A simple bioanalytical method for the quantification of antiepileptic drugs in dried blood spots

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Title: A simple bioanalytical method for the quantification of antiepileptic drugs in dried blood spots

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Abstract

An increasing number of publications on the dried blood spot (DBS) sampling approach for the quantification of drugs and metabolites have been spurred on by the inherent advantages of this sampling technique. In the present research, a selective and sensitive high-performance liquid chromatography method for the concurrent determination of multiple antiepileptic drugs (AEDs) [levetiracetam (LVT), lamotrigine (LTG), phenobarbital (PHB)], carbamazepine (CBZ) and its active metabolite carbamazepine-10,11 epoxide (CBZE)] in a single DBS has been developed and validated. Whole blood was spotted onto Guthrie cards and dried. Using a standard punch (6mm diameter), a circular disk was punched from the card and extracted with methanol: acetonitrile (3:1, v/v) containing hexobarbital (Internal Standard) and sonicated prior to evaporation. The extract was then dissolved in water and vortex mixed before undergoing solid phase extraction using HLB cartridges. Chromatographic separation of the AEDs was achieved using Waters XBridge™ C18 column with a gradient system. The developed method was linear over the concentration ranges studied with r ≥0.995 for all compounds. The lower limits of quantification (LLOQs) were 2, 1, 2, 0.5 and 1 µg/mL for LVT, LTG, PHB, CBZE and CBZ, respectively. Accuracy (%RE) and precision (%CV) values for within and between day were <20% at the LLOQs and <15% at all other concentrations tested. This method was successfully applied to the analysis of the AEDs in DBS samples taken from children with epilepsy for the assessment of their adherence to prescribed treatments.

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Highlights:

- We report a simple method for the analysis of four antiepileptic drugs in DBS samples
- The method was applied to DBS samples collected from children with epilepsy
- Such technique has potential in assessing adherence to AEDs using home sampling

Keywords: Antiepileptic drugs; Dried blood spot; HPLC; Children

Abbreviations:

AEDs	Antiepileptic drugs
DBS	Dried blood spot
CBZ	Carbamazepine
LTG	Lamotrigine
PHB	Phenobarbital
LVT	Levetiracetam
HPLC	High Performance Liquid Chromatography
CBZE	carbamazepine-10,11 epoxide

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1. Introduction

Antiepileptic drugs are the mainstay for the control of seizures in the management of epilepsy [1]. Regular monitoring of AED serum concentrations i.e. therapeutic drug monitoring (TDM), to guide dosage adjustments, is especially useful for children due to the greater pharmacokinetic variability in this population compared to adults. TDM is also important in assessing compliance with the prescribed regimen [2–4].

Dried blood spot (DBS) sampling is a technique used to collect capillary whole blood, either from a finger or heel prick, by spotting the blood onto a filter paper/card. This procedure was first established by Dr. Robert Guthrie in 1963 to measure phenylalanine for the detection of phenylketonuria in newborns [5]. Since then, this technique has been utilised for the population screening of newborns for inborn errors of metabolism and other clinical applications including the detection of a wide range of biological markers in epidemiological studies [6–7], disease surveillance [6–9] as well as toxicological evaluations and screening illicit drug use [10–**12**].

DBS sampling provides various advantages over conventional venous sampling. It is relatively non-invasive, with minimal blood volumes drawn [9]. This makes the technique particularly valuable for collecting samples in infants, children and the elderly [7]. DBS collection can be performed by non-medically trained individuals and by patients themselves after adequate training [7–8]. It also avoids the risks associated with the use of needles and syringes [13]. DBS samples do not need to be centrifuged or separated after collection unlike plasma or serum [7], which in turn also reduces the risks associated with handling of potentially infected materials [13]. A unique attribute of the DBS sampling is that it enables

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samples to be collected by patients themselves or parents/guardians at home and for samples to be posted by regular mail to the laboratory for analysis [8–9]. This allows convenient monitoring at any desired sampling time and for the monitoring results to be readily available at the clinic during a routine check-up [8].

Analysis of single AED concentrations in DBS has been reported for topiramate [14], phenobarbital [15], lamotrigine [16–19], gabapentin [20], phenytoin [**21**] and rufinamide [22]. However, apart from one recent study by Déglon et al. (which determined clobazam and clonaxepam) [**23**], no recent publication reported on the simultaneous determination of the concentration of multiple AEDs in a single DBS sample. The combined determination of different AEDs has the potential to monitor polymedicated patients and offers the possibility to quantify clinical samples of patients treated with any of these compounds in one sequence, with a single set of calibrators and QC samples [**24**]. The analysis of multiple AEDs in a single DBS had been explored by researchers at the Epilepsy Centre, Hemestede in the Netherlands and the MEDTOX® laboratories in the US, but with limited information published [25–28]. Therefore, the aim of the present research was to develop and validate a method to analyse the AEDs of interest, i.e. carbamazepine (CBZ) and its active metabolite carbamazepine-10,11 epoxide (CBZE), levetiracetam (LVT), lamotrigine (LTG) and phenobarbital (PHB) in DBS samples using High Performance Liquid Chromatography (HPLC) with ultraviolet (UV) detection. These AEDs were selected based on a recent audit by our group of the most commonly prescribed AEDs in children with epilepsy in N. Ireland (data not published). Determination of the AEDs of interest in plasma or serum samples using HPLC with UV or diode array detection (DAD) detection has been reported in the literature [29–33]. The analytical methodology was optimised and applied in the analysis of the AEDs in DBS samples of children with epilepsy collected at the clinic and in the patients' home for adherence determination.

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2. Experimental

2.1. Materials

LVT, LTG, PHB, CBZ, CBZE, hexobarbital (HXB) [internal standard], potassium dihydrogen phosphate and dipotassium hydrogen phosphate were purchased from Sigma-Aldrich (Poole, England), the chemical structures of the AEDs are shown in Fig. 1. Methanol and acetonitrile were of HPLC grade and obtained from AGB Scientific Apparatus, Ltd (Belfast, N. Ireland). The water utilised was purified using a Milipore Direct-QTM 5 water purification system (Milipore, Watford, England). Filtration of the phosphate buffer (part of the mobile phase) was performed using FP-VericelTM (0.45µm) membrane filters purchased from Sartorius (Epsom, UK).

2.2. Standards

HXB was used as an internal standard, a stock solution of the IS was prepared by dissolving 25mg in 25mL of methanol and this was further diluted with methanol (1:100). Stock solutions of LVT, LTG, PHB and CBZ were prepared at a concentration of 1mg/mL in methanol. A stock solution of CBZE was prepared at a concentration of 0.5mg/mL in methanol. The stock solutions were further diluted (1:25) with mobile phase consisting of 75% buffer (25mM phosphate buffer pH 6.2), 15% acetonitrile and 10% methanol. The working standards were prepared by further dilutions of the diluted (1:25) stock solutions with the mobile phase described above. Solutions of the calibration standards were prepared from the working standards.

Ten microlitres of each of the analytes of interest (LVT, LTG, PHB, CBZ and CBZE) in methanol were added to 0.95mL human whole blood aliquots to yield final concentrations of the calibration standards (C1-C8) in the concentration range 0.5µg/mL to 10µg/mL for CBZE,

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1µg/mL to 20µg/mL for LTG and CBZ, and 2µg/mL to 50µg/mL for LVT and PHB (Table 1). The lowest calibrator concentrations were arbitrarily chosen as the lower limits of quantification (LLOQs)**.** The low, medium and high quality control (LQC, MQC and HQC) samples were prepared with final concentrations as shown in the Table 1.

2.3. Sample preparation and extraction

Thirty microlitres of the prepared spiked blood standards were spotted onto individual Guthrie cards (Schleicher & Schuell 903®, Aston Ltd, England), dried overnight at room temperature in the dark and stored within a greaseproof paper liner, inside a sealed polypropylene container at -80ºC until required for analyses. For each DBS, a 6mm diameter disk was punched manually and placed in a polypropylene Eppendorf tube (2.0mL capacity). An aliquot (980µL) of extracting solvent containing methanol:acetonitrile (3:1, v/v) and 20µL of the 10µg/mL HXB was added to the disk and the tube was sonicated using a DECON FS200® frequency sweep water-bath sonicator for 15 minutes. The sample mixture was then placed in a disposable glass culture tube and the extract dried under a stream of nitrogen at 40ºC for 30 minutes using a Zymark TurboVap® LV Evaporator Workstation. The residue was then dissolved in 1 mL water and vortex mixed for 30 seconds before undergoing solid phase extraction (SPE).

The SPE procedure was carried out using a Waters Extraction Manifold with Oasis® HLB 1mL cartridges that had been conditioned using 1mL of methanol followed by 1mL of water. The loaded sample was drawn through the cartridge at a maximum flow rate of 1mL/min. The cartridge was then washed with 1mL water and the AEDs eluted with 1mL of methanol:acetonitrile mixture (3:1, v/v) at a maximum flow rate of 1mL/min. The eluate was evaporated under a stream of nitrogen at 40ºC for 20 minutes and reconstituted with 100µL of mobile phase (75% phosphate buffer pH 6.2, 15% acetonitrile and 10% methanol). The

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extract was transferred into an auto sampler vial and 50µL was injected onto the HPLC column.

2.4. Chromatography

HPLC analysis was carried out on a Waters® Alliance HPLC system consisting of Waters® 2695 Separations Module connected to the Waters® 2487 Dual Wavelength Absorbance Detector. Data recording was carried out using Empower™ software. The separation was performed using an XBridge™ C18 column (150 x 4.6mm, 3.5µ; Waters, UK) fitted with an XBridge™ guard column of similar chemistry (20 x 4mm, 3.5µ; Waters, UK).

The mobile phase consisted of a mixture of 25mM phosphate buffer containing 12.5mM sodium chloride, pH 6.2 (A), acetonitrile (B) and methanol (C) delivered using a gradient method (Table 2) at a flow rate of 1mL/min. The mobile phase solutions were degassed and filtered through a 0.45µm filter prior to use. The column temperature was maintained at 45ºC and the wavelength for UV detection was set at 205nm. Total analysis run time was 28 minutes.

2.5. Assay characteristics for method validation

Validation of the developed method was performed to evaluate the following parameters: selectivity, linearity, limits of detection and quantification, accuracy and precision, recovery and stability. Experiments were also conducted to determine the effects of volume of blood used to prepare the DBS on the measured concentration of the AEDs. Validation of the analytical method developed in the present study was according to the guidelines of the International Conference on Harmonisation (ICH) [34].

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2.5.1. Selectivity

Selectivity was investigated using six independent sources of blood from six volunteer subjects. DBS samples were prepared from blank blood and from blood spiked with all of the AEDs of interest at the lower limit of quantification (LLOQ). This was to ensure that there were no interfering peaks present at the retention time of the AEDs of interest. Potential interference from concomitant anti-epileptic medications commonly taken by paediatric patients was investigated by analysing samples which had been spiked with the appropriate drugs, i.e. clobazam, valproic acid, ethosuximide, phenytoin, gabapentin, vigabatrin and topiramate.

2.5.2. Linearity

A five-day calibration was carried out to determine the linearity of the developed assay for eight concentrations of the AEDs spiked in DBS samples. The AEDs were spiked in combination for each of the concentrations as shown in Table 1. The calibration also consisted of a blank and a zero sample (blank DBS with IS added). Calibration plots were constructed for peak area ratio (analyte response/IS response) versus the analyte concentration in order to assess the relationship between the two parameters. Linear regression analysis was performed to determine the slope, intercept and correlation coefficient of the calibration lines. The homoscedasticity assumption for each linear regression analysis was tested using the F-test **[35]**.

2.5.3. Accuracy and precision

Accuracy and precision were determined by replicate analysis of samples containing known amounts of the analyte. They were the quality control (QC) samples prepared at four concentrations (LLOQ, low QC, middle QC and high QC). Five replicates at each QC

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concentration were used to calculate within-day accuracy and precision. Between-day accuracy and precision were determined at each QC concentration over five consecutive days.

The QC samples were analysed against the calibration curve and the concentrations obtained were compared with the known value. The accuracy and precision of the method were expressed as the mean percent relative error (%RE) and percent coefficient of variation (%CV) respectively. The mean accuracy (%RE) and precision (%CV) should be within 15% of the actual value except for LLOQ which should not deviate by more than 20% [36].

2.5.4. Limit of detection (LOD) and lower limit of quantification (LLOQ)

The LOD and the LLOQ were calculated using the following formulae:

$LOD = 3.3\ \sigma / S$ $LLOQ = 10\ \sigma / S$

where σ is the standard deviation of the response, S is the slope of the calibration curve. The slope was estimated from the calibration curve of the analyte and σ from the residual standard deviation of the regression line generated from the Empower™ software.

2.5.5. Recovery

The absolute recovery of an analytical process was determined by comparing the detector response obtained from a known amount of analyte added to, and extracted from, the biological matrix with the detector response obtained for the true concentration of the pure authentic standard representing 100% recovery. Six replicates of three concentrations (low, middle and high QCs) of DBS samples spiked with all AEDs for each of the concentrations

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were extracted and analysed. The detector response obtained was compared with the detector response from solution standards.

2.5.6. Stability

Stability of the AEDs in DBS samples was assessed over a 6-week period at -80ºC and room temperature (25ºC). Stability was also assessed for storage of the samples at 40ºC for 3 days using a GenLab® 50 litre oven with digital control (OV/50/DIG). Three replicates of each AED at the high QC concentration were analysed and compared against freshly prepared spiked blood spots.

2.5.7. The effect of the volume and haematocrit of blood used to prepare the DBS on the measured concentration of AEDs

DBS samples were prepared using varying volumes (20-50µL) of blood, spiked with all the AEDs together to give the final concentrations as follows: CBZ and LTG 12.5µg/mL, PHB and LVT 25µg/mL and CBZE 6.25µg/mL. The samples were processed according to the method described in section 2.3, by taking a 6mm disk from the centre of the DBS. The measured concentrations of the AEDs were compared in triplicate.

In order to examine the effect of varying haematocrit (Hct) levels on the accurate quantification of AEDs, various Hct levels of whole blood were created by adding plasma to or removing plasma from fresh human blood. Blood was prepared at Hct levels of 30, 42.5 and 55% and then spiked with all AEDs at the concentrations described above. DBS samples prepared from spiked blood were processed in the same way and measured concentrations compared in triplicate.

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2.5.8. Statistical methods and data analysis

Analysis of the data was carried out using Microsoft® Excel 2007 (Microsoft Corporation, USA). SPSS® software (version 17.0) was used to present the calibration curve plots. Calibration curve regression analysis was performed using Empower™ software (Waters Corporation, USA).

3. Results and discussion

3.1. Method development and optimisation

Initial analytical method development in this study was based on the article by Vermeij and Edelbroek [32] for simultaneous determination of seven AEDs in serum.

HXB was used as the IS as it was well separated chromatographically from the AEDs of interest. Other compounds were tested for suitability as an IS, i.e. 5-ethyl-(5-para)-2-thio-tolyl barbituric acid and barbital, but were not selected for the final methodology due to poor chromatographic separation.

During method development using standard solutions, chromatographic conditions suggested by Vermeij and Edelbroek [32] were applied. Separation was performed using an Xbridge™ C18 column 3.5µm particle size (15cm x 0.46cm) column, which was preceded by an Xbridge™ guard column of similar chemistry. Xbridge™ C18 columns are designed using Hybrid Particle Technology (HPT) – Ethylene-Bridged (BEH Technology™) Hybrids by Waters®. Such columns are claimed to be superior to other reversed-phase columns using silica-based packing materials, resulting in improved peak shapes for basic compounds and enabling operation under wider pH ranges [37].

Chromatographic conditions, such as the column temperature of 45ºC and phosphate buffer composition (12.5mM, pH 6.2) were used in the analytical method. Acetate and phosphate

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buffers prepared at pH 3 and 5 were also tested during optimisation of the mobile phase compositions, however, phosphate buffer at pH 6.2 as described above was found to be the most suitable in achieving resolution of all peaks of interest.

Initially, a mobile phase consisting of a mixture of methanol (14.5%), acetonitrile (19.5%) and phosphate buffer (66%) delivered isocratically at a flow rate of 0.9mL/min was used. This enabled good resolution of all compounds of interest with the exception of LVT. This was mainly because LVT is highly polar, hence, requiring a mobile phase with very low organic strength to be retained on a reversed phase column [38-39]. To achieve such chromatographic conditions, whilst retaining conditions suitable for the other AEDs of interest, a gradient elution approach was applied. Optimisation of the gradient conditions was carried out and the best separation of the AEDs was attained with gradient parameters as shown in Table 2. Without LVT included in the analysis, UV detection wavelengths of 215nm and 275nm were found to be appropriate. However, a wavelength of 205nm was used to monitor the absorbance of all AEDs analysed, mainly because LVT lacks chromophores and detection was only feasible using very short wavelengths [39].

The UV detection method was feasible in this assay since all of the AEDs of interest are active at relatively high concentrations (µg/ml, rather than ng/ml), hence justifying the use of UV detection as a good and cost-effective option. The use of LC mass-spectrometry [LC-MS(/MS)], on the other hand, has recently gained more attention and acceptance as it offers improved sensitivity (allow measurement of very low concentrations), with shorter run times due to enhanced selectivity. For the purpose described in the current study, however, i.e. routine therapeutic monitoring of AEDs, the use of LC-MS(/MS) technique may actually be considered 'over-engineering' due to the high costs involved and lack of availability of the instrumentation in all clinical laboratories.

Optimisation of AEDs' extraction was carried out by testing mixtures of methanol:acetonitrile at 1:1, v/v and 3:1, v/v as well as acetonitrile:water 1:1, v/v as the extraction solvent. The use

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of acetonitrile:water mixture has been suggested by Janis *et al*. [27] as the extraction solvent of choice to extract AEDs from DBS samples. However, it has been reported that using water for extraction of DBS samples increases the interference from endogenous compounds and should be avoided where possible [8]. This was confirmed by visual inspection of the DBS samples after extraction with the acetonitrile:water mixture (1:1, v/v), which showed that the extract was coloured when compared with extraction using methanol:acetonitrile mixtures. In addition to having a cleaner extract, the use of the methanol:acetonitrile mixture results in protein denaturation and precipitation [8–9], which could be a significant advantage when considering agents which are highly bound to plasma proteins. In this study, it was found that extraction using methanol:acetonitrile (3:1, v/v) gave rise to better recovery (extraction efficiency) of the AEDs when compared with methanol:acetonitrile (1:1, v/v) extraction solvent.

The SPE procedure was optimised using Oasis® HLB cartridges in preference to Oasis® Mixed-mode sorbent cartridges, which are specific for either acidic or basic compounds. This was due to dissimilar chemistry of the AEDs: LVT and LTG are basic compounds, PHB acidic and CBZ neutral [40]. Utilisation of Oasis® HLB cartridges, a hydrophilic-lipophilic balanced copolymer, enabled high recoveries for all compounds of interest. Before loading into the cartridges, the extracted sample was evaporated under a stream of nitrogen at 40ºC using a Zymark TurboVap® LV Evaporator Workstation and reconstituted in water. Reconstitution with 5% of methanolic solution resulted in the loss of the AEDs due to lack of retention of the AEDs during the loading step. This was ascertained by collecting the load sample after passing through the cartridge and injecting it onto the HPLC. For the washing step, various percentages of methanol in water with 2% ammonia or 2% formic acid were tested during the SPE method optimisation. No difference was observed in the recovery of the AEDs indicating that ammonia or formic acid was not needed during the SPE sample clean-up. Consequently, only water was used as the washing solvent.

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The chromatogram for the AEDs of interest, extracted from spiked DBS samples, together with their retention times, is shown in Fig. 2.

3.2. Method validation

3.2.1. Selectivity

The AEDs were found to be well resolved (Fig. 2) using the chromatographic conditions described above. No interfering peaks were observed in the extracted blank DBS chromatograms when overlaid with chromatograms of extracted DBS samples spiked with AEDs at the LLOQ. This indicated that the method exhibited selectivity and the individual AEDs were not affected by the presence of endogenous compounds. Furthermore, selectivity of the analytical method was also evaluated using blank blood samples from six different blood sources. Peaks from endogenous compounds were seen close to the retention time for LVT, which was expected as a result of the low UV detection wavelength (205nm) selected. Nevertheless, this compound was able to be separated and quantified at its LLOQ concentration with acceptable intra- and inter-day accuracy and precision ranging from -0.45% to 13.74% (i.e. within the acceptable limits of <20%). No interferences from the anti-epileptic drugs commonly given to the study patients were observed (see earlier).

3.2.2. Linearity

The F-test revealed a significant difference between the variances of highest and lowest QC concentrations (experimental F-value was significantly higher than tabled F-value at 99% confidence level), thus homoscedasticity assumption was not met. Several calibration models were explored using the Empower™ software to ascertain the most suitable calibration curve for each of the AEDs analysed. These included the ~~non-weighted,~~ 1/x and $1/x^2$ weighted linear regression models. Evaluation of the best fit and percentage deviation of the calculated concentration from the nominal concentration of each of the AEDs indicated

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that the $1/x^2$ weighted linear regression model was the most suitable model. This approach adequately described the relationship between the concentration and peak area response (ratio of the peak area of the AED and peak area of the IS; PAR). The calibration curves for all the AEDs were found to be linear over the concentration range selected. The mean correlation coefficient, slope and intercept values from the five calibration curves are presented in Table 3.

3.2.3. Accuracy and precision

Within and between day accuracy and precision data were determined for each of the AEDs during the 5-day validation experiments at low, middle and high QC concentrations. Precision and accuracy were found to be within ± 15% at all QC concentrations as shown in Table 4.

3.2.4. Limit of detection (LOD) and lower limit of quantification (LLOQ)

The results obtained for the LOD and LLOQ for each compound are shown in Table 5. The values of LOD and LLOQ presented are the highest values obtained from the 5-day calibration results. The validated LLOQ is presented in Table 4 together with the calculated intra- as well as inter-day accuracy and precision. The validated LLOQ for CBZE (0.5 µg/mL) has been shown to be lower than the calculated value (0.78 µg/mL) for the compound. All values were within the acceptable limit of ±20%.

3.2.5. Recovery

The calculated recoveries for each of the AEDs at each concentration of QC standards (n=6) are presented in Table 6. Recovery was found to be consistent and precise with %CV less than 12%. Recovery values at each concentration of QC standards were above 80% for all

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AEDs analysed except for LVT which had recovery values ranging from 61% to 72% (Table 6). However, using the DBS matrix, the percentage recovery was acceptable as the %CV for this compound was consistently less than 12% at each QC concentration.

3.2.6. Stability

The result of the stability studies indicated that the AEDs in the DBS matrix were stable at -80ºC and room temperature (25ºC) over a 6-week storage period. They were also found to be stable after storage at 40ºC for three days. The values were found to range between 0.89±0.02 and 1.04±0.07 indicating stability of the AEDs at the storage conditions employed (Table 7).

The temperature and duration of storage were selected to resemble the actual conditions occurring during actual sample collection and handling in the clinical study. Stability assessment after storage at room temperature was carried out as DBS samples collected in the patients' home would be stored at room temperature prior to mailing them to the laboratory the next day. There would also be the possibility that these samples were exposed to higher temperatures (e.g. storage over weekend in a sun-heated post-box or near a heating device), hence the selection of 40ºC to ensure that the AEDs were stable despite being stored at this temperature.

3.2.7. The effect of the volume and haematocrit of blood used to prepare the DBS on the measured concentration of AEDs

The volume of blood spotted on to the Guthrie cards was varied to evaluate the effect of spotted blood volume on the measured concentration of AEDs. Volumes ranging from 20 to 50µL of blood were chosen to mimic the actual collection of patient samples during the adherence study. Volumes beyond 50µL were not evaluated as it was not expected that volumes greater than 50µL would be obtained from a finger prick in children in the clinical study.

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A fixed volume of 30µL was chosen as the standard volume of blood to be spotted on to the Guthrie cards in the preparation of blood spot calibration standards and quality control samples. This volume was selected as it filled the pre-printed circles on the Guthrie cards and enabled a 6mm diameter punch to be utilised for sample processing.

The measured concentration for each of the AEDs at the differing volumes displayed accuracy and precision, presented as %RE and %CV respectively of less than ±5%. The influence of blood spot volume on the measured concentration of the AEDs is presented graphically in Figs. 3 ~~and 4~~. In general, there was a slight increase in the concentration of the AEDs at 50µL compared to 20µL of blood volume spotted. The highest percentage difference in the measured concentration between 20µL and 50µL of blood volume spotted was, however, only 5.85%, which was observed for LVT. This finding coincides with the report of other investigators who have shown a minor effect of blood volume on measured concentrations in DBS samples [**41**].

The effect of varying Hct levels on measured AED concentrations is shown in Table 8. The results demonstrated minimal effect of Hct within the range of 30-55% on measured concentration of AEDs in DBS. Each of the measured AEDs displayed a difference of less than ±5% from that measured at the middle Hct level (42.5%) within the range studied; Hct values below 30% or beyond 55% were not expected in the children studied.

3.2.8. Clinical application

The developed method was applied to the analysis of LTG, PHB, LVT, CBZ and CBZE in DBS samples collected from children with epilepsy as one of the methods for adherence assessment. Fig. 4 illustrates representative chromatograms obtained from the analysis of each of the AEDs of interest in DBS samples obtained from children at the clinic. Findings from the adherence study using the method described above will be the subject of a separate publication.

4. Conclusion

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A simple analytical procedure for simultaneous analysis of four AEDs and one metabolite in DBS samples has been successfully developed and validated according to the recommended guidelines. AEDs could be quantified with acceptable accuracy and precision using the analytical method developed. The microanalytical method shown here has been successfully applied in analysing DBS obtained from finger pricks in paediatric patients with epilepsy as part of an adherence study. This minimally-invasive sample collection technique has potential in the therapeutic drug monitoring of the AEDs to ascertain adherence or for other relevant purposes, in the paediatric population in the clinical setting.

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Figure Legends:

Fig. 1: Chemical structures of PHB, CBZ, CBZE, ~~PHT,~~ LVT, LTG and HXB (IS)

Fig. 2: Chromatogram showing the result of extraction and separation of spiked blood spot of LVT (10µg/mL), LTG (15µg/mL), PHB (20µg/mL), CBZE (4.5µg/mL) and CBZ (4µg/mL) with IS HXB (2µg/mL) monitored at 205nm

Fig. 3: Influence of blood spot volume on the measured concentration of LVT (25µg/mL), LTG (12.5µg/mL), PHB (25µg/mL), CBZ (12.5µg/mL) and CBZE (6.25µg/mL) [mean conc. ±SD; n=3]

Fig. 4: Representative chromatograms of extracted DBS samples obtained from patients treated with multiple AEDs; (A) LTG and CBZ (found concentrations were 7.4 and 12.7µg/mL, respectively and 2.4µg/mL for CBZE); (B) LTG and PHB (found concentrations were 1.9 and 2.1µg/mL, respectively); (B) LVT and LTG (found concentrations were 45.2 and 10.6µg/mL, respectively); (C) LVT, LTG and CBZ (found concentrations were 35.3, 7.5 and 9.9µg/mL, respectively and 2.0µg/mL for CBZE).

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Table 1: Final AEDs concentrations of calibration standards and quality control samples prepared (µg/mL)

AED	C1	C2	C3	C4	C5	C6	C7	C8	LQC	MQC	HQC
LVT (12-46 µg/mL**)**	2	4	8	10	20	30	40	50	6	15	45
LTG (2.5-15 µg/mL**)**	1	2	4	8	10	15	17.5	20	3	9	18
PHB (10-40 µg/mL**)**	2	4	8	10	20	30	40	50	6	15	45
CBZ (4-12 µg/mL**)**	1	2	4	8	10	15	17.5	20	3	9	18
CBZE	0.5	1	2	3	4	6	8	10	1.5	5	9

[a]Therapeutic intervals of respective AEDs are shown within brackets (µg/mL) [4]

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Table 2: Gradient parameters of the mobile phase used in HPLC-UV analyses of AEDs

Time (min)	A[a] (%)	B[b] (%)	C[c] (%)
0	83	10	7
2	83	10	7
5	75	15	10
13	71	18	11
22	69.5	19	11.5
23 – 28	83	10	7

[a]A – 25mM phosphate buffer, [b]B – Acetonitrile, [c]C – Methanol

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Table 3: Mean slope, intercept and correlation coefficient according to the calibration curves plotted (n=5)

AED	Mean slope ± SD	Mean intercept ± SD	Mean correlation coefficient (r) ± SD
LVT	0.034±0.003	0.032±0.015	0.995±0.003
LTG	0.305±0.005	0.044±0.037	0.998±0.001
PHB	0.147±0.004	0.050±0.026	0.996±0.002
CBZE	0.283±0.006	0.047±0.006	0.997±0.002
CBZ	0.188±0.005	0.020±0.026	0.998±0.001

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Table 4: Results of within day (intra-day) and between days (inter-day) accuracy and precision measurements (n=5)

AED	Nominal conc. (µg/mL)	Within day			Between day		
		Measured conc. ± SD (µg/mL)	Accuracy %RE	Precision %CV	Measured conc. ± SD (µg/mL)	Accuracy %RE	Precision %CV
LVT	2 (LLOQ)	2.27±0.30	13.74	13.25	1.99±0.10	-0.45	5.24
	6 (LQC)	6.33±0.26	5.46	4.06	6.14±0.11	2.25	1.78
	15 (MQC)	15.92±1.00	6.11	6.26	15.10±0.78	0.63	5.20
	45 (HQC)	42.51±5.50	-5.54	12.94	44.41±5.33	-1.31	12.00
LTG	1 (LLOQ)	0.96±0.08	-4.40	8.29	1.00±0.02	-0.06	1.56
	3 (LQC)	3.01±0.09	0.45	2.85	3.01±0.19	0.23	6.34
	9 (MQC)	9.26±0.37	2.87	4.01	9.21±0.39	2.28	4.26
	18 (HQC)	17.99±0.29	-0.07	1.62	17.73±0.64	-1.48	3.63
PHB	2 (LLOQ)	1.96±0.13	-1.81	6.42	1.92±0.08	-3.76	4.19
	6 (LQC)	6.66±0.19	10.97	2.8	6.60±0.33	10.01	4.96
	15 (MQC)	16.89±0.68	12.62	4.03	16.85±0.72	12.31	4.26
	45 (HQC)	44.83±0.85	-0.38	1.9	44.27±1.51	-1.62	3.41
CBZE	0.5 (LLOQ)	0.41±0.04	-17.08	9.36	0.48±0.02	-3.68	3.35
	1.5 (LQC)	1.67±0.05	11.40	2.93	1.66±0.12	10.71	7.27
	5 (MQC)	5.34±0.22	6.72	4.06	5.29±0.24	5.81	4.50
	9 (HQC)	8.82±0.17	-1.97	1.90	8.73±0.32	-3.01	3.68
CBZ	1 (LLOQ)	0.99±0.06	-0.68	5.84	0.99±0.03	-1.36	3.36
	3 (LQC)	3.11±0.08	3.55	2.67	3.11±0.16	3.75	5.09
	9 (MQC)	9.50±0.39	5.54	4.12	9.37±0.44	4.10	4.75
	18 (HQC)	20.58±0.40	14.33	1.92	20.11±0.86	11.70	4.28

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Table 5: Calculated LOD and LOQ for AEDs in DBS samples

AED	LOD (µg/mL)	LOQ (µg/mL)
LVT	0.380	1.150
LTG	0.223	0.676
PHB	0.318	0.963
CBZE	0.300	0.908
CBZ	0.258	0.780

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Table 6: Recovery results for QC standards of the AEDs of interest (n=6)

AED	Nominal conc. (µg/mL)	Percentage recovery ± SD	Precision %CV	Overall percentage recovery ± SD	Overall precision %CV
LVT	6	71.60±2.98	4.15	66.07±5.30	8.02
	15	61.05±2.28	3.73		
	45	65.54±7.77	11.86		
LTG	3	97.93±3.13	3.20	97.19±2.40	2.47
	9	99.13±7.16	7.22		
	18	94.51±1.70	1.80		
PHB	6	99.04±3.41	3.44	94.34±6.38	6.76
	15	96.89±6.67	6.89		
	45	87.08±1.41	1.62		
CBZE	1.5	94.39±3.18	3.37	96.54±2.80	2.90
	5	95.51±6.46	6.77		
	9	99.71±1.81	1.81		
CBZ	3	89.68±3.39	3.78	87.99±6.96	7.91
	9	80.35±5.66	7.04		
	18	93.95±1.74	1.86		

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Table 7: Stability results based on the ratio between two measurements (freshly prepared sample versus sample stored at different storage conditions; mean conc. ±SD; n=3)

AED	Nominal conc. (µg/mL)	Mean±SD ratios		
		40ºC for 3 days	-80ºC after 6 weeks	Room temperature after 6 weeks
LVT	45	0.99±0.02	0.93±0.12	0.98±0.09
LTG	18	1.04±0.05	0.99±0.01	1.01±0.06
PHB	45	1.01±0.03	0.95±0.01	0.97±0.05
CBZE	9	1.04±0.07	0.93±0.06	0.89±0.02
CBZ	18	0.95±0.03	0.89±0.00	0.90±0.05

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Table 8: Effect of varying blood haematocrit on measured concentration of AEDs in DBS (mean conc. ±SD; n=3).

AED	Hct level (%)	Nominal conc. (µg/mL)	Measured conc. ± SD (µg/mL)	%RE	% difference from Hct 45
LTG	30	12.5	12.88±0.02	3.01	3.48
	42.5	12.5	12.44±1.03	-0.45	0
	50	12.5	12.68±0.78	1.45	1.91
LVT	30	25	24.01±0.77	-3.98	-1.24
	42.5	25	24.31±1.67	-2.77	0
	50	25	25.51±1.39	2.03	4.93
PHB	30	25	25.54±0.45	2.16	2.5
	42.5	25	24.92±1.52	-0.33	0
	50	25	25.95±1.78	3.81	4.15
CBZ	30	12.5	12.42±0.20	-0.63	1.82
	42.5	12.5	12.20±0.94	-2.41	0
	50	12.5	12.64±0.83	1.12	3.62
CBZE	30	6.25	5.93±0.48	-5.2	-1.72
	42.5	6.25	6.03±0.54	-3.54	0
	50	6.25	6.29±0.22	0.62	4.31

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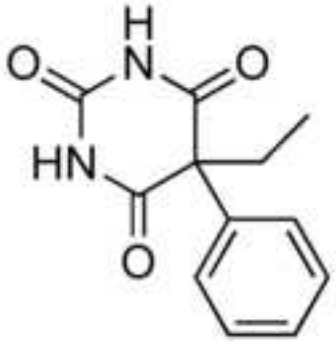
Figure1



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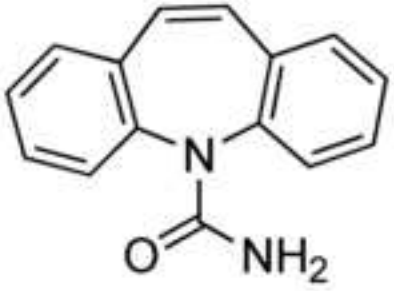
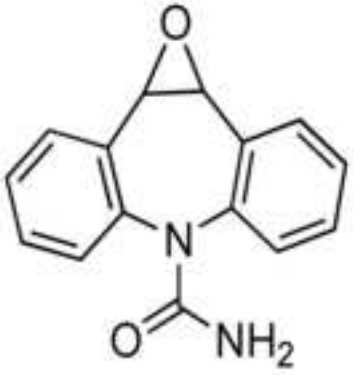


Carbamazepine



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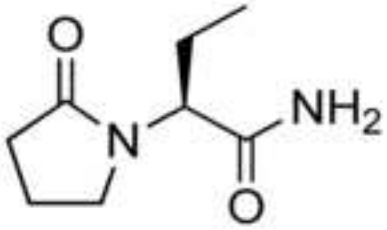

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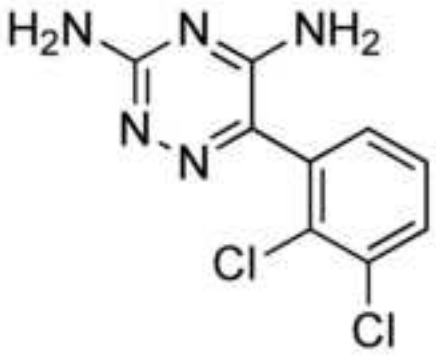

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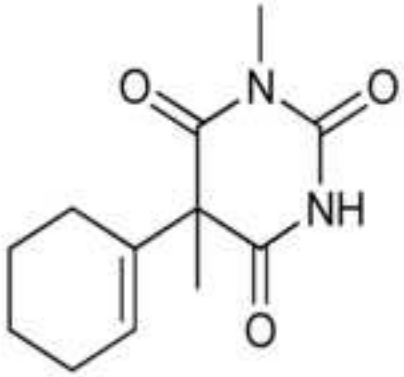
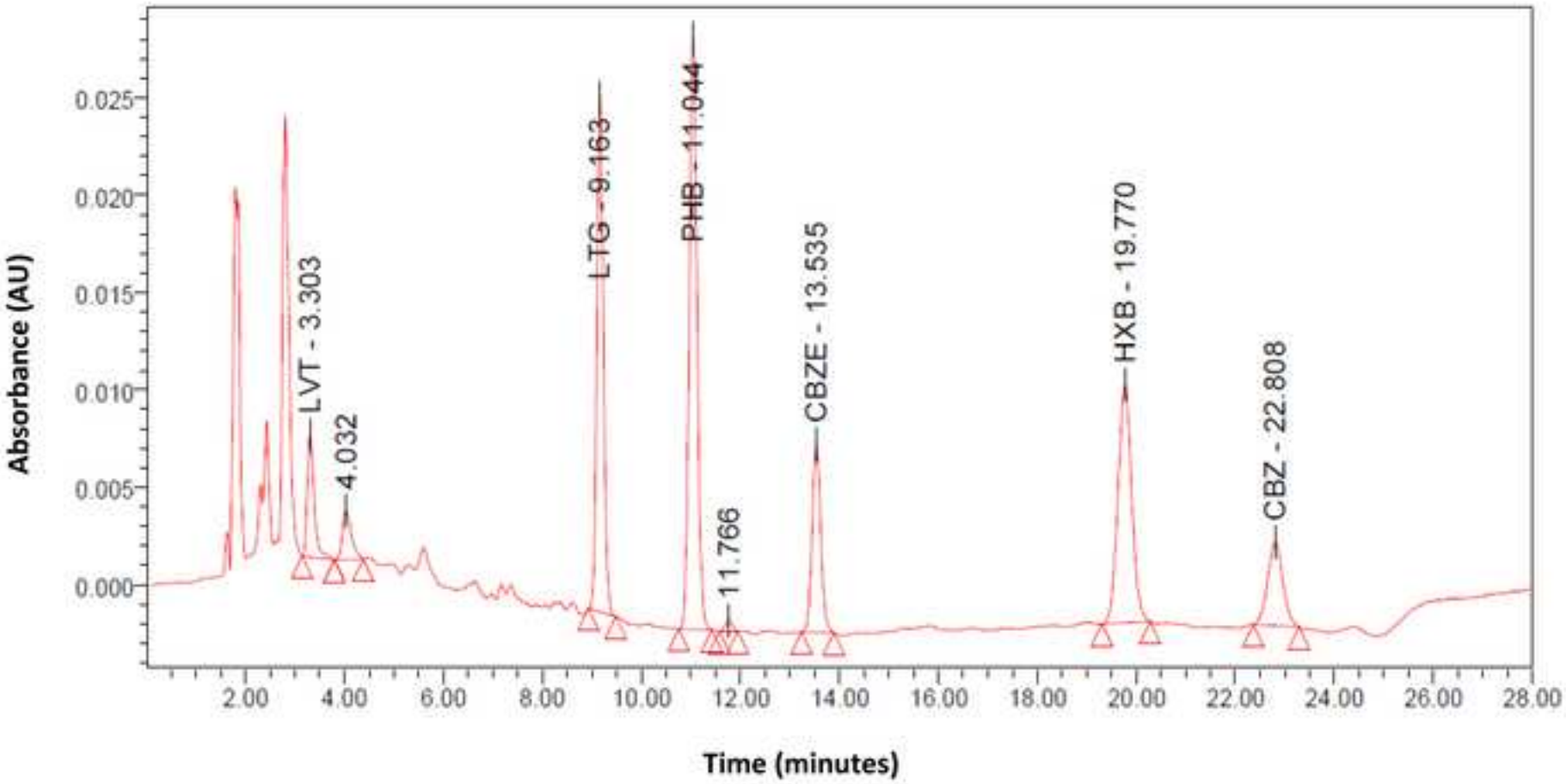


Hexobarbital

Figure2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0.015
0.010
0.005
0.000
LVT - 3.303
4.032
LTG - 9.163
PHB - 11.044
11.766
CBZE - 13.535
HXB - 19.770
CBZ - 22.808
2.00
4.00
6.00
8.00
10.00
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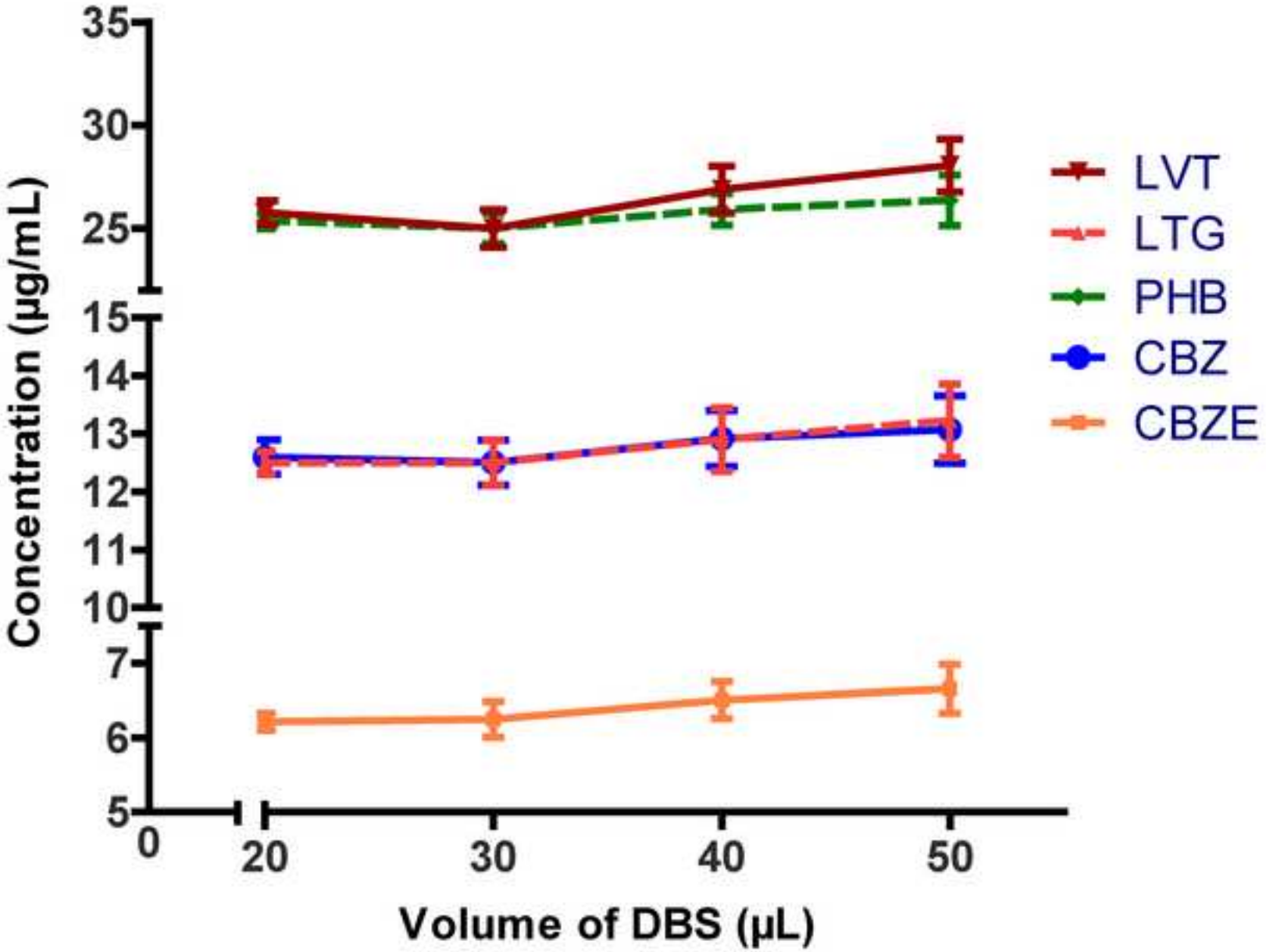
26.00
28.00
Time (minutes)

Figure3


35
30
25
15
14
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12
11
10
7
6
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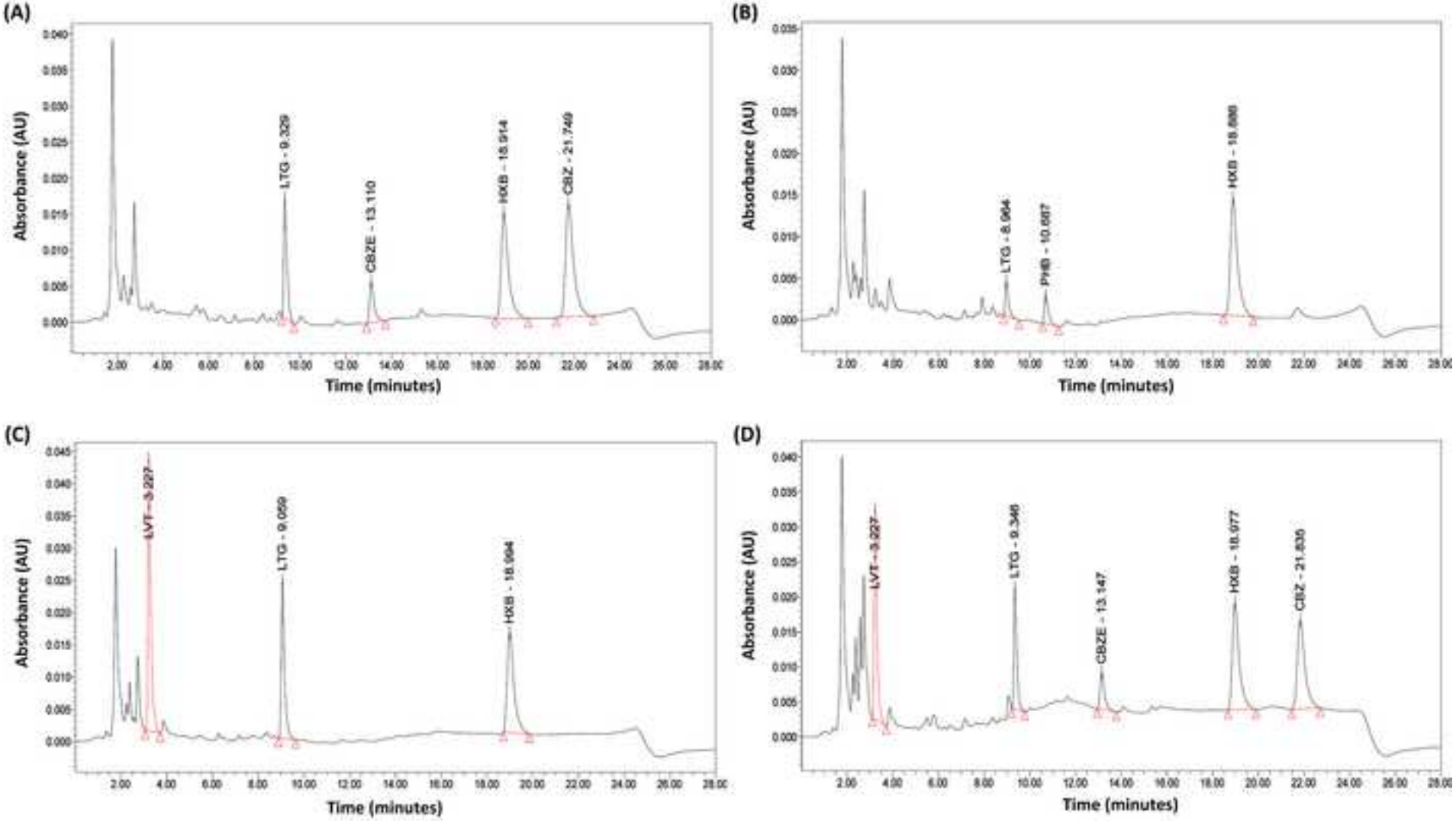
Concentration (µg/mL)
Volume of DBS (µL)
LVT
LTG
PHB
CBZ
CBZE

Figure4


(A)
Absorbance (AU)
LTG - 9.329
CBZE - 13.110
HXB - 18.914
CBZ - 21.749
Time (minutes)
(B)
Absorbance (AU)
LTG - 8.964
PHB - 10.687
HXB - 18.686
Time (minutes)
(C)
Absorbance (AU)
LVT - 3.227
LTG - 9.059
HXB - 18.994
Time (minutes)
(D)
Absorbance (AU)
LVT - 3.227
LTG - 9.346
CBZE - 13.147
HXB - 18.977
CBZ - 21.839
Time (minutes)