Erythrocyte 3-*O*-Methyl-D-Glucose Uptake Assay for Diagnosis of Glucose-Transporter-Protein Syndrome

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Glucose transport into the brain is mediated by a facilitative glucose-transporter protein, GLUT-1. A GLUT-1 defect results in the Glucose-Transporter-Protein Syndrome (GTPS), characterized by infantile epilepsy, developmental delay, and acquired microcephaly. The diagnosis is currently based on clinical features, low to normal lactate levels and low glucose levels (hypoglycorrhachia) in the cerebrospinal fluid, and the demonstration of impaired GLUT-1 function in erythrocytes as described here. Blood samples were collected in sodium-heparin or citrate-phosphate-dextrose solution and uptake of ^{14}C-labeled 3-*O*-Methyl-D-glucose (3OMG) into erythrocytes (0.5 mmol/L 3OMG; 1μCi/mL) was measured at 4C and pH 7.4. Three-OMG influx was terminated at 5-second intervals, washed cells were lysed, and uptake was quantitated by liquid scintillation counting. Patients' uptake (n = 22) was 44 ± 8% of controls (100 ± 22%, n = 70). Statistical analyses showed an uptake cut-off point at 60% uptake, a sensitivity of 86% (95%-confidence interval 78 to 94%), and a specificity of 97% (95%-confidence interval 93 to 100%). Gender, age, and ketosis did not influence 3OMG uptake. This assay provides a reproducible and accurate laboratory test for diagnosing the GTPS. J. Clin. Lab. Anal. 13:116–121, 1999. © 1999 Wiley-Liss, Inc.

Key words: glucose-transporter-protein syndrome; GTPS; De Vivo disease; 3-*O*-methyl-D-glucose; 3OMG-glucose transport; erythrocyte; GLUT-1; glucose transporter; blood-brain barrier; infantile seizures

INTRODUCTION

Transport of D-Glucose, an essential fuel for cerebral metabolism, across the blood-brain barrier and into brain cells is selectively mediated by a sodium-independent, facilitative transport protein, GLUT-1 (1,2). GLUT-1 belongs to a multigene family of facilitative glucose transporters (for reviews see 3–6) and is expressed at various blood-tissue barriers. It is present in high abundance in brain capillaries and astroglial and erythrocyte membranes, representing about 6% of the total red blood cell membrane protein content (7).

In 1991 we described two patients with low glucose concentrations in the spinal fluid (hypoglycorrhachia), infantile seizures, ataxia, hypotonia, acquired microcephaly, and significant developmental delay (8). This condition is recognized as the Glucose-Transporter-Protein Syndrome (GTPS), or DeVivo Disease (9,10), and is caused by a defect in the GLUT-1 protein. The diagnosis is based on clinical features, a low ratio of cerebrospinal fluid glucose versus blood glucose concentrations (ratio ~0.33 ± 0.01) (9), and defective glucose transport via the GLUT-1 protein. As the erythrocyte GLUT-1 protein is identical in molecular weight and antigenic properties to the GLUT-1 protein in brain capillary endothelial cells (11), glucose transport into erythrocytes can be used as a surrogate for glucose transport across the blood-brain barrier. On the basis of previously described assays for ^{14}C-labeled 3OMG-uptake into human erythrocytes (12,13) we developed a diagnostic laboratory test. Here we give the first detailed analyses of our experience with this diagnostic assay in 22 GTPS patients and 70 controls and of improvements made since the test was first described in 1991 (9).

MATERIALS AND METHODS

Clinical Material

The GTPS was suspected on clinical grounds in combination with low glucose concentrations in the cerebrospinal fluid

(hypoglycorrhachia). Twenty-two candidates (11 males, 11 females; 1–19 years of age; mean age, 5 years) were referred to and evaluated by one of the authors (DCD) for the diagnosis of the GTPS (8,9). Blood samples from parents were used as intra-assay controls. The control group included 38 parents (19 males, 19 females) and 32 additional control subjects of varying ages (16 males, 16 females; mean age not determined). In an additional patient (male, age 9 years of age), blood was drawn the day prior to the introduction of a ketogenic diet, and on day four of ketosis, a 3OMG uptake was determined. For each assay blood samples from the nonketonic parents were obtained as intra-assay controls.

Zero-Trans 3OMG Uptake Into Erythrocytes

In the first set of studies, fresh blood samples (3–5 mL) were collected in tubes containing sodium-heparin, immediately stored on wet ice, and assayed within 24 hr. In later studies designed to facilitate long-distance shipment, the blood samples were mixed with CPD solution (5:1, v/v) by gently inverting the tubes several times and immediately stored on wet ice to be processed within 10 days. In three patients and three controls additional assays on CPD blood were performed within 21 days to evaluate the reproducibility of the data as suggested in the literature (14). Blood samples from both parents served as intra-assay controls. Samples were excluded if RBCs were hemolyzed, and none of the subjects had been transfused in recent months.

Solutions used were: (1) citrate phosphate dextrose solution: 2.45g dextrose, 2.63g sodium citrate, 298 mg citric acid, all into 100 mL; and (2) phosphate-buffered saline (PBS), in mmol/L: 137 NaCl, 2.7 KCl, 4.3 $Na_2HPO_4 \cdot H_2O$, 1.4 KH_2PO_4. "Hot" solution was ^{14}C-labeled 3OMG (0.5 μCi/mL) in 0.5 mmol/L 3OMG in PBS. The "stop" solution was prepared by adding 100 μmol/L mercuric chloride and 50 μmol/L phloretin to 500 mL of ice-cold PBS immediately (< 10 min) before use. Reagents: 3OMG, phloretin, and mercuric chloride were from Sigma Chemical Co. (St. Louis, MO), ^{14}C-3OMG (0.1 mCi/ mL; 56.4 mCi/mmol) was from DuPont NEN (Boston, MA).

Preparation of erythrocytes

Procedures were performed at 4°C. Blood specimens (2–5 mL) were centrifuged at 1,000g for 10 min. The supernatant was discarded, and the RBC pellet (~1–2 mL) was washed three times with 50 mL PBS. RBC were then resuspended in PBS at approximately the original blood sample volume by inverting the tubes repeatedly. One hundred μL aliquots were taken for immmediate assay, and 300 μL were used for a cell count.

3OMG uptake

Data points were obtained in duplicate, equilibrium values in triplicate. The 100 μL aliquots of the RBC suspension were briefly vortexed and 200 μL ^{14}C-labeled 3OMG solution was added; uptake was terminated at 5, 10, 15, 20, 25, and 30 sec, and at 25 min for equilibrium time by rapid addition of 1 mL ice-cold stop solution. Zero time values were obtained by adding 200 μL ^{14}C-labeled 3OMG solution to the 100 μL aliquots of the RBC suspension after the addition of 1mL stop solution.

The RBCs were centrifuged at 2,000g for 5 min and the RBC pellet washed twice with 1 mL stop solution. Following the final centrifugation, the erythrocyte pellet was digested with a blend of toluene (40–60%), dimethyl dialkyl quaternary ammonium hydroxide (30–40%), and methanol (5–10%; Soluene® 350, Packard, Meriden, CT) for two hr at 60°C. After cooling to room temperature, samples were bleached with 0.25 mL 30% hydrogen peroxide. The mixture was swirled until all foaming subsided and was subsequently incubated for 30 min at 60°C (15). Hionic Fluor™ scintillation fluid (5 mL/sample; Packard) was added and aliquots were counted with a Packard TR 2300 Scintillation Counter (settings: 5 minute count/sample on the ^{14}C-specific channel; the integrated Dynamic Color Corrected DPM mode provided for absolute activity (DPM) calculations and for correction of color quenching based on transformed Spectral Index of External Standard [tSIE]). The data was expressed as the natural logarithm of intracellular radioactivity in DPM at time T (0–30 sec), divided by intracellular radioactivity in DPM at equilibrium (25 min), versus time in seconds: [ln(1-Ct/Ceq)] vs. (s). The slope derived from linear best-fit analyses (two determinations/datapoint) represented 3OMG uptake. Slope values of subjects presenting with hypoglycorrhachia and the clinical features of the GTPS were compared to the mean slope values of parents and controls, and to their individual intraassay control.

RESULTS

Technical Aspects of the Assay

Washing the erythrocytes three times in PBS resulted in an 80%-reduction of platelets, while erythrocyte and white blood cell input remained unchanged. Hemocytometer counts showed that the resuspended erythrocyte pellet contained on average 4.75×10^6/μL red blood cells. Reproducible results were obtained with $2.0–6.0 \times 10^6$ RBC/μL (data not shown). The reddish-brown color of the red cell lysate produced a high quench and counting instability when directly added to the liquid scintillation cocktail. Counting efficiency was reduced to approximately 30% of the total radioactive isotope input. After treatment with H_2O_2 counting efficiency was improved to approximately 70% (Fig. 1).

Routine clinical laboratory analyses of blood and urine in the patients studied were not indicative of any pathology. With the exception of one control, first-day uptake values compared well for both anticoagulant solutions (Fig. 2). When RBCs were constantly maintained at 4°C, 3OMG uptake in
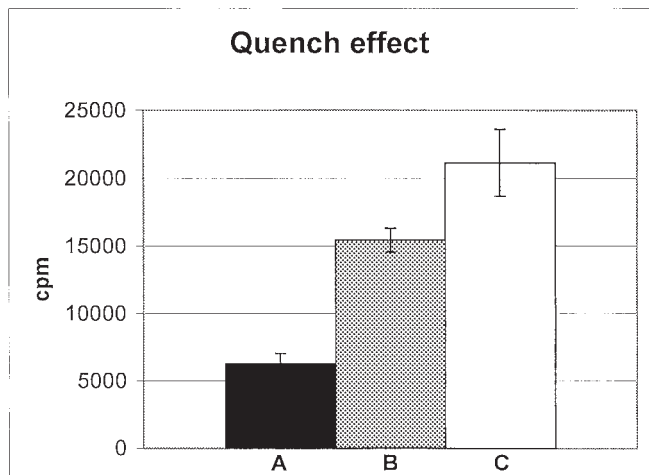
Quench effect

Fig. 1. Quench effect (nine determinations per data point). The reddish-brown color of erythrocytes quenches scintillation counts (A). Counts increase significantly the sample is bleached with H_2O_2 (B). Total counts in PBS without erythrocytes (C). Radiolabel, 1µCi/mL ^{14}C-labeled 3OMG in PBS.

three patients and four controls was reproducible for approximately 10 days (Fig. 3), but declined afterwards.

Diagnostic Value of the Assay

Three-OMG uptake values were obtained from 22 individuals with the clinical diagnosis of GTPS. Seventy individuals served as controls. The data were expressed as the natural logarithm of the ratio of intracellular radioactivity at time T and at equilibrium vs. time (seconds) and 3OMG uptake was expressed as the slope of the curve (Fig. 4).

Controls showed slope values of -0.0313 ± 0.007 (mean $\pm$ SD). Parents' values (-0.031 ± 0.007 mean $\pm$ SD; n = 38)
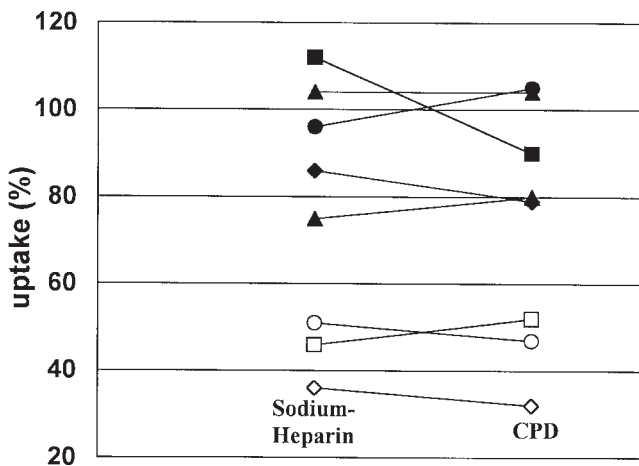
Fig. 2. 3OMG-uptake values (%) using Na-Heparin vs. CPD anticoagulant solution. Data shown (two determinations per data point, day 1) represent uptake values of three patients (open symbols), their parents (closed symbols), and two additional normal controls (▲).

were undistinguishable from the unrelated controls (-0.032 ± 0.007 mean $\pm$ SD; n = 32; t = 1.07, $P = 0.29$).

Patients' slope values were -0.0137 ± 0.0026 (mean $\pm$ SD). No significant gender differences were observed in patients (males: -0.014 ± 0.003 mean $\pm$ SD, n = 11; females, n = 11: -0.013 ± 0.002 mean $\pm$ SD; t = 1.15, $P = 0.26$) or in controls (males: -0.031 ± 0.009 mean $\pm$ SD, n = 35; females: -0.032 ± 0.006 mean $\pm$ SD, n = 35; t = 0.52, $P = 0.60$). When slope values were expressed in % (mean slope value of controls of -0.0313 corresponded to 100%), the mean patients' uptake was 44% $\pm$ 8, ranging from 26–56% (see Table 1).

The impact of ketosis on 3OMG uptake was analyzed the day prior to the introduction of the ketogenic diet and on day 4 of ketosis in one patient and his nonketotic parents. As shown in Figure 5, ketosis did not influence uptake; in the patient, slope values for the nonketotic state were -0.0090 ± 0.0003 (29% uptake) vs. -0.0096 ± 0.0003 (31% uptake) during ketosis. Mean parents' values were -0.0022 ± 0.0006 (70% uptake) and showed a good reproducibility for both assays.

Statistical Analyses

Glucose uptake levels for 92 subjects, 22 with a clinical diagnosis of GTPS and 70 controls, were analyzed to determine their utility as a screen for GTPS. For the 22 patients, two determinations were made for each time point. The reliability of the assay was estimated using an intraclass correlation coefficient (ICC). Two analyses were performed for the 5- and 30-second time points. Reliability was excellent at each time point, with an ICC of 0.95 at 5 seconds and an ICC of 0.99 at 30 seconds.

There was little evidence of any difference in glucose uptake levels between the 35 males and 35 females ($P > 0.2$ based on a two sample t-test), therefore the analysis was not stratified by sex. There was also no difference between the 38 parents and the 32 other controls ($P = 0.34$ based on a two sample t-test). For the 22 patients, there was no significant association between age and slope values (Pearson's r = -0.283, $P = 0.20$), and between hypoglycorrhachia and slope values (Pearson's r = -0.024, $P = 0.78$). Receiver operating characteristic (ROC) curves were used to determine a cut-point for glucose uptake, classifying these subjects as affected or not. The criteria for determining the cut-point were the sensitivity and specificity of the screen with respect to the known diagnostic status of the patients. Examination of these curves yielded a relatively clear cut-point of -0.018 (60%). That is, subjects with slopes greater than -0.018 in magnitude would be classified as patients, and those with magnitude less than -0.018 would be classified as normal (Fig. 6).

This analysis correctly classified 87 of the 92 subjects (95%), corresponding to a sensitivity of 86% (78–94%), a specificity of 97% (93–100%), a positive predictive value of 89% (85–93%), and a negative predictive value of 94% (88–100%) (Table 2).
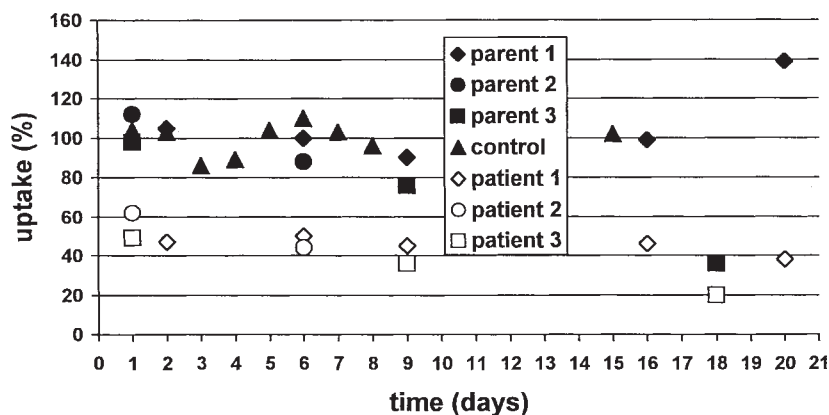
Fig. 3. 3-OMG-Uptake (%) over time using CPD anticoagulant solution (two determinations per data point). Parents 1–3 (closed symbols) served as the intrassay control to patients 1–3 (open symbols). Data of one additional control (▲) is added to the table.
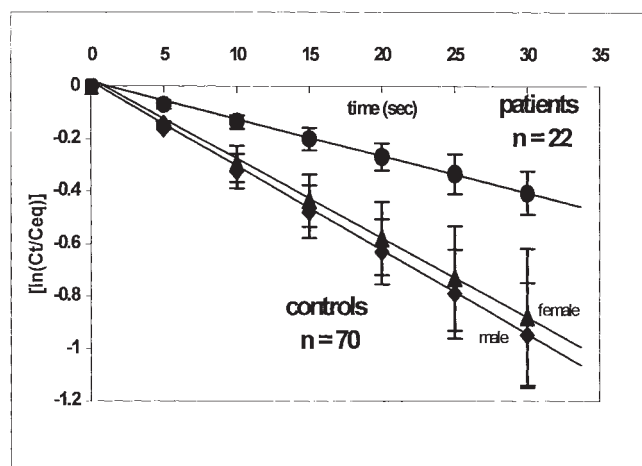
DISCUSSION

The Glucose-Transporter-Protein Syndrome (GTPS), or De Vivo Disease(10), is a rare but treatable metabolic disease presenting early in childhood with seizures and developmental delay. Accurate diagnosis of this condition is important because a high-fat diet providing ketone bodies as an alternative fuel for the brain will result in significant clinical improvement. Since 1991, at least 20 patients have been identified based upon clinical criteria, hypoglycorrhachia, and deficient 3OMG uptake. We utilized 3OMG as a synthetic monosaccharide substrate since it is transported across the erythrocyte plasma membrane but is not further metabolized intracellularly (17–20).

Significant improvements were made on the method initially described (9). Using CPD as an anticoagulant solution allowed blood samples to be maintained for periods up to approximately 10 days, allowing a greater degree of flexibility in the laboratory and the performance of more time-consuming tests. In addition, it now enables us to receive shipments of samples from virtually anywhere in the world. Bleaching the lysate with H_2O_2 yielded higher scintillation counts and resulted in more accurate data. The statistical analysis of the data revealed a good sensitivity (86%) and a high specificity (97%) for the assay.

TABLE 1. Data[a] on 22 GTPS Patients

Pat	Age (yrs)	Sex	CSF/Blood ratio	Slope	Uptake
A	Deficient 3OMG uptake:				
1	9.5	m	0.29	–0.0109	35
2	10	f	0.28	–0.0134	43
3	13.5	m	0.33	–0.0171	55
4	7	m	0.35	–0.0113	36
5	8	f	0.32	–0.0157	50
6	24	m	0.36	–0.0172	55
7	10	f	0.31	–0.0145	46
8	12	m	0.33	–0.0147	47
9	6	f	0.28	–0.0127	40
10	7.5	m	0.34	–0.0152	49
11	14	f	0.33	–0.014	45
12	6	m	0.37	–0.0119	38
13	12	f	0.29	–0.0151	48
14	6	f	0.4	–0.0081	26
15	7	f	0.33	–0.0133	43
16	7.5	m	0.26	–0.0125	40
17	1	m	0.34	–0.0176	56
18	1.5	f	0.4	–0.0107	34
19	3	f	0.23	–0.0133	43
AV	**8.7**		**0.32**	**–0.0136**	**44**
B	Normal 3OMG uptake:				
20	3	m	0.25	–0.0413	132
21	2	f	0.34	–0.0304	97
22	2	m	0.46	–0.035	112
AV	**2.3**		**0.35**	**–0.0356**	**114**

[a]1A, Deficient 3OMG uptake (patient 1–19); 1B, Normal 3OMG uptake (patient 20–22). Average values are in bold.

Fig. 4. Mean 3OMG uptake in GTPS patients (○ = patients, n = 22) and in normal controls (♦ = males, n = 35, s = females, n = 35). The data are expressed as the natural logarithm of the ratio of intracellular radioactivity at time T/equilibrium vs time in seconds (two determinations/datapoint).

A

Pre-ketogenic diet

B
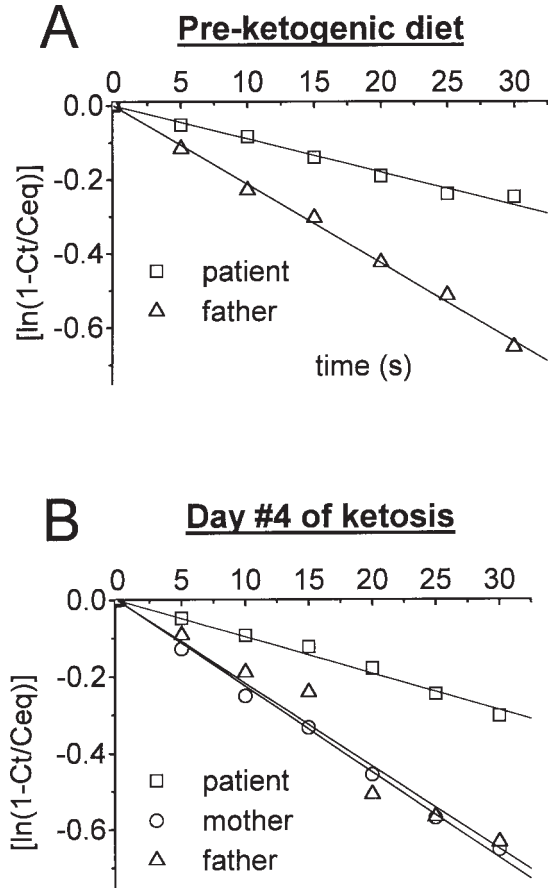
Day #4 of ketosis

Fig. 5. Influence of ketosis on 3OMG uptake into erythrocytes of one patient. **A**, 3OMG uptake one day prior to ketosis (patient) with the patient's father serving as an intraassay control (3 det/datapoint). Slope values: patient, 0.0090 ± 0.00026; father, 0.0212 ± 0.00029. **B**, 3OMG uptake on day 4 of ketosis (patient only) with both parents serving as intraassay controls (3 det/datapoint). Slope values: patient, 0.0096 ± 0.00026; mother, 0.0224 ± 0.00033; father, 0.0217 ± 0.00103.

The 56% reduction in 3OMG transport observed in GTPS patients is in agreement with an approximately 50%-reduction of the mean CSF/blood glucose ratio (normal: 0.65, GTPS: 0.33) (9); and of the GLUT-1 protein immunoreactivity in patients compared to their parents and siblings (8,9,21). Mutations in the GLUT-1 gene produce either hemi- or heterozygosity and are consistent with a dominant loss of function mechanism (21). However, the statistical analyses showed no correlation between uptake values and the degree of hypoglycorrhachia, age, or gender of the 22 patients studied. The parents' values were indistinguishable from the control data and none of the parents was showed clinical features of the GTPS, indicating that this condition is caused by sporadic mutations producing a cellular codominant phenotype of Glut-1 haploinsufficiency(21).

Since dietary ketosis did not influence the rate of glucose uptake by erythrocytes in one GTPS patient (Fig. 5), we suggest that treatment with the ketogenic diet need not be delayed when the diagnosis is suspected clinically.

Although glucose kinetics in human erythrocytes has been studied extensively, little is known about the higher order structure of the carrier. The catalytic properties of glucose transport in erythrocytes cannot be described adequately by any available model (20,22); transport may be complicated under certain conditions by nonideal intracellular distributions of sugars and by the existence of allosteric high-affinity modifying sites on the transport system, which when occupied by sugar modulate the catalytic properties of the system (20). Although our assay did not advance our understanding of these questions, knowing the limitation of the system proved valuable when interpreting the data. In addition to comparing a potential patient's uptake value with the mean of 70 controls, comparing uptake in the patient and his intraassay control proved helpful to reach a diagnosis:
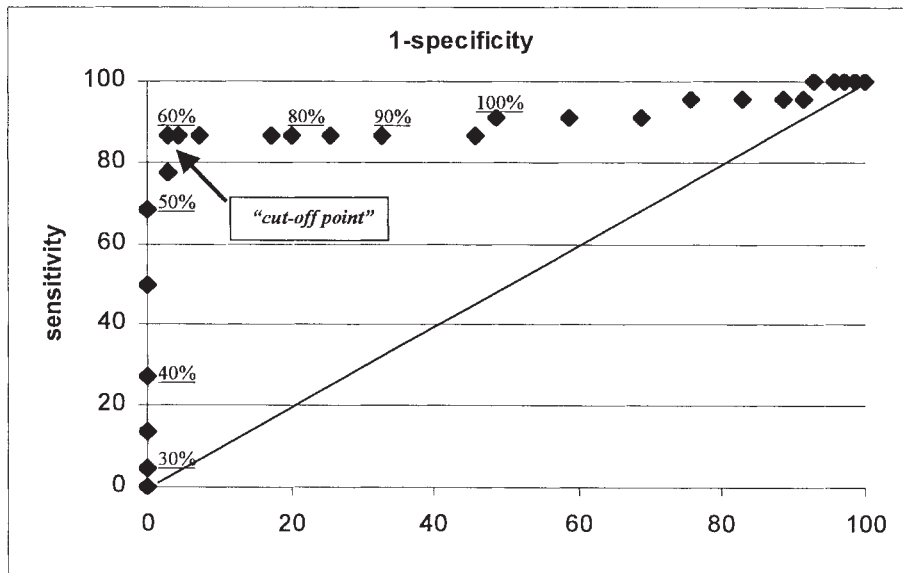
Fig. 6. ROC-Curve of 3OMG-uptake into erythrocytes. 3OMG uptake values in % are underlined and shown next to the datapoints corresponding to sensitivity over 1-specificity. The cutpoint of 60% is highlighted (↖).

TABLE 2.   Classification of Patients and Controls Based on Intra-Assay Uptake Values

Cut-off	Control	Patient	Sum
≥ 60	68	3	71
< 60	2	19	21
Sum	70	22	

Example (Fig 3): uptake of patient 2 on day 1 (61%) was above the statistical cut-off value of 60%; uptake on day 6 (42%) was clearly within the patient range. Within both assays the respective control values were approximately twice the patient's value. Both assays therefore supported the diagnosis of the GTPS.

Three patients carried the clinical diagnosis of the GTPS, but uptake values were entirely within the normal range. Interestingly, all these patients were infants aged 4–6 weeks at the onset of symptoms and have continued to do well after infancy. Treatment with a ketogenic diet was beneficial in all cases and follow-up continues. We suspect that these "false negatives" represent a more benign subgroup of the GTPS. Pathophysiological speculations are an issue-specific mutation involving a fetal isoform of the GLUT-1 protein or mutations interfering with the facilitated diffusion of glucose in more subtle ways than observed to date.

Two other glucose transporter isoforms, GLUT-2 and GLUT-5, have been reported in the erythrocyte membrane (23). However, they do not affect the diagnostic utility of the assay since GLUT-1 is the major glucose transporter in human erythrocytes (24). GLUT-1 functions close to V_{max} under normal physiological conditions where blood glucose values are ~ 5 mmol/L (3), whereas GLUT-2 has a much lower affinity for glucose. GLUT-5, a fructose transporter, does not transport glucose (3–5).

In conclusion, this assay can be utilized as a diagnostic test to confirm the diagnosis of the GTPS. The diagnostic value of the assay should be assessed in the context of clinical seizures and unexplained hypoglycorrhachia.

ACKNOWLEDGMENTS

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