Fluorescence Polarization Immunoassay for Theophylline Modified for Use with Dried Blood Spots on Filter Paper

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We used dried blood spots successfully as alternative specimens for quantifying concentrations of theophylline in the circulation by a modified fluorescence polarization immunoassay (FPIA) with the Abbott TDx instrument. The method described is simple, rapid, and acceptably precise. More importantly, it can provide results comparable with those of the conventional serum assay. Results for 64 pairs of dried blood spots (y) and serum (x) specimens analyzed by the respective FPIA methods yielded the following regression parameters: \( y = 0.94x + 0.71 \), \( r = 0.988 \), and \( S_y = 0.92 \). A major advantage of FPIA is that it requires only basic laboratory skills. When coupled with the use of dried blood spots, this system can be effective in remote theophylline monitoring, particularly suited for domiciliary care.

Because of its narrow therapeutic index, monitoring the concentration of theophylline in serum is an important aspect of safe and effective therapy with theophylline. In the current procedure for specimen collection for therapeutic drug monitoring, the patient must come to a hospital, laboratory, clinic, or physician’s office—a requirement that often means samples will not be collected at the time when symptoms are expressed and during typical living conditions. A method that could be used with samples collected in the home or school would be very desirable.

Dried blood spots (DBS) have been used successfully in other analyses—e.g., for detecting inborn errors of metabolism and congenital adrenal hyperplasia, and in monitoring concentrations of glucose, cholesterol, and therapeutic drugs. Coombes et al. (1, 2) recently reported an adaptation of a fluorimunoassay for theophylline in DBS. A less-practicable gas-chromatographic procedure for theophylline in DBS has also been reported (3).

For patients, DBS collected by fingerprick is a minimally traumatic method of specimen collection that can be performed at home by a parent, or at school by a health-care member. Direct dispatch of the specimen via delivery or through mail service avoids time-consuming visits for venipunctures. Arranging dosage adjustment by telephone contacts also decreases clinic visits. Moreover, physicians can direct that specimens be collected at specific times or under specific conditions, which previously was not possible. This provides additional information on compliance and for assessing treatment failure or toxicity, and encourages increasing parental supervision of medication of asthmatic children.

Here, we have validated the use of DBS collected on filter paper in a popular fluorescence polarization immunoassay (FPIA), and show that such samples can provide results comparable with those obtained for serum.

Materials and Methods

Instrumentation

Proper functioning of the TDx analyzer (Abbott Laboratories, Irving, TX 75061) was validated with "photo," "temp," and "pipe" checks according to the manufacturer’s specifications. Assay slot 12, originally assigned to procainamide, was selected for setting up the DBS theophylline assay; this slot had been activated previously but was not being used to measure procainamide at our institution for lack of requests. The settings for assay 12 were edited as shown in Table 1. The remaining settings such as Crv Fit, MX Dev, Mode, and Gain were not altered from those originally programmed. We did not change the name of the program or settings, MX, BKG, and MN TR, because they were not accessible to the instrument users. We used "Barcode Override" to start an assay run.

Specimens

For clinical specimens in the correlation studies, we collected simultaneously a venous blood sample and three capillary DBS. Whenever possible, we prepared additional DBS from the venous blood. For simplicity, we used the "Blood Collection Form" supplied by the Newborn Screening Program, New York State Department of Health, Albany, NY 12201, which contained a filter-paper strip with preprinted circles. For off-site collection, we provided a DBS collection kit (in a "Ziploc" bag) containing a filter-paper collection form, a "Monolet" lancet (Sherwood Medical, St. Louis, MO 63103), an alcohol wipe, and instructions (Table 2). All serum specimens were analyzed by a usual TDx procedure as soon as collected. The DBS were batch-analyzed; when necessary, they were stored in the open air at 4°C until the pre-assay treatment.

Other assay samples—serum or DBS—were from patients or were pooled whole blood supplemented with theophylline stock standard to the desired concentrations. Both DBS and serum or plasma from the same sample were analyzed.

Table 1. TDx Settings for DBS-Theophylline

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<thead>
<tr>
<th>Location</th>
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<tr>
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<td>MN Span</td>
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</tr>
</tbody>
</table>

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Table 2. Instructions for DBS Collection

1. Clean puncture site (palmar surface of the second or third finger or heel) thoroughly.
2. Wipe with alcohol and allow to dry.
3. Puncture site with the sterile lancet deep enough to assure free flow of blood.
4. Apply the filter paper directly to the wound. Blood flow should be such as to completely fill a circle with a single application. The circle must be completely and uniformly filled. Total saturation of the circle must be evident when the paper is viewed from both sides. Do not apply blood to both sides.
5. Fill all circles completely.
6. Allow blood spots to air dry (10 min).
7. Return the entire form to the "Ziploc" bag.
8. Mail to:

or give to your physician as instructed.

Never touch the filter-paper portion of form with your hands.

Reagents

FFIA reagents. The theophylline antibody, tracer, and pre-treatment solution were purchased from Colony Laboratories, Inc., Richardson, TX 75081 (FFR-Theophylline kit, cat. no. 106001). This material was substituted for those supplied by the TDx manufacturer and was used according to the enclosed instructions except where stated.

Stock standard. Stock theophylline standard, 2040 mg/L, was prepared from an anhydrous preparation (cat. no. 6530; U.S. Pharmacopeial Convention, Inc., Rockville, MD 20852) that had been dried for 4 h at 105°C before being weighed. This stock solution was stable for several months when stored in a dark bottle at 4°C.

Theophylline-free whole blood. Citrated whole blood was obtained from the blood bank. Before using such material, one must confirm that the theophylline concentration in the resulting plasma is less than 0.1 mg/L.

DBS calibrators. First we mixed 5.0 mL of theophylline-free whole blood and 100 μL of the stock standard to obtain a mixture containing 40 mg of theophylline per liter. Subsequently, we prepared several mixtures with concentrations of 3, 10, 5.0, and 2.5 mg/L from this material by serial equal-volume dilutions with the theophylline-free whole blood. We then prepared several lots of DBS calibrators by applying each of the whole-blood mixtures onto the filter-paper portion of the collection form to obtain calibrating filter-paper discs at concentrations of 40, 20, 10, 5.0, 2.5, and 0.0 mg/L. These filter-paper discs, stored at 4°C in open air, were usable for at least three months. To avoid damage to specimens due to condensed moisture, we do not recommend the use of "Ziploc" bags for prolonged storage.

DBS controls. Separate lots of DBS controls were prepared from pools of patients' whole-blood supplemented with an independently prepared stock solution of theophylline to contain about 10 and 20 mg/L. DBS controls were stored under the same conditions as the calibrators.

Deproteinizing solution. Aqueous 5-sulfosalicylic acid, 110 g/L.

Procedures

Instrument calibration. We prepared a new six-point calibration curve every week by assaying the DBS calibrators in duplicates. Acceptance of a curve was determined by the usual manufacturer's criteria—i.e., the root mean square error should be ≤1.0. The net polarization for the calibrators did not change appreciably from week to week. Figure 1 illustrates a typical DBS-theophylline calibration curve obtained by assaying duplicate DBS calibrators. The minimum polarization span between the 0 and 40 mg/L calibrators should be less than 100 arbitrary millipolarization units. The frequency of recalibration was determined by the assayed control values; we recalibrated when results for one of the two control concentrations were outside of the mean ± 2 SD range.

Preparation of DBS. Table 2 shows our instructions for DBS collection from patients. DBS controls, calibrators, and extra samples from venous blood were prepared similarly.

Pre-assay treatment of DBS. We punched out a single disc, 6 mm in diameter, from a DBS and placed it in a flat-bottomed glass test tube. After adding 250 μL of de-ionized, distilled water, we dipped the tubes into an ultrasonic bath. Each disc was sonicated for 3 min or until completely decolorized. We then added 25 μL of deproteinizing solution to the pink extract and mixed vigorously. We transferred all of the pink, cloudy mixture to a microcentrifuge tube, leaving the filter paper disc behind, then centrifuged the extract for 60 s in a microfuge (Brinkmann Instruments, Westbury, NY 11590). We transferred the clear supernate to the specimen-well on the TDx cartridge for analysis. All DBS, including calibrators and controls, were treated identically. A batch of 15 specimens can be prepared for analysis in 10 min.

Results and Discussion

Analytical recovery. Recovery was assessed by triplicate analyses of DBS from pooled whole blood supplemented with known amounts of theophylline ranging from 5 to 30 mg/L.

![Fig. 1. Typical DBS-theophylline calibration curve (arbitrary polarization units)](attachment://image.png)
Recoveries ranged from 97.2 to 103.2% (mean 99.1%), indicating that the pre-assay treatment procedure was satisfactory.

**Effect of hematocrit.** We assessed the effect of hematocrit by assaying DBS prepared from a pooled specimen of blood containing 20 mg of theophylline per liter with an initial hematocrit of 30%. We increased the hematocrit to 66% in some samples by removing part of the plasma. Hematocrit between 30 and 60% did not affect results for theophylline in DBS.

**Distribution of theophylline (erythrocytes vs plasma).** We supplemented a pooled specimen of whole blood with theophylline to give a concentration of 20 mg/L. DBS and plasma were obtained from an aliquot of the specimen immediately after mixing and at various times during the next hour. Table 3 shows that the values for these paired DBS and plasma samples were similar, which implies that the distribution of theophylline between cells and plasma was rapid and indistinguishable (4). We also studied a pooled specimen of whole blood from adults, in which the concentration of theophylline in plasma was 20.5 mg/L and the hematocrit 40%. After centrifugation, the volume of the sample was halved by removal of part of the plasma. We then added isotonic saline to restore the original sample volume. The concentrations of theophylline measured in this saline-diluted plasma and in the corresponding cells were approximately half of the original (10 ± 0.5 mg/L).

**Precision.** We assessed within-run and day-to-day precisions by assaying replicate DBS prepared from two pooled specimens of whole blood with theophylline concentrations of 10 and 20 mg/L. The respective within-run CVs (n = 18) were 5.4 and 4.8%, the day-to-day CVs (n = 25) 6.8 and 5.5%. The CVs obtained were greater than those for the conventional serum FPIA, but lower than reported by Coombes et al. (1).

**Venous DBS vs capillary DBS.** We collected and assayed 31 pairs of venous blood (x) and capillary DBS (y) from patients being treated with theophylline. Regression parameters obtained were: \( y = 1.049x - 0.32, r = 0.963 \), and \( S_{xy} = 0.71 \). There was no significant difference between the two sets of results. Specimens included in this study had concentrations of theophylline ranging from 4.2 to 24.8 mg/L.

**Thermal stability of DBS.** We assayed two lots of DBS twice weekly for four weeks. These materials were stored at 4 °C and at ambient temperatures. During the second week, several samples were exposed to higher temperatures (40 °C) for 24 h. The same material was mailed to a city in the southwestern United States; when it was returned, we assayed the theophylline contents. In all the specimens analyzed, we did not observe significant changes in theophylline contents, indicating that theophylline is stable in a filter-paper matrix for a month.

<table>
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<tr>
<th>Time elapsed, min</th>
<th>Theophylline concn, mg/L</th>
<th>Plasma</th>
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<tr>
<td>0 (^b)</td>
<td>18.4</td>
<td>19.6</td>
</tr>
<tr>
<td>5</td>
<td>18.9</td>
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</tr>
<tr>
<td>60</td>
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</table>

\(^a\)Concentration of theophylline added, 20 mg/l; hematocrit, 47%. \(^b\)Immediately after mixing.

**Nonspecific background fluorescence.** We processed five blank filter-paper discs according to the pre-assay treatment procedure. The extracts obtained showed theophylline concentrations ranging from "low" to 0.1 mg/L. Extracts obtained from DBS containing theophylline-free whole blood gave identical results, indicating that the nonspecific fluorescence was sufficiently low not to affect analyses.

**Comparison of DBS-FPIA vs serum-FPIA.** We assayed 64 pairs of DBS and serum specimens by the respective FPIA methods. DBS were of either capillary or venous origin. Two of the >30 mg/L specimens were drug-supplemented material. Theophylline contents in DBS correlated significantly with those in the serum, as shown in Figure 2. Regression parameters for the DBS method (y) and the conventional serum method (x) were: \( y = 0.94x + 0.71, r = 0.988 \), and \( S_{xy} = 0.92 \).

**Therapeutic ranges.** The usual serum theophylline therapeutic range is 10–20 mg/L for controlling asthma and 7–10 mg/L for neonatal apnea. In a previous report (1), Coombes et al. proposed a much lower range (8.5–16.5 mg/L) for their capillary DBS method, attributing the differences to the methods of specimen collection. Because the results by our two methods correlated well, we did not find it necessary to propose a new therapeutic range, which would have surely confused clinicians.

The concept of using DBS to monitor therapy arises from the practical problems of obtaining specimens from patients at a time when they have symptoms and that are suitable for a convenient and reliable assay method. For this reason we chose FPIA, reportedly among the most popular methods for measuring serum theophylline, followed by enzyme-multiplied immunoassay technique (EMIT®, Syva Co.). Fewer than 5% of the 463 laboratories participating in a recognized proficiency program (5) in the United States use fluoroimmunoassay for quantifying serum theophylline, the analytical method selected by Coombes et al. (1, 2). With the DBS-FPIA procedure and a pre-calibrated TDx analyzer, an assay can be completed within 20 min. In addition, we have reduced the sample extraction time from 90 min, as reported.

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Fig. 2. Results of DBS-FPIA and serum-FPIA for theophylline compared.
by Coombes et al. (1, 2), to only 3 min, with excellent recoveries. We suspect the direct application of sulfosalicylic acid onto the filter-paper discs, as recommended by Coombes et al., may have slowed the release of theophylline back into the solution. We improved the method for standardization, to make it less dependent on the exact volume of whole blood applied onto the filter paper. In the previously reported method (1) exactly 9.8 µL of whole blood was required.

Because operating the TDx-FPIA requires only basic laboratory skills, its use with dried-blood spot specimens can be an effective theophylline-monitoring system particularly suited for outpatient and domiciliary care. This system can also be used in pharmacokinetic profile studies such as that recently reported (6).

References