 % FA]: Instrument [Vortex], but its effect is smaller if compared to the main effects of both ACN and ultrasound sonicator.

Finally, the optimized extraction conditions, as reported in Section 2.5, are shown in the scheme below (Fig. 2).

3.2. UHPLC-MS/MS analysis

UHPLC gradient and MRM acquisition mode allowed the complete separation of the five analytes within 5-min run time. The selectivity of MRM avoided interference from blood components and qDBS-derived impurities, as shown in the MRM chromatograms in Fig. 3.

3.3. Method validation

The optimized analytical conditions were validated according to the EMA guidelines for bioanalytical methods, and the parameters described in Section 2.9 were evaluated [19]. The method was proved to be linear and selective for the whole concentration range studied for CBZ, LCM, LTG, LEV, and VPA (see Table 1 and Fig. 1S on supplementary material). The method's precision and accuracy were evaluated within the same day (intraday) and during a period of three days (interday). The results for intraday and interday precision and accuracy are presented in Table 1, demonstrating satisfactory performance for CBZ, LCM, LTG, and LEV at lowQC and highQC concentrations. Intraday precision and accuracy were within ±15 % for most analytes, with acceptable %CV and %RE, except for VPA, where intraday deviated (CV%: 7,48 % to 32, 78 % and RE%: −23,34 % to −3,41 %, with a high standard deviation. See Table S4). Interday precision and accuracy were within ±15 %, ranging from CV%: 0,05 % to 6,70 % and RE%: −13,64 % to 11,03 %, expect for VPA. Additional data, including concentrations at LLOQ and medium levels, are provided in Table S4 in the supplementary materials. Precision and accuracy were shown to be not affected by HCT levels in the range of 20–70 % (data not shown).

The recovery was calculated by comparing pre- and post-spiked blood-qDBS samples. The results as R%, shown in Table 2, were ≥75 % for CBZ, LCM, LEV, and LTG (average R%: 77,4; 79,7; 92,5; 103,2 respectively). VPA showed an average R% <30 % (29,7 %). See Table S5.

The matrix factor in blood-qDBS samples was evaluated on 6 different controls for each ASM by spiking at 10 μg/ml the solutions obtained after extraction (post-spiked).

The matrix factor was found to be between 80,52 and 102,25 % for all the analytes, except for VPA showing an MF% of 28,20 % (all data are represented in Table S5).

The mean values showed that the matrix effect is negligible (within 20 %, see Table 2 and Table S5) for all the drugs, with the exception of VPA, which has an ion suppression of 70–75 %.

LLOQ and LLOD values resulted comparable than those reported in our previous study on VAMS and those in plasma [23]. LLOQ value ranged from 0,50 ng/mL for CBZ to 120,00 ng/mL for VPA while LLOD ranger from 0,38 ng/mL for CBZ to 9,67 ng/mL for VPA. The detailed values are reported in Table S6. Carry-over was found to be ≤ 20 % for LLOQ and ≤5 % for IS (supplementary material, Fig. 2S). All criteria based on EMA guidelines for method development were satisfied for CBZ, LCM, LTG, and LEV. Due to the lowQC accuracy, recovery, and highQC matrix effect, VPA was excluded from further analysis for the qDBS UHPLC-MS/MS method validation.

3.4. Stability tests

Evaluating blood sample stability on qDBS is essential to determine the time interval allowed between microsampling and extraction followed by UHPLC-MS/MS analysis to avoid degradation of ASMs and their excessive adsorption on the device. This last aspect would make the extraction of the analytes less efficient. In light of the above, the stability of blood-qDBS in sealable polyethylene bags containing desiccant packages at lowQC, mediumQC, and highQC was evaluated over a period of 30 days (7, 15, and 30 days) under different storage conditions described in Section 2.9. Samples were analyzed in triplicate at each time point. Results expressed as mean accuracy and precision are presented in the supplementary material, Table S7. At RT, after 7, 15, and 30 days, accuracy and precision of the dried spiked blood-qDBS were within ±15 %, indicating substantial stability of CBZ, LCM, LTG, and LEV. When stored at −20 °C, the mean concentration was within ±12 % of the nominal concentration after 7 days, showing good stability. The stability of the injected extracts was also assessed at different storage times and conditions. Extracts were analyzed after 24 h and 5 days at 4 °C (autosampler stability) and after 15 days at −20 °C. When stored at 4 °C, the mean concentration of the extracts was within ±15 % of the nominal concentration after 24 h, indicating high stability. After 5 days stored at 4 °C, the accuracy values exceeded ±15 % (−18,80 to 16,69), indicating that degradation occurred. When stored at −20 °C for 15 days, the mean concentration was within ±5 % of the nominal concentration, demonstrating good stability over the longer storage period at low temperatures. As a reference for calculations, fresh calibration

Table 3
Statistical analysis: Bland-Alman and Linear regression results for clinical method validation.

	Blood to Plasma ratio ($R_{b/p}$)	Bland-Altman analysis (plasma vs qDBS)		Linear regression				
		Bias	95 % CI	Equation	Intercept	95 % CI	Slope	95 % CI
CBZ (n=8)	1,01 ± 0,17	0,24	−3,18 to 1,98	y = 1,1930 + 0,7802 x	1,193	−5,7158 to 8,1017	0,7802	−0,05490 to 1,6153
LCM (n=7)	0,92 ± 0,17	0,38	−1,24 to 0,86	y = 0,6444 + 0,8456 x	0,6444	−0,7447 to 2,0335	0,8456	0,6033 to 1,0878
LEV (n=8)	1,03 ± 0,21	0,04	−1,60 to 4,28	y = 1,7543 + 0,9610 x	1,7543	−0,7457 to 4,2543	0,9610	0,7663 to 1,1556
LTG (n=12)	0,9 ± 0,11	0,03	−1,58 to 3,37	y = −0,5993 + 1,2731 x	−0,5993	−2,8910 to 1,6923	1,2731	0,8785 to 1,6678

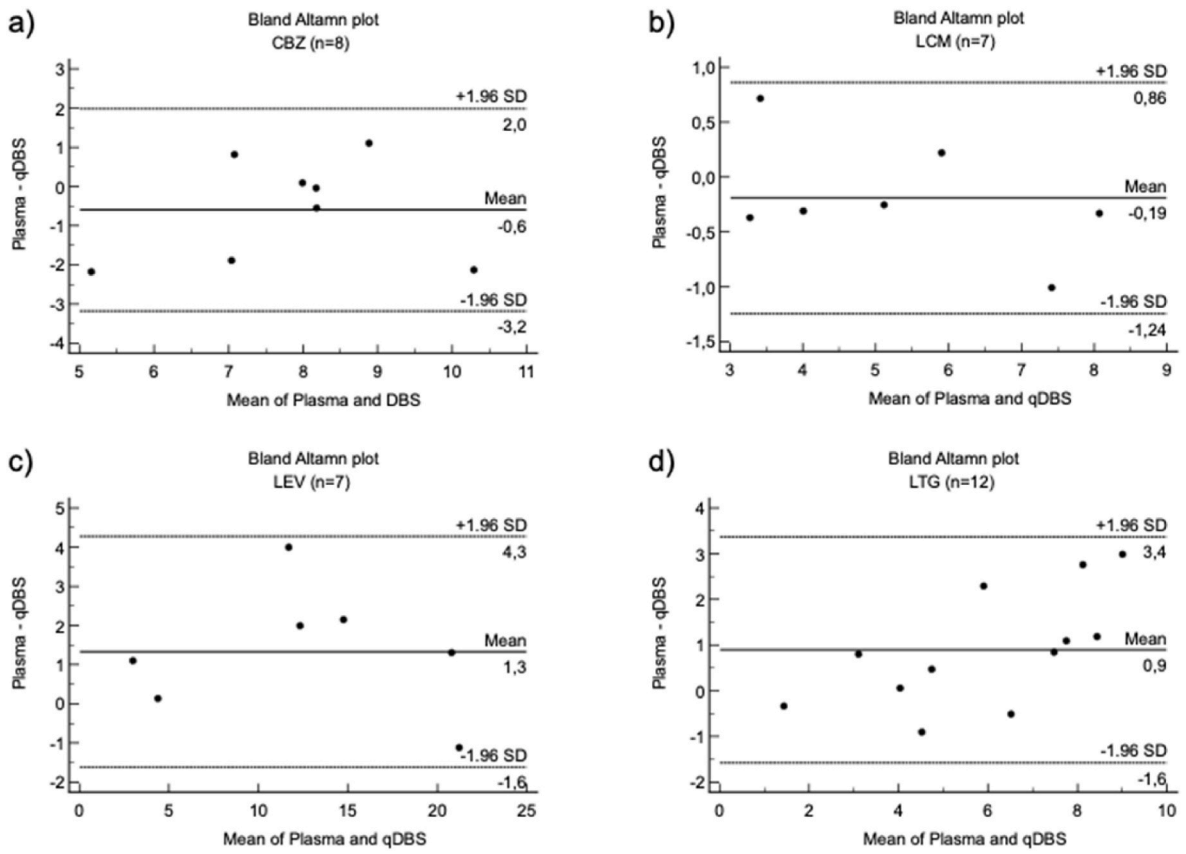

Fig. 4. Bland-Altman plot evaluating the agreement between the two assays for a) CBZ, b) LCM, c) LEV, and d) LTG. The Y-axis represents the difference between paired measurements, while the X-axis shows the average of these measurements. The solid line represents the mean difference between the two methods (bias) and the dashed lines indicate the upper and lower limits of the 95 % confidence interval for the mean difference.

curves was prepared for each analytical session.

3.5. Comparison between plasma and qDBS sample analysis: clinical validation

After the UHPLC-MS/MS analysis, the concentrations obtained from blood-qDBS devices were compared with those found in plasma from a total of 30 PwE. Considering the specific treatment we had 8 CBZ, 7 LCM, 8 LEV, 12 LTG PwE. The blood-to-plasma ratio ($R_{b/p}$) for each ASM was calculated by dividing the drug concentrations in blood-qDBS by those in plasma measured with the Chromsystems kit. The $R_{b/p}$values were found to be according to those already published and with our earlier work on VAMS(18). $R_{b/p}$ was used to convert qDBS results. The $R_{b/p}$values were approximately 1 for CBZ and LEV, 1.01 ± 0.17 and 1.03 ± 0.21, respectively. For LCM and LTG, $R_{b/p}$ values were specifically found to be 0.92 ± 0.17 and 0.90 ± 0.11, respectively (see Table 3).

The Bland-Altman analysis demonstrated a bias close to zero for all ASMs considered, indicating good agreement between plasma and qDBS results. The bias values were 0.24 for CBZ, 0.38 for LCM, 0.04 for LEV, and 0.03 for LTG (see Table 3 and Fig. 4).

In addition, linear regression analysis was conducted to assess the correlation between plasma concentrations and blood-qDBS results. The slopes were close to 1 for all ASMs, indicating strong correlations between the qDBS results and plasma concentrations, which further reinforces the accuracy of the qDBS method for measuring ASMs concentrations. These results indicate that the qDBS method provides reliable and comparable concentration measurements for ASMs when validated against plasma samples (see Table 3 and Fig. 5).

4. Conclusion

The present study describes the development and validation of a qDBS UHPLC-MS/MS method for quantifying four ASMs in PwE, following EMA guideline criteria.

Firstly, we successfully applied a DoE optimal design, which allowed to maximize the statistical efficiency of the experimental results in terms of recovery, accuracy and precision.

The final optimized extraction method was fully validated in terms of linearity, selectivity, precision, accuracy, HCT impact, recovery, matrix effect, carry-over, robustness, and stability, demonstrating acceptable sensitivity down to 0.25 ng/mL for residual ASM blood concentrations. To our knowledge, this is the first study to explore the application of qDBS for ASM quantification.

The results showed comparable concentration levels to those obtained with plasma samples, with qDBS sampling offering a simple and accurate alternative for capillary blood collection that improves on traditional DBS techniques. Our results indicate that the qDBS method for ASMs is unaffected by HCT within a broad range (20–70 %). Additionally, stability tests demonstrated that blood-qDBS samples can be collected and stored at RT for up to 30 days, showing enhanced stability compared to VAMS, for which our previous study set a 7-day stability compromise for 13 ASMs tested [18]. This improved stability could streamline at-home sampling for PwE, allowing sufficient time for sample shipment to the laboratory and facilitating telemedicine applications.

We excluded VPA from the validation due to its low accuracy, precision, and poor recovery. Additionally, we encountered difficulties with repeatability in the analyses, and, as reported in the literature [18,24]. This small compound is challenging to analyze using UHPLC-MS/MS

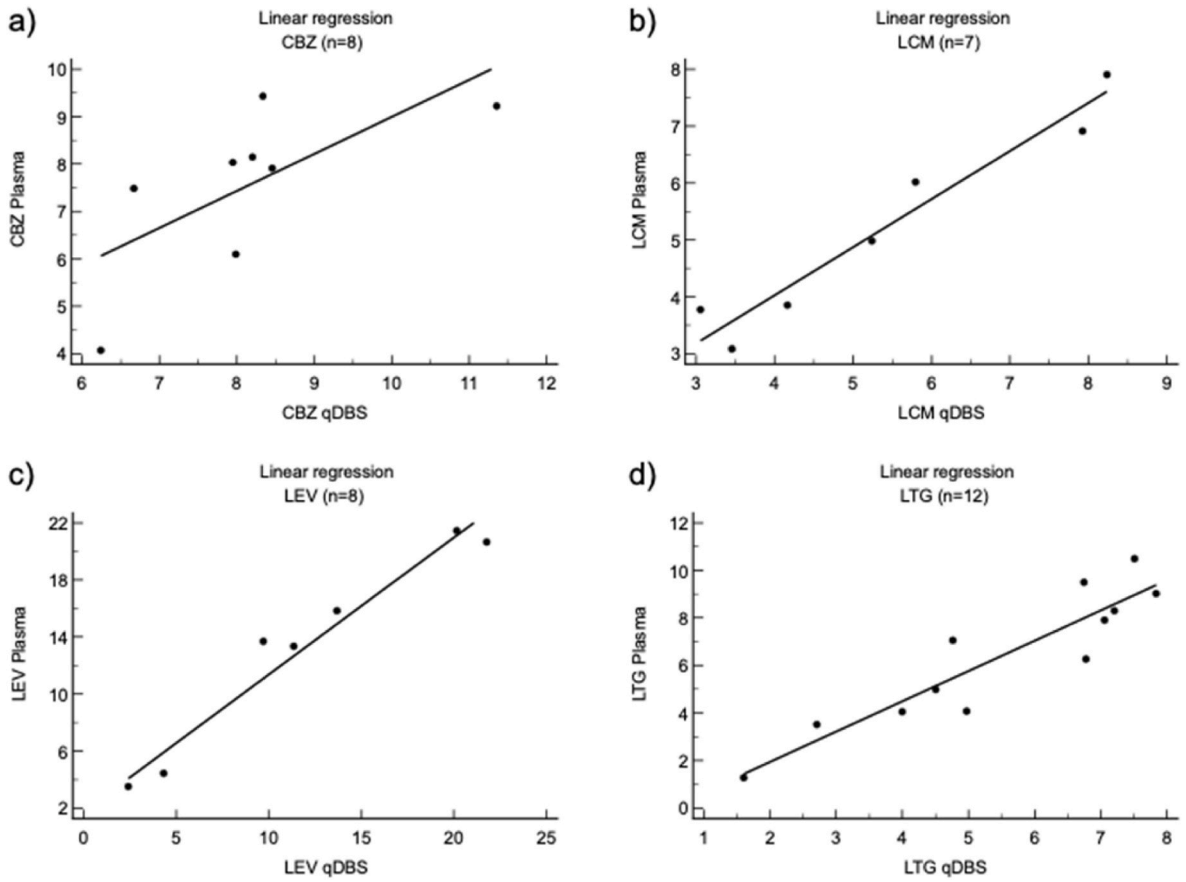

Fig. 5. Linear regression analysis between ASMs concentrations in plasma and in blood-qDBS for a) CBZ, b) LCM, c) LEV, and d) LTG (n = 30 PwE). The solid line represents the regression line.

methods.

To assess correlation, linear regression analysis compared concentration values obtained via the qDBS method with those from plasma samples. Agreement between the two assays was further evaluated using a Bland-Altman plot. The ASM values fell within the 95 % confidence interval.

The UHPLC-MS/MS method combined with qDBS sampling offers a promising approach for telemedicine applications and is becoming increasingly common in patient-centric clinical diagnostics as it provides a safer, more economical, and more comfortable diagnostic solution. Further research is needed to validate this fingerprick method across a broader range of ASMs and to assess its clinical applicability in real-world settings.

CRediT authorship contribution statement

Chiara Cancellerini: Writing – original draft, Validation, Methodology, Formal analysis, Data curation, Conceptualization. **Alice Caravelli:** Writing – review & editing, Validation, Formal analysis, Data curation. **Erika Esposito:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Laura Maria Beatrice Belotti:** Writing – review & editing, Methodology. **Martina Soldà:** Project administration, Data curation. **Nicolas Derus:** Methodology, Data curation. **Alessandra Merlotti:** Methodology, Data curation. **Francesco Casadei:** Methodology, Data curation. **Barbara Mostacci:** Writing – review & editing, Resources. **Luca Vignatelli:** Writing – review & editing, Methodology, Data curation, Conceptualization. **Francesca Bisulli:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Jessica Fiori:** Writing – review & editing, Supervision, Methodology. **Laura Licchetta:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Data curation, Conceptualization.

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2025.128018.

Data availability

Data will be made available on request.

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