Validation of a Free Phenytoin Assay on Cobas c501 Analyzer Using Calibrators From Cobas Integra Free Phenytoin Assay by Comparing its Performance With Fluorescence Polarization Immunoassay for Free Phenytoin on the TDx Analyzer

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For many years, fluorescence polarization immunoassay (FPIA) on the TDx analyzer has been used for determination of free phenytoin concentration. Recently Abbott Laboratories decided to discontinue the TDx analyzer and related assays on this analyzer. Free phenytoin assay is also available from Roche Diagnostics for application on the Cobas Integra analyzer (fluorescence polarization assay) but not on Cobas c510 analyzer. Free phenytoin calibrators from the Cobas Integra free phenytoin assay and the reagents from the KIMS phenytoin assay were used for the determination of free phenytoin on the Cobas

c501 analyzer. The intra-run and inter-run precisions were both <7.2%. The assay was linear from 0.2 to 4 $\mu\text{g/ml}$. The free phenytoin assay on the Cobas c501 was compared with the FPIA assay on the TDx analyzer using sera from 25 patients receiving phenytoin (phenytoin concentration between 0.3 and 3.7 $\mu\text{g/ml}$). The following regression equation was observed: $y = 0.9899x + 0.0408$ ($r = 0.98$, $n = 25$). In conclusion, the free phenytoin assay on the Cobas c501 analyzer is a valid alternative to free phenytoin assay on the TDx analyzer. *J. Clin. Lab. Anal.* 27:1–4, 2013. © 2012 Wiley Periodicals, Inc.

Key words: free phenytoin; fluorescence polarization; Cobas c501

INTRODUCTION

Phenytoin is a classical anticonvulsant, which is considered as the drug of choice for treating patient with partial and generalized tonic-clonic seizure. Phenytoin is 90% bound to serum proteins mainly albumin and the free phenytoin, which is approximately 10% of the total phenytoin concentration, is the pharmacologically active fraction. Kilpatrick et al. reported that unbound phenytoin concentration reflected the clinical status of a patient equally or better than the total phenytoin concentration (1). Booker and Darcey reported that free phenytoin concentrations correlated better with toxicity and the authors observed no phenytoin toxicity in any patients with free phenytoin concentration of 1.5 $\mu\text{g/ml}$ or less (2). For a patient with greatly decreased albumin level, free phenytoin concentration is a better indicator of clinical outcome (3).

The binding of phenytoin to serum albumin can be altered significantly in uremia. The lower protein-binding

capacity of phenytoin in uremia can be related to hypoalbuminemia, structural modification of albumin, and accumulation of uremic compound in blood that displaces phenytoin from protein-binding sites (4–6). Monitoring free phenytoin concentration is clinically important in patients with liver disease and hypoalbuminemia in order to avoid drug toxicity. Lindow and Wijdicks described severe phenytoin toxicity associated with hypoalbuminemia in critically ill patients due to elevated free phenytoin concentrations (7). Zielmann et al. reported that in 76% of 38 trauma patients they studied, the free phenytoin fraction

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Received 16 February 2012; Accepted 15 June 2012

DOI 10.1002/jcla.21536

Published online in Wiley Online Library (wileyonlinelibrary.com).

was increased to as high as 24% compared to 10% free levels observed in otherwise healthy subjects. The authors recommended monitoring of free phenytoin in trauma patients (8). Hong et al. recommended monitoring free phenytoin concentrations in all patients with hypoalbuminemia. The authors further commented that the free phenytoin level measured directly is superior to the calculated free concentration using Sheiner–Tozer equation (9). Iwamoto et al. also reiterated the need of free phenytoin monitoring in patients receiving phenytoin monotherapy because free phenytoin fraction was significantly influenced by age, mean creatinine clearance, and serum albumin concentrations in the patient population they studied (10). Burt et al. commented that total phenytoin is not as reliable as free phenytoin as a clinical indicator for therapeutic and nontherapeutic concentrations. Therefore, the authors recommended that therapeutic drug monitoring of phenytoin should be conducted as monitoring of free phenytoin concentration (11).

In our clinical laboratory, we offer tests for free phenytoin and free valproic acid but free phenytoin is the most frequently ordered test. Soldin reported that in his personal experience, free phenytoin is the most requested free drug level by clinicians (12). For last 20 years, we used the fluorescence polarization immunoassay (FPIA) for the determination of free phenytoin in our clinical laboratory. However, recently Abbott Laboratories, the manufacturer of FPIA assay has announce that both free phenytoin assay and the TDx analyzer will no longer be available. Therefore, we investigated the possibility of alternative method for free phenytoin determination in our laboratory. Free phenytoin assay is available from the Roche Diagnostics (Indianapolis, IN) for application on the Cobas Integra analyzer but not on the Cobas c501 analyzer. We explored the possibility of adopting total phenytoin assay for application on the Cobas c501 analyzer for determination of free phenytoin using calibrators of free phenytoin assay on the Cobas Integra analyzer. We also compared free phenytoin concentrations determined by using the FPIA assay on TDx analyzer with values obtained by the Cobas c501 analyzer.

MATERIALS AND METHODS

The FPIA kits for phenytoin, free phenytoin calibrators, and TDx analyzer were obtained from the Abbott Laboratories (Abbott Park, Chicago, IL). The phenytoin assay (PHNY2) for application on the Cobas c311, c501/502, and free phenytoin calibrators for use on the Cobas Integra system were obtained from the Roche Diagnostics (Indianapolis, IN). The filtration device for preparing protein-free ultrafiltrate (Centrifree Micropartition System, Millipore Catalog # 4104) was purchased from

TABLE 1. Parameters for Free Phenytoin Test Using Cobas c501 Analyzer

Analyze	
Assay	2 Point End
Reaction time	10
Measurement point 1	16
Measurement point 2	60
Wavelength (2 nd)	800
Wavelength (Primary)	600
Sample volume	10.0
R1 reagent volume	93
R2 reagent volume	93
Absorbance limit	32,000
Assay direction	Increase
Cell detergent	Detergent 1
Stirring level	2
Calibration	
Calibration type	RCM
Point	6
Span	6
Range	
Unit	µg/ml
Other	
Standard (1) concentration	0.00
Standard (2) concentration	0.50
Standard (3) concentration	1.00
Standard (4) concentration	2.00
Standard (5) concentration	3.00
Standard (6) concentration	4.00
Standard sample volume	10.0

Millipore Corporation (Billerica, MA). The cutoff concentration of the filter in the device is 30,000 Da.

The free phenytoin calibration system for the application on the Cobas Integra analyzer utilizes six calibrators with phenytoin concentration ranging from 0.0 to 4.0 µg/ml. The phenytoin assay (PHNT2) for the application on the Cobas c501 analyzer is a KIMS (kinetic interaction of microparticles in solution) assay. This assay utilizes a mouse monoclonal antibody against phenytoin. The assay was linear for a serum total phenytoin concentration between 0.8 and 40.0 µg/ml. The free phenytoin assay was developed on the Cobas c501 analyzer using PHNT2 reagent and free phenytoin calibrators using parameters described in Table 1.

Protein-free ultrafiltrate of serum was used for measuring the free phenytoin concentration. For this purpose, approximately 0.8 ml of serum was centrifuged using Centrifree Micropartition System for 20 min at the room temperature at $1,500 \times g$. After centrifugation, the protein-free ultrafiltrate was inspected visually to ensure that it was colorless (a yellow tinge usually appears in the ultrafiltrate if a little portion of serum is leaked to membrane failure). Then, free phenytoin concentration was measured using the protein-free ultrafiltrate.

We determined intra-run and inter-run precision using free phenytoin controls obtained from the Roche

Fig. 1. Regression equation showing comparison of free phenytoin KIMS assay on the Cobas c501 analyzer with the reference phenytoin FPIA assay on the TDx analyzer.

Diagnostics (Cobas free Phenytoin Controls, Catalog # 207590230). Linearity was also verified by running calibrators as unknown. For method validation, we determined free phenytoin concentration in sera of 25 patients receiving phenytoin using the FPIA assay on the TDx analyzer and Cobas c501 analyzer. For these patients, free phenytoin test was ordered. This study was performed using de-identified postdiagnostics specimens according to guideline of University of Texas-Houston Institutional Review Board. We used linear regression analysis for method comparison using Microsoft excel software program. We also studied potential interference of bilirubin and triglyceride with the free phenytoin assay adopted on the Cobas c501 analyzer.

RESULTS AND DISCUSSION

The method validation was carried out following CLIS (clinical and laboratory standard institute) protocol (13). The intra-run precision of the free phenytoin assay was 4.3% for the low control (mean: 0.98 µg/ml; SD: 0.04, $n = 20$) and 1.9% for the high control (mean: 2.99 µg/ml; SD: 0.056, $n = 20$). The inter-run precision was 7.1% for the low control (mean: 1.01 µg/ml; SD: 0.0718, $n = 20$) and 3.3% for the high control (mean: 2.93 µg/ml; SD: 0.096, $n = 20$). The assay was linear for a free phenytoin concentration up to 4.0 µg/ml and the detection limit was 0.2 µg/ml. No carryover was observed in the free phenytoin assay using the Cobas c501 analyzer because when a drug-free specimen was analyzed after analyzing a specimen containing 4.2 µg/ml of free phenytoin (this specimen was originally analyzed using 1:1 dilution

to establish the free phenytoin concentration and during carryover study was analyzed without dilution), the drug-free specimen showed a “none detected” value. The free phenytoin assay was also free from interference of high concentration of bilirubin (total bilirubin concentration up to 20 mg/dl) and high triglyceride (triglyceride concentration up to 1,000 mg/dl). Comparison of the free phenytoin method with the FPIA reference method and the free phenytoin assay on the Cobas c501 analyzer provided the following regression equation (Fig. 1):

$$y = 0.9899x + 0.0408 (r = 0.98, r^2 = 0.9633, n = 25)$$

The slope indicates that the average bias between the reference method and free phenytoin assay on the Cobas c501 analyzer was approximately 1%, and the correlation coefficient was 0.98 indicating that the free phenytoin assay on the Cobas c501 analyzer compared well with the reference method.

Free phenytoin test is useful for special patient population (uremic patient, hypoalbuminemia, critically ill patients, pregnant women, elderly, etc.) and many hospital laboratories offer this test as a part of routine therapeutic drug monitoring service. Although free phenytoin concentration can be accurately determined by ultrafiltration followed by analysis using liquid chromatography combined with mass spectrometry (14), most clinical laboratories use immunoassays for monitoring free phenytoin concentration. According to the recent College of American Pathologists Proficiency Survey (CAP Survey C-C December 2011), only 125 participating laboratories offer free phenytoin assay compared to over

4,000 laboratories that offer total phenytoin assay. However, of 125 laboratories, no laboratory uses chromatographic technique for free phenytoin determination. The most popular method was the FPIA assay for free phenytoin on TDx or TDxFLX analyzer (88 of 125 participating laboratories) followed by 32 laboratories that use Cobas Integra free phenytoin assay, which is also a fluorescence polarization assay.

CONCLUSIONS

We conclude that adaptation of free phenytoin assay on Cobas c501 analyzer using PHNY2 reagent and calibrators of free phenytoin assay from Cobas Integra provided comparable results with FPIA free phenytoin assay on the TDx analyzer. Therefore, this assay can be used for routine monitoring of free phenytoin in clinical laboratories.

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