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Microassay for Screening Newborns for Galactosemia with Use of a Fluorometric Microplate Reader

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We describe a microassay for measuring galactose (Gal) and galactose 1-phosphate (Gal-1-P) in dried blood spots. After a coupled enzyme reaction involving galactose dehydrogenase (GADH, EC 1.1.1.48) and alkaline phosphatase (AP, EC 3.1.3.1) in a microplate well, NADH fluorescence is measured by a highly sensitive fluorometric microplate reader, capable of rapid measurement of fluorescence (2 min per 96 samples). Within- and between-run CVs for measurements of Gal at 90 mg/L with Gal-1-P at 130 mg/L were both <5% (n = 8), and analytical recoveries for Gal at 90 mg/L and Gal-1-P at 130 mg/L were 98% and 92%, respectively. Five hundred dried blood-spot samples can be assayed within 2 h, with full calculation of results by an on-line microcomputer. This rapid and reliable assay system is very useful for the routine screening of newborns for galactosemia.

Newborn screening for galactosemia has been effectively introduced to the phenylketonuric screening program in many countries (*1*). The measurement of galactose (Gal) and galactose 1-phosphate (Gal-1-P) in dried blood spots is used mainly to screen for three types of galactosemia: deficiencies of UDPglucose–hexose-1-phosphate uridylyltransferase ("transferase"; EC 2.7.7.12), galactokinase (EC 2.7.1.6), and UDPglucose 4-epimerase ("epimerase"; EC 5.1.3.2).[4] Several methods have been reported for this purpose. The method of Paigen et al. (*2*), a microbiological assay that detects above-normal concentrations of Gal and Gal-1-P, is difficult to maintain and cannot be used for full quantification. Fujimura et al. (*3, 4*) developed an enzymatic fluorometric assay for determination of Gal and (or) Gal 1-P, based on the GADH-NAD^+/NADH reaction system combined with use of AP, which they devised for routine newborn screening with a Technicon AutoAnalyzer. Hoffman et al. (*5*) also adapted this assay technique to a dual-channel AutoAnalyzer system, with simultaneous measurement of phenylalanine. These AutoAnalyzer continuous-flow systems are simple enough, but they have the potential risks of leaking, packing, bubbling, etc. Such interruptions of the assay, if frequent, cause serious problems for routine newborn screening.

Here, using a modification of the above enzymatic assay, we report a new approach to screening for galactosemia. This method is technically easier and can more rapidly obtain reliable results with the use of a fluorometric microplate reader/microcomputer system.

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[4] Nonstandard abbreviations: Gal, galactose; Gal-1-P, galactose 1-phosphate; GADH, galactose dehydrogenase; AP, alkaline phosphatase; transferase, UDPglucose–hexose-1-phosphate uridylyltransferase; and epimerase, UDPglucose 4-epimerase.

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Materials and Methods

Apparatus

We measured fluorescence with a Model MTP-100F microplate reader (Corona Electric, Katsuta, Japan) in a well of Microstrips (1 × 8 stripped microplate well, no. 9502157; Labsystems, Helsinki, Finland) supported by a holder. A microcomputer, Type PC-9801VF (NEC, Tokyo, Japan), interfaced to the microplate reader was used for data processing. The instrument settings for the MTP-100F were as follows: photomultiplier gain 4.5, sensitivity (Em-Ex-Response) 3-3-3, excitation/emission wavelength 360/450 nm.

Reagents

Gal and Tris were purchased from Wako Pure Chemicals, Osaka, Japan. Gal-1-P, NAD^+ (lithium salt, no. 837067), GADH (5 g/L, no. 104981), and AP (10 g/L, no. 108154) were all obtained from Boehringer, Mannheim, F.R.G. All reagents were stored at 4 °C. The stock solutions of the enzymes were diluted to the appropriate concentrations with de-ionized water when used.

Specimens and Sampling

Dried blood spots on filter paper cards, taken from newborns the fourth and seventh postnatal day and sent to us for the Guthrie test (*6*), were used. A blood disc 3 mm in diameter was punched out from each dried-blood spot. Dried-blood-spot standards for Gal (40, 80, 200 mg/L; Fujilevio, Tokyo, Japan) were used for the routine screening. The concentration units in the dried blood spots were all expressed in terms of whole blood. Aqueous solutions of Gal and Gal-1-P were also used for the linearity and analytical-recovery studies.

Procedures

The analytical procedure was based on the reaction system of Fujimura et al. (*3, 4*), modified for this microassay as follows:

Hemoglobin denaturation and extraction. Ten microliters of a mixture of methanol:acetone:de-ionized water (35:35:10 by vol) was dispensed to each disc in the wells of a 96-well, U-bottomed microtiter plate and dried for 30 min at 37 °C. After addition of 50 μL of de-ionized water and a subsequent 10-min shaking at room temperature, 40 μL of the extract was transferred to a well of a Microstrip with a multichannel micropipette.

Reaction and measurement. To each well we added 30 μL of reagent mixture consisting of 10 μL of 0.2 mol/L Tris buffer (pH 8.0), 10 μL of 10 mmol/L NAD^+ solution, 9 μL of 0.1 g/L GADH reagent, and 1 μL of 0.5 g/L AP reagent. After incubation for 1 h at 37 °C, we added to each well 130

μL of a 0.2 g/L Triton X-100 solution. Fluorescence was measured with the microplate reader as described above. For Gal determination alone, we replaced AP in the reaction mixture by the same volume of de-ionized water.

Calculation. The sample concentrations derived from a regression curve with mean and SD values were calculated by an on-line microcomputer, and galactosemia-positive samples were identified automatically in each assay. The concentration of Gal-1-P was calculated as the difference in fluorescence intensities for the complete reagent system (sum of Gal and Gal-1-P) and for the AP-free reagent system (Gal alone).

Cutoff values. All samples were initially measured to determine the sum of Gal and Gal-1-P for the complete assay system, the primary cutoff value being 80 mg/L (calculated as total Gal). For samples exceeding this cutoff value, Gal and Gal-1-P in dried-blood spots on the initial cards were measured simultaneously by combined use of the complete and the AP-free reagent systems. If Gal was >60 mg/L or Gal-1-P was >150 mg/L by this follow-up test, a second filter-paper card was requested. If the result for this was also positive by the same criteria as described above, medical evaluation was performed.

As an additional confirmatory test, together with the simultaneous measurement of Gal and Gal-1-P, two spot-tests were performed for samples exceeding the primary cutoff value, one for transferase, the Beutler test (*7*), and the other for epimerase (*8*).

Results

Microplate reader. Most of the microplate readers for fluorometry measure reflect light; however, this type of microplate reader is known to be seriously affected by scatter of the excitation light. We tried to use several microplate readers of that type, but none was sensitive enough to measure the desired concentration of Gal in dried-blood spots. The MTP-100F is a highly sensitive fluorometric microplate reader, devised to measure fluorescence by transmitting the excitation light through the side of the Microstrip well, then measuring the emission light vertically from the bottom of the well. Performance data for the MTP-100F for 200 μL of NADH in 2 mmol/L Tris buffer solution (pH 8.0) were as follows: linearity up to 15 nmol, detection limit 0.1 nmol, CV 1% (n = 96) for 1 nmol of NADH per well. Use of Microstrips (1 × 8 wells) in a retaining holder (12 columns) was as easy to handle as a rigid 96-well microplate. However, when new Microstrips were used without pretreatment, we sometimes encountered unreasonably high fluorescence intensity, attributable to contamination from small pieces of packing paper in the well. Therefore, before use, we washed the Microstrips with neutral detergent, then rinsed them with tap water and then de-ionized water. We could re-use Microstrips repeatedly if we washed them as described between uses.

Calibration curve. A linear calibration curve was obtained by duplicate analysis of dried-blood-spot standards of Gal at three concentrations: 40, 80, and 200 mg/L. The regression equation was FL = 2.69[Gal] + 18.0 mg/L, where FL denotes the fluorescence intensity in arbitrary units (with the reagent blank subtracted). The detection limit for both Gal and Gal-1-P was 10 mg/L. Linearity was confirmed up to 750 mg/L for Gal, 1000 mg/L for Gal-1-P. When it is necessary to measure higher concentrations, the extracted solution should be diluted to the appropriate concentration.

Precision and analytical recovery. Precision and recovery were evaluated by repeat analyses of dried-blood spots that had been prepared by adding aqueous solutions of Gal and (or) Gal-1-P to heparinized whole blood. In both the complete and the AP-free assays, within- and between-run (day-to-day) CV values ranged from 3.6% to 7.3% (Table 1). Analytical recoveries were in the range of 91% to 98% for Gal and 85% to 103% for Gal-1-P (Table 2).

Results of screening. During the period from April 1987 to January 1989, we screened 34 608 newborns in Sapporo City for galactosemia by this method. The follow-up test, simultaneous measurement of Gal and Gal-1-P, was performed for 471 samples (1.36%) with use of additional blood discs from the initial cards, and a second card was requested for 32 samples (0.09%). Two of these infants had persistently increased values for either Gal or Gal-1-P in dried-blood spots on the second card. Medical evaluation entailed measurements of transferase, galactokinase, and epimerase activities in erythrocytes, and a general liver-function test was performed for these two infants. One infant was confirmed galactosemic with epimerase deficiency; the other—whose values were accompanied by a fourfold increase of tyrosine, 78.9 mg/L and 71.2 mg/L, in dried blood spots on the initial and second card, respectively—was diagnosed as having peliosis hepatitis. The concentrations of Gal and Gal-1-P in dried blood spots from these patients are shown in Table 3, with the data for hypergalactosemic patients with epimerase deficiency and glycogen storage disease type XI, who had been detected before this study.

Discussion

This microassay system has the following major advantages for screening newborns for galactosemia. First, measurement is rapid, with a fully calculated result. Overall assay time for 500 samples is <2 h, whereas the AutoAnalyzer system requires >10 h and the method of Paigen et al. requires 16 h. The decreased assay time is important because prompt detection is especially necessary for the successful treatment of patients with transferase deficiency. Second, both the AutoAnalyzer system and the method of Paigen et al. require a visual inspection of peak heights or bacterial growth zones to detect the positive samples. Misidentification in the visual inspection is one of the most likely errors in screening programs. In contrast, our system provides automated identification of the posi-

Table 1. Precision Data for the Complete and the AP-Free Assay with Dried-Blood Spots

	Added		Complete assay		AP-free assay	
	mmol/L	mg/L	FL[a]	CV, %	FL[a]	CV, %
Within-run (n = 8)						
Gal	0.5	90	264	7.0	264	7.3
	1.0	180	464	4.9	488	4.0
Gal-1-P	0.5	130	261	6.8	—[b]	—
	1.0	260	534	5.3	—[b]	—
Mixture[c]	1.0		456	3.6	278	3.6
Between-run (n = 8)						
Mixture[c]	1.0		450	4.2	263	4.0

[a] Mean fluorescence intensity, arbitrary units. [b] No significant intensity was observed. [c] Equimolar Gal and Gal-1-P were added, shown as the total concentration.

Table 2. Analytical Recoveries of Gal and Gal-1-P from Dried-Blood Spots

Added, mmol/L	Gal			Gal-1-P		
	Expected	Found	Recovery, %	Expected	Found	Recovery, %
	mg/L			mg/L		
Gal						
0.5	90	88	98	0	ND[a]	—
1.0	180	166	92	0	ND	—
Gal-1-P						
0.5	0	ND	—	130	120	92
1.0	0	ND	—	260	249	96
Mixture[b]						
1.0	90	85	94	130	134	103
2.0	180	164	91	260	220	85

[a] <10 mg/L. [b] Equimolar Gal and Gal-1-P were added, shown as the total concentration.

Table 3. Concentrations (mg/L) of Gal and Gal-1-P in Dried-Blood Spots from Hypergalactosemic Patients

Patient	Primary screening			Recalled		
	Total[a]	Gal	Gal-1-P	Total[a]	Gal	Gal-1-P
Epimerase deficiency						
1	278	29	360	250	24	330
2[b]	378	48	474	—	—	—
Peliosis hepatitis	81	75	29	147	118	147
Glycogen storage disease, type XI[b]	747	723	33	1200	1180	33
Neonatal control (Mean ± SD, n = 55)	24 ± 15		13 ± 6	15 ± 11		

[a] Sum of Gal and Gal-1-P, expressed as Gal. [b] Detected before this study.

tive samples with the use of the on-line microcomputer. Third, reagent costs for 20 000 analyses currently are about 100 000 Yen (US$ 700) a year—half of that for the AutoAnalyzer system, and one-third that for the method of Paigen et al.

The Beutler test (*7*) has been widely used as the primary test for transferase deficiency, and the most common abnormality with this test is low transferase activity in compound heterozygotes, those with classic galactosemia and the Duarte variant. Although this heterozygote is usually benign, as reported by Levy et al. (*9*), some subjects have high blood Gal concentrations for a brief period after birth (*10*). Therefore, some heterozygotes of Duarte/galactosemia would also be detected by our method if the increase in blood Gal exceeds our primary cutoff value.

Often there are deviates from galactosemia screening itself. Some patients with hepatic disease have increased Gal in their blood (*4*), as shown by our patients with peliosis hepatitis, and also glycogen storage disease type XI, a rare case of which had been detected before this study (Table 3). Also, liver dysfunction is an early clinical complication of galactosemia. A falsely negative result has been shown with the Beutler test when a blood sample containing citrate as an anticoagulant agent, from an infant with transferase deficiency complicated liver disease, was used (*11*). The falsely negative result was caused by an unexpected formation of NADPH through greatly increased isocitrate dehydrogenase ($NADP^+$) (EC 1.1.1.42) in plasma. In our assay, NADPH fluorescence may influence the proper values of Gal and Gal-1-P; however, no falsely negative result will occur.

The incidence of transferase deficiency is from 1 in 26 000 to 89 000 in western countries according to worldwide galactosemia screening programs (*1*), but relatively rare in Japan: 1 in 667 000.

Concerning the cutoff value, 80 mg/L for Gal plus Gal-1-P (calculated as total Gal) at the primary test is generally the accepted value for detection of all three types of galactosemia, and the rate of the second card requests, 0.09%, is low enough for screening metabolites in newborns.

The proposed method is easy, and reading of results is also quick and easy, with no influence of analyst subjectivity. Furthermore, it can be applied readily in wide-scale newborn screening for galactosemia.

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