Optimisation *via* experimental design of LC method for simultaneously determination of four antiepileptic drugs and active metabolite in human plasma

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Absract: This paper presents the chemometrically assisted optimisation and validation of the UV-HPLC method for the simultaneous determination of four antiepileptic drugs (carbamazepine, lamotrigine, valproic acid and levetiracetam) and carbamazepine 10,11 epoxide (active metabolite of carbamazepine) in human plasma. To reach desired chromatographic separation of analytes,   *Fractional factorial 3^4* design was used for simultaneously optimisation of some chromatographic parameters: acetonitrile content in the mobile phase, pH of the buffer solution in the mobile phase and time of gradient elution. Separations of analytes were performed on a Zorbax Eclipse XDB C-18(150x4.6 mm; 5μm) column, with a gradient elution system comprising acetonitrile (ACN) - phosphate buffer (pH 3.0). Two procedures were tested for the pretreatment of human samples: protein precipitation and solid phase extraction. The last preparatory method provides the best results of extraction recoveries and allowed for the determination of all anaytes. The developed method was linear over the concentration ranges studied with r ≥0.995 for all compounds. Accuracy (%) and precision (CV,%) values for within and between day were <15% at all concentrations tested. This method was successfully applied to the plasma samples from epileptic patients and it seems to be suitable tool for routine therapeutic drug monitoring of AEDs.

Keywords: antiepileptic drugs, fractional factorial design, plasma, therapeutic drug monitoring, validation

I.   INTRODUCTION

Epileptic seizures are one of the most prevalent neurological disorders, affecting approximately 1% of the population in developed countries [1,2]. Among several therapeutic approaches, pharmacotherapy based on a wide variety of antiepileptic drugs (AEDs) is the first-line treatment option to achieve seizure control [3]. Since the introduction of potassium bromide and paraldehyde (1850-1880), several generations of antiepileptic agents have been introduced into the clinical usage, greatly improving the life quality of many people suffering from seizures [4]. Carbamazepine, ethosuximide, phenobarbital, phenytoin and valproate are the most frequently used conventional antiepileptics. The therapeutic failure in 20-25% of patients has stimulated intensive research on novel antiepileptic drugs and over the past 20 years, no fewer than 14 AEDs have been licensed for use as "add-on" [5,6]. These are: felbamate, gabapentin, lamotrigine, levetiracetam, oxcarbazepine, tiagabine, topiramate, vigabatrin, pregabalin, rufinamide, stiripentol, zonisamide [7]. The most prescribed one from the newer AEDs are lamotrigine, levetiracetam and topiramate [8].

Monotherapy is the choice regimen to treat newly diagnosed epilepsies. If an epileptic condition is refractory to an initial monotherapy regimen, patients should be switched to monotherapy with another AED or to polytherapy [9]. This often leads to complex and unpredictable pharmacokinetic and pharmacodynamic interactions [10,11], with possible clinical consequences in terms of adverse side effects. For these reason careful monitoring of serum/plasma levels (TDM - therapeutic drug monitoring) of AEDs plays important role in optimising epilepsy treatment [12, 13,14] . Appropriate and rational utilisation of TDM may improve drug therapy by maximizing seizure control and minimising the risk of adverse drug reactions and therefore, may also have a cost-saving effect [15,16,17]. TDM is usually performed on serum or plasma, and these matrixes can be used interchangeably because no differences in AEDs concentration have been demonstrated between them [18]. In general, in clinical practice determination of plasma concentration is carried out for more AEDs by commercial immuno-chemical kits due to their easy of use and the results are received quickly. Chromatographic techniques, such as gas chromatography and high-performance liquid chromatography (HPLC) are more versatile, sensitive and specific, and this makes them a very interesting alternative to immunoassays. Also the advantages of HPLC methods is that can allow for the simultaneous separation and

quantitation of many compounds in a single run which is important when is need the TDM of AEDs in polytherapy [19].

During the last years, several high-performance liquid chromatographic methods coupled to ultraviolet detection (HPLC-UV) or mass spectrometry detection (HPLC-MS and HPLC-MS/MS) for the simultaneous determination of AEDs and some of their metabolites have been reported in literature [20,21,22,23,24,25].

The present work aimed at developing and optimization of a rapid and sensitive bioanalytical method for simultaneous separation and quantification the two novel (lamotrigine - LTG and levetiracetam - LEV), two traditional AEDs (valproic acid - VPA and carbamazepine - CBZ and its pharmacologically active metabolite, carbamazepine 10,11 epoxide - CBZ-EP) in human plasma, using the chemometric approach. The main advantage of such approach is simultaneous optimization of influencing factors and response variables which enables prediction of chromatographic retention time and optimum conditions for separation. Finally, the validation of the proposed analytical method was conducted in accordance to the recommendations of the guideline *Bioanalytical method validation* [26].

II. EXPERIMENTAL

2.1. Chemicals and reagents

CBZ, CBZ-EP, VPA, LTG, LEV and nitrazepam (internal standard,IS), were purchased from Sigma Aldrich, USA. Methanol and acetonitrile, HPLC grade, were purchased from Merck, Germany. Potassium dihydrogen phosphate and phosphoric acid for buffer preparation were analytical grade and were also obtained from Merck, Germany. For all analyses HPLC grade water purified with a TKA_LAB Reinstwasser system (Niederelbert, Germany) was used. OASIS® HLB cartridges (30mg/1mL) used for the solid-phase extraction (SPE) procedure were supplied by Waters (Milford, MA, USA).

2.2 Human plasma sampling

Plasma was collected from healthy volunteers (drug-free plasma for the method validation) and from the epileptic patients undergoing chronic AEDs therapy. Blood samples were collected in the morning, just before the first daily drug administration, into heparinised tubes and centifuged at 3000 rpm for 15 min. The supernatant plasma was transferred into test tubes and frozen at -20 ^{0}C until analysis. Prior to analysis, plasma samples were thawed and allowed to equilibrate at room temperature.

The participation of each subject was voluntary and could be cancelled by any individual at any time during this study (according to the Helsinki II declaration). The Ethics Committee at the Faculty of Pharmacy and the Faculty of Medicine, Ss. Cyril and Methodius University – Skopje, approved the research protocol for this study and all volunteers signed the Study Informed Consent form.

2.3 Instrumentation and chromatographic conditions

The assay was performed on Shimadzu LC-30 Nexera. The chromatographic separation was performed using Zorbax Eclipse XDB C-18 (150 x 4.6 mm; 5 µm) column, with a gradient elution system comprising acetonitrile (ACN) - phosphate buffer (pH 3.0). The analyses were run at a flow rate of 1 mL/min, volume of injection 50 µL and temperature 25 ºC. Detection was made at 210 nm.

2.3.1 Optimisation of HPLC method using experimental design

Fractional factorial 3^4 design was used for the optimisation of chromatographic conditions. The influence of four experimental factors (pH of buffer solution in the mobile phase, initial and final content of acetonitrile in mobile phase during gradient elution and the gradient time) was investigated at three factor levels (Table 1). All remaining factors such as the flow rate of mobile phase (1mL/min), wavelength of UV detection (210 nm), temperature of the column (25 ^{0}C) and volume of injection (50 µL) were kept at constant level. Thirty nine planned experiments were performed. MODDE 10.1 Software (Umetrics, Umea, Sweden) was used for developing of chemometrics model.

Table 1.

2.4. Preparation of standards and quality control samples

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.

Seven calibration standards (CS) containing the IS at constant concentration (10 µg/mL) were prepared by spiking working solutions with aliquots of blank human plasma (drug-free human plasma). The resulting

plasma concentrations range were: 1.0 - 25.0 μg/mL (LTG); 3.0 -70.0 μg/mL (LEV); 1.0 - 25.0 μg/mL; (CBZ); 0.5 -15.0 μg/mL (CBZ-EP); 25.0 - 250 μg/mL (VPA).

Quality control (QC) samples were prepared at four concentration level: lower limit of quantitation (LLOQ) (1.0 μg/mL LTG; 3.0 μg/mL LEV; 1.0 μg/mL CBZ;   0.5 μg/mL CBZ - EP; 25.0μg/mL VPA); low QC sample (5.0 μg/mL LTG; 7.5 μg/mL LEV; 5.0 μg/mL CBZ; 2.5 μg/mL CBZ - EP; 100 μg/mL VPA); medium QC (10.0 μg/ mL LTG; 30.0 μg/mL LEV; 10.0 μg/mL CBZ ; 5.0 μg/mL CBZ-EP; 150 μg/mL VPA); and high QC sample (20.0μg/mL   LTG; 60.0 μg/mL LEV; 20.0 μg/mL   CBZ; 10.0 μg/ mL CBZ - EP; 200 μg/ mL VPA) , in same way as described above for the CS, and stored at -20^0 C until analysis.

2.5. Sample preparation
2.5.1 Protein precipitation (PP)
Ratio of spiked plasma and organic solvent (methanol, acetonitrile or trichloracetic acid) was 1 to 4. Solutions were mixed and centrifuged at 13 000 rpm, for 10 min, and after 10 min of standing, the supernatant was injected into the HPLC [27].

2.5.2 Solid - phase extraction (SPE)
The SPE procedure was performed using Waters Oasis® HLB cartridges (30 mg/1mL). This sample pretreatment procedure was carried out according   the following steps: conditioning with 1 mL methanol, equilibration with 1mL water; loading of a mixture of 200 μL plasma +   50 μL IS standard solution + 200 μL 4% H_3PO_4 ; washing with 1 mL water and 1 mL 5% methanol and elution with 250 μL 90% methanol [28] . The eluent was transferred to microvials and inject into the HPLC.

2.6 LC method validation
The LC method was validated according to the guideline *Bioanalytical method validation* [26] regarding selectivity, linearity, precision, accuracy, recovery and stability. Blank human plasma samples from seven different subjects were used in order to assess method selectivity, analysing the eventual interference of matrix endogenous substances at the retention times of LTG, LEV, CBZ, CBZ-EP, VPA and IS.

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

Within-day precision and accuracy were investigated analysing five sets of QC samples in a single day (n= 5), while inter-day precision and accuracy were obtained by repeated analysis 5 times of each QC samples in a single analytical run performed at two different days   (n= 15).

The recovery (extraction yield) of the compounds from human plasma samples submitted to the treatment previously described was investigated at the four concentration levels (lower limit of quantitation, low, medium and high QC samples). It was calculated by comparing the peak areas of analytes obtained from the SPE processed blank plasma sample previously spiked with analytes versus peak areas obtained from an SPE processed blank plasma sample, and then spiked with analytes, representing 100 % recovery [29].

Stability test 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 10 h and after 30 days at -20 ^{0}C (long term stability) .

III.   RESULTS AND DISCUSSION
3.1.Development and optimisation of chromatographic separation
During the method development, several chromatographic conditions were tested in order to achieve the best separation of the analytes within the shortest run time. 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. This enabled separation of all compounds of interest, but for the long retention time (R_t), especially for 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). To achieve   good separation and peak shape with a shorter retention time suitable for all AEDs of interest, a gradient elution approach was applied. Gradient steps were predicted from chemometric assessment of experimental data. The evaluation of the full scan spectra showed that LEV and VPA are weak UV absorber due to the lacks of chromophores. The maximum absorption of LEV and VPA the 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. All of these compounds have a polycyclic structure with different *pKa* value (LEV - 4.95; LTG - 5.7; CBZ - 13.9; VPA - 4.9). However, some of the compounds are basic in nature, like lamotrigine, and some are acidic like VPA, indicating the diverse nature of the selected drugs. In addition, LTG and LEV are new generation antiepileptic drugs which are

structurally different from other conventional AEDs.. Based on the differences of the *pKa* value and structure of the investigated compounds, pH value of the buffer solution in the mobile phase, in range of 3.0 - 6.5 was chosen as experimental range of these parameters during chemometrics optimisation.

The preliminary tests were used to define the experimental range of the investigated chromatographic factors. The experimental design was further applied for optimisation of chromatographic separation as well as for monitoring the impact of each chromatographic parameter on the retention time. The application of experimental design allowed estimation of the influence of the investigated experimental factors on the retention time (R_t). The experiments were conducted according to the plan of experiments and the obtained data are presented in Table 2 .

Table 2.
The influence of experimental factors on the chromatographic separation and R_t on the each analytes expressed as regression coefficients, displayed through Coefficients overview plot (Fig. 1).

Figure 1.
It was observed that initial content of ACN in the mobile phase during gradient elution had the largest influence on retention time of LEV, LTG,CBZ-EP, CBZ and less influence on retention time of VPA and IS. Largest initial content of ACN in the mobile phase during gradient elution decrease the retention time of the all investigated supstances. The final content of ACN in the mobile phase during gradient elution influenced on the separation at the same way as initial content of ACN in the mobile phase i.e inversely. These factor had a significant influence on the retention times of CBZ, CBZ-EP, IS and VPA and less influence on retention time of LTG especially on LEV. The regression coefficients obtained for pH value of buffer solution in the mobile phase, pointed that these factor had a greatest impact on R_t of VPA and LTG and less influence on R_t of LEV and other analytes, inversely at all, except on R_t of LTG. Gradient time had a greatest impact on the retention time of CBZ, CBZ-EP, VPA, LTG while the less impact on retention time of LEV.

Considering that interferences of biological matrix (plasma) are might be expected, the desired value of the retention time for LEV should be greater than 2.5 minutes. Since initial content of ACN in the mobile phase and the pH value of the buffer solution in the mobile phase had the greatest influence on the retention time of LEV, Response Contour Plot was constructed to the overall area of these important factors, while the final content of ACN in the mobile phase and gradient time was set to constant value (70% and 15 min, respectively). These values for the experimental factors were chosen because satisfactory retention time for LEV was achived. The results obtained using Response Surface methodology shows that optimal retention time is achieved if initial content of ACN in the mobile phase is between 10 - 10.5 %, at a pH value of the buffer solution in the mobile phase from 3.0 to 5.5 (Fig. 2).

Figure 2.
Response contour plot was constructed also for VPA at the same conditions as explained above, because VPA is the one of the analytes that determine the duration of run time. The results obtained from this contour plot, confirmed that initial content of ACN inversely less influence on retention time of VPA (Fig. 3), appropriate with accepted value (10%) which is satisfactory and very important for LEV. On the other hand, as can be seen from Fig.4, the final content of ACN has more influence on the retention time of VPA.

Figure 3.

Figure 4.
Analysis of the Response Contour diagrams confirmed that the initial and final content of acetonitrile in the mobile phase of 10% and 70%, respectively, the pH value of the buffer solution in the mobile phase of 3.0 and gradient time from 15 min was justified .

The optimal chromatographic conditions, which corresponds to adequate separation between the investigated AEDs and in addition, shortest as possible retention time, were obtained using **gradient elution system comprising acetonitrile (ACN) - phosphate buffer (pH 3.0)**.

3.2 Optimisation of the sample preparation procedure
The sample preparation step is an important part of bioanalytical method, therefore it is necessary to develop simple extraction procedure that generates good recovery and clean extracts. In order to find the most efficient method for sample preparation, three precipitation methods, using acetonitrile, methanol or trichloracetic acid and a solid- phase extraction (SPE) were evaluated and compared.

Sample pre-treatment methodology was initially investigated by plasma precipitation with acetonitrile, methanol and trichloracetic acid. Only the protein precipitation with methanol gave satisfactory extraction, but

with low recovery. Since SPE procedures are usually associated to high and reliable extraction of AEDs from human plasma [30,31], several SPE conditions were tested, including washing steps and eluting solvents. The use of SPE procedure described above (loaded a 250 µL of sample, washing with 1ml water and 1 ml 5% methanol and eluting with methanol) demonstrated to be the best option as it allowed a more effective elimination of interfering substances and avoided sample dilution, improving the selectivity and sensitivity of the analytical method.

3.3. Method validation
3.3.1. Selectivity
A reliable separation of the five analytes and internal standard using chromatographic conditions reported above was obtained.Representative chromatograms of blank plasma with IS and spiked plasma samples with LTG, LEV, CBZ,CBZ-EP, VPA and IS, are presented in Fig.5. No interfering peaks were observed at the retention times of analytes or IS.
Figure 5.

3.3.2 Linearity
The calibration curves prepared with human plasma for all compounds at the concentration ranges defined in section 2.4 were linear ($R^2 > 0.995$) and show consistent correlation between analyte - IS peak area ratios and corresponding plasma concentration. The regression equations of the calibration curves and the corresponding regression coefficients attained for each analytes are summarized in Table 3.

Table 3.

3.3.3 Accuracy and precision
According to bioanalytical method validation guidelines, the acceptable criterion for precision, which is expressed as percentage of coefficient of variation (% CV), must be equal to or lower than 15%; whereas accuracy, which is expressed as the deviation of experimental from nominal concentration values in percentage (%), must be within ±15%. The within-run and between-run accuracy and precision are reported in Table 4. which shown that acceptance criteria were fulfilled for all tested samples at four concentration levels.

Table 4
3.3.4. Recovery
Two different sample preparation methods were tested as described in the sample preparation section. The three protein precipitation techniques were found effective in removing proteins from human plasma. With acetonitrile and acid precipitations, low analyte recovery were obtained. Protein precipitation with methanol gave satisfactory extraction yield only for some compounds, except for CBZ-EP which gave the very low recovery values. In order to improve the extraction yield of the method, another plasma sample pretreatment procedure was developed, based on SPE as a described above. However, SPE with the Oasis[®] HLB gives the best results and cleanliness of the sample. The overall recovery values obtained using the PP with methanol and SPE with Oasis[®] HLB cartridges are presented in Table 5. The recoveries of AEDs and the metabolite at four concentration levels ranged from 15.8% to 68.3% for PP with methanol and 85.2% to 99.6% for SPE. These demonstrate that the proposed SPE procedure gives better results than those obtained with PP .

Table 5.
3.3.5. Stability
The stability of the five AEDs and the metabolite was assessed under the different conditions and the results are reported in Table 6. Stock solutions, stored at 2 – 8 °C, were found to be stable for one month.

Table 6.
The obtained results indicate that the analytes were stable under any of the storage conditions described above and that no stability related problems would be expected during the routine plasma sample analysis.

3.4. Application to patient plasma samples
Having in mind that epileptic patients are often treated with polytherapy, to evaluate the selectivity of the proposed method, plasma samples taken from patients simultaneously treated with carbamazepine, lamotrigine, valproate, levetiracetam and topiramate (commonly prescribed drugs in Macedonia) were analysed. In the Fig.6 were presented chromatograms of patients that are under polytherapy. The chromatogram corresponding to a plasma sample from a patient received LEV (1000 mg per day), CBZ (800 mg per day) and VPA (1200 mg per day), is shown in Fig.6A. As can been seen, baseline separation of the analyte peaks is

achieved and no interferences is detected. The analyte levels found in this sample were: 8.29 µg/mL for LEV, 9.85 µg/mL for CBZ, 0.42 µg/mL for CBZ-EP and 48.94 µg/mL for VPA.

The chromatogram corresponding to a plasma sample from another patient treated simultaneously with LEV (750 mg per day), LTG (300 mg per day) and Oxcarbazepin(1200 mg per day) is shown in Fig.6B. The analyte levels found in this sample were:7.98 µg/mL for LEV and 1.32 µg/mL for LTG.

Finally, in Fig.6C was shown the chromatogram corresponding to a plasma sample from a patient treated with VPA (1000 mg per day), CBZ (800 mg per day), LEV (1750 mg per day). The analyte levels in this sample were found to be: 59.92 µg/mL for VPA, 6.63 µg/mL for CBZ, 0.96 µg/mL for CBZ-EP and 7.04 µg/mL for LEV. No chromatographic interference was observed in the retention times of LEV, LTG, CBZ, CBZ-EP, VPA and IS, hence the method could be used even when these antiepileptic drugs were co-administered.

IV.   CONCLUSION

In this study, a gradient HPLC-UV method for the simultaneous determination of LEV, LTG, CBZ, CBZ-EP and VPA in human plasma samples was developed and optimized. Time of analysis and resolution were simultaneously optimized by applying chemometrics tools: *Fractional factorial 3^4 design.* The results of the study demonstrate the benefit of applying this approach in selecting optimum conditions for the determination of AEDs in plasma samples. Total chromatographic analysis time per sample was approximately 15 min. The validation study supported the selection of the assay conditions by confirming that the assay was specific, accurate, linear and precise. The method was found to be simple, sensitive and can be applied successfully in routine analysis for the estimation of LEV, LTG, CBZ, CBZ-EP and VPA in human plasma.

V.   REFERENCES

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Tables. **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 acetonitrile in mobile phase (V/V,%)	10	15	20
final content of acetonotrile in mobile phase (V/V,%)	40	60	70
gradient time	15	20	30

Table 2. Performed experiments (Fractional factorial 3^4 design)

No	Rt (LEV)	Rt(LTG)	Rt(CBZ-EP)	Rt(CBZ)	Rt(IS)	Rt(VPA)
1	2.924	7.022	10.898	14.242	15.609	19.370
2	2.924	7.022	10.898	14.242	15.609	19.370
3	2.861	7.449	12.171	15.905	17.474	21.254
4	2.825	5.936	8.657	11.130	12.110	14.503
5	2.195	4.279	6.649	8.364	9.230	11.357
6	2.143	4.589	7.259	9.611	10.765	13.600
7	2.117	5.668	12.201	18.720	21.398	27.813
8	2.147	5.165	9.474	13.603	15.322	19.450
9	1.873	3.194	5.603	7.867	9.015	11.838
10	1.890	3.249	6.086	8.923	10.345	13.996
11	1.903	3.264	6.361	9.620	11.234	15.238
12	1.934	3.580	7.480	12.351	14.778	20.276
13	2.097	4.923	7.952	10.734	12.089	14.916
14	2.097	4.923	7.952	10.734	12.089	14.916
15	2.089	4.329	6.292	8.186	9.146	11.143
16	2.105	5.923	12.308	18.795	21.833	27.187
17	1.928	3.691	7.377	12.100	14.671	19.485
18	1.913	3.723	8.191	14.377	17.763	23.959
19	1.924	3.258	5.446	7.780	9.016	11.559
20	1.931	3.256	5.161	7.097	8.095	10.220
21	2.718	6.168	8.852	11.096	12.120	14.366
22	2.694	7.071	11.095	14.064	15.596	18.578
23	2.537	7.088	10.773	13.889	15.418	18.742
24	2.820	7.480	11.874	15.696	17.432	20.793
25	2.097	4.923	7.952	10.734	12.089	14.916
26	2.097	4.923	7.952	10.734	12.089	14.916
27	2.097	4.923	7.952	10.734	12.089	14.916
28	1.922	4.799	6.398	9.680	11.474	3.802
29	1.922	4.799	6.398	9.680	11.474	3.802
30	1.932	5.268	7.346	12.063	14.686	3.957
31	1.918	4.405	5.590	7.899	9.126	3.588
32	2.550	9.256	11.752	15.432	17.236	7.230
33	2.532	6.848	8.083	10.066	11.081	5.945
34	2.639	7.350	8.592	10.817	11.994	6.257
35	2.642	9.770	12.498	16.481	18.443	7.573
36	2.077	8.076	11.870	18.140	21.219	5.903
37	2.224	6.832	8.858	12.402	14.202	5.427
38	2.074	6.285	7.842	10.627	12.004	5.169
39	2.080	5.328	6.336	8.203	9.141	4.626

Table 3. Mean calibration parameters (n = 7) of LEV, LTG, CBZ, CBZ-EP, and VPA in human plasma

Calibration Parameters			
Analyte	Concentration range (µg/mL)	Equation	R^2
LEV	3.0 - 70.0	y = 0.150x + 0.035	0.9983
LTG	1.0 - 25.0	y = 0.732x - 0.526	0.9985
CBZ	1.0 - 25.0	y = 0.553x - 0.233	0.9985
CBZ-EP	0.5 - 15.0	y = 0.416x - 0.244	0.9976
VPA	25.0 - 250	y = 0.035x - 0.010	0.9984

Table 4. Precision and accuracy of the analytes in human plasma samples

Analyte	Nominal concentration (µgmL¹)	Within -run assay (n=5)			Within -run assay (n=5)		
		Measured concentration (µgmL¹)	Accuracy (%)	Precision (CV%)	Measured concentration (µgmL¹)	Accuracy (%)	Precision (CV%)
LEV	3.0	2.98	99.26	0.38	2.98	99.42	3.47
	7.5	7.79	99.57	2.58	7.53	100.42	1.10
	30.0	29.83	98.89	6.92	29.59	97.23	7.88
	60.0	60.01	100.98	1.23	58.65	97.76	0.20
LTG	1.0	1.02	101.52	1.62	0.98	98.87	1.06
	5.0	4.83	96.64	0.36	4.90	98.02	0.11
	10.0	9.84	98.36	0.21	9.76	97.57	0.45
	20.0	20.83	104.13	2.94	19.60	98.02	0.28
CBZ	1.0	1.06	105.78	1.79	0.98	98.54	3.52
	5.0	4.98	99.71	0.23	4.95	98.91	0.31
	10.0	9.93	99.28	0.87	9.73	97.29	0.22
	20.0	20.08	100.41	0.26	19.54	97.69	0.49
CBZ-EP	0.5	0.52	104.34	2.12	0.49	97.50	1.28
	2.5	2.47	98.80	0.46	2.44	97.66	0.23
	5.0	4.87	97.41	2.17	4.89	97.88	1.07
	10.0	9.96	99.61	0.33	9.93	99.32	0.33
VPA	25.0	24.31	99.24	2.94	24.96	99.83	3.97
	100	99.81	99.81	0.65	98.41	98.41	1.89
	150	148.63	99.08	0.93	152.31	101	0.96
	200	198.97	99.49	0.23	196.19	98.09	1.55

Table 5. Recovery results with different sample preparation method

Analyte	Concentration (μgmL^1)	PP with methanol		SPE with Oasis HLB	
		Recovery(%)		Recovery(%)	
LEV	3.0	60.5		85.2	
	7.5	62.6		89.6	
	30.0	65.8		88.5	
	60.0	68.3		89.4	
LTG	1.0	58.9		85.6	
	5.0	59.6		85.9	
	10.0	57.3		92.4	
	20.0	60.9		90.6	
CBZ	1.0	34.4		98.5	
	5.0	36.5		98.8	
	10.0	36.4		99.2	
	20.0	39.5		98.9	
CBZ-EP	0.5	15.8		85.3	
	2.5	20.6		89.5	
	5.0	23.5		90.4	
	10.0	23.9		99.5	
VPA	25.0	35.9		96.8	
	100	38.5		99.6	
	150	29.6		99.2	
	200	36.5		98.5	

Table 6. Stability (values of accuracy in percentage) of LEV, LTG, CBZ, CBZ-EP and VPA under different conditions (n=3)

| Analyte concentration (μgmL^1)		Stability conditions			
		Short term stability (24h at room temp.)	Autosampler stability (after 12h)	Three freeze - thaw cycles	Long term stability (30days at -20°C)
LEV	3.0 / 60.0	96.5 / 102.4	95.9 / 98.4	100.2 / 96.9	102.1 / 98.8
LTG	1.0 / 20.0	98.0 / 103.1	96.12 /101.0	105.3/93.2	102.0 / 98.4
CBZ	1.0 / 20.0	98.9 / 98.1	97.5 / 102.1	98.6 / 100.4	96.8 / 103.0
CBZ-EP	0.5 /10 .0	99.2 / 97.4	97.8 / 97.2	99.8 / 96.5	101.3/101.4
VPA	25 .0/ 200	98.0 / 100.4	94.2 / 97.8	93.2 / 97.7	103.9/100.5

Figures
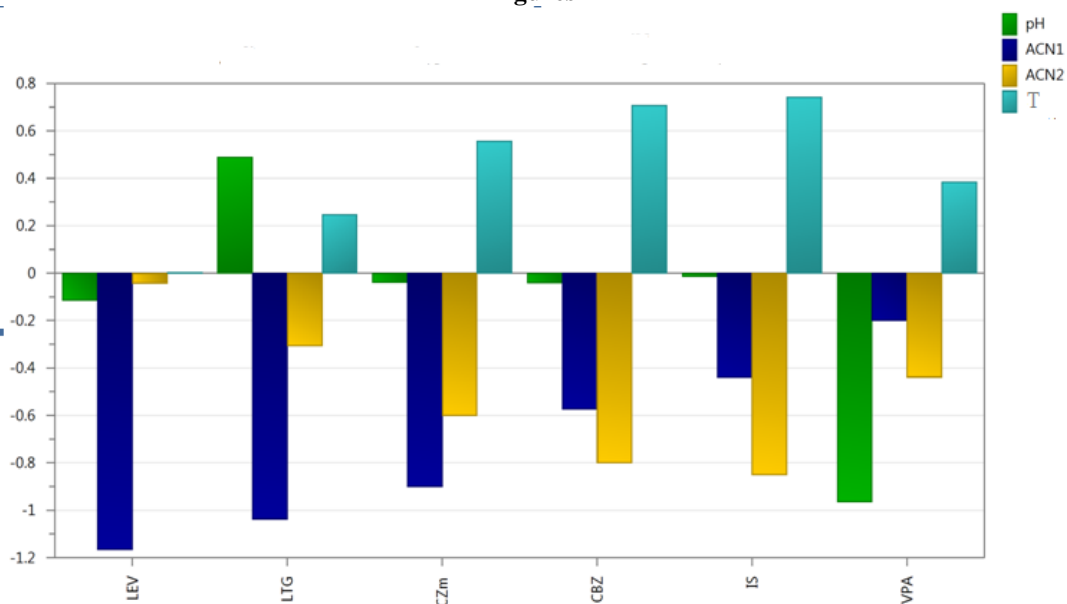
Fig. 1. Coefficient overview plot (ACN1:initial content of ACN in mobile phase; ACN2:final content of ACN in mobile phase; pH : value of buffer solution in mobile phase; T: gradient time.
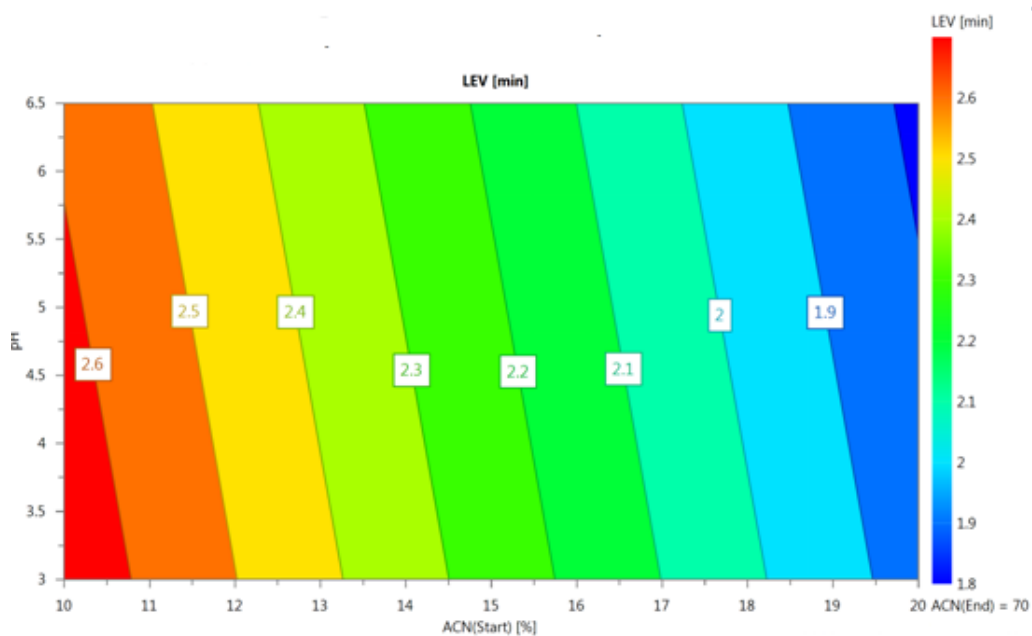

Fig.2 Influence of initial content of ACN in the mobile phase and the pH value of the buffer solution in the mobile phase on the retention time of LEV(Response contour diagram.)
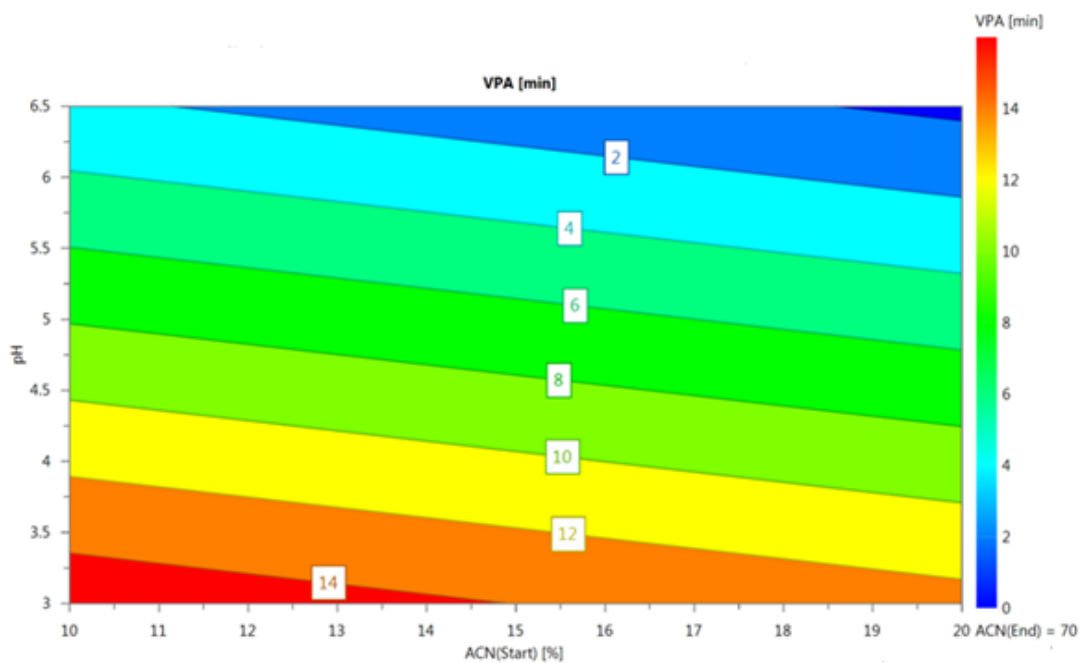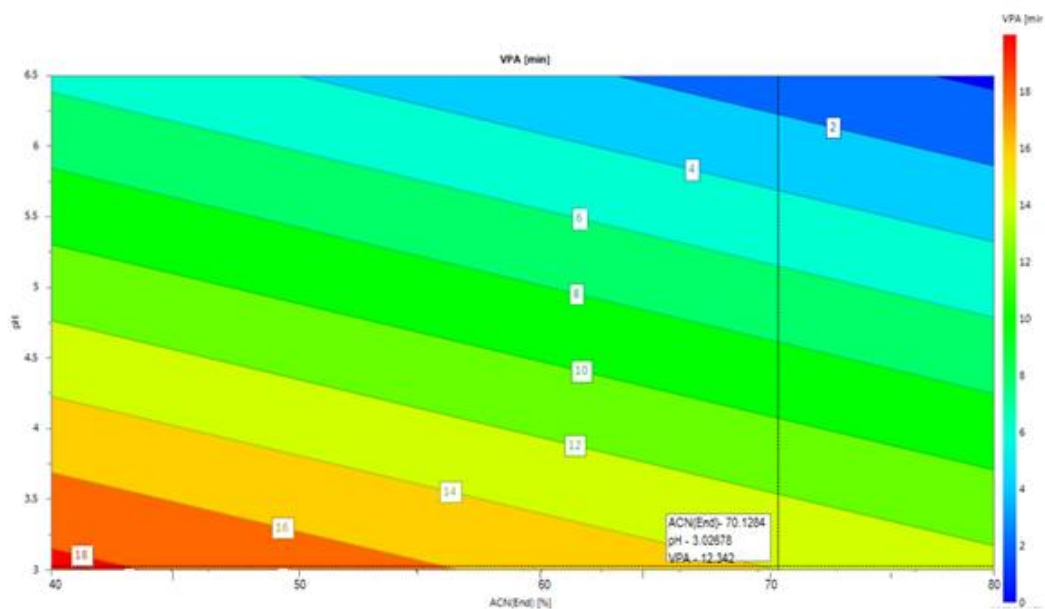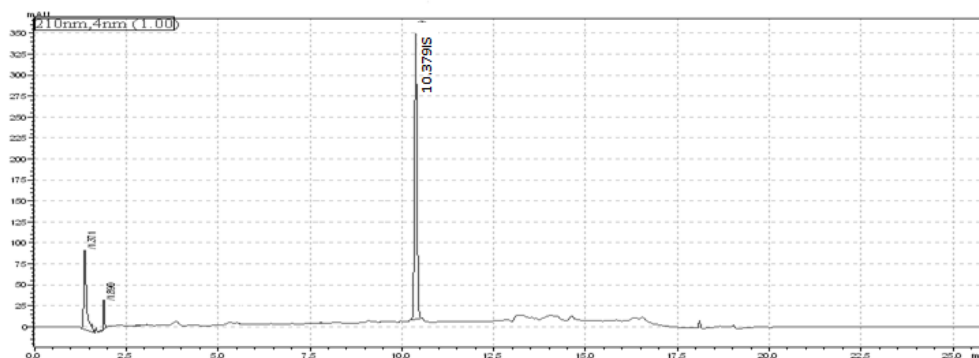
Fig. 3 Influence of initial content of ACN in the mobile phase and the pH value of the buffer solution in the mobile phase on the retention time of VPA(Response contour diagram).

Figure 4. Influence of final content of ACN in the mobile phase and the pH value of the buffer solution in the mobile phase on the retention time of VPA(Response contour diagram)
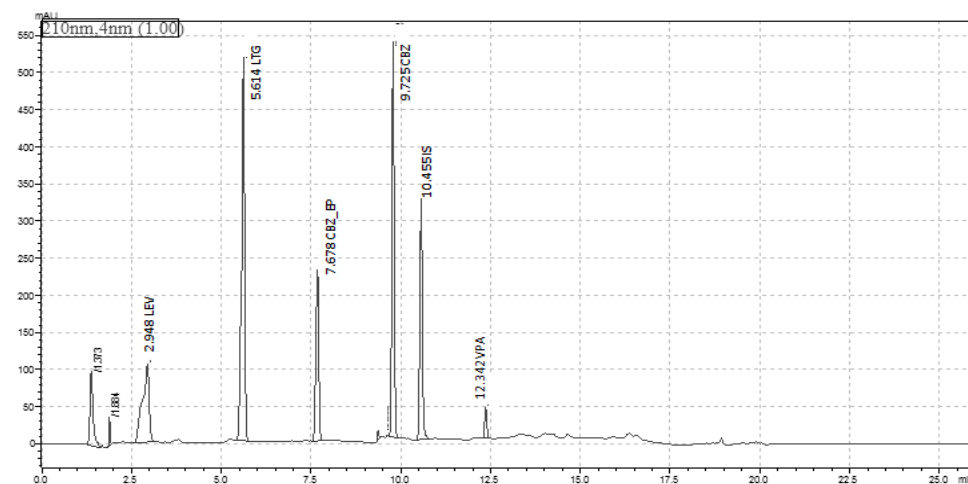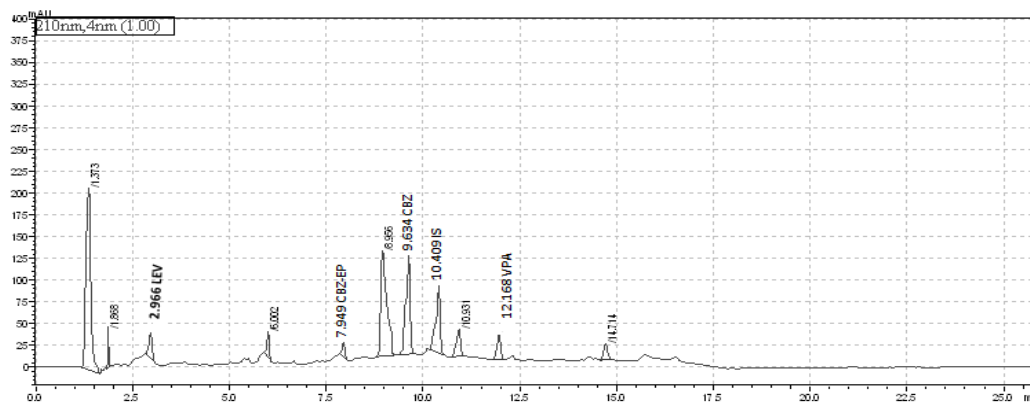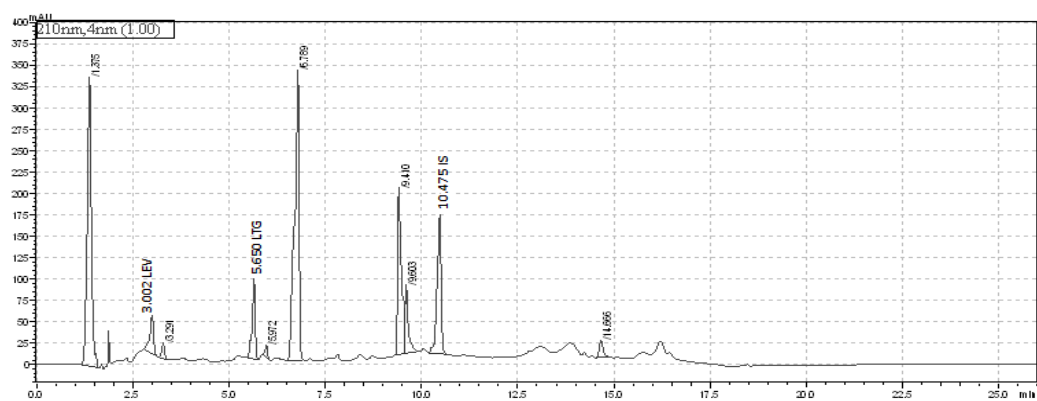
A)

B)

Fig. 5 Chromatograms obtained from: A) blank plasma (drug free plasma) with IS ,
 B) blank plasma spiked with 7.5µg/mL LEV , 5.0µg/mL LTG, 5.0µg/mL CBZ , 2.5µg/mL CBZ-EP and 100 µg/mL VPA and IS (10µg/mL).
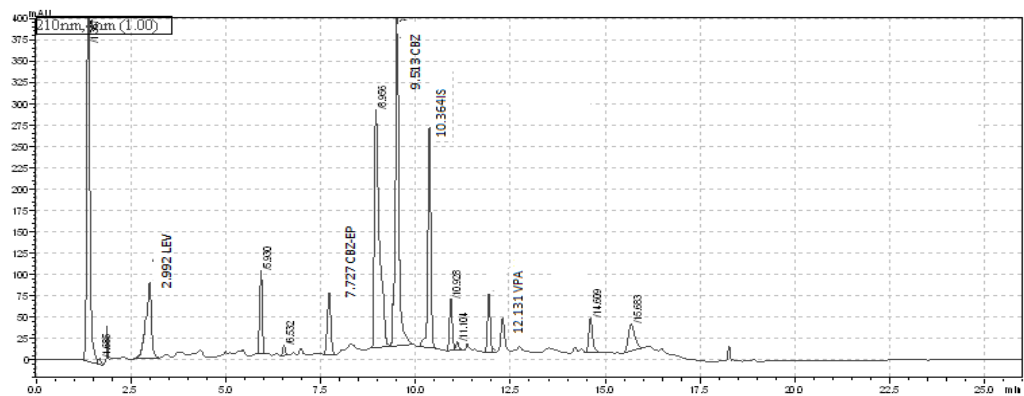
A)

B)

C)

Fig. 6 Chromatograms of plasma sample from : A) patient treated with 1000mg per day of LEV, 800mg per day of CBZ and 1200mg per day of VPA; B) patient treated with 750mg per day of LEV, 300mg per day of LTG and 1200mg per day of oxcarbazepin; C) patient treated with 1000mg per day of VPA, 800mg per day of CBZ and 1750mg per day of LEV.