Multilayer Film Analysis for Glucose in 1-μL Samples of Plasma

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With the ultramicroanalytical system described here we can measure glucose in 1 μL of plasma or serum. The sample is placed on a dry, multilayer film element (Eastman Kodak), where a colored spot about 3.5 mm in diameter develops. The reflectance of these spots is measured with a reflectance digital matrix photometer that was conceived, designed, and constructed in our laboratory. The spot is illuminated with monochromatic light and its image is projected by a camera lens onto the photosensitive surface of a linear photodiode array containing 512 individual photodetectors. The photodetector signals are processed by a computer to obtain the reflectance and diameter of the spot. The latter is proportional to sample volume. Because the reflectance of the spot does not depend greatly on sample volume, accurate pipetting is not required. The coefficients of variation of repeatable glucose analyses were, for 400, 3000, and 5120 mg/L, 1.7, 2.3, and 2.8%, respectively. The correlation coefficient (r) between glucose analyses by our method (y) and with the Ektachem 400 (x) was 0.9918; the regression equation was y = 1.07x – 54.3 mg/L.

Additional Keyphrases: pediatric chemistry • glucose assay as an example • ultramicroanalysis

The recent development by Eastman Kodak Co. and Fuji Photo Film Co. of dry, multilayer slides for clinical glucose analyses represents new concepts in the technique of routine clinical-chemical analyses (1, 2). In both systems, the reflectance on the slides is measured by reflectance photometry and 10 μL of sample is required to completely cover the reactive area of the slides. The systems differ in that the Kodak slide requires plasma or serum and the Fuji slide, whole blood. We observed that if smaller samples, for instance 1 μL of plasma, are applied to the Kodak glucose slide, small, circular, and highly reproducible colored spots are formed, which measure about 3.5 mm in diameter. It occurred to us that combining this dry, multilayer film technique and our high-resolution reflectance digital-matrix photometer (3) might make it possible to perform routine clinical chemistry analyses on samples of about 1 μL.

Briefly, a 1-μL plasma or serum sample is placed on a Kodak multilayer film element. After incubation at room temperature, monochromatic light is used to illuminate the colored spot that develops in the area of sample application, and its image is projected by a camera lens onto the photosensitive surface of a linear photodiode array. The signals are processed by a computer to provide data on the reflectance of the spot, which is proportional to sample concentration (Figure 1).

Materials and Methods

Instrument Components

The design of our reflectance digital matrix photometer is described elsewhere (3, 4). It is based on the use of a solid-state photodiode array (RL-512-G; E. G. and G. Reticon Co., Sunnyvale, CA 92086), an interface circuit, and a computer. A 540-nm, three-cavity interference filter was used.

Materials

Protein-based references. Ektachem calibrators (cat. no. KP74351J; Eastman Kodak Co., Rochester, NY 14650).

Multilayer film elements. Ektachem Clinical Chemistry Slides for glucose (Eastman Kodak Co.).

Procedures

Sample application. Approximately 1 μL of sample or reference was applied to the slides with a 2-μL syringe or 5-μL disposable pipet (Accupette, P4518-SX; Dade Diagnostics, Aguada, PR 00602), with care not to indent or scratch the spreading layer of the slide. The reaction was allowed to proceed at room temperature for 7 min.

Instrument operation. Before the reflectance of the spot on a slide was measured, the signal for each photodetector was stored for both the dark signals and the blank signals representing 100% reflected light. The blank signals were obtained from an unused slide. The slide with the plasma sample was placed in the instrument and manually positioned so that its image was projected onto the center of the photodiode array. This was done with the aid of an oscilloscope, which was connected to the video output of the photodiode array system. At 7 min from the time the plasma sample was placed on the slide, the reflected light seen by each photodetector was measured and converted to reflectance. All measurements are the average of four replicate measurements taken within 1 s.

Data processing, computations, and calibration. The photometric transformations used were based on the work of Williams and Clapper (5) as modified by Curme et al. (1). The dark signals for each photodetector were stored as a linear array D(j) (Equation 1) and the blank signals as B(j) (Equation 2).

\[
D(j) = (D_1, D_2, \ldots, D_{512})
\]

(1)

\[
B(j) = (B_1, B_2, \ldots, B_{512})
\]

(2)

For calibration, we used appropriate reference solutions to develop the colored spots. The photodetector signals for each photodetector were stored as linear array S(j) (Equation 3).

\[
S(j) = (S_1, S_2, \ldots, S_{512})
\]

(3)

The reflectance density for each photodetector, corrected for flare light, Rf, was stored as linear array Dr(j) (Equation 4).

\[
Dr(j) = \log \left( \frac{(B_1 - D_1)/(S_1 - D_1 - Rf)}{\log(B_2 - D_2)/(S_2 - D_2 - Rf)} \right) \ldots \log \left( \frac{(B_{512} - D_{512})/(S_{512} - D_{512} - Rf)}{\log(B_1 - D_1)/(S_1 - D_1 - Rf)} \right)
\]

(4)

Rf was determined by an iterative technique with use of the value giving the best straight-line fit for the calibration curve. Numerical integration of the Williams–Clapper transform yields Equation 5. Transformed reflection densities were computed for 25 (p1–p25) photodetectors that were located in the central portion of the spot and stored in linear array Dt(j).
Fig. 1. Plot of percent reflectance vs photodiode number of a scan of a spot produced by 1.0 μL of plasma placed on a Kodak Ektachem slide for glucose.

The measurement was taken across the center of the spot.

\[ D_t(j) = \left( -1.194 + 0.469 \times D_r(p_1) + 0.422 \left( 1 + 1.179 \times \varepsilon(3.379 \times D_r(p_1)) \right) \right) + \left( -1.194 + 0.469 \times D_r(p_2) + 0.422 \left( 1 + 1.179 \times \varepsilon(3.379 \times D_r(p_2)) \right) \right) + \ldots + \left( -1.194 + 0.469 \times D_r(p_25) + 0.422 \left( 1 + 1.179 \times \varepsilon(3.379 \times D_r(p_25)) \right) \right) \]

(5)

An average, \( D_a \), of these 25 values was used in calibration and computations for specimens. Concentration can be computed from reflection data by the expression:

\[ C = \text{Beta} \times (D_a - D_b) \]

(6)

Slight rearrangement of Equation 6 gives the equation for a straight line where \( 1/\text{Beta} \) is the slope and \( D_b \) is the y-intercept.

\[ D_a = \left( 1/\text{Beta} \right) \times C + D_b \]

(7)

We performed a linear regression using Equation 7 with the reference solutions. Unknowns were also computed by using this equation and regression parameters.

**Results**

**Required sample volume.** Volumes ranging from 0.1 to 2.0 μL of 400 and 3290 mg/mL glucose calibrators were applied to separate slides and the reflectance measured as described above. Figure 2 is a plot of the amount of reflectance in the center of the spots vs sample volume for two different concentrations. Samples of 1 μL or greater appeared to produce a nearly constant color in the central portion of the spot. Between the sample volumes of 0.8 to 1.5 μL, the percent reflectance for the 400 mg/mL calibrator decreased only from 69.6% to 68.9%, representing on a calibration graph 394 and 403 mg/mL, respectively, and for the 3290 mg/mL calibrator the percent reflectance decreased only from 13.4% to 12.4%, representing 3218 and 3404 mg/mL, respectively.

Fig. 2. Plot of percent reflectance of spot centers vs. sample volume in microliters at two different glucose concentrations: A, 400; B, 3290 mg/mL.

**Precision.** Replicate analyses (n = 17) of three samples containing different glucose concentrations gave the following results: sample 1: mean = 395 mg/mL; SD = 6.8; CV =

Fig. 3. Three-dimensional viewing transformations of the colored spots produced on Kodak glucose slides by the following plasma sample volumes: A, 0.2; B, 0.5; C, 1.0; D, 2.0 μL.

The glucose concentration in all samples was 3290 mg/mL. Dt is transformed reflection density.

Fig. 4. Plot of percent reflectance of spot centers vs time for three glucose concentrations: A, 400; B, 3290; C, 5450 mg/mL.
change in sample volume resulted in only a 1% change in the resulting reflection density. Our studies demonstrate that for a given glucose concentration, the color developed in the center of the spots is fairly constant between sample volumes of 0.8 to 1.5 μL (Figure 2B). We observed that color development became progressively more dependent on sample volume as the sample volume was decreased below 0.2 μL. In addition, the computed glucose concentration became progressively more dependent on sample volume as the glucose concentration increased.

An important advantage to using a photodiode array detector is that the borders of the colored spot can easily be defined and as a result, the spot diameter can be used to estimate the sample volume. This mechanism can serve as a valuable check to assure that an adequate sample volume has been applied to each slide.

The system we have described is a prototype and designed specifically to demonstrate the feasibility of performing analyses on 1 μL of sample. Several important improvements could be made to further optimize system performance. The lamp's light output was not regulated and the light pipe did not produce a highly parallel and homogeneous illumination of the sample. A specific lens system may enhance optical resolution. Samples were applied manually and for timing we used a stopwatch. The sample was positioned in the detector manually. The analyses were performed at room temperature with no temperature regulation. Undoubtedly, with further improvements in these areas an even higher precision of the technique can be expected.

We have shown that the combination of a reflectance digital matrix photometer with the modern multilayer film technology makes it possible to analyze minute plasma samples for glucose in the range of 1 μL. This new approach may open a new dimension in the miniaturization of ultramicrochemical analyses in clinical practice, particularly in pediatrics, where it is often difficult to obtain samples of the size required by current methodology.

References