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ARTICLES

Comparison of Differential Pulse Voltammetry (DPV)—a New Method of Carbamazepine Analysis—with Fluorescence Polarization Immunoassay (FPIA)[1]

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Abstract—Carbamazepine is a widely used anti-epileptic drug with narrow therapeutic range. Many methods have been developed for monitoring the serum drug level. Differential pulse voltammetry (**DPV**), an electrochemical method advantaged by simple, inexpensive, and relatively short analysis time, has recently been developed for carbamazepine detection. We used a newly developed DPV method with glassy carbon as a working electrode to determine the carbamazepine level. The performance of DPV is compared with the widely used Fluorescence Polarization Immunoassay (**FPIA**) technique in precision, accuracy, linearity and detection limit. The precision, linearity and accuracy of the DPV and the FPIA techniques were comparable at most clinical used levels. The detection limit was 1 ug/mL for the DPV technique and 0.5 μg/mL for the FPIA technique. The performance of the DPV technique was within the FDA guidelines for bioanalytical methods, which ensures the clinical applicability of the DPV technique. The DPV technique may have the potential to be a good alternative for carbamazepine analysis.

Keywords: electro-chemical method, differential pulse voltammetry, drug monitoring, immunoassay, carbamazepine.

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Carbamazepine, 5H-Dibenz[b,f]azepine-5-carboxamide, is an anti-epileptic drug widely used for treatment of simple and complex partial seizures, trigeminal neuralgia, and bipolar affective disorder. It selectively inhibits the high frequency epileptic foci without affecting the normal neural activity by blocking sodium channels [1–3]. The usual adult therapeutic levels are between 4 and 12 μg/mL [4]. Serious side effects, such as coma, seizures, respiratory failure and cardiac conduction defects, develop more frequently when the serum level of carbamazepine is higher than 15 ug/mL [5–7]. In one report, approximately 13% of patients died after massive carbamazepine overdose [8]. Thus, it is critical to monitor the serum drug level.

Many analytic techniques have been applied in the determination of the carbamazepine level, such as high performance liquid chromatography (HPLC), gas chromatography, gas chromatography combined with mass spectrometry and fluorescence polarization immunoassay (FPIA) [9–14]. Though the HPLC technique has the advantages of rapid run times and excellent resolution, the expense of the equipment and the high maintenance required to keep it running optimally limit the application in clinical labs. Gas chromatography has the advantage of very high resolving power provided by the capillary columns that are typically used. However, the compounds must be not only sufficiently volatile to be introduced in the gas phase when the sample is injected into the GC but also stable so that they do not degrade at the temperatures required to vaporize them. As a consequence, gas chromatography is generally limited to non-polar and slightly polar molecules, which make up about 20% of the known organic molecules [15]. In the clinical laboratory, immunoassays are more widely used to monitor the carbamazepine concentrations in serum or plasma due to the simplicity of use [16]. Of these immunoassays, FPIA is accepted in most clinical labs because it provides accurate and sensitive measurement of small toxicology analytes, such as therapeutic drugs, narcotics, and some hormones, than others.

Electrochemical methods have been proved to be sensitive and reliable for detection of several electroactive drugs such as abacavir and sildenafil citrate. In comparison with other analytical techniques, electrochemical techniques are simple, inexpensive, and have

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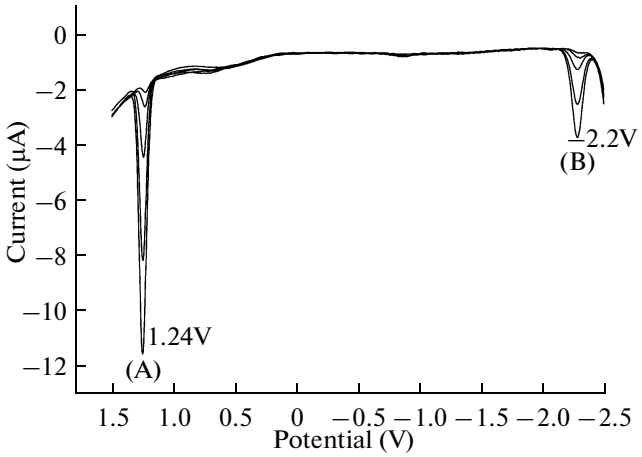

Fig. 1. Typical DPV potential of carbamazepine occurs at +1.24 V (A) and -2.2V (B).

relatively short analysis time [17–19]. Only a few studies have investigated carbamazepine with electrochemical techniques such as cyclic voltammetry, differential pulse polarography and coulometry [20, 21] and none of them compared the electrochemical technique with the widely used FPIA technique. In this study, we evaluated the performance of differential pulse voltammetry (DPV), one of the electrochemical methods, in carbamazepine analysis and compared it with the performance of FPIA.

EXPERIMENTAL

Sample preparation. Pure carbamazepine in powder form was purchased from MP Biomedicals, Inc. (Germany). Tetrabutylammonium Perchlorate (TBAP) was obtained from Toyo Kasei Kogyo Co., Ltd. (Japan). The tested samples were prepared in 7 different concentrations (0, 2, 4, 8, 12, 20 and 23.6 ug/mL) by dissolving carbamazepine in 0.1 M TBAP/ acetonitrile. All the other reagents used were of analytical grade. Doubly distilled water was obtained by purification through a Millipore water system.

Voltammetric measurements. Differential pulse voltammetry (DPV) was performed in a standard three-electrode electrochemical cell, in which a BAS glassy carbon electrode (area = 0.07 cm^2) was used as a working electrode, the glassy carbon electrode was polished with 0.05 μm alumina on Buehler felt pads and was ultrasonicated for 2 min to remove the alumina residue and a platinum wire was used as the auxiliary electrode. For analytical application, the following parameters were employed: DPV pulse amplitude, 50 mV; pulse width: 0.05 (s); sample width: 0.0167 (s); pulse period: 0.2 (s); scan rate: 20 mV/s^{-1}. DPV was performed with a CHI421A (CHI Model 660 series electroanalytical workstation). All cell potentials were measured by using a homemade Ag/AgCl, KCl (sat.) reference electrode. The buffer solution contained 0.1 M $(C_4H_9)_4NClO_4$ to support electrolytes in CH_3CN. During the voltammetric measurement a constant flux of N_2 was kept over the solution surface in order to avoid the diffusion of atmospheric oxygen into the solution of carbamazepine.

With DPV techniques, the current is measured at two points for each pulse: just before the application of the pulse and at the end of the pulse. The difference between the two measured currents for each pulse is plotted against the base potential. We used a series of fixed amplitude potential pulses and increased the base potential slowly. The carbamazepine has two reducible sites protic solvents, the stibene based ethylenic bond and the amide's CO double bond. The anodic peaks exhibited is attributed to the phenolic OH group in the carbamazepine structure. One OH group in each structure is oxidized to imnoquinone group with the involvement of one electron and one proton. The typical current-potential curve of carbamazepine is shown in Fig. 1. The +1.24 V peak was adapted in our study for analysis since the peak is higher than that of −2.2 V. The concentration-related currents are represented in Fig. 2. According to the calibration curve shown in Fig. 2, the concentration of the tested samples can be calculated from the current.

FPIA technique. The FPIA technique was performed by a qualified technician using an Abbott TDx analyzer with standard operating procedure. All the required reagents were purchased from Abbott Laboratories (Chicago, IL, USA).

Method validation. *Precision*. Tested samples of 4, 8, 12 ug/mL of carbamazepine were analyzed 20 times by the DPV technique and four tests per run for 5 runs by the FPIA technique to evaluate the between-run variation. Comparison of the with-in run variation is not possible since the DPV technique can only test one sample per run. The results are expressed as coefficient of variation (CV, %).

Accuracy. Tested samples of 2, 4, 8, 12, 20 and 23.6 ug/mL of carbamazepine were analyzed 5 times by the DPV technique and FPIA technique, respectively. The results are expressed in bias, the difference between the mean of each concentration and the reference concentration, and in the percent recovery, which is calculated by dividing the bias by the reference concentration. The positive bias indicates that the mean concentration is higher than the reference concentration, whereas the negative bias indicates that the mean concentration is lower than the reference concentration.

Linearity. The 7 different concentrations of the tested samples were tested three times with each technique. The mean of the three results was correlated with the theoretical concentration of each sample. The results are expressed as the coefficient of determination (R^2, *RSQ*).

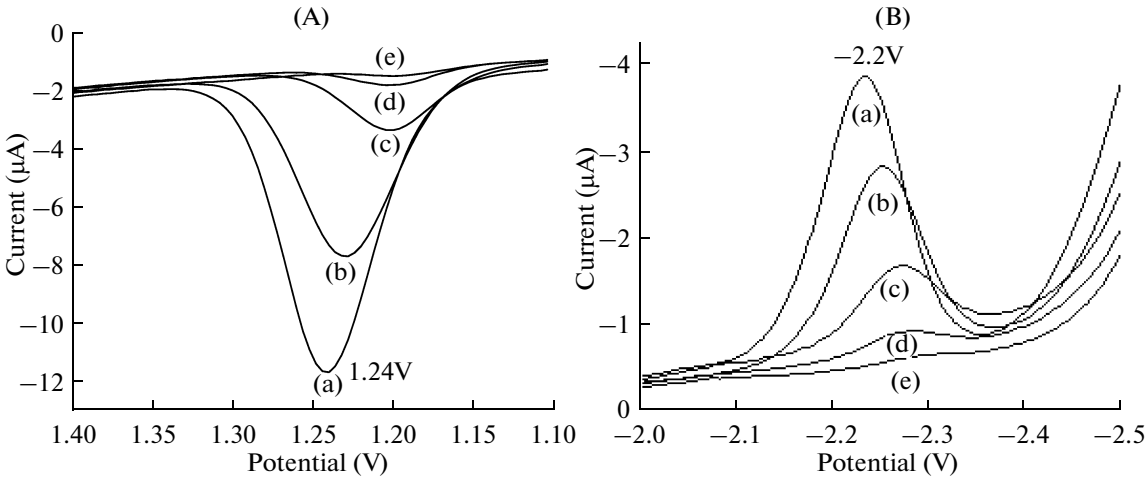

Fig. 2. DPV voltammograms of carbamazepine under optimum conditions: (*a*) 2.0×10^{-4} M; (*b*) 1.0×10^{-4} M; (*c*) 4.0×10^{-5} M; (*d*) 1.0×10^{-5} M; (*e*) 1.0×10^{-6} M at +1.24 V (A) and -2.2 V (B).

Detection limit. The tested sample with 0 ug/mL was analyzed 10 times with each technique. The tested sample of 2 ug/mL was diluted serially and analyzed 10 times with each technique as well. Then the mean and the CV of the results were calculated. The minimal concentrations indicating that the mean − 3SD was higher than the mean + 3SD of the blank results were interpreted as the detection limit of each technique.

Statistical analysis. According to the FDA guidelines for bioanalytical methods [22], it is recommended that the accuracy and precision of the assay should be within 15% of the actual value except at the lower limit of quantitation (**LLOQ**), where it should not deviate by more than 20%.

Using Pearson's correlation, a linear relationship was calculated between the results obtained with the FPIA technique and the reference concentration as well as between the results obtained with the DPV technique and the reference concentration. A *t*-test was used for the calculation of the statistical significance of *r* (Pearson's Correlation Coefficient). A *P* value of less than 0.05 was considered to be statistically significant. The coefficient of determination (R^2, *RSQ*) was also calculated.

Agreement between the two methods and agreement of each method with the reference value concentration are presented using the Bland–Altman approach, by plotting the percent difference between the two methods versus the mean concentration determined. We considered outliers values to be outside two standard deviations from the mean.

RESULTS AND DISCUSSION

The results of the precision, accuracy, linearity and the detection limit of the DPV and the FPIA techniques are listed in table. The coefficient of variation for the DPV technique at the concentrations of 4, 8, and 12 ug/mL was 3.27, 2.35 and 2.87%, respectively. The coefficient of variation for the FPIA technique at the concentrations of 4, 8, and 12 ug/mL was 3.26, 2.65 and 2.44%, respectively. The precision of the DPV and FPIA techniques was comparable.

The bias for the DPV technique at the concentrations of 2, 4, 8, 12, 20, and 23.6 ug/mL was −0.38 ug/mL (−18.89%), −0.30 ug/mL (−7.38%), 0.53 ug/mL (6.62%), 0.59 ug/mL (4.88%), −0.29 ug/mL (−1.44%) and −0.14 ug/mL (−0.61%), respectively. The bias for the FPIA technique at the concentrations of 2, 4, 8, 12, 20, and 23.6 ug/mL was −0.03 ug/mL (−1.4%), −0.13 ug/mL (−3.28%), 0.19 ug/mL (2.32%), −0.52 ug/mL (−4.31%), 1.55 ug/mL (7.76%) and 0.03 ug/mL (0.13%), respectively. The performance of both techniques was within the FDA guidelines for bioanalytical methods [22]. Although the bias in the DPV technique seems bigger than that in the FPIA technique, it ensures the clinical applicability of the DPV technique.

In comparison with the reference samples, the RSQ was 0.993 for the DPV technique and 0.994 for the FPIA technique. The *t*-test for the statistical significance of *r* (Pearson's Correlation Coefficient) in both the DPV and FPIA techniques was $P < 0.001$. The detection limit was 1 ug/mL for the DPV technique and 0.5 ug/mL for the FPIA technique.

The Bland–Altman approach for both the DPV and reference value concentration (Fig. 3) and the FPIA and reference value concentration (Fig. 4) showed good agreement.

Electrochemical analysis determines the concentration of an analyte by measuring changes in current in response to an applied voltage with respect to time using an electrode. According to Faraday's Law, the change reflects the amount of analytes that undergo oxidation or reduction. The most commonly used electrochemical techniques are constant potential

The results for the precision, accuracy, linearity and detection limit of the DPV and FPIA techniques

	DPV	FPIA
Precision (CV, %)		
4 ug/mL	3.27	3.26
8 ug/mL	2.35	2.65
12 ug/mL	2.87	2.44
Accuracy (Bias, ug/mL; %)		
2 ug/mL	−0.38 (−18.89%)	−0.03 (−1.40%)
4 ug/mL	−0.30 (−7.38%)	−0.13 (−3.28%)
8 ug/mL	0.53 (6.62%)	0.19 (2.32%)
12 ug/mL	0.59 (4.88%)	−0.52 (−4.31%)
20 ug/mL	−0.29 (−1.44%)	1.55 (7.76%)
23.6 ug/mL	−0.14 (−0.61%)	0.03 (0.13%)
Linearity (RSQ)	0.993	0.994
Detection limit (ug/mL)	1	0.5

amperometry, high-speed chronoamperometry, fast cyclic voltammetry (**FCV**) and differential pulse voltammetry (DPV). Constant potential amperometry monitors the change of current over time by applying a constant potential. The time resolution of constant potential amperometry is very good. However, the chemical selectivity is limited because all species with oxidation potentials below the applied voltage will be oxidized and thus will change the current. Chronoamperometry is a square-wave-pulsed voltammetric technique. By calculating the ratio of the peak oxidation current versus the peak reduction current, some limited information about the identity of the analytes can be obtained. Fast cyclic voltammetry is a linear sweep voltammetric technique, which measures the current response over a range of potentials. Additional information about the electrolyzed species can be obtained by using a background-subtracted voltammogram. Fast cyclic voltammetry is, as its name implies, relatively fast (about 100 ms/scan). However, the signal-to-noise ratio decreases as the scan rate increases. Differential pulse voltammetry is a hybrid form of linear sweep and pulsed voltammetries. Though the scan rates are relatively slow (200 seconds/scan, about 20 mV/s), since multiple pulses in the waveform are required, the selectivity of differential pulse voltammetry is higher than that of other types of voltammetry.

According to our results, the precision and linearity of the DPV technique are as good as those of the FPIA

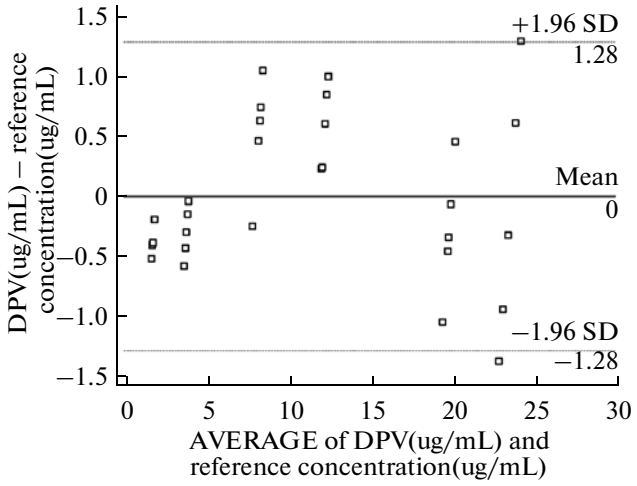

Fig. 3. Bland-Altman plots of the DPV results and reference value concentrations. The solid line represents the mean difference, and the dashed line represents 1.96 SD.

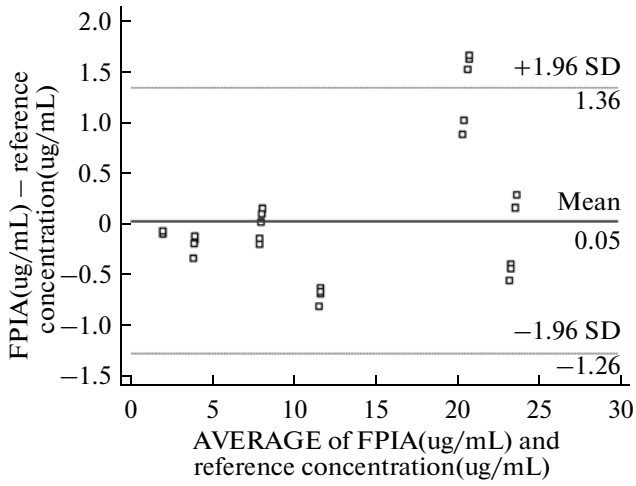

Fig. 4. Bland-Altman plots of the FPIA results and reference value concentrations. The solid line represents the mean difference, and the dashed line represents 1.96 SD.

technique. Though the accuracy of the DPV technique is not as good as that of the FPIA technique at the level of 2 ng/mL, it is still within the 20% limit of the FDA guidelines for the lower limit of quantitation (**LLOQ**) [22], which allows higher bias than in other concentrations due to the nature of the higher variation of analytic techniques and the lower effect on clinical diagnosis at the lower concentration. Thus, this disadvantage should have no adverse effect in clinical application. At the other concentrations that play more important roles in clinical monitoring, the bias recommendation of the FDA guidelines is 15%. The accuracy of the DPV technique is not only comparable with that of the FPIA technique, but is also within the FDA guidelines at the concentrations between 4 ug/mL and 23.6 ug/mL. Similar condition is also noted for the detection limit. The detection limit of the DPV technique was higher than that of the FPIA technique. However, in clinical application, the therapeutic level of carbamazepine is 4–12 ug/mL. Whether the detection limit is 0.5 ug/mL (for the FPIA technique) or 1 ug/mL (for the DPV technique) makes no apparent difference in clinical application.

In addition to the aforementioned advantages, the electrochemical methods also advantaged by the required instrumentation is relatively inexpensive, simple, and the required reagents are of longer duration for the validity. The methods are capable of determining elements accurately at trace and ultra-trace levels [23] in relatively short analysis time so as to continuously monitor the serum level [24]. Besides, electrochemical methods have the potential ability to determine multi-elements and drugs at the same time [25, 26]. Although the possibility of simultaneous electrochemical determination of structurally similar isomers may be problematic for DPV in traditional electrodes, with the advancement of electrodes, such as the recently developed multi-wall carbon nanotubes modified electrodes, the ability of simultaneous determination of isomers have been proved in dopamine and serotonin [27], nitrophenol isomers [28], hydroquinone, catechol and resorcinol [29, 30], and catechol and hydroquinone [31]. Almost all substance contained N, S, O would interfere with DPV result. Pre-treatment of test samples is therefore very important. With well pre-treatment of samples, DPV can determine the target drugs in high sensitivity. Erk et al. used DPV in the determination of moxifloxacin in human plasma, which was pre-treated with acetonitrile to precipitate human plasma proteins. Good recovery was achieved in different drug concentrations [32].

Immunoassays depend mainly on antibody-antigen reactions. They are widely applied in the clinical laboratory, and include the radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), fluorescence polarization immunoassays (FPIA) and cloned enzyme donor immunoassay (CEDIA). However, it is well known that the stability of antibodies may be affected by many factors, such as temperature, repeated freezing and thawing, dilution and freshness. Thus the storage and handling of antibodies requires special care. In contrast, the reagents that are required for DPV analysis are relatively stable. In addition, the cross-reaction with substances that have similar chemical structures may result in erroneous results [33–35]. Kozer et al. had reported a case who was misdiagnosed as amphetamine abuse by positive urinary drug screen with FPIA because he took overdosed mexiletine, an antiarrhythmic agent, in a suicide attempt [33]. In other words, the immunoassays in commercial use may not be able to accurately distinguish some compounds within a class [34, 36]. Therefore, therapeutic drug monitoring with immunoassay may require the antibodies with low cross-reactivity to the structurally similar species in cases with simultaneous prescription of drugs in the same class or in cases where the functioning metabolites have chemical structures similar to those of the original compounds. However, these high-quality antibodies with better specificity may increased the expense of assaying.

About 75% of carbamazepine in plasma is bound to protein [37]. After it is metabolized extensively by the hepatic mixed-function oxidase system, the primary metabolite of carbamazepine is 10,11-epoxide, which is quite stable, pharmacologically active, and found in the plasma and tissues. 10,11-epoxide is then metabolized further to 10,11-dihydroxide. 10,11-dihydroxide may be eliminated in the urine or it may be conjugated with glucuronic acid [38]. Routine monitoring of the 10,11-epoxide metabolite is recommended during carbamazepine therapy, as serum carbamazepine levels alone may not be adequate to detect toxicity in some patients. Total serum carbamazepine-10,11-epoxide levels above 9 umol/L are associated with greater side effects than lower levels [4, 39]. Since the electrochemical technique with multi-wall carbon nanotube-modified electrodes can determine structure-similar isomers simultaneously, it may have the potency to measure carbamazepine as well as the pharmacologically active metabolites by interpreting different peaks in the DPV. Evaluation of the ability of DPV to detect the level of carbamazepine-10,11-epoxide itself and simultaneously with the carbamazepine level is now ongoing.

In this study, we used the reference concentration sample for DPV validation. However, in clinical application, the tested material, such as serum, contains many other elements besides the pure chemical material that we used. Before the DPV technique can be applied in the clinical monitoring of carbamazepine, it needs further evaluation. Based on the encouraging results of this study, we plan to use serum as a test sample in future research.

The performance of the DVP technique for carbamazepine evaluation is comparable with that of the FPIA technique and is also within the recommended

FDA guidelines. With the advantages of lower cost, shorter analysis time, and relatively less sensitivity to environmental change, such as temperature and acidity, the DVP technique has the potential for concurrent functional metabolite analysis and continuous monitoring. In conclusion, we consider the DVP technique may be a good alternative for carbamazepine analysis.

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