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An ImmunoChip prototype for simultaneous detection of antiepileptic drugs using an enhanced one-step homogeneous immunoassay^{1,2,3,4}

Xiaoyun Yang^a, Jarmila Janatova^{a,*}, JoEtta M. Juenke^b, Gwendolyn A. McMillin^{b,c}, and Joseph D. Andrade^{a,*}

^a Department of Bioengineering, University of Utah, Salt Lake City, UT 84112-9202, USA

^b ARUP Institute of Clinical and Experimental Pathology, Salt Lake City, UT 84108, USA

^c Department of Pathology, University of Utah School of Medicine, Salt Lake City, UT 84132, USA

Abstract

The development and characterization of a one-step homogeneous immunoassay-based multi-well ImmunoChip is reported for the simultaneous detection and quantitation of antiepileptic drugs (AEDs). The assay platform utilizes a Cloned Enzyme Donor Immunoassay (CEDIA), and a Beta-Glo assay system for generation of bioluminescent signal. Results of the one-step CEDIA for three AEDs (CBZ, carbamazepine; PHT, phenytoin; VPA, valproic acid), in the presence of serum, correlate well with the values determined by Fluorescence Polarization Immunoassay. CEDIA intra-assay and inter-assay coefficients of variation are lower than 10%. A microfabrication process, xurography, was utilized to produce the multi-well ImmunoChip. Assay reagents were dispensed, and lyophilized, in a three-layer pattern. The multi-well ImmunoChip prototype was used to detect and quantify AEDs in serum samples containing all three drugs. Luminescent signals generated from each well were recorded with a Charged Coupled Device (CCD) camera. The assays performed on an ImmunoChip were fast (5 min), requiring only small volumes of both the reagents (< 1 µl per well) and the serum sample. The ImmunoChip assay platform described herein may be well suited for therapeutic monitoring of drugs and metabolites at the point-of-care.

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²*Abbreviations used:* POC, point-of-care; CEDIA, cloned enzyme donor immunoassay; AEDs, antiepileptic drugs; VPA, valproic acid; CBZ, carbamazepine; PHT, phenytoin; R1, CEDIA Reagent 1; R2, CEDIA Reagent 2; EA, enzyme acceptor; ED, enzyme donor; FPIA, fluorescence polarization immunoassay; HPLC, high pressure liquid chromatography; CCD, Charged Coupled Device; CLIA, Clinical Laboratory Improvement Amendments; CLSI, Clinical and Laboratory Standards Institute; TDM, therapeutic drug monitoring; CVs, coefficients of variation; CBZ-E, carbamazepine-10,11-epoxide; HPPH, 5-(4'-Hydroxyphenyl)-5-phenylhydantoin.

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* Corresponding author. Tel.: +1-801-583-5210; Fax: +1-801-585-5361.

USPS Mail Address of the corresponding author: Dr. Jarmila Janatova, Adjunct Professor, Department of Bioengineering, University of Utah, 50 So. Central Campus Drive, Room 2480, Salt Lake City, UT 84112-9202, USA, Tel.: +1-801-583-5210, Fax: +1-801-585-5361

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Keywords

Homogeneous Immunoassay; CEDIA; Bioluminescence; ImmunoChip; Therapeutic Drug Monitoring; Antiepileptic Drugs

The introduction of arrays of immobilized biological compounds in micro-sized spots on a substrate (microarrays) has played an important role in modern biology and medicine [1,2]. Microarrays offer several advantages over conventional analytical devices. They are small, their manufacture can be automated, and only relatively small volumes of sample and assay reagents are required. These features make it possible to miniaturize microarray sensors for application at the point-of-care (POC) setting. The major advantage offered by microarrays is the parallel analysis of multiple analytes.

The development of a disposable and quantitative analytical device for the measurement of multiple analytes, such as metabolic markers of chronic diseases, therapeutic drugs and their pharmacologically active metabolites, in small sample volumes has been under study in our laboratory for several past years [3,4]. Such a device, designated as a ChemChip, could be used for the diagnosis and management of clinical conditions at POC and eventually in-home environments. In order to access analytes not readily measurable by enzyme-based reactions [3], our effort has been extended to the development of a ChemChip, based on an immunological principle, called an ImmunoChip.

Cloned Enzyme Donor Immunoassay (CEDIA) [5], one of a few homogeneous immunoassays available, appeared to be suitable for application to an immunosensor, as it uses the competitive binding assay principle, without the need for separation steps. During the first phase of an ImmunoChip project, a one-step bioluminescence-based homogeneous immunoassay platform has been developed [6,7], using a CEDIA kit to detect the antiepileptic drug (AED) valproic acid (VPA) in an aqueous matrix, using a luciferin-based, highly sensitive substrate for β -galactosidase [8].

In this communication, the optimized luminescence-based one-step CEDIA platform [7] was extended to provide detection and quantitation of three widely used AEDs in serum, namely, VPA, carbamazepine (CBZ) and phenytoin (PHT). Results generated by CEDIA were compared with values obtained by another immunoassay format, fluorescence polarization immunoassay (FPIA) [9].

The main and final objective of the project has been to design, and test, an ImmunoChip prototype, employing the previously developed immunoassay platform [7], for the simultaneous therapeutic drug monitoring (TDM) of the three AEDs present in small serum sample volumes.

To prototype microstructures for the ImmunoChip, an immunoassay-based biosensor, a novel microfabrication method [10], called "xurography," was employed. The one-step CEDIA procedure [7] together with monoclonal antibodies specific to each AED were used to simultaneously detect and quantify specific target drugs in the multi-well ImmunoChips.

An immunosensor assay platform, developed and tested in this study with three AEDs, could be extended to other areas of therapeutic drug monitoring (TDM), to provide POC monitoring of other therapeutic drugs, their active metabolites, and also metabolic markers of chronic diseases.

Materials and methods

Measurement of CBZ, PHT and VPA concentrations in serum

Reagents for the luminescence-based homogeneous immunoassay—The CEDIA reagents were a part of the CEDIA kits (Carbamazepine II, Phenytoin II, and Valproic Acid II kits) purchased from Microgenics Corporation (Fremont, CA). Commercial R2 reagents were substituted by a special R2 reagent, containing only AED-enzyme donor conjugate and no substrate. Both CEDIA reagents, R1 (anti-AED antibody, enzyme acceptor) and R2 (AED-enzyme donor conjugate), were stored at 4 °C. The Beta-Glo assay system (Promega Corp., Madison, WI) consists of two parts that are combined at the time of assay to form Beta-Glo reagent, which contains biolumigenic substrate. The Beta-Glo reagents were stored at –20 °C and thawed at room temperature (RT; about 22 °C) immediately prior to assay.

Stock solutions of AEDs—CBZ stock solution (420 µg/ml) was prepared by dissolving carbamazepine (Sigma-Aldrich Corp., St. Louis, MO) in ethanol. PHT stock solution (630 µg/ml) was obtained by dissolving phenytoin (Sigma-Aldrich Corp.) with acetone. Valproate sodium (Depacon, 500 mg/ml) was diluted with 20 mM Hepes, pH 7.5, to obtain VPA stock solution (3150 µg/ml).

Preparation of AEDs solutions in normal serum—The stock solution of each of the three AEDs (see above) was diluted sequentially with drug-free serum to yield the final concentrations of each AED (therapeutic range for each drug is shown in the brackets) as follows: 2.5, 5.0, 10, 15 and 20 µg/ml for CBZ (5 – 12 µg/ml); 5, 10, 15, 20, and 30 µg/ml for PHT (10 – 20 µg/ml); and 25, 50, 75, 100 and 150 µg/ml for VPA (50 – 100 µg/ml). The final concentrations of ethanol or acetone in the final AEDs solutions were less than 1%.

Analyses of AEDs by one-step CEDIA in the presence of serum—Drug-free serum samples spiked with AEDs were analyzed using the following protocol: CEDIA reagents (R1, R2), AED solutions and Beta-Glo reagent were incubated separately in a water bath for 1 minute at RT. Each AED solution (0.51 µl) was transferred into R1 (2.56 µl), and immediately R2 (1.93 µl) was added to the mixture of R1 and AED. Then the CEDIA reaction mixture (5 µl) was transferred to a polystyrene tube (12 × 55 mm, Elkay Products Co., Inc., Springfield, NJ) containing freshly prepared Beta-Glo reagent solution (5 µl). Luminescent signal (RLU, relative light units) was measured as a function of time at RT using a Luminometer TD 20/20 (Turner Designs, Inc., Sunnyvale, CA), at a sensitivity setting of 40.2%. The concentrations of all the three drugs (CBZ, PHT and VPA) were measured, individually, using the procedure described above.

Determination of AEDs concentrations in serum samples by CEDIA and FPIA—Residual de-identified serum samples from patients, treated with one or more AEDs, were analyzed by the CEDIA procedure (described in the previous paragraph) and FPIA. The latter was performed at ARUP Laboratories, a CLIA-certified clinical national reference laboratory,⁵ according to the manufacturer's instructions. The sources of the reagents for FPIA were as follows: CBZ and VPA (Abbott Laboratories, Abbott Park, IL), and PHT (Seradyn Inc., Indianapolis, IN).

ImmunoChip fabrication—A new rapid prototyping method [10] was used to produce the multi-AEDs ImmunoChip. A Graphtec FC5100A-75 knife plotter (Graphtec, Santa Ana, CA) was used to cut ImmunoChip patterns (Fig. 1A) in a 180 µm thick adhesive-backed vinyl film

⁵ARUP (Associated and Regional University Pathologists) Laboratories, 500 Chipeta Way, Salt Lake City, UT 84108-1221, USA; <http://www.arup-lab.com>

(Scotchlite Plus Reflective 680 series from 3M, St. Paul, MN). The holes were “weeded” out with tweezers and the “perforated” vinyl film was then transferred to 15 mm square glass cover slips (VWR Corp., West Chester, PA), which were 150 μ m thick and became the clear bottom for the 1 μ l wells. This prototype of an ImmunoChip consists of 3×3 arrays of 2.50 mm diameter holes spaced 2.00 mm apart.

Reagent dispensing [11] and lyophilization—Individual ImmunoChip wells were filled with assay constituents via a computer controlled XYZ stage with a syringe pump and solenoid dispensing system. In order to aspirate and dispense multiple reagents, six VHS-M/2 solenoid valves (The Lee Company, Westbrook, CT) were purchased and connected to a computer controlled syringe pump (P/N 0162573 PSD/2, Hamilton Co., Reno, NV) fitted with an eight distribution port valve (P/N 0200018, Hamilton Co.) and a 500 μ l syringe (P/N 81247, Hamilton Co.). A LabVIEW program (National Instruments Corp., Austin, TX) was used to control the XYZ stage moving via the computer’s serial COM port. The six dispensers were attached to a vertical stepper motor translation stage (VT-80-25-2SM, Phytron, Inc., Williston, VT). A dispensing platform that was to hold the ImmunoChips was attached to an XY horizontal stepper motor translation stage (VT-80-150-2SM, Phytron, Inc.).

To prevent evaporation during dispensing, and mixing of the constituents of one-step CEDIA procedure before lyophilization, dry ice was placed under the platform, holding ImmunoChips (Fig. 1B), to freeze a layer of each reagent as soon as it was dispensed. Reagents were delivered into the individual wells, and frozen, in separate layers sequentially (from the bottom to the top): (1) Beta-Glo solution (500 nl) containing excipients (100 mM sucrose + 50 mM glycine + 10 mM GSH), (2) R2 (188 nl) with excipients (100 mM sucrose + 50 mM glycine) and (3) R1 (250 nl) with excipients (100 mM sucrose + 50 mM glycine).

After dispensing, the ImmunoChips with frozen layers of the reagents were placed in the sample chamber of a VirTis Genesis 12 pilot plant lyophilizer (SP Industries, Inc., Gardiner, NY), at a shelf temperature of -50°C . Primary lyophilization was performed in a vacuum at less than 100 mTorr with the condenser chamber cooled to -70°C for 24 hours. The temperature of the shelf chamber was then increased in 25°C increments every 12 hours until it reached 22°C for the secondary lyophilization stage, which lasted from 12 to 24 hours.

Sample analysis and ImmunoChip imaging—Serum sample (25 μ l) containing all three AEDs was dispensed on the center of the nitrocellulose filter paper (Whatman, Middlesex, UK), which was placed above the wells of the array. Serum sample, applied to the middle, wicked along and through the filter paper and into each well, dissolving the reagents and initiating the CEDIA and bioluminescent reactions.

The bottoms of the ImmunoChips were viewed by placing them on a window above a closeup macro camera lens, which collected the luminescent signals from the individual wells and projected it onto an Andor iXon DV 885 Charged Coupled Device (CCD) (Andor Corp., Belfast, Northern Ireland). The signals were recorded in a light-tight imaging environment. The luminescent signal for each assay was recorded during a 5-minute exposure (CCD temperature = -40°C , binning = 4×4 pixels). Images were analyzed with ImageJ (rsb.info.nih.gov/ij/) and MatLab (The MathWorks, Inc., Natick, MA) systems to subtract background noise and integrate the CCD counts across the area of each well for each exposure.

Results and discussion

Performance of one-step CEDIA platform to measure AEDs in serum

Numerous methods have been used to detect and quantify VPA, CBZ, and PHT, in serum. These include spectrophotometry [12], gas chromatography [13], high performance liquid

chromatography (HPLC) [14], radioimmunoassay [15], enzyme-linked immunoassay [16], fluorescence polarization immunoassay [9], and tandem liquid chromatography-mass spectrometry [17]. The widespread use of these AEDs as mono- or combined therapy in the management of different seizure types, in combination with well-established therapeutic ranges [18], resulted in a high demand for rapid quantitation of drug concentrations in serum.

The luminescence-based one-step CEDIA platform [7] provides a sensitive, simple and relatively fast technique for detection and quantitation of CBZ, PHT and VPA in the presence of serum. Data generated with increasing concentrations of each drug are shown in Fig. 2 and illustrate how the respective calibration curves may look using this technique. The intra-assay and inter-assay imprecision data for measurement of the three AEDs using this one-step CEDIA procedure are summarized in Table 1. Intra-assay imprecision was determined by assaying serum samples at six different concentrations of each drug, five times in a single day. To define inter-assay imprecision, serum samples at six different concentrations of each drug were measured on five different days over a two-week period. The highest calculated CVs, of intra-assay and inter-assay, measured in serum samples were 6.9% and 9.3% for CBZ, 5.3% and 9.6% for PHT, and 5.6% and 9.2% for VPA.

Assays demonstrating intra-assay imprecision of 5–10% are acceptable for clinical use, according to the Clinical and Laboratory Standards Institute (CLSI) protocols [19].

Evaluation of the luminescence-based one-step CEDIA procedure

Using residual de-identified patient specimens, the results generated with the optimized onestep CEDIA platform were compared to data obtained by FPIA. Results were generated for 10 samples for CBZ and 12 samples for PHT and VPA, respectively. Each sample was divided into 5 aliquots. Every aliquot was tested 5 times in a single day. Five aliquots from the same sample were tested on different days over a two-week period. A total number of tested points was 300 for CBZ (10 samples, 5 replicates each day, run on 6 days), and 360 each for PHT and VPA, respectively.

Bland-Altman approach [20], based on graphical techniques and simple calculations, was used to describe the correlation of the data obtained by one-step CEDIA and FPIA for measurement of CBZ (Fig. 3A), PHT (Fig. 3B) and VPA (Fig. 3C). Agreement of the values obtained by the two methods can be summarized by calculating the bias, estimated by the mean difference d and the standard deviation of the differences (s). If there is no consistent bias, most of the differences are expected to lie between $d-2s$ and $d+2s$ and follow a normal distribution. According to these criteria, the results depicted in Fig. 3 demonstrate that the data obtained for the three AEDs by one-step CEDIA agree well with those measured by FPIA.

Specificity of the one-step CEDIA for measuring CBZ and PHT

Active metabolites of CBZ and PHT were specifically considered to evaluate the potential for positive bias in results due to the well-recognized potential of these metabolites to cross-react with detection antibodies in immunoassays. Carbamazepine-10,11-epoxide (CBZ-E) is the active metabolite of CBZ, both having similar structures. Specificity of the one-step CEDIA for measuring CBZ was tested with patient serum specimens containing CBZ and CBZ-E (Table 2). Our results indicate that when the CBZ-E concentration is higher than 10 $\mu\text{g/ml}$, its cross-reactivity is evident, and total CBZ concentrations increase. However, the assay has low cross reactivity to CBZ-E, when this metabolite is present in low concentration (CBZ-E less than 5 $\mu\text{g/ml}$). Accumulation of CBZ-E is patient specific, so significantly accumulated levels of CBZ-E above 5 $\mu\text{g/ml}$ may falsely influence the CBZ results and should be viewed with caution. The main metabolite of PHT, 5-(4'-Hydroxyphenyl)-5-phenylhydantoin (HPPH), is exhibiting a 5% cross-reactivity at its concentration of 500 $\mu\text{g/ml}$ in the CEDIA Phenytoin II

assay, as tested at the Microgenics Corp. [21]. Further evaluation of the specificity and accuracy of the ImmunoChip will require comparison of this method to a chromatographic and/or mass spectrometric methodology.

Simultaneous detection of multiple AEDs using multi-well ImmunoChip

Upon demonstrating that the one-step CEDIA procedure: (1) could quantitatively detect individual AEDs in serum samples (Fig. 2), (2) correlates well with a comparison method (Fig. 3), we then investigated the detection of multiple AEDs present in the same serum sample using a multi-analyte multi-well ImmunoChip (Fig. 4). Within each row of the three wells (Fig. 1A; the rows from top to bottom: 1, VPA; 2, PHT; and 3, CBZ), assay reagents specific to a particular drug were dispensed, as illustrated in Fig. 1B, and lyophilized, as described in the Materials and methods.

Serum samples were assayed in triplicate for the presence of the three AEDs using an ImmunoChip. The luminescence patterns that resulted when serum samples containing a mixture of the three AEDs were dispensed over the ImmunoChip through a bonded nitrocellulose filter paper is presented in Fig. 4A. The concentrations ($\mu\text{g/ml}$) of respective AED in serum samples are listed in the table below each ImmunoChip.

As illustrated in Fig. 4A, assays of AEDs on each ImmunoChip produced different luminescent signal intensities at the appropriate site where particular capture antibodies were dispensed. A comparison of six ImmunoChips (from left to right in Fig. 4A) reveals that the light intensity in each well increases with higher concentrations of the respective drug tested. The calibration curves presented in Fig. 4B demonstrate the feasibility of detection and quantitation of the three AEDs simultaneously in a multi-AEDs ImmunoChip.

Performance of an ImmunoChip prototype

The current study fulfilled one of our major goals. It demonstrated the feasibility to build a multi-analyte multi-well immunosensor intended for the TDM at the POC setting. As with each prototype, there were still some limitations identified. Unlike a one-step CEDIA in the fluid phase, an ImmunoChip prototype has had a relatively large CV between different batches. This appears to be, at least partly, due to the small reagents volumes used in this prototype, as the assay reagents are unlikely to coat each well uniformly, and insufficient wetting of the sides may concentrate reagents in that area. This could also contribute to the inhomogeneous signal detected in each well (Fig. 4A). There also may be variations, from batch to batch, with respect to a starting point of the CEDIA and bioluminescent reactions, which is assumed to be at the end of the rehydration of lyophilized assay reagents with serum samples. Under the experimental settings used in this study, there was a lapse of about 30 sec between dispensing serum sample and starting signal recording by CCD camera. The time required for reconstitution, however, depends on the condition of lyophilized reagents, such as the shape, homogeneous form, or excipient types and their concentrations used. Taken these factors into consideration, the starting point of reaction taking place in different wells of the same ImmunoChip or wells on different ImmunoChips may have variations, resulting in relatively larger differences of the signal values. To further improve its utility, the ImmunoChip, developed and used in this study, requires further optimization and testing before it could be made more widely available.

Conclusions

The purpose of this study was to use array technology to make an ImmunoChip for the rapid and simultaneous analysis of multiple AEDs. The luminescence-based one-step CEDIA procedure [7], which serves as the ImmunoChip platform, requires no separation steps,

consumes small quantities of reagents and samples, and has short analysis time with easy handling. This platform provides a precise, and relatively fast technique for detection and quantitation of CBZ, PHT and VPA in serum samples simultaneously. The total analysis time for each serum sample was 30 sec for the sample dispensing, and 5 min for an exposure to the CCD camera. For each serum sample, three AEDs could be measured in triplicate on one ImmunoChip. CEDIA results, generated using residual patient specimens, correlated well with values obtained by FPIA. In light of the demand for diagnostic assays that can be carried out in small laboratories, doctors' offices, and point-of-care, the ImmunoChip opens the door to miniaturized biodiagnostics.

This work details several novel points related to current microarray assays. First, this is one of the first studies to describe the use of a microarray platform to achieve the simultaneous detection of AEDs in serum. Second, the rapid nature of the assay system offers considerable advantages over conventional microarrays that require long incubation times and washing steps. Finally, microfabrication using xurography and noncontact dispensers has the capacity to produce arrays of drugs in small spots. This microfabrication method showed great potential for rapidly prototyping microstructures to perform accurate sample delivery and reagent mixing. In addition, its short processing time and low cost material requirement make it feasible for high throughput automated fabrication. This multi-analyte ImmunoChip has broad implications for a wide range of substances, such as other therapeutic drugs, drugs of abuse, peptide hormones and metabolic markers of chronic diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. ImmunoChip design and dispensing of the assay reagents. (A) A 3×3 multi-well ImmunoChip set-up and dimensions. (B) Dry ice was placed under the platform (XYZ stage), supporting a number of ImmunoChips, to achieve freezing of the assay reagents as soon as they were dispensed. In each well, the assay's reagents were dispensed in the order (from the bottom to the top): (1) Beta-Glo solution with excipients (500 nl); (2) R2, containing ED-AED conjugate, and excipients (188 nl); and (3) R1, consisting of anti-AED antibody and EA, and excipients (250 nl). The reagents specific to the three AEDs were dispensed in individual rows (VPA, PHT, CBZ) in triplicate. ED, enzyme donor and EA, enzyme acceptor, are two genetically engineered fragments of β -galactosidase, essential for the CEDIA reaction. Ex, excipients present in the individual reagents are described in Materials and methods.

Fig. 2.

Determination of VPA, CBZ and PHT concentrations, in the presence of serum, by onestep CEDIA with Beta-Glo Assay System. In this set of experiments, drug-free normal serum was spiked with VPA, CBZ or PHT, as described in Materials and methods. Calibration curves represent slopes of kinetic curves (120~180 seconds) as a function of AED initial concentration. Crossed bars designate therapeutic ranges of the respective AED. Data are based on 4 replications for each concentration of respective drug measured (mean $\pm$ SD). CBZ (open diamond) and PHT (open square) employ lower X-axis; VPA (open circle) uses upper X-axis. RLU: Relative light unit(s).

Fig. 3. Difference between the data, obtained by one-step CEDIA and FPIA, against their mean for (A) CBZ, (B) PHT, and (C) VPA. The residual de-identified patients' specimens were analyzed by both the one-step CEDIA procedure, and FPIA. The results were evaluated using the Bland-Altman approach [20].

Fig. 4. Simultaneous detection of three AEDs using multi-well ImmunoChips. Serum samples, used in testing the performance of ImmunoChip, were prepared from a drug-free normal serum, to which a mixture of VPA, CBZ and PHT, was added at the concentrations ($\mu\text{g/ml}$), listed in the table below the CCD images of ImmunoChips. (A) CCD images for VPA, PHT and CBZ assay performed on ImmunoChips. Serum containing different concentrations of the three AEDs rehydrated the lyophilized reagents (Fig. 1B) in each well, and initiated CEDIA and bioluminescence reactions, resulting in luminescent signal. (B) Calibration curves represent integrated CCD counts from a 5-minute exposures as a function of AED concentrations. CBZ (open diamond) and PHT (open square) employ lower X-axis; VPA (open circle) uses upper X-axis.

Intra-assay and inter-assay imprecision data from measurements of CBZ, PHT and VPA, by one-step CEDIA in the presence of normal human serum.

Table 1

Conc. (µg/ml)	CBZ ^a		Inter CV(%)	Conc. (µg/ml)	PHT ^b		Inter CV(%)	Conc. (µg/ml)	VPA ^c		Inter CV(%)
	Intra CV(%)				Intra CV(%)				Intra CV(%)		
0	5.7	9.3	0	0	4.5	7.3	0	0	5.6	5.3	
2.5	6.9	5.7	5	5	4.1	2.4	25	25	4.3	9.2	
5	2.9	6.6	10	10	1.5	9.6	50	50	1.7	4.1	
10	2.6	1.2	15	15	3.3	3.1	75	75	3.0	2.3	
15	3.4	8.6	20	20	5.3	8.6	100	100	2.1	2.4	
20	2.9	4.2	30	30	1.7	7.2	150	150	2.9	1.4	

^{a,b,c} Normal serum, on day zero, was spiked with either CBZ, PHT or VPA stock solution, to reach the final concentration of: 0, 2.5, 5, 10, 15 and 20 µg/ml for CBZ; 0, 5, 10, 15, 20 and 30 µg/ml for PHT; and 0, 25, 50, 75, 100 and 150 µg/ml for VPA, respectively. The aliquots of the individual samples, each containing the respective drug at the given concentration, were tested 5 times in a single day, on 6 nonconsecutive days within a two-week period. **n** = 120 for all the three AEDs.

Cross-reactivity detected by one-step CEDIA in the patient serum sample during the measurement of CBZ in the presence of CBZ-E.

Table 2

CBZ(µg/ml)	5	5	5	9	9	9	16	16	16	16
CBZ-E (µg/ml)	5	10	20	5	10	20	5	10	20	20
Cross-reactivity (%) ^a	2	12	21	3	8	13	2	4	5	5

^a $\text{Cross-reactivity (\%)} = \frac{(\text{Value of } ([\text{CBZ}] + [\text{CBZ-E}]) - \text{Value of } [\text{CBZ}])}{\text{Value of } [\text{CBZ}]} \times 100\%$