Short Reports

Development of Stable Film Test for Rapid Estimation of Blood or Plasma 3-Hydroxybutyrate

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A film test for the rapid detection of plasma/serum 3-hydroxybutyrate (3-OHB) has been developed. The film contains NAD, nitro blue tetrazolium, 3-OHB dehydrogenase, and diaphorase, and the surface is coated with modified biomembrane and can detect 50–1500 μM 3-OHB within 2–3 min. One drop or 50 μl of plasma/serum or blood is applied to the film, and the violet color is read via reflectance meter after 2 min. Plasma/serum samples >1500 μM 3-OHB can be measured by dilution with saline. In blood with 40% hematocrit, the color developed is 50% less than with plasma/serum, and this was adjusted in the reflectance meter. A good correlation (r = 0.99) was observed between results with automated and film methods and between visual methods and reflectance meter. In insulin-dependent diabetes mellitus, all 3 subjects with positive ketonuria (++), 8 of 12 subjects with mild ketonuria (+), and 7 of 25 subjects without ketonuria exhibited elevation of 3-OHB in blood >200 μM. The results indicate that 3-OHB film is valuable not only in the emergency room for the differential diagnosis between ketoacidotic and nonketotic hyperosmolar coma but also as a marker for insulin dependency, energy dependency on fatty acid compared with glucose, and metabolic control of diabetes. *Diabetes Care* 13:522–24, 1990

The major ketone body that increases in the blood of patients with diabetes on low-calorie or high-fat diets, or in fasting, exercise, or stressful situations is 3-hydroxybutyrate (3-OHB) (1). The levels are mainly regulated by insulin, insulin-antagonistic hormones, and food intake (2). Proneness to ketosis is an important marker for the diagnosis of insulin-dependent diabetes mellitus (IDDM) (3). In contrast to the elevation of 3-OHB, the degree of elevation of acetoacetate and acetone is usually less remarkable (4). The latter two are roughly detectable by nitroprusside sticks, which do not react against 3-OHB. Therefore, a rapid test for the estimation of 3-OHB is needed. We have previously reported a paper method that detects 3-OHB within 3 min, but the paper test has not been stable enough for clinical use (5). We now report a stable film test for the rapid estimation of blood or plasma/serum 3-OHB.

RESEARCH DESIGN AND METHODS

Blood samples containing various concentrations of 3-OHB were prepared by adding D-3-OHB (Sigma, St.

Louis, MO; sodium salt contains equal moles of D and L, and only D form is biologically active) to the normal blood. The samples were used after being left at room temperature for 5 h until equilibration had been obtained.

Children aged 6–15 yr who participated in the diabetes camp sponsored by Kinkitsubominokai were studied. Mean duration of diabetes was 4 yr. The children were treated with 0.32–1.3 U/kg of intermediate-acting insulin before breakfast. In ~50% of the subjects, additional intermediate-acting insulin (0.06–0.7 U/kg) was injected before dinner. In some of them, short-acting insulin was added in the morning or evening injection. HbA_{1c} and HbA_1 ranged from 6 to 14 and 8 to 17%, respectively (normal upper ranges are 5 and 8%, respectively). Their urinary excretion rates of C-peptide reactivity were all <5 μg/day.

3-OHB is enzymatically converted to acetoacetate, and the formed NADH reduces nitro blue tetrazolium by diaphorase, resulting in formazan formation, which has a violet color. In total, 1.5–3.5 mg NAD, 30–70 U 3-OHB dehydrogenase, (Sanwakagaku Kenkyusho, Higashi-ku, Nagoya, Japan), 60–140 U diaphorase, and 1.5–3.5 mg nitro blue tetrazolium were used for the production of 100 sheets of polyester film sticks (0.5 × 8 cm). On the tip end, ~2 mm, the stick was coated with thin film 7 mm long containing the reaction reagents. The film is coated with an artificially modified natural polymer that excludes substances with molecular weight >10,000, so that blood enzyme cannot penetrate into the film layer and does not interfere with the color development. Film and reflectance meter are commercially available from Sanwakagaku Kenkyusho. One drop or 50 μl of blood, plasma, or serum was applied to the film surface. After 2 min, the violet color was read either visually from a standard color chart or with a reflectance meter (Fig. 1). By reflectance meter, 3-OHB can be detected from 50 to 1500 μM in plasma/serum and up to 3000 μM in blood. Intra-assay coefficients of variation for samples ranging from 50 to 1500 μM by reflectance meter were <2–4%. In addition, the same range of coefficients of variation (2–3.5%) was observed for interassay samples (800 μM) stored for 12 and 18 mo in a refrigerator, indicating that the film tests are stable for at least 1 yr in a refrigerator. Normal upper range of serum 3-OHB was 74 (mean + 2SD) by the colorimetric assay and in fact has never exceeded 100 μM by the film tests for samples obtained after overnight fasting.

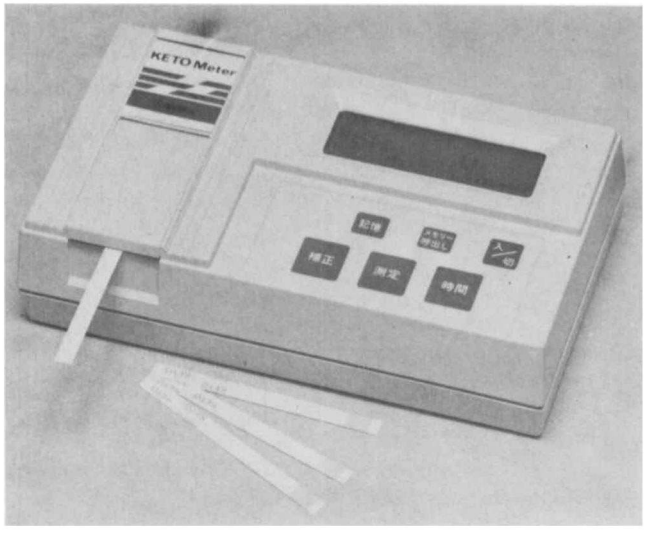

FIG. 1. Reflectance meter for 3-hydroxybutyrate.

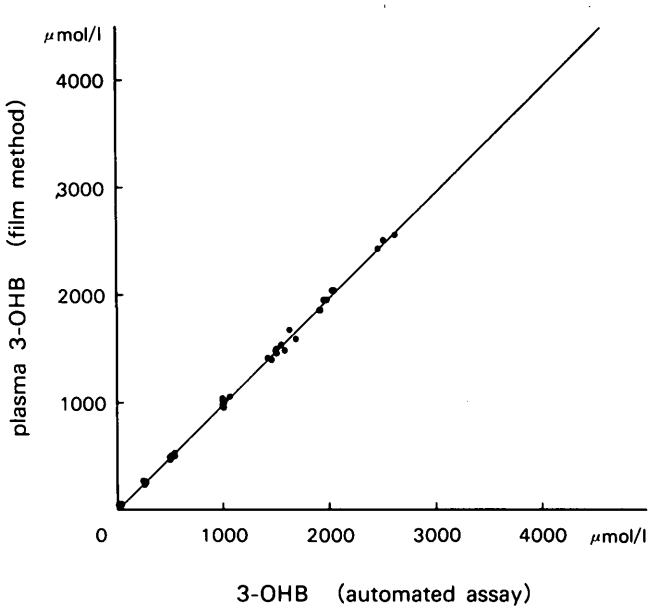

FIG. 2. Correlation between film and automated methods for determination of plasma 3-hydroxybutyrate (3-OHB). 3-OHB was determined by film test via reflectance meter. n = 54, r = 0.999, y = 1.00x − 2.85.

A direct enzymatic method for the evaluation of ketone bodies was used to compare the results with the film method (6). For a conventional semiquantitation of ketone bodies, Ketostix (Miles, Sankyo, Tokyo) was used. Glucose was determined in plasma by an immobilized enzyme method (Diagluca, Toyobo, Osaka). The correlation study between the visual and meter-reading tests was performed by two investigators: one read from the meter while the other judged the color development independently.

RESULTS

A good correlation (r = 0.99) was observed between the film test read by reflectance meter and the automated chemical assay (Fig. 2). Films with 3-OHB >1500 μM were diluted with saline, and the developed color was read by reflectance meter. A good correlation (r = 0.99) and high correspondence were observed for the values determined by film methods via both reflectance meter and visual judgment. The film method can detect 3-OHB as low as 50 μM by both reflectance meter and

visual judgment. A good correlation (r = 0.99) was observed between the plasma and whole-blood samples. An increase of hematocrit by every 1% >40% reduces the color development by 1.6%. Thus, the value obtained in a blood sample with hematocrit deviating from 40% should be corrected accordingly. NaF (5–25 mg/ml), EDTA (2.5–10 mg/ml), heparin (50–200 U/ml), uric acid (10–30 mg/dl), acetoacetate (0.3–1.5 mM), lactate (5.5–11.1 mM), and pyruvate (0.3–1.5 mM) had no interfering effect on the color development. Acetoacetate at 3 mM reduced color development by 10–20%. Therefore, if an unusually elevated acetoacetate level is anticipated, samples should be measured after dilution. L-Ascorbate acid was the only compound that produced a false-positive color, but this was eliminated by adding L-ascorbate oxidase to the film.

In IDDM, all 3 subjects with positive ketonuria (+ +) showed obvious elevation of 3-OHB >200 μM. Eight of 12 subjects with mild ketonuria (+) and 7 subjects of 25 without ketonuria exhibited moderate elevation of 3-OHB in plasma (Fig. 3). In total, 18 of 40 subjects exhibited abnormal 3-OHB, whereas ketonuria was noted with Ketostix in 11 of 40 subjects, indicating that determination of plasma 3-OHB by film is more sensitive in detecting ketonemia in IDDM than a nitroprusside reaction. 3-OHB levels were higher before breakfast, 100–3000 μM, than before lunch, 20–200 μM, in IDDM. Because the normal upper level of 3-OHB before breakfast is 100 μM at most, all the diabetic children exhibited elevated plasma 3-OHB. Before dinner, 3-OHB levels diminished below the normal level (80 μM), although hyperglycemia prevailed.

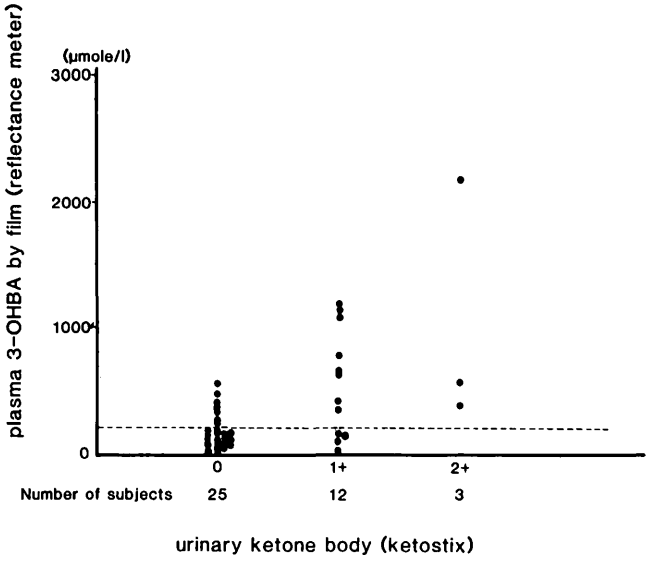

FIG. 3. Relationship between plasma 3-hydroxybutyrate (3-OHBA) determined by film (reflectance meter) and conventional Ketostix results in urine before breakfast.

DISCUSSION

Development of a film test for 3-OHB in a blood sample is thought to be one of the breakthroughs in the management of diabetes. In film, a reaction layer can be separated from a color-developing layer by devising the order of coating, thus contributing to the longer stability compared with a filter test. In IDDM with mild ketonuria (+), 4 of 12 subjects failed to exhibit elevated blood 3-OHB levels. Acetoacetate started appearing in urine at lower levels (0.1 mM) than 3-OHB (0.6 mM) because of better tubular resorption for 3-OHB. Therefore, in mild ketonemia, especially with preponderant elevation of acetoacetate, use of Ketostix in the urine sample is a sensitive method to detect ketonemia early. 3-OHB increases in fasting hypoglycemia due to insulinopenia, but no elevation has been noted in insulin-induced hypoglycemia.

The film method is particularly useful in the emergency room for comatose patients, in diabetes wards, or in outpatient clinics as metabolic markers assessing diabetic control other than glucose and glycosylated hemoglobin (7).

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