Phenytoin is an anticonvulsant drug used to treat most types of seizures; achieving steady-state concentrations of this drug can require 4 to 8 days. Total phenytoin (TP) has a narrow therapeutic index with steady-state concentrations in the range of 39.6 to 79.2 µmol/L. Signs of toxicity include nausea and nystagmus (>79.2 µmol/L); ataxia (>118.8 µmol/L); lethargy; central nervous system (CNS) depression; coma; increased seizure activity; and, potentially, death occur at plasma concentrations >158.4 µmol/L.¹ ²

There is a generally strong correlation between TP concentrations and seizure control in patients with uncomplicated seizures. However, patients can experience signs of toxicity when measured TP concentrations are within the therapeutic range.³ ⁴ The risk of phenytoin toxicity increases with age,³ ⁴ multiple comorbidities,⁵ hypoalbuminemia,⁶ critical illness,⁷ ⁸ and/or polypharmacy.¹⁰ Free phenytoin (FP) levels can help to maximize seizure control while minimizing toxicity.¹ ⁶ Therapeutic FP steady-state concentrations range from 4.0 to 8.0 µmol/L; measurements that exceed 20 µmol/L can be lethal.¹ ⁶

Since the 1990s, Abbott Diagnostics (Abbott Park, IL) has offered an FP assay on the TDx/FLx Immunochemistry Analyzer, a compact bench-top immunochemistry analyzer with a menu devoted largely to therapeutic drug monitoring, toxicology, fetal lung maturity, and endocrinology immunoassays. However, in 2008, Abbott Diagnostics notified laboratories that it would discontinue the TDx/FLx

**Abbreviations**

TP, total phenytoin; CNS, central nervous system; FP, free phenytoin; FLMII, fetal lung maturity II; CAP, College of American Pathologists; LLOQ, lower limit of quantitation; QC, quality control; AMR, analytic measurement range; FPIA, fluorescence polarization immunoassay; EMIT, enzyme-multiplied immunoassay technique; CMIA, chemiluminescent microparticle immunoassay; CV, coefficient of variation; TE, total error; FDA, United States Food and Drug Administration

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Laboratory QA

analyzer and would phase out all assays manufactured for that platform. Accordingly, laboratories have sought alternatives to the widely used fetal lung maturity II (FLMII) assay;11 transfer of other Abbott assays to alternate analytic platforms has been completed or is in progress by laboratories such as ours. FP assays are available on a limited number of analytic platforms; however, the performance of these methods varies based on proficiency testing results reported among participant summaries provided by the College of American Pathologists (CAP).12

During our search to replace the TDx/FLx FP assay, we noted that the lower limit of quantitation (LLOQ) of the ARCHITECT iPhenytoin reagent, as claimed by the manufacturer, is 2.0 µmol/L. This LLOQ is sufficiently low to measure the concentrations of FP. However, the iPhenytoin reagent is not designed to measure FP. Thus, we evaluated the performance of the ARCHITECT iPhenytoin assay for measuring FP concentrations in filtered serum specimens submitted to our laboratory.

Materials and Methods

Specimens/Analytic Materials

Serum-based calibrators manufactured by Abbott Diagnostics were used for this study. Assayed quality control (QC) material with known phenytoin concentration (Liquichek Immunoassay Plus Control; BioRad Laboratories Inc., Hercules, CA) was used to validate the method. Patient specimens submitted to the laboratory for phenytoin testing were de-identified and retained for correlation studies. Consent was not required to perform testing because the specimens we used were selected from clinical-use collections and had been de-identified before use in the correlation study. We treated CAP proficiency testing survey materials as patient specimens for each event, after reconstituting those specimens according to instructions from the manufacturer. All materials used in this study for FP measurements on the ARCHITECT platform are herein-after referred to as specimens.

Specimens (500 µL) were added to Centrifree ultracentrifuge tubes (Millipore Corporation, Bellerica, MA; catalog number 4104; molecular weight cut-off, 30 kDa), capped the tubes, and centrifuged them at 1500 g for 15 minutes in an IEC Centra CL2 ultracentrifuge (Thermo Fischer Scientific Inc., Waltham, MA) fitted with a fixed-angle rotor. We transferred ultrafiltrates to testing cups before analysis. We measured all specimens using the predicate TDx/FLx FP assay and the ARCHITECT iPhenytoin assay within an hour of sample preparation, as would occur during testing of specimens from patients. We alternated the testing sequence by specimen to reduce systematic bias (ie, TDx/FLx FP and then ARCHITECT iPhenytoin assay, or vice versa).

Reagents

For the evaluation, we used the TDx/FLx FP assay reagents that we currently use in our laboratory and we also acquired ARCHITECT iPhenytoin reagent kits. Abbott Diagnostics provided 1 reagent kit, to be used in the ARCHITECT i1000 analyzer, for our iPhenytoin assay evaluation; we purchased the other kits that we required to complete the studies. We used iPhenytoin kits to perform testing on the 2 ARCHITECT i1000 analyzers. All reagent components were within the ranges of labeled stability, storage, and expiration dates that were specified by the manufacturer.

Calibration and Calibration Verification

We calibrated the ARCHITECT iPhenytoin assay according to the instructions given by the manufacturer for the dynamic range of the assay as labeled (ie, we conducted initial calibration for the intended use/TP assay). The assay was calibrated using the assayed materials provided by the manufacturer, and we then tested assayed QC materials to establish performance criteria for our daily use prior to FP testing.

Analytic Measurement Range (AMR) Verification

The calibrators we tested for the iPhenytoin assay cover TP measurement as concentrations of 0, 9.9, 19.8, 39.6, 79.2, and 158.4 µmol/L. As a prevalidation step, we first verified the LLOQ (2.0 µmol/L), as claimed by the manufacturer, with diluted, unfiltered calibrator to achieve TP concentrations of 2.0 and 4.0 µmol/L (per product labeling). We prepared and measured each targeted concentration 5 times. This dilution strategy (initially without filtration) was used during the internal validation testing according to the specifications provided by the manufacturer [oral communication, Abbott Diagnostics Customer Service, October 15, 2011]. We considered an acceptable recovery of TP (measured concentration/expected concentration × 100%) to be within 10% of expected concentrations. These studies were conducted prior to FP validation testing.
To verify the LLOQ of the FP testing conditions, we repeated this study at the 2.0- and 4.0-µmol/L target concentrations using filtered calibrators (as would be prepared on the TDx/FLx platform for FP testing). The recovery targets of intended FP concentrations were within 10% of stated means and calculated as described earlier.

After we completed the FP LLOQ feasibility test, we conducted full-range calibration verification using all calibrators except the zero calibrator, after filtration. We calculated the percentage of FP present in the filtered calibrators and the recovery of FP to determine the filtered calibrator acceptability and the AMR of the FP application.

**Imprecision Testing**

Performance and imprecision for the FP application were established through daily testing of filtered, assayed QC materials that we have used routinely in performing the TDx/FLx FP assay. The mean (SD) targets determined by BioRad Laboratories Inc. were as follows: level 1, 6.1 (1.2) µmol/L; level 2, 12.4 (2.0) µmol/L; and level 3, 19.5 (3.2) µmol/L. We prepared control materials that were consistent with those used in daily practice on the TDx/FLx device and in the same manner as patient specimens, as described earlier. Our use of the Liquichek material for FP testing was consistent with the instructions from the manufacturer of the assay. The recovery targets were within 1.5 SD of the stated means, and the imprecision was less than 15.0% (1 CV) for daily use ranges.

**Bias Assessment**

We compared the results of the TDx/FLx FP and the ARCHITECT iPhenytoin assays through patient-specimen correlation testing. The therapeutic interval for the TDx/FLx FP assay was 4.0 to 8.0 µmol/L. We used de-identified clinical specimens submitted for phenytoin testing and stored per the instructions from the manufacturer, which allowed for room temperature storage (20°C to 25°C) for up to 2 days, refrigerated storage (20°C to 80°C) for up to 8 days, or frozen (−20°C or colder) for up to 5 months. We thawed 30 of these specimens, filtered them, transferred them to specimen cups, and measured the FP levels on the TDx/FLx and ARCHITECT/i1000 platforms. The bias target between methods was less than 0.5 µmol/L (<25% of the LLOQ).

We tested proficiency specimens (2 per event for 3 events over 1 calendar year) in the same manner as patient specimens, as part of our continuous validation efforts. The target performance was within 2 SD index (SDI) units of peer group means (fluorescence polarization immunoassay [FPIA] for the predicate-method comparator and enzyme-multiplied immunoassay technique [EMIT] for a second-method comparator). We selected these peer groups because fewer than 10 participating sites reported results for the chemiluminescent microparticle immunoassay [CMIA] FP method; Abbott Laboratories recommended using this peer group prior to 2013.

**Statistical Analysis**

We performed statistical calculations using Microsoft Office Excel, version 2003 (Microsoft Corporation, Redmond, WA). We calculated percent recovery, arithmetic mean, SD, coefficient of variation (CV), slope, intercept, correlation coefficient (r²), standard error (SE) of the estimate, percentiles (0.5th and 99.5th to test commutability of the therapeutic interval), and QC failure rates. We compared cumulative error (CV for the cumulative data set for the 2 analyzers) with the Liquichek Immuonosay Plus Control product-insert specifications and represented this finding as the total error (TE). We calculated SDI using the following equation: SDI = (laboratory result – consensus group mean)/consensus group SD. The number of samples tested yielded a statistical power of greater than 99%. The Sigma calculation was calculated as TE – Bias/CV%.

**Results**

Mean recoveries for filtered calibrators were 95% at 1.9 µmol/L and 100% at 4.0 µmol/L.

Acceptable LLOQ results using filtered calibrators prompted us to analyze the measurable calibrators (excluding the 0 calibrator) after filtration, to determine the percentage of FP that was present in the specimens. All 5 materials tested yielded FP results near 15% of the TP concentration target (15.2% [0.8%]) on both ARCHITECT analyzers. The resulting AMR for the FP method, using filtered calibrators as described by the manufacturer, was 2.0 to 25.0 µmol/L on both analyzers.

Control materials were tested daily at all 3 commercially available levels (Table 1) recoveries were 96.1% [5.0%] for level 1, 99.2% [5.0%] for level 2, and 99.3% [5.7%] for level 3). CVs were 5.2%, 5.0%, and 5.8% for QC levels 1, 2, and 3, respectively, after a 1-month study. Biases for the 3
levels of QC were -3.9%, -0.9%, and -0.7%, respectively, versus TDx/FLx). Using a total allowable error of 10%, the estimated $\sigma$ values for QC levels 1, 2, and 3 results using the iPhenytoin reagent were 9.2, 9.8, and 9.9, respectively.

Linear regression analysis of FP concentrations in clinical specimens were: TDx/FLx v Architect “1”, slope = 0.98, y-intercept = 0.29, $r^2$=0.98; TDx/FLx v Architect “2” slope = 0.97, intercept = 0.46, $r^2$=0.98. (Figure 1). The regression analysis included the portion of the AMR (2.0-20.0 µmol/L) that is most relevant to FP clinical testing and included FP concentrations in the toxic range. Mean (SD) recovery was 103.7% [10.6%] in patient specimens versus the results from the TDx/FLx.

The bias we observed between the FP results obtained via the TDx/FLx assay and the iPhenytoin reagent method on the ARCHITECT in patient specimens was minimal and not clinically significant (0.1 [0.3] µmol/L). No outliers were rejected from the data. The 0.5th- and 99.5th-percentile calculations did not differ by more than 0.1 µmol/L for analytic platforms. The percentile-based range for TDx/FLx FP results was 2.0 to 18.8 µmol/L; for the 2 ARCHITECT analyzers, the range was 2.0 to 18.7 and 2.0 to 18.8 µmol/L.

In Figure 2, we present CAP survey performance results for 3 sets of survey challenges in 2012 (2 challenges per survey). Although some bias was observed between our results and the peer group mean, all SDI calculations were acceptable per CAP standards. Increased bias, nearing

<table>
<thead>
<tr>
<th>Variable</th>
<th>Level 1 (n = 69)</th>
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<tr>
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<td>12.4 (2.0)</td>
<td>19.5 (3.2)</td>
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<tr>
<td>Mean (SD), µmol/L</td>
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<td>12.6 (0.6)</td>
<td>20.1 (1.2)</td>
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<td>CV, %</td>
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<tr>
<td>Mean bias, % vs TDx/FLx</td>
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<td>-0.9</td>
<td>-0.7</td>
</tr>
</tbody>
</table>
| CV, coefficient of variation; QC, quality control.
| * The ARCHITECT instrument is manufactured by Abbott Laboratories, Abbott Park, Illinois; Liquichek Immunoassay Plus Control, BioRad Laboratories Inc., Hercules, CA. Failure = Westgard 1-3s (or greater occurrences during daily QC testing.
| * Accounts for the single QC failure.
+2.0 SDI, was identified on ARCHITECT test 2 in the third survey example (Figure 2). By the time all survey results had been returned, the results of our investigation showed that calibration had occurred in the interim; repeat testing of the proficiency material yielded an acceptable result consisting of SDI of less than 2.0.

Discussion

Our results confirm that the ARCHITECT iPhenytoin assay is suitable for monitoring therapeutic FP concentrations. FP results obtained using this method correlated with results obtained via the TDx/FLx platform. QC results were stable on the ARCHITECT platform; proficiency testing results have been acceptable since the ARCHITECT method was implemented in our laboratory in January 2012. Assay performance consistently yielded a s of greater than 9.0; this performance is considered to be at or among the best of its class.

In this correlation study, potential variations in specimen quality attributed to sample storage (eg, freeze-thaw cycles, shipment, and storage) were minimized by performing the tests on both platforms within a 1-hour time frame. Specimen instability can introduce bias and outliers. Moreover, comparisons with results from external laboratories may be invalid if different platforms and/or assay reagents are used for FP measurements, including liquid chromatography–tandem mass spectrometry, which is not widely used in hospital laboratories for therapeutic drug monitoring of phenytoin.

Measuring FP using the iPhenytoin assay required no procedural changes other than adding a specimen filtration step. The AMR for the iPhenytoin assay did not require modification and is sufficient for a clinically relevant FP AMR. The FP fraction we measured in calibrators was approximately 15%, which is slightly higher than the FP fraction reported in human studies (10%). However, FP fractions greater than 15% have been reported in patients on phenytoin therapy.3,4,6,8-10,13 The AMR that we have established and confirmed with the filtration method includes FP concentrations in the nontherapeutic, therapeutic, and potentially toxic or fatal ranges. The QC materials we used have target FP concentrations at clinically relevant concentrations (level 1 mean = 6.1 µmol/L, consistent with therapeutic FP concentration; level 2 mean = 12.6 µmol/L, consistent with increased and potentially toxic FP concentration; level 3 mean = 20.1 µmol/L, consistent with toxic and potentially fatal FP concentration). These QC materials yielded CVs at all 3 levels that were below the target CV of 15.0% suggested by the manufacturer. The use of the iPhenytoin reagent on the ARCHITECT platform, when performed with appropriate calibration and QC for measuring FP concentration, provided consistently acceptable FP results (Table 1).

Figure 2
Calculated standard deviation index results using the iPhenytoin assay (Abbott Laboratories, Abbott Park, IL) for free phenytoin measurement. A, Y-axis range for 2A (versus fluorescence polarization immunoassay [FPIA]). B, Y-axis range for 2A (versus enzyme-multiplied immunoassay technique [EMIT]) represents an acceptable SD-index window, per the College of American Pathologists (CAP).
Transfer of FP testing from the TDx/FLx platform to the ARCHITECT platform was relatively seamless at clinical and operational levels. Calculated percentile results via the /Phenytoin method were identical to those we calculated via the predicate TDx/FLx method, using results from the correlation study. Since specimen treatment and testing was similar between the 2 platforms, we were confident that the therapeutic range we had been using would be transferable to the new FP method on the ARCHITECT platform. Our technical staff reported improved workflow after the migration of FP testing to the ARCHITECT platform. Physicians working in our institution have expressed that they are comfortable with the reliability of the new FP assay and have noticed the improvement in turnaround time. Validation and implementation of the FP assay on the ARCHITECT platform has improved laboratory efficiency and has allowed the laboratory to continue in-house FP testing. 

References