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Original Scientific Paper

Application of analytical quality by design approach to bioanalytical methods - simultaneously quantification of antiepileptic drugs in human saliva

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Abstract

Bioanalytical methods play a significant role in therapeutic drug monitoring (TDM) with the main objective, the implementation of a rational therapy. The obtained results from bioanalytical method should be consistent, reliable and with high quality, in order to apply for therapeutic monitoring in routine clinical practice. Analytical Quality by Design - AQbD is required as an essential tool during the process of development, optimisation, validation and routine application of bioanalytical method. The aim of this paper is to introduce the concept of analytical quality by design (AQbD), in the development of robustness bioanalytical method for simultaneous determination of antiepileptic drugs (lamotrigine, levetiracetam, valproic acid, carbamazepine and its active metabolite carbamazepine-10,11-epoxide) in human saliva. The proposed and validated methods have been successfully applied for determination of those antiepileptic drugs in saliva collected from epileptic patients that are under the therapy. The results and conclusions confirmed that during the development and optimisation of bioanalytical method, the application of AQbD provide better reliability and quality of obtained bioanalytical data.

Keywords: bioanalytical methods, analytical quality by design, lamotrigine, antiepileptic drugs, saliva.

1. Introduction

Bioanalytical methods are a set of procedures that include the collection, processing, storage and analysis of a biological matrix (blood, plasma, urine, saliva and tissues) for measuring concentration of drug and their metabolites. Determination of the drug concentration in the biological matrix is important in therapeutic drug monitoring - TDM because it plays important role in rational therapy, improve the clinical effects, reduce or avoid side effects, and reduce the cost of therapy. TDM mostly is used in drugs that have the correlation between drug concentrations in biological matrix and their therapeutic effect. Antiepileptic drugs-AELs are one of the most common drugs used in TDM (Patsalos et al, 2008). Many studies recommended saliva as an alternative biological matrix for therapeutic drug monitoring of many AEDs because of the measurement of drug concentration in saliva present the free fraction (unbound to plasma proteins) of drugs in plasma that have a clinical significance (Grim *et al*, 2003; Mecarelli *et al,* 2007; Patsalos, 2013). The measurement of free drug concentration is desirable but it is difficult to achieve in practice because the processes as the equilibrium of dialysis and ultracentrifugation are time-consuming, require large sample volumes, and many filters that are expensive (Soldin, 1999).
Based on literature data and numerous studies(Trnavska *et al*, 1991 Tsiropoulos *et al,* 2000; Patsalos *et al*, 2010; Krasowski, 2010) justified the use of saliva as an alternative medium for TDM of Lamotrigine - LTG and Levetiracetam - LEV, and due to the several advantages of saliva as a biological matrix compared to plasma (the collection of saliva is simple and non-invasive, avoiding discomfort or stress in patients, painless, cheaper than venepuncture, many complication are avoided, suitable for children , opportunity to perform at home without need for qualified personnel), as well the fact that there are no published data in

determining the AELs concentration of new generation (LTG and LEV that are usually taken in combination with Carbamazepine - CBZ and Valproic acid - VPA) in saliva, for the population in North Macedonia , contributed to examine the possibility of measuring and monitoring the concentration of these antiepileptics in saliva.

In recent years, the development and optimisation of bioanalytical methods have become a challenge, due to the strict regulatory requirements for sensitivity, accuracy, and reliability of sample analysis, because the results obtained with these methods are significant in assessing safety, efficacy, and therapeutic outcome of a drug. For these reasons, it is necessary to use sensitive and selective analytical methods that will allow accurate determination of the drug and its metabolites in low concentrations (Singh *et al*, 2008; Sharma *et al*, 2011*).* Throughout this process: development, optimisation, and validation of the bioanalytical method, application of the Analytical Quality by Design (AQbD) approach is a very useful tool. The steps of the AQbD approach involves: defining the analytical target profile, critical methods parameters (CMPs), risk assessment, method operable design region (MODR), method control strategy, life cycle management of the method (Nethercote *et al*, 2015). During the last years, several studies demonstrated that AQbD helps in development of robust and cost-efficient LC methods for determination different analytes in bulk drugs, pharmaceutical formulations and bioanalytical samples (Beg *et al*, 2011; Garg *et al,* 2013; Murthy *et al,* 2012; Schmidt *et al,* 2013; Skrdla *et al,* 2009; Kurmi *et al,* 2014)

The aim of this paper is to present the developed of a rapid, sensitive UV-HPLC method using analytical quality by design (AQbD), for simultaneously determination of antiepileptic drugs in human saliva.

2. Materials and methods

2.1. Standards and reagents

Lamotrigine - LTG; Levetiracetam - LEV; Carbamazepine - CBZ; Carbamazepine 10,11 epoxide CBZ-EP; Valproic acid - VPA and Nitrazepam (internal standard, IS), were purchased from Sigma Aldrich, USA. Methanol and acetonitrile, HPLC grade, analytical grade potassium dihydrogen phosphate and phosphoric acid for buffer preparation were obtained from Merck, Germany. For all analyses HPLC grade water purified was used. OASIS® HLB cartridges (30mg/1mL) used for the solid-phase extraction (SPE) procedure were supplied by Waters (Milford, USA).

2.2 Apparatus and chromatographic conditions

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The assay was carried out on Shimadzu LC-30 HPLC system Nexera, equipped with degasser (DGU-20A_5), solvent delivery pump (LC-30AD), autosampler (SIL-30AC), column oven (CTO-30A) and UV-VIS detector (SPD-20A UFLC). The chromatographic separation was performed on Zorbax Eclipse XDB C-18 (150 x 4.6 mm,5 μm) column using gradient elution system with acetonitrile (ACN) and phosphate buffer (pH 3.0) in mobile phase, at a flow rate of 1 mL min^{-1}. The temperature was 25 ºC, volume of injection 50 μL and UV detection was set at 210 nm.

2.3. Biological matrix

Saliva samples from both, healthy volunteers (drug-free saliva for the method validation) and patients with epilepsy (under the polytherapy with LTG, LEV, CBZ and VPA), were obtained from Clinic of Neurology, Faculty of Medicine, University "Ss Cyril and Methodius", Skopje, Macedonia. This research was approved by the Ethics Committee of the Faculty of Pharmacy and the Faculty of Medicine, Ss. Cyril and Methodius

University, Skopje, North Macedonia. All volunteers signed the Study informed consent form in accordance to Helsinki II declaration. Saliva samples were collected in the morning, just before the morning dose of

drug. Patiens were instructed to rinse the mouth with water before collecting saliva in order to remove any remnants of food or medication. The same procedure was applied during collecting saliva from healthy volunteers. About 5ml of unstimulated saliva is collected in appropriate tubes and then the saliva samples were frozen and storage in -20^0 C until analysis.

2.4. Preparation of Standard Stock Solutions

Stock standard solution of LTG (500 µg/ mL), LEV (500 µg/mL), CBZ (500 µg/mL), VPA (500 µg/mL), CBZ-EP (500 µg/mL) and IS (500 µg/mL) were individually prepared by dissolving appropriate amounts of each compound in methanol. Working solutions were prepared daily from stock solutions by dilution with purified water to obtain the concentration as per the need. Stock solutions were stored at 2–8 °C and were stable for 3 month.

2.5. Preparation of Bioanalytical Samples and Extraction Procedure

Seven calibration standards (CS) and four quality control samples (QC) containing the IS at constant concentration (50 µg/mL) were prepared by spiking working solutions with aliquots of of 200 µL blank human saliva (drug-free human saliva). The resulting saliva concentrations range were: 1,0 - 100 µg/mL(LTG); 10,0 -300 µg/mL (LEV); 1,0 - 100 µg/mL;(CBZ); 0,5 -150,00 µg/mL(CBZ-EP); 10,0 - 500 µg/mL(VPA).

Preparation of samples was based on the solid-phase extraction (SPE) using Oasis® HLB cartridges (30 mg/1ml). Frozen saliva samples collected from healthy volunteers and epileptic patients were allowed to thaw at room temperature before being centrifuged at 3000 rpm for 15 min. This procedure is done in order to overcome the difficulties resulting from the high viscosity of freshly obtained saliva (highly mucous, stringy and sticky consistence). The 200 µL saliva (supernatant) of epileptic patients was transferred into the Eppendorf tube and spiked with 50µl IS (100 µg/ mL) and 250µl of 4% H3PO4, while the supernatant from blank saliva was previously spiked with each working solutions of LTG, LEV, CBZ, CBZ-EP , VPA and IS. The mixture was vortex-mixed for 30 sec and loaded into Oasis® HLB cartridges, previously conditioned according to the following steps : conditioning with 1 mL methanol, equilibration with 1mL water; loading a sample of saliva ; washing with 1 mL water and 1 mL 5% methanol ; drying 30 min. under vacuum (-20kPa) and elution with 500 µl 90% methanol . The eluent was transferred to microvials and inject into the HPLC.

2.6. Development and optimisation of HPLC method applying Analytical Quality by Design(AQbD)

Initially, during the development of the bioanalytical method of AEDs, were defined the summary of the quality characteristics of the analytes and the analytical method. During the Risk assessment was identifying the method variables that primarily influence on analytical attributes. In a risk assessment plan was constructed the Ishikawa diagram that helps in establishing the cause-and-effect relationship among the method variables and analytical attributes. During the optimization of chromatographic conditions was investigated influence of four experimental factors (pH of buffer solution, initial and final content of acetonitrile in the mobile phase during gradient elution) in three factor levels, using *Fractional factorial* 3^4 experimental design shown in Table 1.

MODDE 10.1 Software (Umetrics, Umea, Sweden) was used for developing the chemometrics model.

Table 1. Investigated chromatographic factors and their levels

Experimental factors	Factors level		
	1	2	3
pH of buffering solvent	3	4	6.5
Initial content of ACN in mobile phase (V/V,%)	10	15	20
Final content of ACN in mobile phase (V/V,%)	40	60	70
Gradient time	15	20	30

2.7. Method validation

The proposed method was validated according to EMA bioanalytical method validation guidelines regarding selectivity, precision, accuracy, recovery and stability (EMA 2011)

Selectivity was evaluated by comparing the chromatograms of blank human saliva samples (drug-free saliva) obtained from 7 different sources and those obtained from saliva samples spiked with analytes (LTG, LEV, CBZ,CBZ-EP, VPA) and IS.

The linearity of the method was evaluated within the defined saliva concentration ranges, with seven calibration standards. Calibration curves were constructed by means of the least-squares method, obtained by plotting the analyte - IS peak area ratios versus the respective analyte concentrations.

Accuracy and precision were evaluated for the QC samples within single (within-run) and different runs (between -run). Within-run accuracy and precision were determined for five samples per concentration level of QC in a single run (n=5). Between-run accuracy and precision were assessed by five determinations per concentration per run at four concentrations of QC samples from three runs analyzed on two different days (n=15).

Extraction yield (recovery***)*** was assessed at four concentration levels (lower limit, low, medium and high QC samples). The absolute recovery values of the analytes were calculated by comparing the peak area ratio of blank plasma spiked with the analyte and processed by the clean-up procedure *versus* the peak areas obtained from the blank plasma that was first processed bythe clean-up procedure and then spiked with analytes.

Stability studies were performed on three replicates of low and high QC samples after 24 h at room temperature (short term stability), after three freeze-thaw cycles, autosampler stability for 12 h and after 90 days at -20 ^{0}C (long term stability) .

3. Results and discussion

3.1 Development and optimisation of chromatographic conditions by applying AQbD

For developing a robust bioanalytical method we approached the application of AQbD , in order to demonstrate and guarantee that the method can be used in routine analysis and give reliable and quality results (Khurana *et al*, 2015).

During development of the bioanalytical method the first step was the selection of target analytical profile - ATP that includes: choice/selection of analytes, selection of analytical techniques and the selection of the method requirements (number of analytes that will be determined, mobile phase, method of elution, concentration, and method for sample preparation). LTG, LEV, CBZ, CBZ-EP and VPA were selected as analytes, while UV-HPLC was selected as a technique, in our case. For the optimisation of the HPLC method, were taken in consideration the chromatographic conditions (pH, composition of the mobile phase)

which value influence in chromatographic separation,that is related with the physico-chemicals characteristic of the analytes.

In preliminary experiments were tested several composition of the mobile phase (different types of organic solvents and different ratio of organic and aqueous phase) in order to achieve the best, satisfactory separation of analytes within shortest run time, as an important fact for routine control. Initially, mixture of acetonitrile-phosphate buffer in the ratio 20:80 (*V/V*) delivered isocratically at a flow rate of 1mL/min was used as a mobile phase. Under these conditions, is not achieved a good separation of all analytes in a short time with a satisfactory retention time (R_t), especially for CBZ, VPA and IS(R_t of LEV - 3,117; R_t of LTG - 3,687; R_t of CBZ - 31,908; R_t of IS - 44,375 and R_t of VPA - 81,540).

As a useful tool in identifying critical variables/parameters of the method that affecting in the ATP (to identify risk assessment and risk managment) was constructed an Ishikawa diagram (fish bone diagram). The fishbone diagram is established by considering the various variables/parameters of the method that convincingly affect in the attributes - in the performance of the HPLC method for simultaneous determination of the selected antiepileptic drugs, within shortest time and the best separation . From the preliminary performed research and experiments during the development of the method,were determined which parameters will be optimise and which are the critical factors(variable), as shown in "Figure 1". The selected critical variables/factors were subjected to further processing by applied appropriate experimental design.

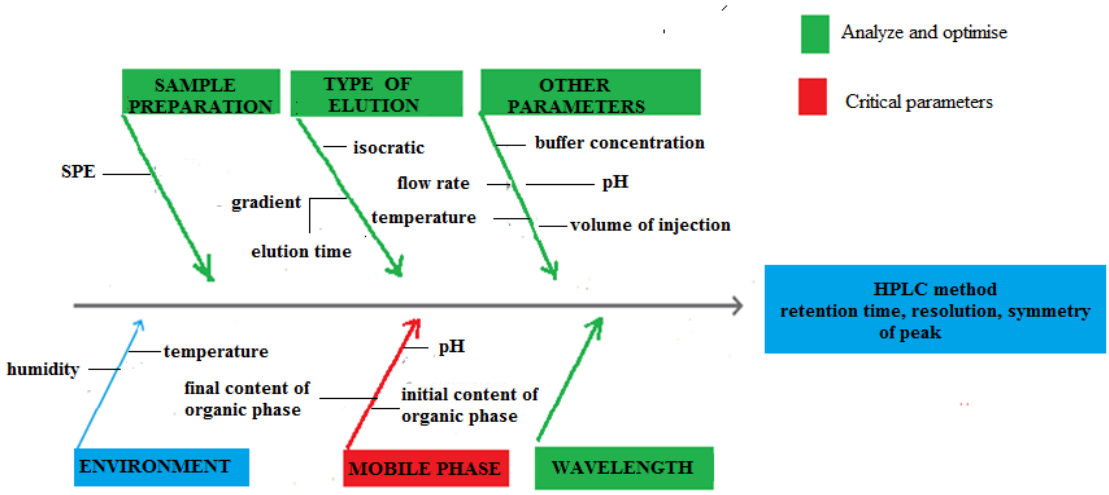


Figure 1. Ishikawa diagram (fish bone diagram)

As a critical factors of the optimisation of the method were: pH of mobile phase, content of acetonitrile-ACN in mobile phase (because the preliminary investigations demonstrated that increasing the initial and final content of ACN significantly shortens the analyze time) and gradient time .

During the selecting of the wavelength, were evaluated the full scan spectra of the analytes and was showed that LEV and VPA are weak UV absorber due to the lacks of chromophores. The maximum absorption of LEV and VPA was obtained at 205 nm and 213 nm, respectively. After analyzing the UV spectra of antiepileptic drugs, 210 nm was chosen as the optimum wavelength for the simultaneous determination of the selected antiepileptic drugs. In the optimisation strategy, pH value of the mobile phase is one of the first parameter that should be studied. Because the differences of the *pKa* value (LEV - 4.95; LTG - 5.7; CBZ - 13.9; VPA - 4.9).and structure of the investigated analytes, pH value of the buffer solution in the mobile phase, in range of 3.0 - 6.5 was chosen as experimental range during chemometric optimisation. The application of experimental design allowed estimation of the influence of the investigated experimental factors (pH of mobile phase, initial and final content of ACN in mobile phase, gradient time)

on the retention time (R_t). The 39 planned experiments were performed that shortens the optimization time of the method.

The influence of experimental factors on the chromatographic separation and R_t on the each analytes was shown in "Figure 2" as Coefficients overview plot .

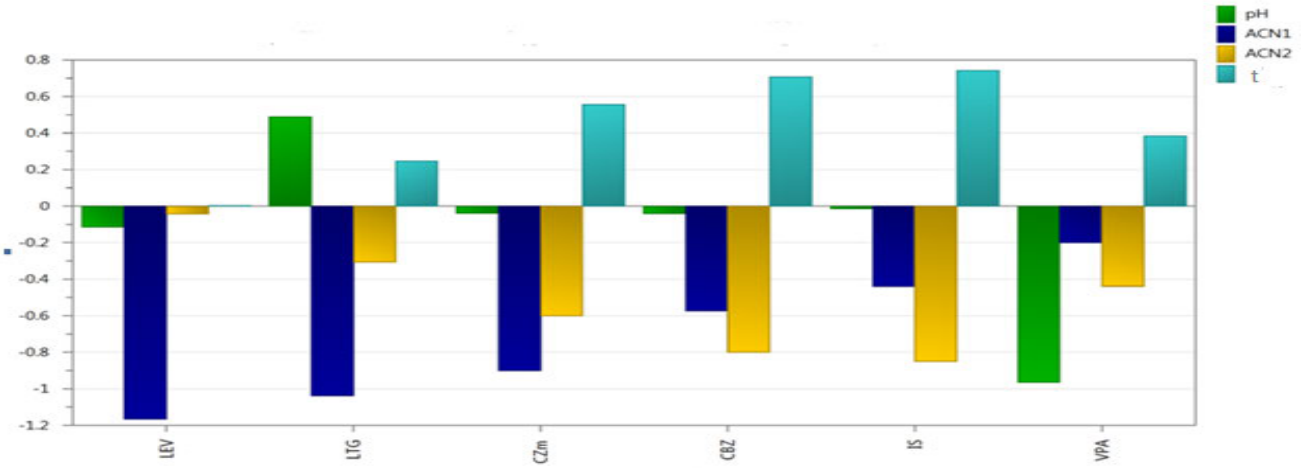
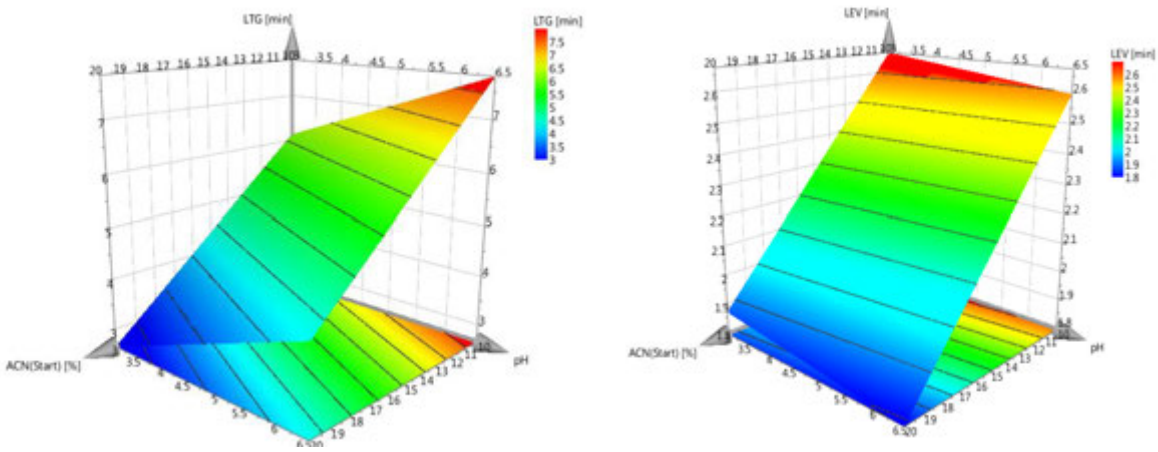


Figure 2. Coefficient overview plot (ACN1: initial content of ACN in mobile phase; ACN2: final content of ACN in mobile phase; pH value of buffer solution in mobile phase; T: gradient time

It was observed that initial content of ACN in the mobile phase during gradient elution and pH value of buffer solution influenced more on retention time of LEV, while final content of ACN in the mobile phase has more influence on retention time of VPA. These two analytes were two main substances that the values of different experimental factors influenced more on their retention time.

The influence of initial content of ACN in mobile phase on the retention time of each analytes was shown in "Figure 3".



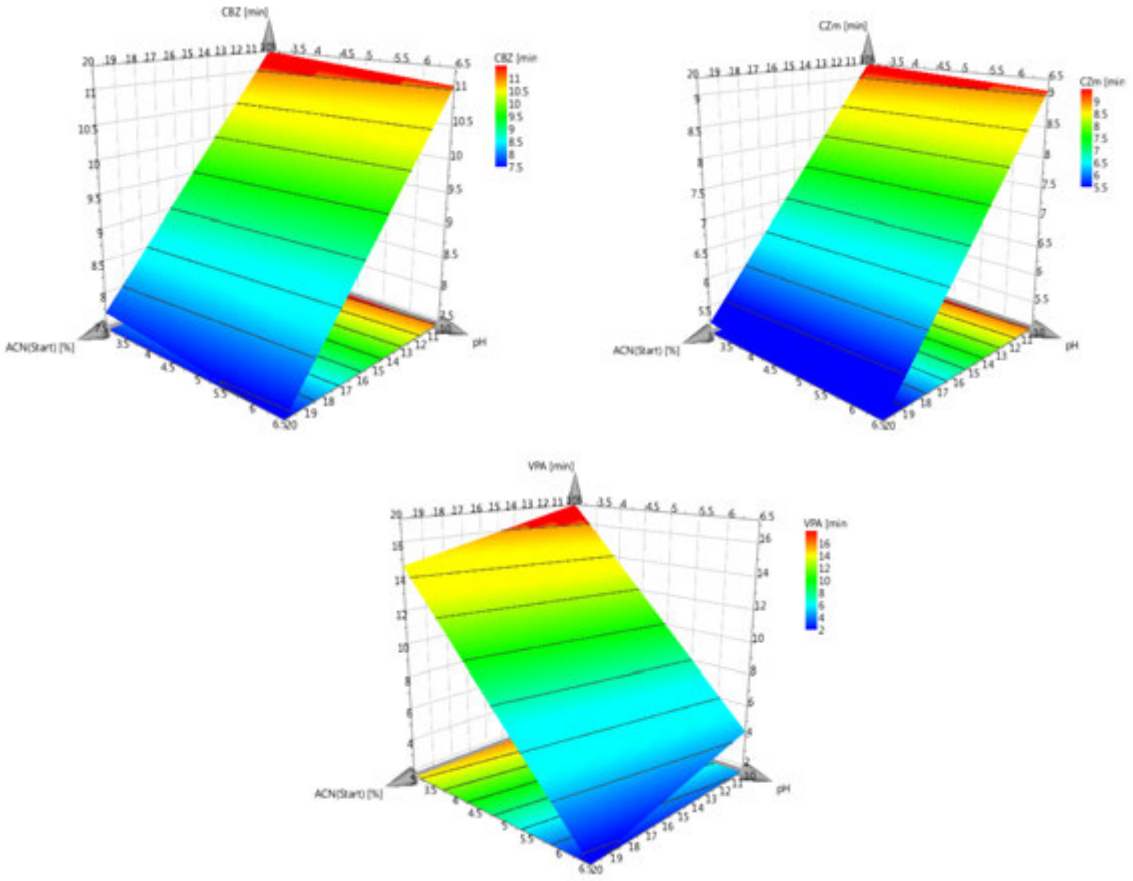


Figure 3. Response area of chromatographic system expressed by changing retention time of each analytes

Analysis of the diagrams of experimental design confirmed that the 10% of initial content and 70% of final content of acetonitrile in the mobile phase, respectively, the pH value 3.0 of the buffer solution in the mobile phase and gradient time from 15 min was justified.

Obtained results provided by the Fractional factorial 3^4 design according a statistical analysis of the degree of overlap are linearly, with exception of the LEV and VPA where is the larger deviation , but R^2 is over the 0.90 that confirms that experimental design is good, as shown in "Figure 4".

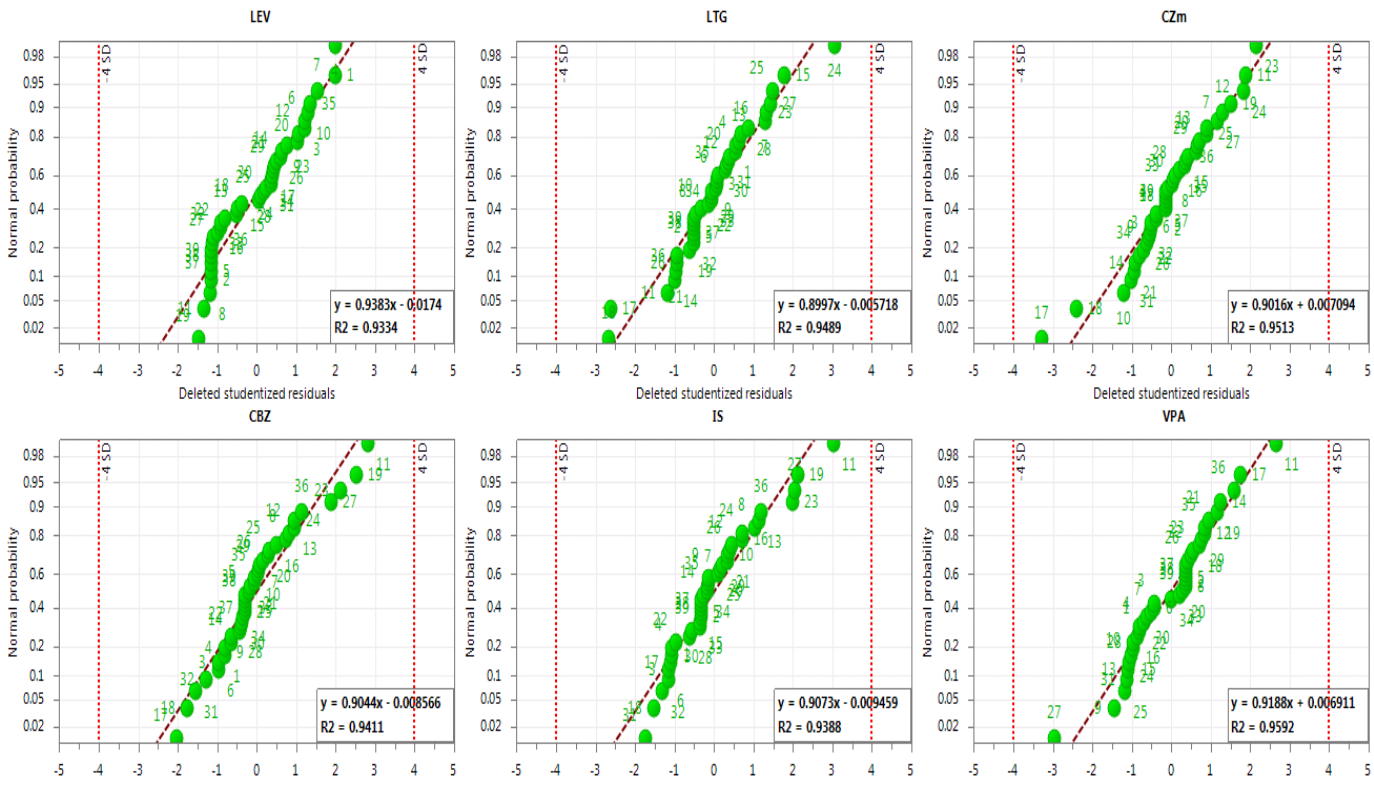


Figure 4. Statistical analysis the degree of overlap of results obtained by Fractional factorial 3^4 design

3.2. Optimisation of SPE procedure - sample preparation procedure

The sample preparation is an important part of bioanalytical methods. We used solid-phase extraction (SPE) as procedure for sample preparation because it is easy and simple to perform and usually gives good extraction yield with good selectivity (Subrammanian *et al,*2008) .OASIS ® HLB column (30mg/1ml) were used to perform SPE.

During the optimisation of the SPE procedure different types of washing the column and elution were evaluated. The best washing of column was achieved with water and 5% methanol in water, which allows complete removal of salts and plasma proteins that, can interfere with the analytes. During selected the solvent for elution, it was taken in the consider the fact that all analytes are soluble in methanol. Therefore, different percentage values of methanol in water (70% and 90% methanol in water) and pure methanol were evaluated. Also different volumes of saliva were evaluated. The best results for recovery for all analytes, up of 85%, were obtained using SPE procedure that is described in the sample preparation section.

3.3. Method validation

Under the proposed chromatographic conditions, no interfering peaks were observed in the retention times of analytes or IS. That shows the selectivity of the method in "Figure 5".

a)

b)

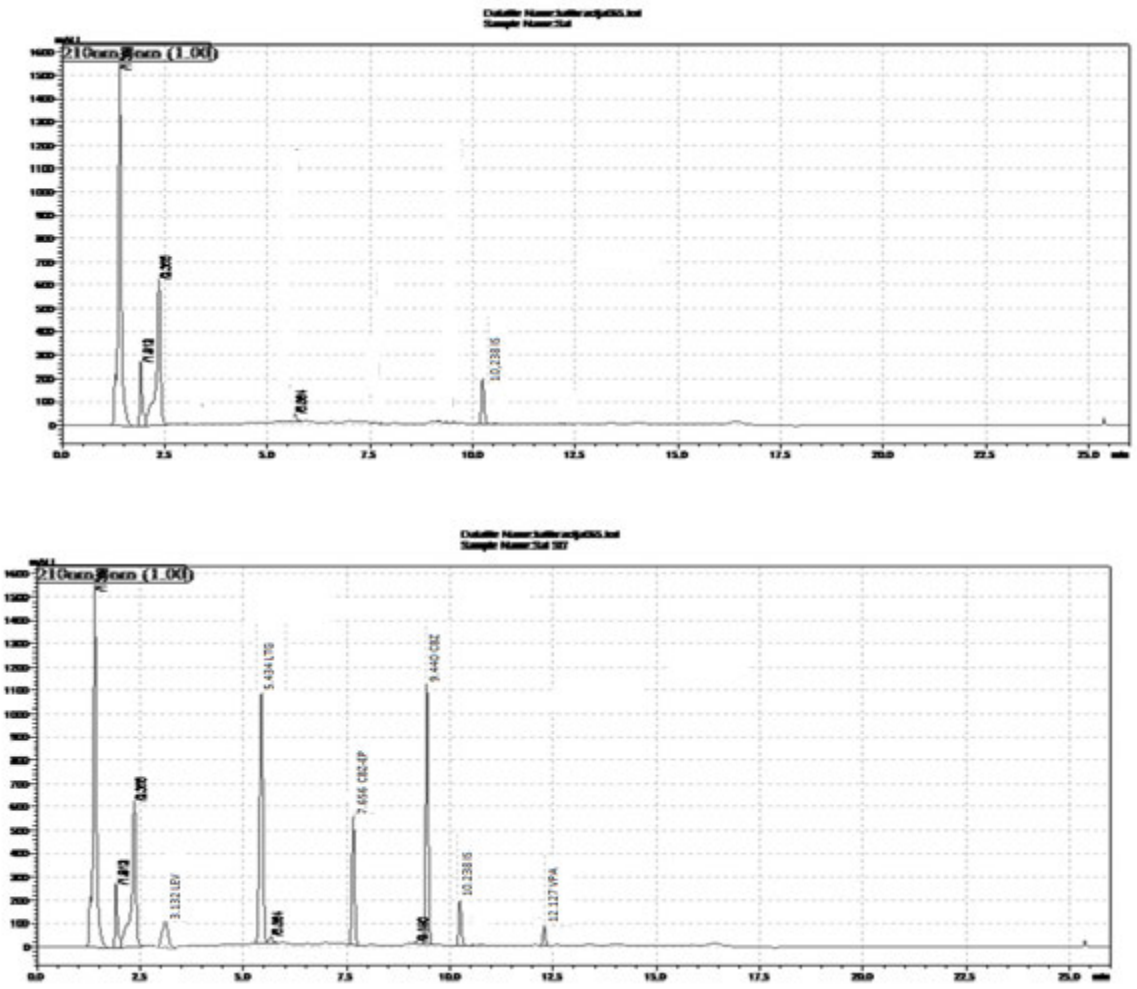


Figure 5. Chromatograms obtained from: a) blank saliva; b) blank saliva spiked with 20,0 µg/mL LEV; 2,5µg/mL LTG; 2,65 µg/mL CBZ; 1,25µg/mL CBZ-EP; 20,0µg/mL VPA and 1,0µg/mL IS

Calibration curves were set up by plotting the analytes - IS peaks area ratio versus the respective analytes concentrations. The regression equations and the corresponding regression coefficients r^2 for each analytes are summarized in Table 2.

Table 2. Calibration parameters (n=7) of LTG, LEV, CBZ, CBZ-EP and VPA in human saliva

Analyte	Calibration parameters		
	Concentration range (µg/mL)	Equation	r^2
LEV	1,0-30,0	y = 0,476x - 0,427	0,997
LTG	0,1-10,0	y = 0,054+0,136	0,995
CBZ	0.1-10,0	y = 0,055x +0,028	0,998
CBZ-EP	0,05-5,0	y = 0,054x - 0,014	0,997
VPA	1,0-50,0	y=0,026x+0,028	0,998

The results for accuracy and precision were within recommended limits that are reported in Table 3.

Table 3. Precision and accuracy of the method for simultaneously quantification of analytes in human saliva

Analyte	Nominal conc. (µg/mL)	Within -run assay(n=5)			Between-run assay (n=30)		
		Measured concentration (µg/mL)	Accuracy (%)	Precision (CV,%)	Measured concentration (µg/ml)	Accuracy (%)	Precision (CV,%)
LEV	1,0	1,03	103,25	4,21	1,04	104,61	4,85
	5,0	4,98	99,42	0,36	4,88	98,89	0,76
	20,0	19,91	99.58	0,36	19,69	98,49	6,22
	25,0	25,83	103,71	1,86	25,83	103,33	1,86
LTG	0,1	0,09	97,22	1,37	0,09	97,22	1,37
	0,5	0,49	99,13	1,74	0.49	98,06	9,43
	2,5	2,52	100,76	0,14	2,54	101,72	1,55
	5,0	4,96	99,27	1,11	4,95	98,94	2,10
CBZ	0,1	0,54	102,11	0,102	0,09	98,68	0,97
	0,5	0,47	93,86	2,14	0,49	99,78	1,83
	2,5	2,55	101,99	1,34	2,54	101,99	1,34
	5,0	4,97	99,45	1,58	5,14	102,82	1,55
CBZ-EP	0,05	0,048	97,24	3,93	0,047	97,23	3,93
	0,25	0,24	99,07	0,49	0,247	99,07	0,49
	1,25	1,23	98,19	3.01	1,23	98,52	2,75
	2,5	2,58	103,25	1,09	2,57	101,15	0,81
VPA	1,0	0,98	98,46	0,74	0,98	98,46	1,74
	5,0	4,55	98,96	3,80	4,98	99,72	0,68
	10,0	10,73	107,34	0,95	10,73	107,34	0,95
	20,0	19,94	99,75	0,87	19,94	99,74	0,87

The recovery values of the extraction procedure at four concentration levels were in range: 86,5% - 90,5% for LEV; 98,7% - 99,3% for LTG; 97,8% - 98,5% for CBZ; 97,6% - 99,3% for CBZ-EP and 98,2% - 98,9% for VPA. These shows that SPE with OASIS® HLB gives the good results and cleanliness of the sample. Stability studies indicate that stock solutions and saliva samples were stabile under different storage conditions as shown in Table 4.

Table 4. Stability of LEV, LTG, CBZ, CBZ-EP and VPA under different conditions (n=3)

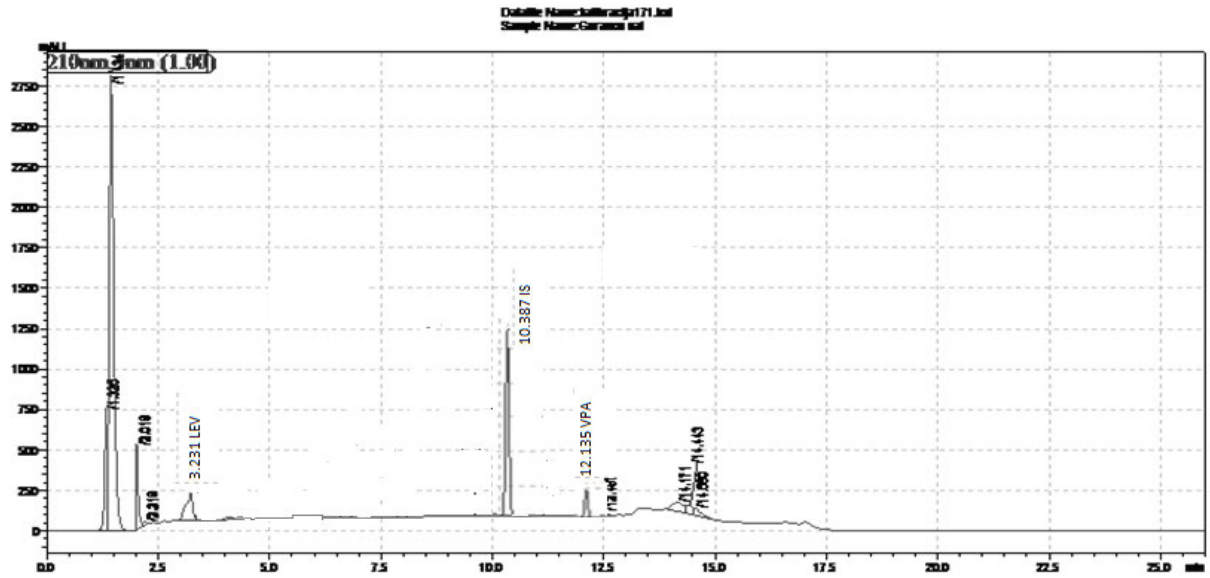
Analyte	Nominal conc. (μg/mL) LLOQ/HQC	Initial conc. t=0 (μg/mL)	Accuracy(%)	Precision (CV,%)
	Short term stability (24h at room temp.)			
LEV	1,0 / 25,0	1,02 / 25,05	98,6 / 97,8	2,21 / 1,84
LTG	0,1 / 5,0	0,11 / 5,01	103,90 / 100,60	2,96 / 1,11
CBZ	0,1 / 5,0	0,12 / 4,98	98,9 / 97,9	1,97 / 2,53
CBZ-EP	0,05 / 2,5	0,049 / 2,53	97,54 / 100,11	2,93 / 1,55
VPA	1,0 / 20,0	1,03 / 20,02	102,51 / 100,56	3,65 / 1,38
	Autosampler stability(after 12h)			
LEV	1,0 / 25,0	1,01 / 25,2	97,5 / 100,12	2,45 / 1,89
LTG	0,1 / 5,0	0,14 / 5.03	99,3 / 101,32	1,56 / 2,54
CBZ	0,1 / 5,0	0,11 / 5,04	95,8 / 102,35	4,3 / 2,4
CBZ-EP	0,05 / 2,5	0,048 / 2,52	97,4 / 97,61	2,3 / 3,6
VPA	1,0 / 20,0	1,04 / 20,4	94,15 / 98,75	3,37 / 1,82
	Three freeze-thaw cycles			
LEV	1,0 / 25,0	0,089 / 24,95	99,25 / 102,34	1,45 / 1,68
LTG	0,1 / 5,0	0, 098 / 4,98	98,58 / 99,23	1,89 / 1,56
CBZ	0,1 / 5,0	0,09/ 5,0	98,6 / 98,82	2,63 / 1,78
CBZ-EP	0,05 / 2,5	0,049 / 2,48	103,8 / 96,53	1,65 / 3,42
VPA	1,0 / 20,0	1,04 / 20,2	93,17 / 98,65	4,16 / 2,34
	Long term stability (30 days at -20ºC)			
LEV	1,0 / 25,0	0,091/ 24,96	97,03 / 100,45	3.05 / 1,28
LTG	0,1 / 5,0	0,11 / 5,0	97,82 / 98,24	2,54 / 1,56
CBZ	0,1 / 5,0	0,12 / 4,99	98,71 / 99,24	2,45 / 1,36
CBZ-EP	0,05 / 2,5	0,048 / 2,47	102,21 / 103,82	1,65 / 1,98
VPA	1,0 / 20,0	1,04 / 19,99	103,91 / 100,50	3,95 / 1,12

3.4. Analysis of patient saliva

The proposed method was applied to the simultaneous determination of LTG, LEV, VPA, CBZ and its active metabolite CBZ-EP, in saliva samples collected from epileptic patients (50 patients are enrolled in the study) that are under the polytherapy with AEDs of old and new generation. In the " Figure 6." were presented chromatograms corresponding to a saliva samples from two patients, one who received LEV(2500mg/day) and VPA (2000mg/day) and the other received Levetiracetam (3000mg per day), Lamotrigine (25mg per day), Oxcarbazepine (2400mg per day) and Phenobarbital (10mg per day) By interpolation on the respective calibration curve , the following concentrations were obtained: for the first

patient (a) 3,73 µg/mL for LEV and 3,18 µg/mL for VPA, and second patient (b) 0,52 µg/mL for LTG and 10,58 µg/mL for LEV.

a)



b)

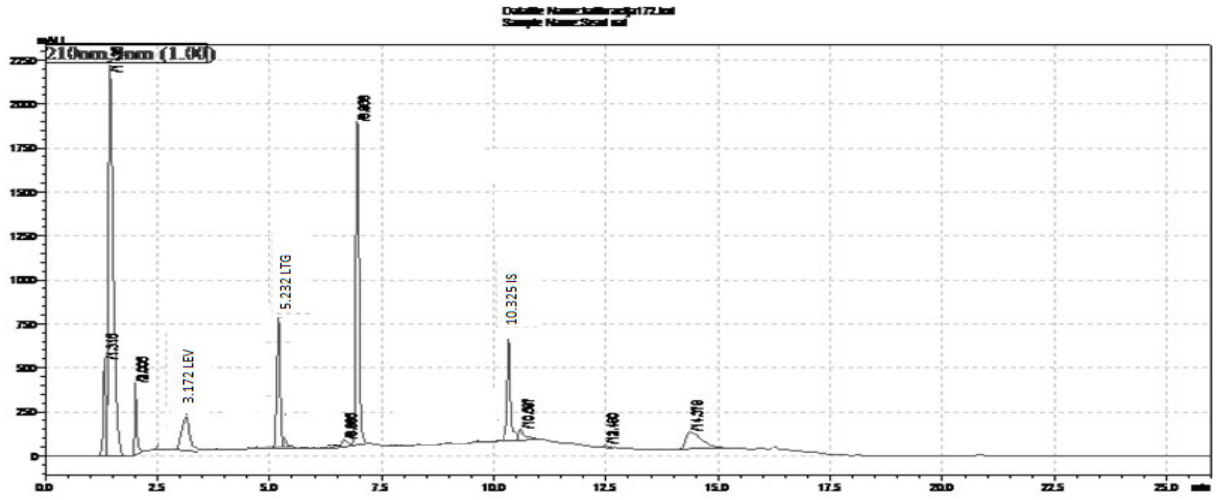


Figure 6. Representative chromatogram of saliva samples from patient treated with: a) 2500mg/day of LEV and 2000mg/day of VPA ; b) 3000mg/day of LEV, 25mg/day of LTG, 2400mg/day Oxcarbazepin and 10mg/day of phenobarbital.

Because the epileptic patients are often treated with polytherapy, it was important to evaluate the probable chromatographic interferences from potentially co-administered antiepileptic drugs. It was evident that no chromatographic interference was observed from co-administered drugs in the retention times of LEV, LTG, VPA and IS . The method gives promising results with saliva samples and was shown to be useful for easy TDM of these AEDs in epileptic patients.

4. Conclusion

A simple, rapid, sensitive and accurate HPLC method has been successfully developed employing AQbD approach, for simultaneous determination of LTG, LEV, CBZ, CBZ-EP and VPA in human saliva. From the results obtained of this study can concluded that during the development and optimisation of bioanalytical method, applying the AQbD provide greater reliability and quality of the bioanalytical data. The proposed method was successfully applied to saliva samples obtained from epileptic patients that are under the therapy with these AEDs.

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