Improved Substrate-Labeled Fluorescent Immunoassay for Theophylline in Dried-Blood Spots on Filter Paper

To the Editor:

We previously described a substrate-labeled fluorescent immunoassay for measuring the theophylline concentration in filter paper dried-blood spots (DBS), suitable for at-home therapeutic drug monitoring, based on use of the Ames/Miles "Fluorostat" instrument, which involved manual additions of reagents (Ames TDA) and polyclonal antibodies (I). Recently, the introduction of an automated and programmable fluorometer (the Ames/Gilford "Optimate," which more than halves TDA reagent consumption) and monoclonal TDA reagents has coincided with a change in the filter paper available for the assay. We therefore studied the effect of these changes on the original procedure and report a modified method suitable for the assay on both the manual and the automated analyzers.

Capillary DBS (γ) and plasma samples (x), collected from the same patient at the same time, were assayed for theophylline by using the Fluorostat analyzer, monoclonal TDA reagents, and the original protocol (I). The relationship between the two sets of results (n = 38) was highly significant (r = 0.9755, y = 0.9172x + 0.28), but the slope was 0.13 greater than that established for assays with the polyclonal antibody.

To explain this difference, we investigated the pH of the assay reaction mixtures, and found the DBS mixture to be significantly less basic (pH 7.90, SD 0.018, n = 20) than the plasma mixture (pH 8.27, SD 0.017, n = 15). To have the pH in the DBS assay approach that of the serum assay we decreased the volume of reagent used to elute the DBS disc (5′-sulfosalicylic acid, 50 g/L) to 270 μL and, after elution, combined 200 μL of the eluate with 200 μL of the manufacturer's concentrated bicine assay buffer. This modification resulted in a reaction mixture having a pH of 8.20 (SD 0.017, n = 15) and produced the 51-fold dilution of patient's sample required for the assay (the 6-mm DBS disc of PKU-31 paper, now distributed by Whatman Ltd., contains 11.0 μL of whole blood). The relationship between theophylline in DBS (γ) and serum samples (x) by this modified procedure remained highly significant (n = 30, r = 0.9971, y = 0.8547, x = 0.08), and the slope was nearer to our original result. The pH difference in the reaction mixture between the serum and DBS assays had also existed in our original procedure involving the polyclonal reagents but apparently had had no effect. We suspect the monoclonal reagents, unlike the polyclonal, are pH sensitive but cannot confirm this because the polyclonal reagents are no longer available. To automate the DBS procedure for the Ames/Gilford Optimate we programmed the analyzer to "re-use the plasma dilution" (option 16 of the theophylline assay program). The diluted eluate (obtained as described above for the modified Fluorostat procedure) is placed in the dilution cup, and the assay is completed by proceeding as pre-programmed by the manufacturer for the plasma assay. The relationships obtained (2) validated the Optimate results (Table 1).

The therapeutic range for serum theophylline in asthma patients is typically 10 to 20 mg/L; the equivalent therapeutic range for capillary DBS is 8.6 to 17.1 mg/L. Other laboratories should determine their own relationship for serum/DBS values, which will depend on the characteristics of their particular batch and batch of filter paper.

DBS assay reproducibility was adequate for clinical purposes. The modified Fluorostat procedure gave within-batch CVs (n = 10) of 4.6% and 4.3% and between-batch CVs (n = 10) of 7.1% and 6.9% for DBS theophylline concentrations of 9.2 and 19.8 mg/L, respectively. The Optimate procedure gave within-batch CVs (n = 10) of 5.1%, 6.6%, and 5.2% for DBS theophylline concentrations of 6.9, 11.9, and 18.8 mg/L, respectively, and between-batch CVs (n = 12) of 6.9% and 5.8% for DBS theophylline concentrations of 11.7 and 23.4 mg/L, respectively.

The advantages to the clinician of using capillary DBS samples that can be collected and mailed from the patient's home to the laboratory for the therapeutic drug monitoring of theophylline and phenytoin in children, adults, and the elderly have been described elsewhere (1, 4–6). The automated Optimate analyzer offers the laboratory many advantages. Reagent and buffer additions as well as fluorescence measurements are automatically performed and timed, decreasing operator involvement and hence assay cost. Fluorescence is read earlier—4.5 min vs 20 min on the Fluorostat—thereby shortening assay time. Nonspecific fluorescence is also minimized. Consumption of antibody/ enzyme and fluorogenic drug reagent is decreased from 50 μL to 20 μL per test. This is important when determining profiles, in which duplicate samples typically are collected when peak and trough concentrations of a circulating drug are expected, or when the patient is likely to suffer symptoms (e.g., the "early morning dipper").

Storage of standard-curve data (typically for at least three to four weeks) also decreases reagent consumption because most assay batches will then consist of quality-control and patients' samples only. The Optimate analyzer has so far performed reliably, has been well accepted by laboratory personnel, and thus seems well suited to monitor theophylline therapy with either conventional plasma samples or dried spots of capillary blood.

References


Table 1. Correlation between Theophylline Results for Plasma and Blood-Spot Samples as Determined by Various Analytical Methods

<table>
<thead>
<tr>
<th>Sample and method compared</th>
<th>n</th>
<th>r Regression equation</th>
<th>Error of regression about y</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma, HPLC (3) Plasma, Optimate</td>
<td>50</td>
<td>0.9950</td>
<td>y=0.9607x + 0.071</td>
</tr>
<tr>
<td>Plasma, Optimate DBS, Optimate</td>
<td>45</td>
<td>0.9955</td>
<td>y=0.8200x + 0.03</td>
</tr>
<tr>
<td>DBS, Fluorostat Optimate</td>
<td>40</td>
<td>0.9958</td>
<td>y=0.9543x + 0.073</td>
</tr>
</tbody>
</table>


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Screening for Benzodiazepines in Urine after Hydrolysis of Glucuronide Conjugates

To the Editor:

The EMT Urine Benzodiazepine Assay (Syva Diagnostics, Palo Alto, CA) is marketed for the detection of benzodiazepine misuse. The recommended limit of reliable detection with 95% confidence has been variously stated as 0.3 or 0.5 mg/L with oxazepam as a reference.

When we obtained several falsely negative results for random urine samples from patients in this hospital who had received 30-mg doses of oxazepam the previous night, we conjectured that most of the drug was excreted as conjugated metabolites, which were not being detected by the test system. In investigating this possibility, we found that the sensitivity of the method could be markedly increased by incubating urine samples with β-glucuronidase (β-glucuronide glucurononylhydrolase; EC 3.2.1.31) from Helix pomatia ("crude solution"; Sigma Chemical Co., St. Louis, MO).

Urine samples collected over 36 h after a patient had completed a two-day course of oxazepam (30 mg, twice daily) were treated by adding 0.05 mL of crude enzyme (as supplied) to 3.0 mL of urine and incubating at ambient temperature for 2 h. Absorbance was read at 340 nm according to the recommended procedure, after a delay time of 30 s and again 60 s later. The changes in absorbance were as follows:

<table>
<thead>
<tr>
<th>Urine sample</th>
<th>Before hydrolysis</th>
<th>After hydrolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-dose</td>
<td>681</td>
<td>675</td>
</tr>
<tr>
<td>Post-dose</td>
<td>817</td>
<td>1557</td>
</tr>
<tr>
<td>12 h</td>
<td>882</td>
<td>1321</td>
</tr>
<tr>
<td>24 h</td>
<td>707</td>
<td>1252</td>
</tr>
<tr>
<td>36 h</td>
<td>867</td>
<td>1144</td>
</tr>
</tbody>
</table>

Oxazepam calibrator, 0.3 mg/L

The recommendations of the manufacturer direct that, in general, ΔA values should exceed those obtained with the calibrator before a positive result can be reported with 95% confidence. By this criterion, the present results show that patients on a "therapeutic regime" of oxazepam are likely to show negative results, unless the urinary drug conjugates are first hydrolyzed. After hydrolysis, the therapeutic use of oxazepam is detectable for at least 36 h after the last dose.

The EMT benzodiazepine assay, as marketed, will sometimes give a positive result after a 10-mg dose of diazepam; however, after a 5-mg dose the drug is not detectable in urine (1). After the enzyme treatment step, we found in a single 5-mg dose of diazepam was detectable in a urine sample collected 24 h after the dose. In some circumstances, a change in the post-hydrolysis ΔA value (relative to the pre-hydrolysis value) may be a more sensitive indicator of benzodiazepine use than comparison with a calibrator of unknown relevance—a possibility we are investigating further.

Reference

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Influence of Uremia on Four Assays for Theophylline: Improved Results with a Monoclonal Antibody in the TDx Procedure

To the Editor:

Recent reports indicate that the fluorescence polarization immunoassay for theophylline in the TDx (Abbott Laboratories, North Chicago, IL) gives higher values for serum from patients with chronic renal failure than does homogeneous enzyme immunoassay (EMT; Syva Co., Palo Alto, CA) (1, 2) or "high-performance" liquid chromatography (HPLC) (1–3). Here we report confirmation of these observations...