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Robust isocratic high performance liquid chromatographic method for simultaneous determination of seven antiepileptic drugs including lamotrigine, oxcarbazepine and zonisamide in serum after solid-phase extraction

T.A.C. Vermeij, P.M. Edelbroek *

Epilepsy Institute of the Netherlands (SEIN), Heemstede, The Netherlands

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Abstract

A rapid, simple and robust method is presented for the simultaneous determination of seven antiepileptic drugs (AEDs), including primidone, phenobarbital, phenytoin, carbamazepine with its two major metabolites carbamazepine-10,11-epoxide and carbamazepine-10,11-(*trans*)-dihydrodiol and the new AEDs lamotrigine, hydroxycarbazepine (active metabolite of oxcarbazepine) and zonisamide in serum by high performance liquid chromatography (HPLC)-diode array detector (DAD). After solid-phase extraction, separation is achieved on an Alltima 3C18 analytical column using isocratic elution with a mixture of acetonitrile, methanol and phosphate buffer at 45 °C. The method is exhaustively validated, including experimental design in combination with statistical evaluation (ANOVA) to study the robustness of chromatography and sample preparation. Commonly co-administered antiepileptic drugs do not interfere with the method. Intra-day precision (RSD < 1.9%), linearity, lower limit of quantitation (LOQ < 0.065 mg/l) and robustness make the method suitable for daily therapeutic drug monitoring and pharmacokinetic studies.

Keywords: Anticonvulsants; Zonisamide; Carbamazepine; Oxcarbazepine; Lamotrigine; Phenytoin; Primidone; Phenobarbital; Drug analysis; HPLC; Validation; Design of experiments

1. Introduction

Phenytoin (PHT), phenobarbital (PHB), carbamazepine (CBZ), primidone (PRM), valproic acid (VPA) and ethosuximide (ESM) are well-known classical antiepileptic drugs (AEDs). Since the 1990s a number of new AEDs, such as lamotrigine (LTG), oxcarbazepine (OXC) and zonisamide (ZNS) were registered worldwide and are currently used as an add-on or monotherapy in patients with epilepsy [1–3].

LTG has an anticonvulsant profile similar to that of PHT and CBZ, but it is structurally unrelated to them. It is extensively metabolised and excreted predominantly as a glucuronide conjugate via the urine. The elimination of LTG is significantly altered by concomitant therapy with drugs such as CBZ, PHB, PHT that induce or VPA that inhibit the uridine diphosphate glucuronosyltransferases.

OXC is a keto-analogue of CBZ and is rapidly converted into the racemic 10-monohydroxy derivative (HCB), which is the pharmacologically active substance.

ZNS is metabolised by the cytochrome P450 3A4 isoenzyme and concurrent medication with liver enzyme-inducing antiepileptic drugs decreases its plasma half-life, lowering its concentration to dose ratio [4,5]. ZNS shows a high affinity for human erythrocytes – eight times higher than that for plasma proteins – which is dependent of the plasma concentration.

For optimal drug treatment of epilepsy the monitoring of concentrations of these new drugs in serum can be useful [6–8]. However, the increasing number of antiepileptic drugs makes it difficult to offer such a service in clinical routine on a regular

* Corresponding author at: Laboratory of Clinical Chemistry and Clinical Pharmacology, Epilepsy Institute of the Netherlands (SEIN), P.O. Box 540, 2130 AM Hoofddorp, The Netherlands. Tel.: +31 23 558131; fax: +31 23 558839.
E-mail address: pedelbroek@sein.nl (P.M. Edelbroek).

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and economic base. Therefore integration of as much as possible antiepileptic drugs within one analytical method, which can also be automatized is an usable approach, especially in patients on polytherapy with antiepileptic drugs.

For the simultaneous analysis of AEDs in human serum or plasma by high performance liquid chromatography (HPLC) several methods are reported [9–15]. However, these methods only include analysis of a number of classical AEDs and/or use a liquid–liquid extraction method, which is difficult to automatize.

In this paper, we describe a method for the simultaneous analysis of ZNS, PRM, PHB, LTG, HCB, PHT, CBZ and its two major metabolites carbamazepine-10,11-epoxide (CBZE) and carbamazepine-10,11-(*trans*)-dihydrodiol (CBZD).

After solid-phase extraction (SPE) from serum an aliquot of the sample is injected onto the HPLC. Drugs and internal standard are separated on a reversed phase column under simple isocratic conditions and monitored using a diode array detector (DAD).

The method was validated according to the guidelines of the International Conference on Harmonisation (ICH) [16], including a robustness study by means of an experimental design in combination with statistical evaluation of the data (ANOVA). The described method is also appropriate for analysis of other antiepileptic compounds, including ESM, sulthiame (STH), felbamate (FBM), heptobarbital (HPB), phenacemide (PAC), *N*-desmethylmethsuximide (NDMS) (the active metabolite of methsuximide (MSM)), phensuximide (PSM), hexobarbital (HXB) and OXC. However, because of the very limited need of analysis of these compounds, they were not included in the calibrator mixture and the robustness study.

2. Experimental

2.1. Reagents and standards

Zonisamide was a gift from Elan Biopharmaceuticals Inc. (San Diego, CA, USA); CBZ, CBZE, CBZD and HCB were donated by Ciba-Geigy (Division of Novartis, Basel, Switzerland); LTG was a gift from Glaxo-Wellcome Inc. (Division of GlaxoSmithKline, London, UK); PHT was obtained from Katwijk Farma (Leiden, The Netherlands); PHB was purchased from Merck (Darmstadt, Germany); PRM from Imperial Chemical Industries (London, UK); and the internal standard 5-ethyl-5-para-tolyl barbituric acid (ETB, gold label) from Sigma–Aldrich (St. Louis, MO, USA).

Methanol (Lichrosolv), sodium hydroxide, sodium chloride and concentrated (37%) hydrochloric acid (all p.a.) were obtained from Merck. Acetonitrile (Chromasolv), potassium dihydrogen phosphate and dipotassium hydrogen phosphate (both Microselect, Fluka) were from Sigma–Aldrich. Ultra pure water was prepared using the Millipore-Q-plus water purification system (Millipore, Bedford, MA, USA).

Stock molar phosphate buffer (pH 7.0) was prepared by dissolving 68 g potassium dihydrogen phosphate and 87 g dipotassium hydrogen phosphate in 1000 ml of water. For mobile phase preparation 25 ml of this stock solution was diluted to 1000 ml with 12.5 mM sodium chloride (0.73 g/l) in water. pH was adjusted to 6.2 with diluted hydrochloric acid.

Table 1
Concentrations of AEDs and metabolites in calibration serum samples [mg/l]

Analyte	CS1	CS2	CS3	CS4	CS5	CS6	CS7
PRM	0.54	1.09	2.72	5.45	10.9	16.4	21.8
ZNS	0.55	1.09	2.73	5.46	10.9	16.4	21.8
CBZD	0.23	0.46	1.16	2.32	4.64	6.96	9.28
LTG	0.54	1.08	2.70	5.41	10.8	16.2	21.6
HCB	0.92	1.94	4.86	9.72	19.4	29.2	38.9
PHB	1.26	2.52	6.29	12.6	25.2	37.7	50.3
CBZE	0.16	0.31	0.78	1.57	3.14	4.70	6.26
PHT	0.77	1.54	3.86	7.71	15.4	23.1	30.8
CBZ	0.37	0.74	1.86	3.71	7.42	11.1	14.8

Internal standard solution (10 mg/l ETB) was prepared by diluting 1 volume of ETB stock solution (1 mg/ml in methanol) with 100 volumes of 0.1 M phosphate buffer, pH 7.

Calibration serum (CS) was prepared in seven concentrations (CS1–CS7) by spiking blank newborn calf serum with all seven AEDs and the two metabolites at the highest concentration and subsequent diluting with blank calf serum. Concentrations for all components are listed in Table 1.

2.2. Instrumentation

HPLC analysis was carried out on a P4000 quaternary solvent delivery system equipped with an AS3500 autosampler with column oven and a UV6000LP diode array detector (Thermo Separation Products Inc., San Jose, CA, USA). Integration and system parameters were controlled by Spectrasystem PC1000 software (Thermo Separation Products Inc.).

For sample preparation we used a Baker spe-12G Column Processor (Mallinckrodt-Baker, Deventer, The Netherlands) equipped with a Barnant vacuum/pressure station (Alltech). Drugs were extracted from serum using 30 mg Oasis HLB disposable extraction columns (Waters, Milford, MA, USA) with 1 ml sample volume capacity.

2.3. Sample preparation

Prior to SPE 0.1 ml of serum is diluted with 0.5 ml of the internal standard working solution. After conditioning the extraction column with 1 ml methanol followed by 1 ml water the sample mixture was poured into the column reservoir. The sample was then drawn through the column with a maximum flow-rate of 1 ml/min. Consequently the column was washed with 2 ml of water at maximum speed. The drugs were then eluted with three times 0.1 ml of a mixture of acetonitrile/methanol (7/3, v/v). After diluting the eluate with 1 ml of water 50 μl was injected onto the HPLC system.

2.4. Chromatography

Separation was performed on an analytical column (15 cm × 0.46 cm) packed with Alltima 3C18 (Alltech Nederland, Breda, The Netherlands) and kept at a constant temperature

of 45 °C. The column eluate was monitored at fixed wavelengths of 215 and 275 nm. The mobile phase consisted of a mixture of methanol (14.5 vol.%), acetonitrile (19.5 vol.%) and 25 mM phosphate buffer containing 12.5 mM of sodium chloride, pH 6.2 (66 vol.%), and was delivered isocratically at a flow-rate of 0.9 ml/min. If necessary the final pH of the mobile phase was adjusted to 6.7 ± 0.1 with diluted solutions of sodium hydroxide or hydrochloric acid.

3. Results and discussion

3.1. Sample preparation

Application of a solid-phase extraction technique makes sample preparation very simple, rapid and accurate. The method is pre-eminently suitable for automation with an on-line solid-phase extraction and injection device (e.g. ASPEC from Gilson Inc., Middleton, WI, USA). The use of Oasis HLB extraction columns contributes to the method's simplicity: only flow-rates during sample absorption and elution steps are somewhat critical but not during conditioning and washing steps. No care has to be taken to prevent columns from running dry.

3.2. Chromatography

In Fig. 1, chromatograms are presented of (a) a blank human serum sample, (b) a calibration sample in new-born calf serum and (c) a patient on LTG, CBZ and PHB medication.

Retention times of the seven AEDs, the two metabolites of CBZ and the internal standard are presented in Table 2a. Retention times of nine other – less frequently prescribed – AEDs and two metabolites are presented in Table 2b. These compounds are quantitatively co-extracted and with few restrictions our method is also suitable for therapeutic monitoring of these drugs.

The table shows that the retention times of ZNS and FBM are comparable. However, if ZNS is co-administered with felba-

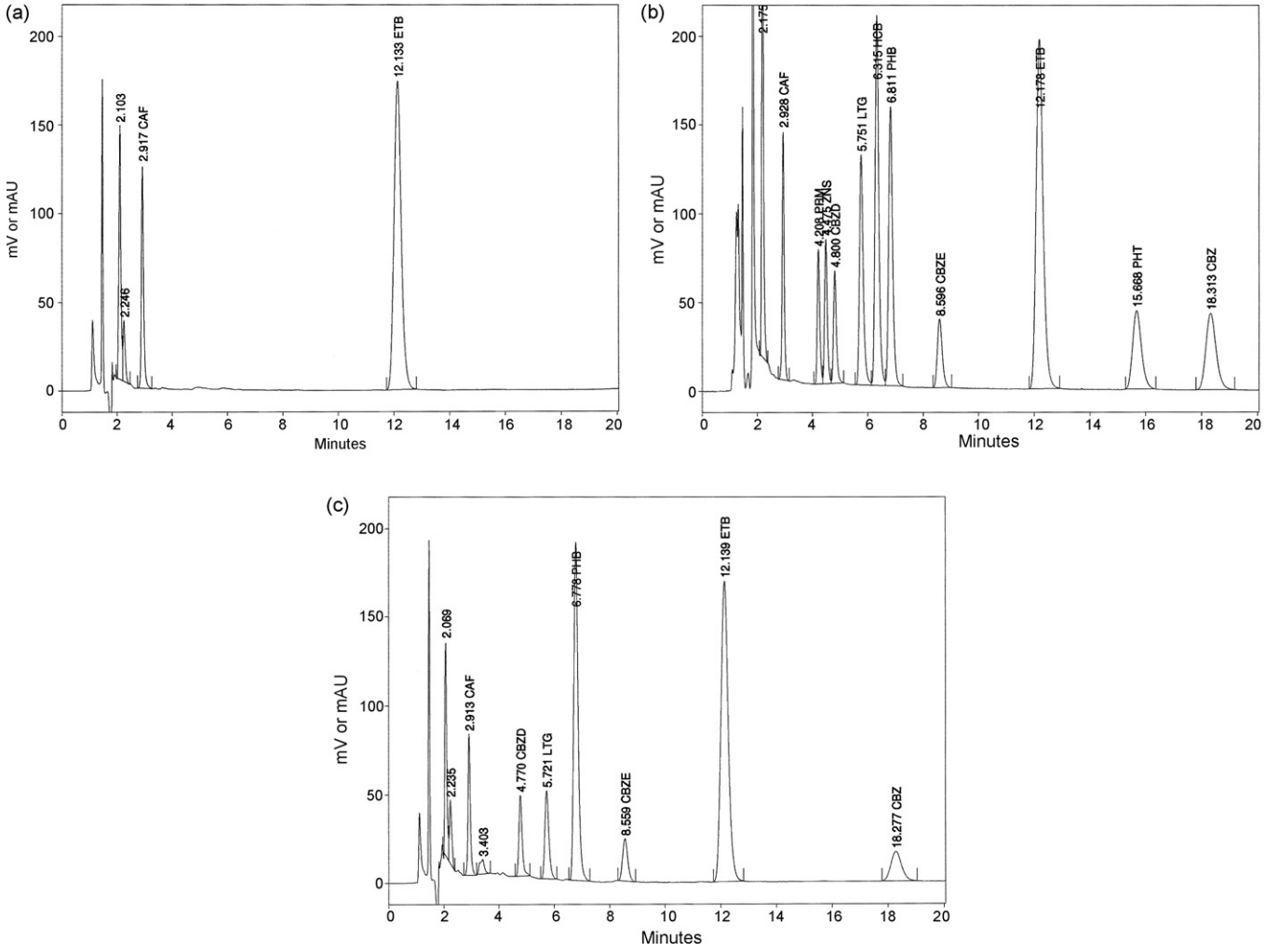

Fig. 1. HPLC-chromatograms of (a) a blank human serum sample. (b) A calibration sample containing 10.90 mg/l of primidone (PRM), 10.90 mg/l of zonisamide (ZNS), 4.64 mg/l of carbamazepine-10,11-*trans*-diol (CBZD), 10.82 mg/l of lamotrigine (LTG), 19.43 mg/l of hydroxycarbazepine (HCB), 25.15 mg/l of phenobarbital (PHB), 3.13 mg/l of carbamazepine-10,11-epoxide (CBZE), 15.42 mg/l of phenytoin (PHT) and 7.41 mg/l of carbamazepine (CBZ). Retention times are 4.21 min for PRM, 4.47 min for ZNS, 4.80 min for CBZD, 5.75 for LTG, 6.32 for HCB, 6.81 for PHB, 8.60 for CBZE, 15.67 for PHT, 18.31 for CBZ and 12.18 min for the internal standard ETB. (c) Serum sample of a patient on lamotrigine, carbamazepine and phenobarbital medication. Concentrations are 4.8 mg/l for LTG, 39.2 mg/l for PHB, 3.5 mg/l for CBZ, 2.4 mg/l for CBZE and 4.6 mg/l for CBZD.

Table 2

Analyte	Retention time (min)	%Recovery ($n=12$)
(a) Retention times and extraction recoveries of AEDs and metabolites		
PRM	4.20	102
ZNS	4.45	101
CBZD	4.80	103
LTG	5.75	92
HCB	6.30	102
PHB	6.80	100
CBZE	8.60	102
ETB	12.15	100
PHT	15.65	98
CBZ	18.30	100
(b) Other antiepileptic compounds		
Acetazolamide (AZM)	<2.15	
Caffeine (CAF)	2.90	
Phenylethylmalonamide (PEMA)[a]	3.10	
Sulthiame (STH)	3.65	91
Ethosuximide (ESM)	3.70	99
Felbamate (FBM)	4.45	94
Heptobarbital (HPB)	4.85	92
Phenacemide (PAC)	5.10	101
N-Desmethylmethsuximide (NDMS)[b]	7.80	93
Phensuximide (PSM)	7.80	101
Oxcarbazepine (OXC)[c]	10.35	76
Hexobarbital (HXB)	14.60	93

[a] PEMA is a metabolite of PRM.
[b] NDMS is the active metabolite of methsuximide (MSM).
[c] OXC is only found in low concentrations.

mate (FBM) it can be analysed with our method by selecting the detection wavelength of 275 nm. A FBM serum level of 40 mg/l corresponds with only 0.6 mg/l of ZNS at this wavelength. Analysis of FBM in the presence of ZNS is not possible with our method.

During method development addition of trace amounts of sodium chloride appeared to improve robustness of chromatography. The presence of sodium and chloride ions prevents changes in chromatographic behaviour of some drugs when these ions are added to the mobile phase while adjusting the pH with sodium hydroxide or hydrochloric acid.

3.3. Recovery

Absolute recovery from serum for all seven drugs and the two metabolites were calculated by comparing peak areas of extracted calibration serum samples ($n=12$) with those in a reference mixture in mobile phase. As listed in Table 2 SPE-extraction with acetonitrile/methanol (7/3, v/v) as an eluent resulted in recoveries for all components ranging from 98 to 103%. Only the LTG recovery is somewhat lower with 92%; compared to methanol, pure acetonitrile as an eluent resulted in smaller final sample volumes, but PHT recovery was approximately 15–20% lower than that of the other compounds. Application of a 7/3 (v/v) mixture of acetonitrile and methanol as elution solvent resolved this problem. Additional advantage was the final sample composition being almost compatible with the mobile phase.

Recoveries of nine other antiepileptic compounds as listed in Table 2b were also investigated and appeared to be always >91% ($n=6$), except for OXC which was only approximately 76%.

3.4. Intra- and inter-day precision

Intra-day precision was determined by subsequent analysis of calibration serum at three levels within the therapeutic range of the drugs. Results show low coefficients of variation between 0.6 and 1.9%. Inter-day precision was calculated after analysing control samples at two levels during a 2-month period of routine use of the method by three different technicians on 20 different days. The coefficients of variation ranged from 0.9 to 3.3%. All data are shown in Table 3.

Intra-day precision of the less frequently prescribed antiepileptic compounds listed in Table 2b was also investigated and appeared to be <2.5% ($n=6$).

3.5. Linearity, accuracy and sensitivity

For determination of linearity, accuracy and sensitivity of the method, calibration serum samples at six levels – completed with a blank – were analysed in six-fold. Quantitation of AED levels was achieved after one-point calibration on CS5 in 10-fold, using

Table 3
Intra- and inter-day precision

Analyte	Cal. sample	%RSD	
		Intra-day ($n=12$)	Inter-day ($n=20$)
PRM	CS4	1.3	1.9
	CS5	1.4	
	CS6	1.1	1.9
ZNS	CS4	1.3	2.0
	CS5	1.3	
	CS6	1.3	1.9
CBZD	CS4	1.3	3.0
	CS5	1.3	
	CS6	1.0	1.9
LTG	CS4	1.2	1.8
	CS5	1.9	
	CS6	1.2	1.2
HCB	CS4	1.2	1.1
	CS5	1.2	
	CS6	1.1	1.4
PHB	CS4	0.6	0.9
	CS5	1.0	
	CS6	0.7	1.3
CBZE	CS4	1.2	3.3
	CS5	1.2	
	CS6	0.9	1.4
PHT	CS4	1.3	1.3
	CS5	1.5	
	CS6	0.8	1.6
CBZ	CS4	1.2	1.2
	CS5	1.5	
	CS6	0.8	1.5

Table 4
Linearity parameters

Analyte	Range (mg/l)	Intercept (SE)	Slope (SE)	R^2 (SE)
PRM	0–21.8	0.1304 (0.0416)	0.9913 (0.0038)	0.9993 (0.2002)
ZNS	0–21.8	0.0364 (0.0539)	1.0248 (0.0050)	0.9989 (0.2596)
CBZD	0–9.28	0.0370 (0.0171)	0.9899 (0.0037)	0.9993 (0.0823)
LTG	0–21.6	0.0619 (0.0411)	0.9924 (0.0038)	0.9993 (0.1979)
HCB	0–38.8	0.2125 (0.0708)	0.9845 (0.0037)	0.9993 (0.3410)
PHB	0–50.4	0.1854 (0.0953)	0.9883 (0.0038)	0.9993 (0.4590)
CBZE	0–6.26	0.0212 (0.0118)	0.9894 (0.0038)	0.9993 (0.0568)
PHT	0–31.0	0.0982 (0.0674)	0.9905 (0.0044)	0.9991 (0.3245)
CBZ	0–14.8	0.0492 (0.0290)	0.9917 (0.0039)	0.9992 (0.1399)

an internal standard method on peak heights. Linearity parameters and their standard errors (SE) were determined using the least squares linear regression analysis method and are given in Table 4, showing high linearity of the method and covering a range up to approximately twice the upper limit of the therapeutic ranges for all drugs.

Accuracy for each compound presented as a percentage of the target value ranges between 99 and 106% in the four highest concentrations, covering the therapeutic and supertherapeutic range. The lowest three concentration levels showed little higher coefficients of variation (1.7–4.9%) and 5–15% deviations from target values were observed in the two lowest concentration levels, probably due to interferences with endogenous substances. Mean RSD's and accuracies together with their standard deviations are presented in Table 5a.

For each component the limit of quantification (LOQ) and the limit of detection (LOD) were calculated based on signal-to-noise ratio. LOQ and LOD were defined as successively 10 and 6 times the standard deviation of the noise divided by the signal (peak height per concentration unit). In Table 5b, LOQ and LOD data are presented for each analyte.

Table 5
Accuracy and sensitivity parameters

Analyte	%RSD ($n=6$), mean ± SD	%Accuracy ($n=6$), mean ± SD
(a) Accuracy		
PRM	2.1 ± 1.1	105.7 ± 6.7
ZNS	2.1 ± 1.1	106.7 ± 4.2
CBZD	2.1 ± 1.2	103.0 ± 4.3
LTG	1.9 ± 1.2	102.8 ± 4.9
HCB	2.0 ± 1.2	104.4 ± 6.6
PHB	1.9 ± 1.3	102.5 ± 4.2
CBZE	2.0 ± 1.4	102.2 ± 4.0
PHT	2.5 ± 1.5	102.8 ± 5.1
CBZ	2.2 ± 1.2	103.2 ± 5.2

Analyte	LOQ	LOD
(b) Sensitivity (mg/l) based on signal-to-noise ratio		
PRM	0.029	0.017
ZNS	0.027	0.016
CBZD	0.014	0.009
LTG	0.014	0.008
HCB	0.017	0.010
PHB	0.029	0.017
CBZE	0.014	0.009
PHT	0.065	0.039
CBZ	0.030	0.018

3.6. Specificity

As mentioned before retention times of some less frequently administered AEDs were also investigated. AZM, STH and ESM elute before PRM and do not interfere in the analysis. The same applies to CAF and its more polar metabolites that are often present in human serum samples. Under these chromatographic conditions the retention time of FBM approximately equals that of ZNS but through selection of another detection wavelength – 275 nm – analysis of the latter is possible. HPB and PAC elute very close to each other and may interfere with analysis of CBZD. Retention times of NDMS and PSM are equal and UV absorbance spectra show little differences, so both compounds can only be estimated in absence of each other. Analysis of STH in the presence of ESM is possible through selection of 265 nm as a detection wavelength. Other AEDs, benzodiazepines and relevant metabolites were tested for interference with the method: valproic acid, levetiracetam, topiramate, stiripentol, vigabatrin, gabapentin, pregabalin, clobazam, *N*-desmethyl clobazam, diazepam, *N*-desmethyl diazepam, oxazepam, temazepam, clonazepam and nitrazepam could not be shown in the chromatogram. The use of a diode array detector is a useful aid in diminishing the chance on interferences by checking peak purity. Identity of each analyte is further confirmed by matching UV-spectra with references in the spectral library. Furthermore application of chromatography at constant temperature considerably contributes to the specificity of the method: retention times within one series of analysis are very reproducible with RSD's smaller than 0.3%.

Within the first 8 min of the chromatogram some endogenous substances may be present. However, these peaks only result in false positive AED levels <0.1 mg/l.

3.7. Robustness study

Robustness is an important part of method validation and, as defined by the ICH, it is a measure of the method to remain unaffected by small variations in the specified optimal method parameters and provides an indication of its reliability during normal usage. Experimental design is a useful tool in this kind of studies as it facilitates the investigation of several parameters

at the same time while reducing the number of experiments. Preceding the actual testing a selection of essential factors and the levels at which to test them has to be performed and a decision must be made about to consider which responses. Once experiments have been carried out, analysis of variance (ANOVA) can be used to evaluate the results [17–19]. We used a software tool called Essential Regression and Experimental Design for Chemists and Engineers, developed as an add-in for Microsoft Excel® and which comes with an electronic book package [18], for setting up experimental designs and for evaluating the results.

Robustness testing was carried out in the pre-validation process of method development, during investigation of accuracies and precision, and was divided into two parts. Concerning the chromatography part of the method we selected seven factors which we found likely to be significant in practical use of the method: methanol and acetonitrile content of the mobile phase, pH and molarity of the phosphate buffer, molarity of sodium chloride, flow-rate and column oven temperature. Initially experiments were performed on two types of analytical columns, i.e. a Polaris 3C18-A cartridge system (Varian Inc., Middelburg, The Netherlands) and a conventional Alltima 3C18 HPLC column with equal dimensions. Although mobile phase composition was different, robustness of chromatography obtained the same results. Eventually we chose the Alltima column for practical reasons: when performance decreases these columns can simply be restored by replacing the top packing with clean silica material. The selected factors and their investigated upper and lower limits are shown in Table 6a. A fractional factorial design was generated for the study of seven factors. Including 3 centerpoints this resulted in 11 experimental conditions under which we subsequently analysed a calibration serum containing the analytes at their mean therapeutic concentration levels in 10-fold. This resulted in precision data (RSD) of the analytes that we used as quantitative response. Mean plate numbers, asymmetry factors, resolutions and RSD of retention times were calculated to evaluate effects on the qualitative response. Concerning quantitative response of only PHT the acetonitrile content of the mobile phase appeared to be a statistically significant factor ($p < 0.05$) in the linear response regression model. Nevertheless RSD for PHT was always <0.63%, for the other components always <0.81%. Regarding qualitative responses molarities of phosphate and sodium chloride are never a significant factor. With respect to peak resolution oven temperature, pH, flow and acetonitrile content of the mobile phase appeared to be significant factors for several peaks ($p < 0.05$). During the experiments only three times a resolution was found between 1.15 and 1.25, meaning more or less partial overlapping of some peaks. Critical peak pairs were PRM/ZNS, HCB/PHB and PHT/CBZ. In daily practice separation of those pairs can be improved by adjusting one or more of the significant factors, temperature being the most effective and convenient way. Three parameters – acetonitrile, pH and temperature – appeared to be statistically significant ($p < 0.01$) concerning peak shape, i.e. asymmetry and plate number. However, during the actual experiments of this study asymmetry factors were always between 0.95 and 1.30 and column plate count – calculated on the latest eluting peak – was always between 91.000 and 107.000 plates/m. It can be concluded that between the chosen limits column efficiency is never dramatically decreased. None of the variables affected the reproducibility (RSD) of retention times.

Table 6
Selected parameters and their variations[a]

	−	0	+
(a) Chromatography robustness			
MeOH in mobile phase (vol.%)	14.0	14.5	15.0
MeCN in mobile phase (vol.%)	19.0	19.5	20.0
Molarity Phosphate buffer (mM)	20	25	30
Molarity NaCl (mM)	10.0	12.5	15.0
Final pH of mobile phase	6.6	6.7	6.8
Flow (ml/min)	0.8	0.9	1.0
Oven temperature (°C)	43	45	47
(b) Sample preparation robustness			
Volume of phosphate buffer added to sample (ml)	0.4	0.5	0.6
Molarity of phosphate buffer (mM)	75	100	125
pH of phosphate buffer	6.5	7.0	7.5
Sample volume (ml)	0.05	0.10	0.15
SPE wash volume (ml)	1.5	2.0	2.5
SPE elution volume (ml)	0.20	0.35	0.30
Acetonitrile/methanol ratio in elution solvent (v/v)	8/2	7/3	6/4
Eluate dilution volume (ml)	0.8	1.0	1.2
Injection volume (μl)	40	50	60
SPE column batch number	B	A	C

[a] 0: centerpoint; − and + are, respectively, lower and upper limits.

Robustness of sample preparation was investigated using one qualitative variable – the batch number of the Oasis HLB extraction columns – and nine quantitative variables with upper and lower limits as shown in Table 6b. This time a Plackett-Burman design was generated, reducing the number of experiments to 14, including 2 centerpoints. Again a calibration serum sample containing the analytes at their mean therapeutic concentration levels was subsequently analysed in six-fold. Resulting coefficients of variations were considered as most important quantitative response factors.

Batch number of the extraction columns, molarity of the phosphate buffer and acetonitrile to methanol ratio of the elution solvent appeared the most significant factor ($p < 0.001$) affecting repeatability of all components, except LTG. Less significant parameters ($p < 0.01$) were elution volume, phosphate volume and sample volume. However, between the tested limits the coefficient of variation was always below a very acceptable 3.6%.

4. Conclusion

We present a simple, rapid, well-validated and robust method for the simultaneous determination of zonisamide, primidone, phenobarbital, lamotrigine, 10-hydroxycarbazepine, phenytoin, carbamazepine, carbamazepine-10,11-epoxide and carbamazepine-10,11-(*trans*)-dihydrodiol in human serum. Experimental design is a very helpful tool in testing robustness of analytical methods during the pre-validation phase. The preceding exploration of its limits is very useful in identifying potential problematic factors and how to control them. The

method is in daily use for routine therapeutic drug monitoring for a considerable time without any problems. The experimental results with respect to linearity, accuracy, precision, specificity and sensitivity demonstrate the reliability of the procedure for its intended application.

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