Serum Creatinine Concentration and the Discrepancy Between Enzyme Multiplied Immunoassay Technique and Gas-Liquid Chromatographic Phenytoin Levels*

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ABSTRACT

In patients on phenytoin therapy and with renal failure, the enzyme multiplied immunoassay technique (EMIT), unlike gas-liquid chromatographic techniques (GLC), can give falsely elevated serum phenytoin concentrations. In 25 patients (creatinine ≤1.5 mg per dl), discrepancies between the serum EMIT and GLC phenytoin concentrations were accounted for by the inherent imprecision of the methods. However, in 16 of 18 uremic patients (creatinine ≥1.7 mg per dl), the discrepancies were greater than could be accounted for by imprecision. For these 18 patients the correlation (r = 0.49) between the discrepancies and the creatinine concentrations appeared to be statistically significant (P < 0.05). The linear regression relationship was inadequate for accurate correction of the EMIT values using the creatinine concentrations. Another original observation was that in most cases renal dialysis decreases the EMIT to GLC discrepancy. However, post-dialysis serum samples with a creatinine concentration >1.7 mg per dl usually still showed a falsely elevated EMIT phenytoin concentration.

Introduction

Phenytoin (DPH) is one of the most widely used anticonvulsant drugs. Vastly different steady-state serum concentrations, ranging from sub-therapeutic to toxic, are obtained when a group of individuals suffering from seizure disorders
are given the same dose of DPH. Concurrent dosing with other medications or intercurrent, non-neurologic disease states can alter the steady-state serum concentration of DPH of an individual without a change in dose.

Monitoring the serum concentration of DPH enables the physician to ensure that for each individual a steady-state concentration in the therapeutic range is attained and maintained. Methods used to measure serum DPH concentrations include radioimmunoassay (RIA), gas-liquid chromatography (GLC), high-pressure liquid chromatography (HPLC) and homogeneous enzyme immunoassay (EMIT). The EMIT system has become a widely used method for measuring serum DPH in clinical chemistry practice because of the speed and simplicity of the EMIT procedure. However, several recent reports have shown that in patients with renal failure the EMIT method, unlike GLC or HPLC, can give falsely elevated values for serum phenytoin.

The studies reported here were undertaken to determine: (1) whether or not there is a strong correlation in uremic patients between the degree of azotemia, as reflected by the serum creatinine concentration, and the degree of discrepancy between the EMIT and the GLC measurement of DPH; (2) whether or not, with renal dialysis, the alterations in serum creatinine concentration are correlated with alterations in the discrepancy between the DPH concentrations obtained by EMIT and GLC; and (3) if these correlations exist, can the serum creatinine concentration be used to correct the EMIT DPH values obtained for patients with renal disease?

Materials and Methods

The specimens used in this study were those obtained for the routine determination of serum phenytoin or creatinine or both. Samples from patients receiving renal dialysis were obtained just prior to and just after dialysis. Creatinine concentrations were determined by a modification of the Jaffe procedure using the Centrifichem 400.* Serum phenytoin was measured both by an EMIT and a GLC procedure.

The EMIT phenytoin reagents, lot letter codes J, K, and L, were purchased in a kit.** All calibrator standards were obtained from the company and were prepared according to their specifications. The calibration curves were determined using a curve-fitting program obtained from the company for use with a calculator.† All EMIT assays were performed using a centrifugal analyzer‡ using the guidelines specified by Syva.

The GLC analyses of phenytoin were performed by the following procedure, developed in our laboratory. To one ml of sample were added four ml of deionized water and one ml of an internal standard solution of 5-(p-methyl-phenyl)-5-phenyl hydantoin (MPPH). After mechanical stirring of this mixture, one ml of a charcoal suspension was added, mixed, and centrifuged. After the supernatant was poured off, the drug-bound charcoal was washed once with distilled water. One milliliter of methanol and seven ml of anhydrous diethyl ether were added. The tube was shaken for one minute, centrifuged, and the ether-methanol supernatant decanted. After the solvent was evaporated to dryness, 80 μl of acetone were used to reconstitute the residue. Samples (1.0 μl) were injected into the chromatograph. A gas chromatograph§ was equipped with a flame ionization detector and a stainless steel

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*Union Carbide, Pleasantville, NY.
** The Syva Company, Palo Alto, CA.
† Model 9815, Hewlett Packard, Avondale, PA 19311.
‡ Model 9815, Hewlett Packard, Avondale, PA 19311.
§ Model 5711A, Hewlett Packard, Avondale, PA 19311.
The gas chromatographic method described has been compared to the well-established GLC method of Kupferberg. Both methods gave statistically identical results.

For a given set of linear regression data when the value of $X$ is set equal to $X$ (the mean of the $X$ values), the predicted value of $Y$ and its 95 percent confidence interval can be calculated as follows. The predicted value of $Y$ at $X$ is $\bar{Y}$. The standard deviation of the predicted value of $Y$ when $X$ equals $X$ is

$$\text{S.D.}(\bar{Y}) = \sqrt{\frac{N}{(N-2)}} \left( \frac{s_y^2}{(1-r^2)} \right) \left( 1 + \frac{1}{N} \right)$$

where $N$ is the sample size, $s_y$ the variance of the $Y$ values and $r$ is the correlation coefficient. At $X$, the predicted value of $Y$ and its 95 percent confidence interval are

$$\bar{Y} \pm t(S.D.)(\bar{Y})$$

where the $t$ value is obtained for $N-2$ degrees of freedom and 95 percent probability.

### Results

The day-to-day variation for the measurements of phenytoin with GLC and EMIT techniques was established by using two different control sera which were prepared by supplementing a drug-free serum pool with two different amounts of phenytoin. Day-to-day analysis of the control serum supplemented with the higher amount of phenytoin gave values of $28.8 \pm 0.6$ mg per L (mean $\pm$ S.D.) $N = 50$ with GLC and $27.6 \pm 1.1$ N = 25 by the EMIT technique. Using this control serum EMIT/GLC phenytoin ratios were observed that ranged from 0.80 to 1.12 (median 0.96, N = 25). With the serum supplemented with the lower amount of phenytoin, day-to-day analysis gave values of $12.3 \pm 0.3$ (mean $\pm$ S.D.) $N = 50$ with GLC and $11.8 \pm 0.5$ N = 25 by the EMIT technique. Using this control serum, EMIT/GLC phenytoin ratios were observed that ranged from 0.82 to 1.11 (median 0.98, N = 25). Essentially the same range of ratios was observed for the two controls with approximately 2-fold different concentrations. Thus, when the EMIT/GLC ratio for a particular sample fell within the range 0.80 to 1.12 (henceforth designated as the reference range), any differences between the phenytoin concentrations measured by EMIT and GLC were ascribed solely to the inherent imprecision in the two methods.

In 25 patients with serum creatinine concentrations $\leq 1.5$ mg per dL (median 0.9, range 0.4 to 1.5 mg per dL), the values of the EMIT/GLC ratio for serum phenytoin were all within the reference range (median 0.98, range 0.83 to 1.12). The correlation ($r$) between the EMIT and GLC measurement of phenytoin in these 25 patients was 0.998, $\text{EMIT} = 0.98 \text{ GLC} + 0.007$. The EMIT/GLC ratios and creatinine concentrations were not correlated, $r = 0.087$, (EMIT/GLC) = 0.02 Cr + 0.96.

Of 18 uremic patients (creatinine $\geq 1.7$ mg per dL) being given phenytoin, 16 had an EMIT/GLC ratio outside of the reference range of 0.80 to 1.12 (table I). It should be noted that the phenytoin concentrations in the majority of these samples were lower than the concentration range, approximately 12 to 28 mg
TABLE I

Comparison of Serum Concentrations of Phenytoin Assayed by Enzyme Multiplied Immunoassay Techniques and Gas-Liquid Chromatographic Technique in 18 Patients with Serum Creatinine $\geq$ 1.7 mg per dL

<table>
<thead>
<tr>
<th>Patients</th>
<th>Creatinine (mg/dL)</th>
<th>EMIT/GLC</th>
<th>EMIT (mg/L)</th>
<th>GLC (mg/L)</th>
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<tr>
<td>P.L.</td>
<td>1.7</td>
<td>1.16</td>
<td>5.1</td>
<td>4.4</td>
</tr>
<tr>
<td>P.F.</td>
<td>2.4</td>
<td>1.10*</td>
<td>8.9</td>
<td>8.1</td>
</tr>
<tr>
<td>P.S.</td>
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<td>1.14</td>
<td>8.7</td>
<td>7.6</td>
</tr>
<tr>
<td>L.W.</td>
<td>2.7</td>
<td>1.08*</td>
<td>7.3</td>
<td>6.7</td>
</tr>
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<td>J.S.</td>
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<td>1.14</td>
<td>10.6</td>
<td>9.3</td>
</tr>
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<td>1.22</td>
<td>28.0</td>
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<td>J.G.</td>
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<td>1.27</td>
<td>19.0</td>
<td>15.0</td>
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<td>D.D.</td>
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<td>6.3</td>
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<td>1.33</td>
<td>9.6</td>
<td>7.2</td>
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<td>M.H.</td>
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<td>12.5</td>
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<td>14.1</td>
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<td>T.G.</td>
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<td>1.68</td>
<td>10.6</td>
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<td>L.B.</td>
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</table>

*Differences in EMIT and GLC accounted for by imprecision of methods, reference range 0.80 to 1.12.

per L, for which the reference range for the EMIT/GLC ratio was determined. One would expect the precision of the measurements to be poorer with lower concentrations, and thus it is possible that the reference ratio range may be somewhat broader with concentrations less than 12 mg per L. It is doubtful that this possibility was a major factor in the observation that of the 18 samples with an elevated creatinine concentration, 16 had ratios above 1.12. For example, the sample with the lowest phenytoin concentration, 2.2 and 1.7 mg per L by the EMIT and GLC methods, respectively, gave an EMIT/GLC ratio of 1.29, a ratio considerably higher than 1.12, the upper limit of the assigned reference range. The 16 ratios outside of the reference range of values had values extending from 1.14 to 2.12.

In the 18 uremic patients correlation ($r = 0.49$) between the EMIT/GLC ratios for phenytoin and the creatinine concentration appeared to be significant ($P < 0.05$). However, the regression rela-
tionship, $(\text{EMIT/GLC}) = 0.04 \text{ Cr} + 1.09$, was not adequate for accurate prediction of the discrepancy between EMIT and GLC values from the creatinine concentrations. For example, the predicted value and its 95 percent confidence interval for the EMIT/GLC ratio at the mean value of creatinine, 6.78 mg per dL, is $1.34 \pm 0.96$. (The value of $s^2$ for the linear regression equalled 0.0614.) The phenytoin concentrations measured in sera from these 18 uremic patients by EMIT and GLC were highly correlated, $r = 0.966$ ($\text{EMIT} = 1.25 \text{ GLC} + 0.69$).

Phenytoin and creatinine concentrations were measured in 11 pre- and post-renal dialysis serum samples from five patients (table II). There was correlation ($r = -0.54$) between the changes in the EMIT/GLC ratio for phenytoin and the changes in the creatinine concentration owing to renal dialysis ($\Delta (\text{EMIT/GLC}) = -0.11 (\Delta \text{Cr}) + 0.74$). Although this correlation appeared to be statistically significant ($P < 0.1$), the regression relationship was not adequate for accurate prediction. The correlation between EMIT and GLC phenytoin concentrations in the 22 pre- and post-dialysis samples was quite high, $r = 0.948$ ($\text{EMIT} = 1.12 \text{ GLC} + 1.00$).

Serum from chronic renal dialysis patients who had never received phenytoin showed no measurable phenytoin level by either the GLC or EMIT assays for phenytoin. Thus, as observed by others, the substance causing the measured EMIT phenytoin concentrations to be erroneously high in uremic patients is not inherent in the sera of uremic patients. Phenytoin added to sera from patients with either normal or impaired renal function was completely recovered with both assay techniques. Thus, as noted by others, the interfering substance was not falsely depressing the GLC values.

When the primary metabolite of phenytoin, 5-[(4-hydroxyphenyl)-5-phenyl hydantoin (HPPH), was added to sera
from individuals with normal or impaired renal function, the EMIT assay measured approximately 1.3 mg per L of phenytoin for every 20 mg per L of HPPH added. However, no phenytoin was detected by GLC, with the same HPPH supplemented serum samples indicating that the chromatographic technique readily separated phenytoin and HPPH.

**Discussion**

When the serum creatinine concentration of patients was ≤1.5 mg per dL, any difference between the DPH values obtained by EMIT and GLC could be totally accounted for by the inherent imprecision of the two analytical procedures. However, 16 of 18 patients (or 89 percent) with serum creatinine concentrations ≥1.7 mg per dL had differences in the DPH concentrations determined by EMIT and GLC that could not be totally accounted for by the inherent imprecision of the two methods (table I). In these 16 cases, the ratios of EMIT/GLC were all greater than 1.12, the apparent upper limit of the ratio owing to imprecision. Previous investigators have shown that such discrepancies are the result of an erroneously elevated EMIT value.

Nandedkar et al showed that the interfering substance that causes the erro-
neously high EMIT DPH values when the serum creatinine concentration is elevated is a dialyzable substance. Burgess et al.\(^5\) stated that the EMIT/GLC ratios for serum phenytoin concentrations were not altered by renal dialysis. Our observations do not verify that statement. In most cases a rather large change in the EMIT/GLC ratio was observed as the result of renal dialysis (table II). The possible reason for the discrepancy between the statement by Burgess et al.\(^5\) and our observations will be discussed later.

In nine of the 11 pairs of samples from patients undergoing renal dialysis, the discrepancy between the EMIT and GLC values for serum DPH decreased after dialysis; in two cases the discrepancy (ratio) was essentially unchanged by renal dialysis (table II). These observations strongly indicate that the interfering substance and DPH dialyze at different rates. In four of the nine samples in which the discrepancy between EMIT and GLC values for serum DPH decreased following renal dialysis, the difference in the EMIT and GLC values after dialysis could be totally accounted for by the imprecision inherent in the two assays, despite post-dialysis concentrations of serum creatinine greater than 1.5 mg per dL (table II). However, because the majority of the samples tested still showed a real discrepancy between the EMIT and GLC values after dialysis, it cannot be assumed that renal dialysis will always eliminate the discrepancy and that post-dialysis serum samples will give accurate DPH values by EMIT. Based on our observations presented earlier, that when the serum creatinine concentration is \(\leq 1.5\) mg per dL the EMIT and GLC phenytoin concentrations are not discrepant, it appears that unless the post-dialysis creatinine concentration is \(\leq 1.5\) mg per dL, the post-dialysis serum sample should probably not be assayed for DPH by the EMIT procedure. (Our suggestion that the post-dialysis sample would show no significant discrepancy if the creatinine concentration was \(\leq 1.5\) mg per dL is a projection because none of the post-dialysis samples studied showed a decrease to \(\leq 1.5\) mg per dL (table II)).

Phenytoin has two major metabolites. The drug is first hydroxylated to form 5-(4-hydroxyphenyl)-5-phenyl hydantoin (HPPH), and then the hydroxyl group is conjugated by glucuronidation (conjugated HPPH). Only about four percent of a dose of DPH escapes metabolism via this route.\(^3\) The two metabolites are excreted into the urine; in patients with normal renal function who are receiving DPH, the concentrations of the two metabolites in serum is quite low.\(^7\) However, in patients with renal failure who are receiving DPH, the HPPH level in serum is several-fold higher and the conjugated HPPH level in serum is 10-fold to 100-fold higher than in patients with normal renal function.\(^3,12\)

Aldwin and Kabakoff\(^1,2\) have studied the cross-reactivity of the EMIT DPH antibody to HPPH and conjugated HPPH. Cross-reactivity to HPPH may account for a small portion of the falsely elevated EMIT DPH serum values in uremic patients; apparently the cross-reactivity with the HPPH glucuronide accounts for most, 80 to 90 percent, of the false elevation.\(^2\) Different lots of the EMIT DPH antibody show different degrees of cross-reactivity\(^1\) and the Syva Company currently selects antibodies for a minimal response to HPPH-glucuronide.\(^2\) This selection may account for the lower EMIT/GLC ratios observed by us than were observed by past workers with discrepant samples. In the 16 discrepant samples reported here, the EMIT/GLC ratio ranged from 1.14 to 2.12 with a median of 1.30 (table I). In the past studies,\(^5,6,13,14\) most of the EMIT/GLC ratios were in the two-fold to three-fold range for discrepant samples.

The current practice of the Syva Com-
pany to select EMIT phenytoin antibodies with the lowest response to the HPPH-glucuronide may account for the discrepancy between the statement by Burgess et al\textsuperscript{5} that renal dialysis did not alter the EMIT/GLC phenytoin ratio and our observation that in most cases renal dialysis lowered the ratio (table II). Previously available lots of phenytoin antibodies may have become saturated with the HPPH-glucuronide at a much lower concentration of this metabolite than do currently available antibody preparations. Thus with older lots of antibody, saturation with HPPH-glucuronide could still have occurred even after this metabolite’s concentration was lowered by dialysis. If this were the case, the degree of discrepancy between the EMIT and GLC phenytoin measurements (the EMIT/GLC ratio) would not be changed by dialysis. The observations presented here, that the EMIT/GLC ratios obtained by us were generally lower than those observed by Burgess et al\textsuperscript{5} and that dialysis usually reduced these ratios, indicate that the phenytoin antibodies used in the present study required higher concentrations of HPPH-glucuronide for saturation. In only two cases in our study, in the samples with the highest pre- and post-dialysis concentrations of serum creatinine, did the EMIT/GLC ratio not decrease after dialysis (table II). It is reasonable to assume that these samples also contained the highest concentrations of HPPH-glucuronide and that the EMIT/GLC ratio was not altered because the metabolite concentrations after dialysis were still saturating even for the currently available antibody preparations.

For a given serum total phenytoin concentration, the decreased binding to albumin in uremia leads to a higher free concentration of phenytoin than would occur in a patient with normal renal function.\textsuperscript{8} Therefore, with uremic patients the total concentration is usually maintained at the low end of the therapeutic range, 5 to 20 mg per L, used for patients with normal renal function (tables I and II—GLC values). With a given concentration of HPPH-glucuronide the lower the total serum phenytoin concentration the greater is the error in the EMIT measurement of phenytoin.\textsuperscript{1} Thus even though current EMIT antibodies show a decreased response to HPPH-glucuronide it is not surprising that samples from uremic patients still show a significant false elevation of the EMIT phenytoin concentration. Perhaps the problem of the false elevation could be solved by saturating the current antibodies in the reagents with HPPH-glucuronide.

In summary, the creatinine concentration can not be used to correct the falsely elevated serum phenytoin concentration obtained when the EMIT procedure is applied to samples from uremic patients. Thus, because there is no simple means to correct the erroneously elevated EMIT DPH concentration in uremic patients, we recommend that the EMIT DPH assay be used with caution to measure DPH in serum samples with creatinine concentrations greater than 1.5 mg per dL until greater DPH antibody specificity is achieved.

References


6. Flachs, H. and Rasmussen, J. M., Renal disease