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Microdetermination of Galactose and Galactose 1-Phosphate in Dried Blood Spots

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Newborn screening for galactosemia (galactose-1-phosphate uridyltransferase deficiency), as well as for other defects in galactose metabolism (galactokinase deficiency and uridine diphosphogalactose 4-epimerase deficiency), requires a method of determining both galactose and galactose 1-phosphate in dried blood. We have developed a sequential quantitative method for the microdetermination of galactose and galactose 1-phosphate that can be applied to 3-mm-diameter disks of dried blood and that can be used with a Technicon Autoanalyser II equipped with a fluorometer.

Galactose is determined by the fluorescence of NADH following treatment with β-galactose dehydrogenase and with the consequent reduction of NAD. The complete system includes alkaline phosphatase for the hydrolysis of galactose 1-phosphate, so that the total amounts of a galactose and galactose 1-phosphate are determined. For the measurement of galactose alone, alkaline phosphate is omitted from the system. The difference in fluorescence between that from the complete system and that from the alkaline phosphatase-omitted system yields the concentration of galactose 1-phosphate.

Newborn screening for galactosemia (galactose-1-phosphate uridyltransferase deficiency) and for other defects in galactose metabolism (galactokinase deficiency and uridine diphosphogalactose 4-epimerase deficiency) is important for early diagnosis and effective treatment of these diseases (1,2). The most effective screening procedures are those that identify increases in galactose and in galactose 1-phosphate. However, there are problems with each of the available methods. The galactose assay of Rommel *et al.* (3) and the assay of Kirkman *et al.* (4) for galactose 1-phosphate necessitate at least 0.2 ml of blood and cannot be applied to filter-paper specimens. The metabolite inhibition assay of Guthrie (5) measures only galactose and is difficult to maintain (1). The *Escherichia coli*–phage assay of Paigen and Pacholec (6), though sensitive to galactose and when modified (2) sensitive to galactose 1-phosphate, may also be somewhat difficult to maintain and cannot be used for quantitation or for low levels of metabolite.

We have developed a quantitative chemical method for the sequential measurement of galactose and galactose 1-phosphate in 3-mm-diameter disks from filter paper with dried blood specimens. This method can be automated using a Technicon Autoanalyser II and can be used for both routine newborn screening and for confirmatory testing of infants suspected of having a deficiency.

MATERIAL AND METHODS

Reagents. Reagents were purchased as follows: galactose and quinine sulfate from the Katayama Chemical Company; β-galactose dehydrogenase, alkaline phosphatase, galactose 1-phosphate–$K_2 \cdot 5H_2O$, uridine diphosphoglucose–$Na_2 \cdot 5H_2O$, glucose 1-phosphate, glucose 6-phosphate, glucose 1,6-diphosphate, and NAD from the Boehrin-

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ger-Mannheim Company; D- and L-fucose, galactiol, uridine diphosphogalactose–$3 \cdot 5H_2O$, and *p*-nitrophenyl phosphate from the Sigma Company.

Standards and test samples. Standard specimens containing varying concentrations of galactose (Katayama Chemical Co.) and galactose 1-phosphate (Boehringer-Mannheim Co.) were prepared by adding the respective compound to whole blood obtained from healthy adults in the fasting state. Prior to the addition of the monosaccharide, the hematocrit of the blood was adjusted to 55% by the removal of plasma. Standards containing galactose alone, galactose 1-phosphate alone, and galactose plus galactose 1-phosphate were developed in concentration of 0.1, 0.25, 0.35, 0.5, 0.75, 1.0, and 2.0 mM of each compound. The precise concentration was determined for galactose by the method of Rommel (3) and for galactose 1-phosphate by the method of Kirkman *et al.* (4). A special filter paper, produced by Toyo–Roshi Company, for the Guthrie test, was impregnated with these blood specimens to develop filter-paper standards.

Hemoglobin denaturation. Prior to the nonautomated assay, the filter paper with the blood specimens must be treated so that the hemoglobulin is denatured and does not diffuse into the reagent mixture. Such diffusion produces nonspecific and variable fluorescence. Two treatment procedures are effective. One utilizes formate vapor as previously described (7). The second procedure is a simpler one. A 3-mm disk is punched out from the filter paper with the blood specimen and is placed into a small test tube. A freshly prepared mixture (50 μl) of methanol:acetone:water (35:35:30) is added to the tube, is kept at room temperature or at 37°C overnight and then is removed by a stream of air. Hemoglobin denaturation is not necessary when the assay is automated with the Technicon Autoanalyser since this apparatus contains a dialyzing membrane which excludes macromolecules such as hemoglobin.

Manual assay. The basic principle of the assay is the conversion of galactose to galactonolactone by β-galactose dehydrogenase and the concomitant reduction of NAD to the fluorescent NADH. The complete system for determination of galactose and galactose 1-phosphate consists of 10 μl of 13 mM NAD, 9 μl of 0.05 mg/ml β-galactose dehydrogenase, 1 μl of 5 ng/ml alkaline phosphatase, and 10 μl of 1 M Tris–HCl buffer, pH 8.0. The total volume is 30 μl. In the system for galactose determination alone, alkaline phosphatase is omitted and the volume is replaced by 1 μl of distilled water. For a control system NAD is omitted from the complete system and the volume is replaced with 10 μl of distilled water. Optimal conditions for the reagents in the system were established by separate experiments. The appropriate reagent system is added to each test tube containing a 3-mm filter-paper disk and to one tube without a disk that serves as a blank. The tubes are capped to prevent loss due to evaporation and are incubated at 37°C for 1 h. Following the incubation 3 ml of twice-distilled water are added to each tube to stop the reaction and the fluorescence is immediately determined by fluorometry with excitation at 340 nm and emission at 450 nm. Prior to using the fluorometer, the transmittance is adjusted to 100% with either a 0.29 μM quinine sulfate solution (8.4 mg in 40 liters of 0.01 M phosphate buffer, pH 7.4) or a 1.17 μM solution. The fluorometer used is either Type 204 from the Hitachi Company or Type 502 from the Simazu Company.

Automated assay. To facilitate mass screening of new born infants, an automated method was developed. Autoanalyser Type II (Technicon Co.) with a nephro fluorometer Type II or Type III is used. The system for the automated procedure is illustrated in Fig. 3. Enzyme mixtures and the NAD solution are kept at 4°C.

RESULTS

Standard Curve

The calibration curve of galactose or galactose 1-phosphate is shown in Fig. 1, and

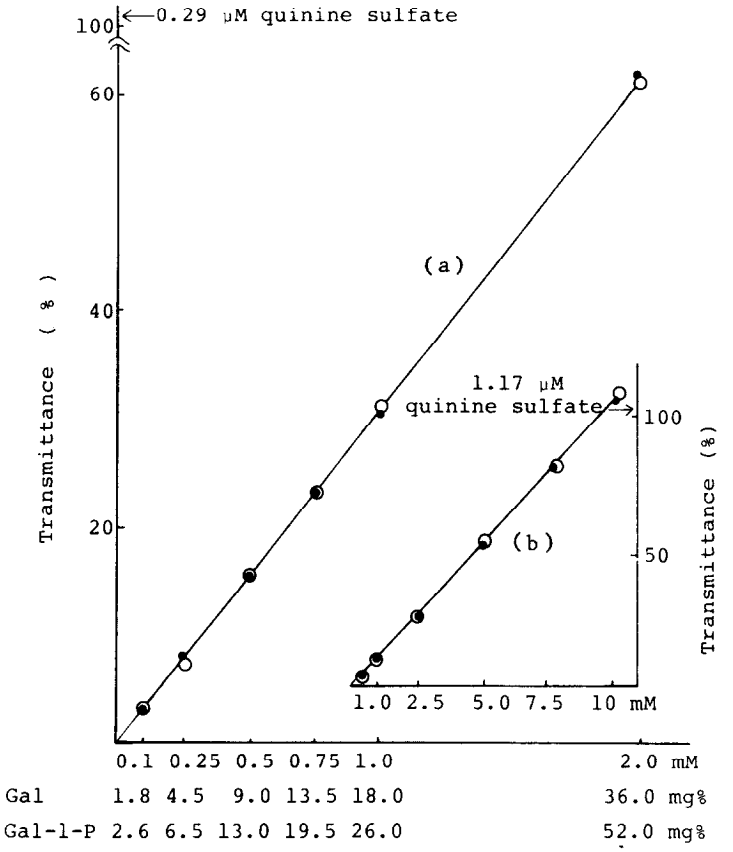


FIG. 1. Calibration curve of galactose (Gal) (●) and galactose 1-phosphate (Gal-1-P) (○). (a) Hitachi 204 and (b) Shimazu 502.

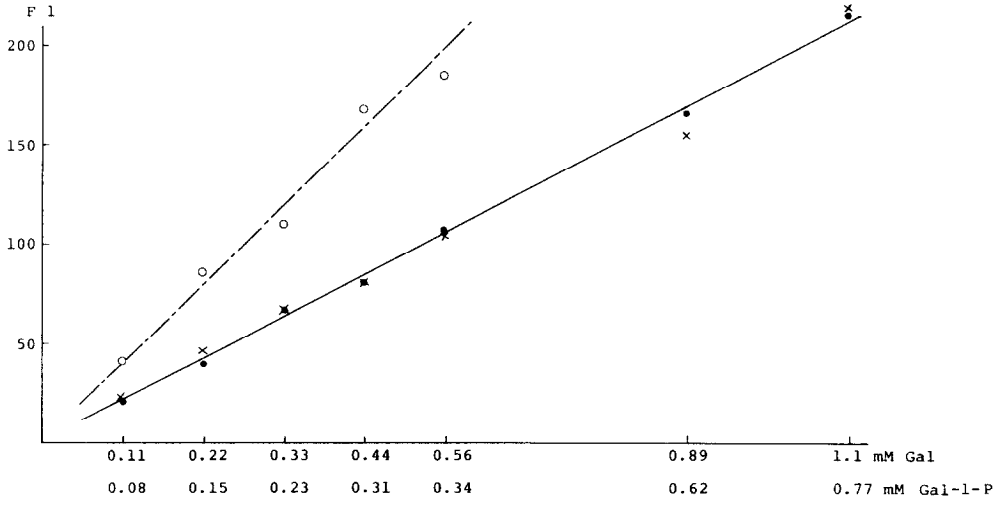


FIG. 2. Standard curve of galactose (Gal) and galactose 1-phosphate (Gal-1-P) in dried blood spot. Galactose (●), galactose 1-phosphate (x), and galactose plus galactose 1-phosphate together (○).

TABLE 1

SUBSTRATE SPECIFICITY OF VARIOUS COMPOUNDS FOR THE PRESENT ASSAY SYSTEM[a]

Substrate[b]	Concentration: 10 mM	1 mM	0.1 mM
Part A			
D-Galactose	100	100	100
D-Fucose	95	91	88
L-Fucose	0	0	0
Galactitol	0	0	0
Part B			
Gal-1-P	100	100	100
UDP-Gal	0	0	0
Glc-1, 6-DP	0	0	0
Glc-1-P	0	0	0
Glc-6-P	0	0	0

[a] Various compounds were tested at different concentrations with the present method, and the amount of NADH formed was normalized with respect to the amount of NADH formed when either D-Galactose (Part A) or Gal-1-P (Part B) was tested.

[b] Gal-1-P, galactose 1-phosphate; Glc-1,6-DP, glucose 1,6-diphosphate; Glc-1-P, glucose 1-phosphate; Glc-6-P, glucose 6-phosphate.

the calibration curve when galactose and galactose 1-phosphate are included in the same specimen is shown in Fig. 2. The linear curves were obtained with 3-mm disks of filter-paper blood specimens containing 0.1–10 mM galactose and/or galactose 1-phosphate. The calibration curve for concentrations greater than 10 mM was also linear. A 3-mm disk contains approximately 2 μl of blood, so that the actual amounts of galactose or galactose 1-phosphate in a 0.1-mM standard disk is about 2×10^{-10} mol. With this system, 1×10^{-10} mol was easily measurable. Prior to obtaining these standard curves the optimal conditions for alkaline phosphatase activity were studied including the optimum pH, the effect of buffers, and the effect of Zn^{2+} and Mg^{2+}; the optimal conditions were chosen as described under Manual assay.

Substrate Specificity

The substrate specificities of alkaline phosphatase and β-galactose dehydrogenase were analyzed with D- and L-fucose, galactitol, and various hexose phosphates at concentrations of 0.1, 1, and 10 mM of each compound. The results are shown in Table 1. The combination of alkaline phosphatase and β-galactose dehydrogenase did not react with either L-fucose or galactitol, but it did react with D-fucose. However, D-fucose is not present in humans. Also, uridine diphosphogalactose and other hexose phosphates did not react in this system. Consequently, there would appear to be no substanital interference with galactose and galactose 1-phosphate reactions.

Automated Assay

As illustrated in Fig. 3, a simple flow system was developed for automation with the Technicon Autoanalyser. Galactose and galactose 1-phosphate were accurately measured. Two sections of the graph obtained with filter-paper blood standards and with specimens from newborn infants are shown in Fig. 4. The results of the determination of galactose in dried blood are shown in Fig. 4a and the results of the determination of galactose plus galactose-1-P are shown in Fig. 4b. When only galactose was measured in the automated system, saline instead of alkaline phosphatase was added. For the results shown in Fig. 4 the speed of the automated assay was 55 samples/h.

The results of an automated assay determining 110 samples/h is shown in Fig. 5. When this automated assay is applied to a routine screening, the assay speed of 55 samples/h is not adequate. Thus, in a routine screening, an accelerated assay might be more suitable. In the accelerated assay, there was a carryover effect of elevated samples. No differences were found between the treated and the untreated disk in this automated system with Technicon Autoanalyser Type II, and untreated disks could be used with the Type C membrane dialyzer. In comparing nephrofluorometer Types II and III, Type III demonstrated the greater sensitivity.

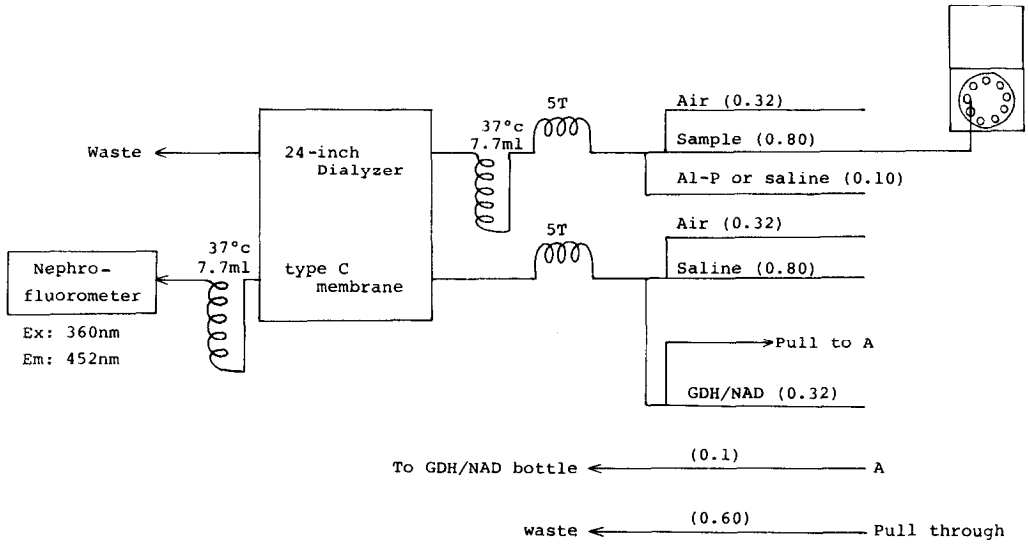


FIG. 3. A flow diagram of an automated assay of galactose and galactose 1-phosphate in dried blood. Ex, excitation wavelength; Em, emission wavelength.

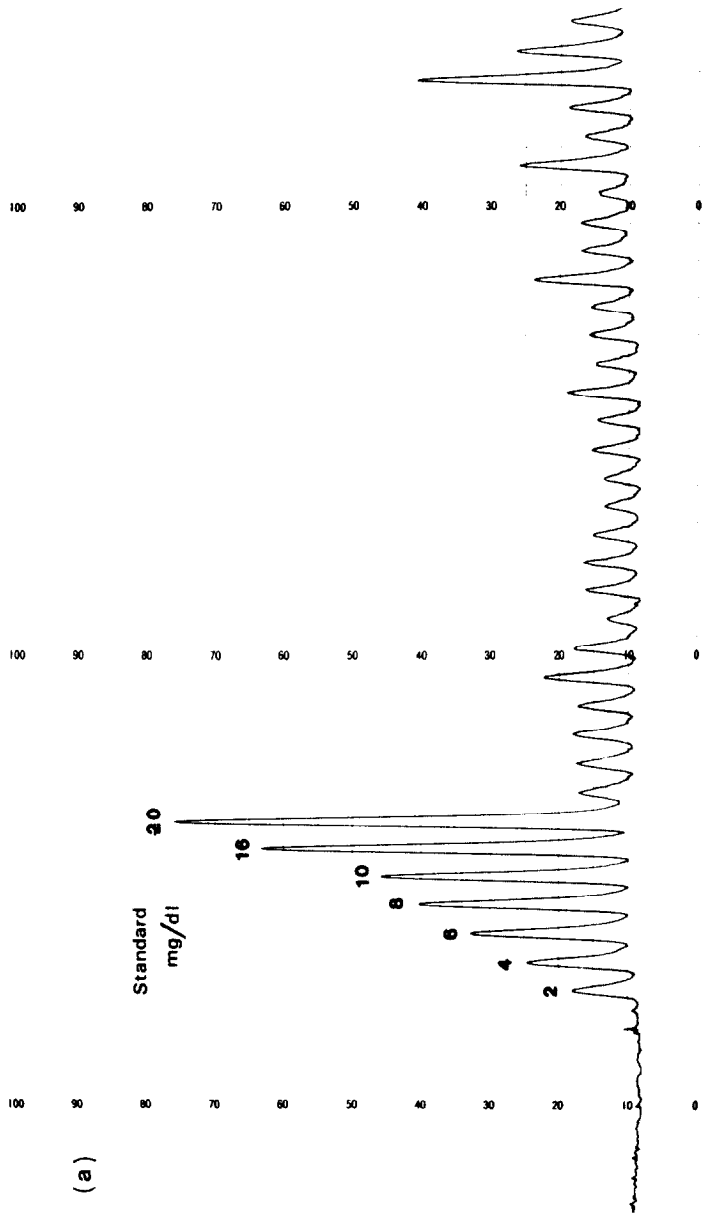
Stability of Galactose and Galactose 1-Phosphate

To study the stability of galactose and galactose 1-phosphate in filter-paper blood specimens, disks containing 0.25, 0.5, 0.75, 1.0, and 2.0 mM of galactose 1-phosphate and 0.5 mM galactose were stored either at −20°C, at 4°C, or at room temperature for varying periods of time and then were analyzed. Following storage at room temperature (20–30°C), galactose and galactose 1-phosphate were stable for one week, but galactose gradually deterioated after two weeks. The origin concentration was reduced by 10% *at* four weeks, by 20% *at* five weeks, and by 30% *at* six weeks. Galactose 1-phosphate was stable at room temperature for four weeks, but showed deterioration after several freezings and thawings.

DISCUSSION

Screening or galactosemia and other defects in galactose metabolism is an important part of multiple newborn screening for metabolic disorders (1,2). This screening must be performed with a filter-paper specimen of dried blood since this is the type of specimen obtained for newborn screenings throughout the world. Though a spot assay specific for galactose-1-phosphate uridyltransferase activity has been widely used (8), there are disadvantages with this method that are overcome with the more general and more reliable screening for increased concentrations of galactose and galactose 1-phosphate (1). The method described in the present paper allows for sequential measurement of galactose and galactose 1-phosphate by a manual analysis or by an automated procedure using a 3-mm disk from a filter-paper blood specimen. It can be used for routine mass newborn screenings and for accurate confirmatory and investigational studies.

This method has particular advantages in the measurement of galactose 1-phosphate, which compound seems to be the most important one for early detection of transferase deficiency (1,2,9). Currently the method of Kirkman *et al.* (4) is the most popular means for quantitation of galactose 1-phosphate. However, several milliliters of blood are necessary for this procedure and this amount of blood is difficult to obtain from infants


100 90 80 70 60 50 40 30 20 10 0
100 90 80 70 60 50 40 30 20 10 0
20
16
10
8
6
4
2
Standard
mg/dl
100 90 80 70 60 50 40 30 20 10 0
(a)

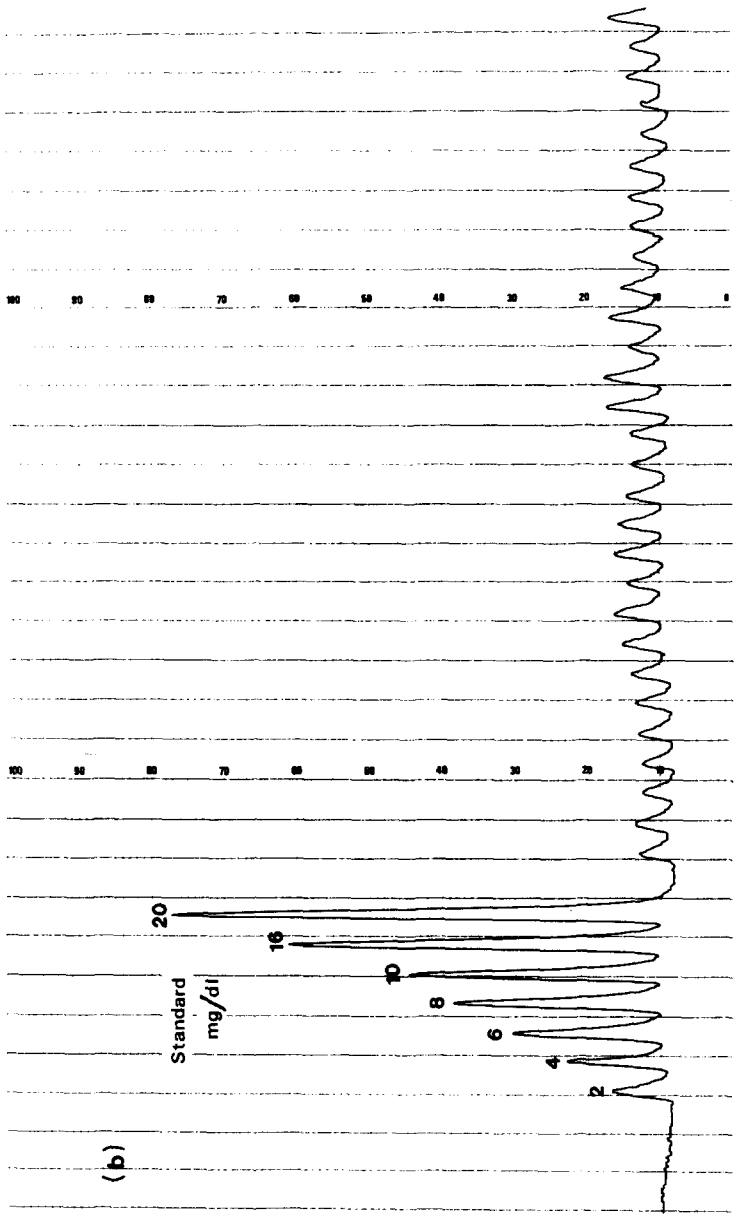


FIG. 4. Chart of automated assay of standard disks of newborn samples. Result of automated assay of (a) galactose and of (b) galactose plus galactose 1-phosphate. Assay speed, 55 samples/h.

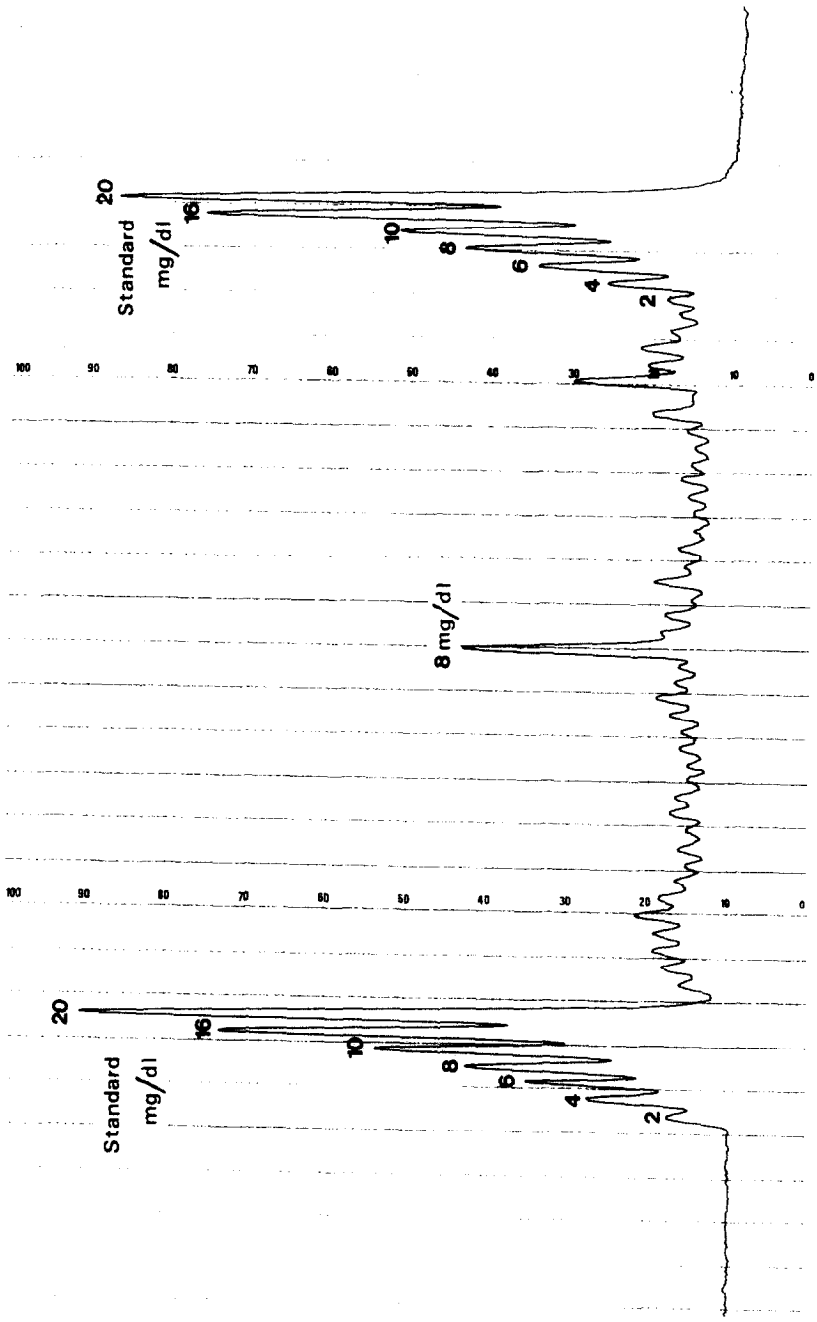


FIG. 5. A result of an accelerated assay of galactose plus galactose 1-phosphate. Assay speed 110 samples/h.

and children. Furthermore, the procedure itself is somewhat complicated and the range of measurable concentrations is relatively narrow. The procedure we have described is more sensitive, has a wider range of assayable amounts, and is less complicated than the method of Kirkman *et al.* The minimum measurable amount is 1×10^{-10} mol of galactose or of galactose 1-phosphate and the range is 1–360 mg/dl of galactose and 2–500 mg/dl of galactose 1-phosphate in a 3-mm disk of a filter-paper blood specimen.

Automated methods for measuring galactose in filter-paper dried blood specimens have been reported. The size of the disk necessary for these methods, however, is large; a 14-mm diameter is required in the method of Tengstrom (10) and a 7.9-mm diameter in the method of Grenier and Laberge (11). Furthermore, the lower limit of sensitivity in these methods is about 20 mg/dl galactose and there is a nonspecific fluorescence which interferes with the reproducibility. In our method both galactose and galactose 1-phosphate are measured in a filter-paper disk of dried blood with only a 3-mm diameter and there is good reproducibility because nonspecifc fluorescence is eliminated either by hemoglobin denaturation (manual method) or by hemoglobin exclusion (automated method).

An accelerated automated method could determine 110 sample/h and should be effective as a tool for mass screening. However, as shown in Fig. 5, some carryover effects could not be eliminated. Though the extent of the carryover effects was not serious and though we did not have any troubles in our routine program, it may be possible to pick out the samples that had a carryover from the previous sample and to do them over again at a slower speed. In our routine screenings in such a case, we put a tube containing pure water between each sample and with this modification we had the same separation for each peak as shown in Fig. 4.

Concerning the specificity of our method, D-fucose could be a substrate in this system, but this compounds does not exist in human fluids. Galactose 6-phosphate could be converted to galactose by alkaline phosphatase, but this compounds is not known to exist in human fluids. Therefore, the specificity of our method is good enough not only for mass screening of newborns but also as a diagnostic technique in a clinic.

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