

Stable Isotope Dilution Analysis of Galactitol in Amniotic Fluid: an Accurate Approach to the Prenatal Diagnosis of Galactosemia

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Summary

A stable isotope dilution assay for galactitol in amniotic fluid has been developed using selected ion monitoring chemical ionization gas chromatography-mass spectrometry of the hexaacetate derivative. [1,1-²H₂]Galactitol was synthesized for use as the internal standard. Galactitol is a component of normal amniotic fluid with a mean concentration of 0.70 ± 0.18 $\mu\text{mol/liter}$ ($n = 5$). The amniotic fluid of a fetus with galactosemia had a concentration of 7.96 $\mu\text{mol/liter}$. Mannitol, sorbitol, and inositol were also found to be normal constituents of amniotic fluid. This stable isotope dilution assay is a rapid accurate method for measurement of galactitol in amniotic fluid for prenatal diagnosis of galactosemia.

Abbreviations

GCMS, gas chromatography-mass spectroscopy
SIM, selected ion monitoring
D₂, ²H₂ label
CI, chemical ionization

Genetically determined disorders of galactose metabolism include classical galactosemia, galactose-1-phosphate uridylyltransferase (EC 2.7.7.12) deficiency, galactokinase (EC 2.7.1.6) deficiency, and uridine diphosphate galactose-4-epimerase (EC 5.1.3.2) deficiency (6, 7, 12). Each of the enzymes is known to be present in cultured skin fibroblasts and in cultured amniotic fluid cells. The disorders of galactose metabolism have serious implications for the untreated affected child. Galactosemia may lead to death in early infancy, usually from sepsis, and this could occur even in infants detected in programs of routine neonatal screening. Further manifestations are failure to thrive, jaundice, and hepatic cirrhosis. Later, cataracts and mental retardation develop. Patients with galactokinase deficiency develop cataracts. If detection is early, postnatal dietary treatment is effective although there may be some irreversible damage, especially to the central nervous system. Prenatal diagnosis can assist in determining the need for restrictive dietary management during gestation (11, 13). Prenatal diagnosis has been reported using enzymatic assays (5, 10).

The accumulation of galactitol in body fluids of patients with disorders of galactose metabolism raises the possibility of the development of a rapid, precise chemical method of prenatal diagnosis by direct analysis of the amniotic fluid for galactitol. Allen *et al.* (1) have reported the accumulation of galactitol in amniotic fluid and have published (2) a method for the prenatal diagnosis of galactosemia using GC for the determination of

galactitol in amniotic fluid. There are two major disadvantages of this method. The first was the use of mannitol as an internal standard, because this compound is a normal constituent of amniotic fluid. Furthermore, the use of GC instrumentation alone has a limited resolving capacity and the detection system is relatively nonspecific, allowing the possible interference by a coeluting compound.

We have developed a stable isotope dilution method for galactitol, utilizing an on-line GCMS system operated in the CI SIM mode. CI-SIM-GCMS circumvents the limitations of previous methodology and provides stable isotopically labeled [1,1-²H₂]galactitol as an internal standard not found in physiological fluids.

MATERIALS AND METHODS

Normal amniotic fluids were obtained between 16 and 18 weeks of pregnancy by amniocentesis for analysis of chromosomes and stored at -20°C until analyzed. The fluid from a pregnancy with a fetus with galactosemia as indicated by a deficiency of galactose-1-phosphate uridylyltransferase was sent for analysis as sterile fluid frozen in dry ice and stored prior to analysis at -20°C . D-Galactitol, D-mannitol, D-sorbitol, and myoinositol were obtained from Sigma, St. Louis, MO. Sulfosalicylic acid was from Aldrich, Milwaukee, WI.

Synthesis of [1,1-²H₂]Galactitol. For synthesis of the internal standard, 50 mg of [6,6-²H₂]galactose, 98% isotopic purity (MSD Isotopes, Pointe Claire-Dorval, Quebec, Canada) was suspended in 0.5 ml of 50 mmol/liter NaOH containing 50 mg NaBH₄. The reaction was allowed to proceed with agitation at room temperature for 4 hr. The reaction was terminated by the addition of acetic acid (2 mol/liters), and the mixture was applied to a column (1.5 cm ID \times 2.0 cm) containing Dowex 50 (H⁺ form, Bio-Rad Laboratories, Richmond, CA) with a cover layer of the borate-specific resin IRA-743 (1.5 \times 6.5 cm, Sigma, St. Louis, MO). The column was subsequently washed with 50 ml of water, the eluate was collected, and the solvent was removed under vacuum. The residue was resuspended in water and purified further by gel chromatography on a Bio-Gel P-2 column (1.0 cm ID \times 90 cm, Bio-Rad Laboratories, Richmond, Ca), collecting 1.0-ml fractions. Each fraction was monitored for carbohydrate by removing an aliquot and testing for charring with 50% H₂SO₄ at 100°C for 10 min. The fractions containing the reduced product were pooled and the water was removed under vacuum. The residue was suspended in water, filtered through a 0.2- μm filter, and lyophilized. The yield was 39.6 mg of D₂-galactitol or about 80%.

The chemical and isotopic purities of the purified [1,1-²H₂]galactitol were compared to those of D-galactitol and the [6,6-

$^2\text{H}_2$]galactose starting material. One micromole of each compound was acetylated in duplicate as described below for the preparation of amniotic fluid samples and 50 nmol were injected into the GCMS. The SP-2340 column was programmed from 200 to 275°C at 8°C/min with 20 ml/min of helium as the carrier gas and with ammonia as the reagent gas at a pressure of 9 pascals in the source. CI spectra from m/z 50 to 650 were obtained every 2 sec and peak areas were determined from the total ion chromatograms. After subtracting the reagent and solvent blank peaks, the two peaks corresponding to the hexaacetate and pentaacetate derivatives constituted 99.53% of the peak areas for galactitol and 99.52% of the peak areas for D_2 -galactitol. The total of the peak areas of the two derivatives for D_2 -galactitol were 100.45% of those of galactitol, indicating identical purities. The peak area of the hexaacetate was 90.5% of the combined peak areas of the hexaacetate and pentaacetate for galactitol and 91.8% for D_2 -galactitol, indicating similar derivatizations. The relative intensities of the isotope cluster of the ammonium adduct pseudomolecular ion in the CI spectrum of galactitol hexaacetate were 18.71% for m/z 453/452 and 4.61% for m/z 454/452. These were similar to the theoretically expected relative intensities of 20.78 and 4.46%, indicating minimal hydrogen losses. The corresponding relative intensities for the CI spectrum of D_2 -galactitol hexaacetate were 18.63% for m/z 455/454 and 4.47% for m/z 456/454. The close similarities to the relative abundances for galactitol hexaacetate showed that there were no significant isotope effects of the deuterium. The isotopic purity of the D_2 -galactitol hexaacetate calculated from the intensities at m/z 452, 453, and 454 was 99.84% with two deuteriums, 0% with one deuterium, and 0.16% with no deuteriums. This corresponded closely to the isotopic purity of the pentaacetate of the D_2 -galactose starting material determined similarly to be 99.67% with two deuteriums, 0% with one deuterium, and 0.33% with no deuteriums.

Preparation of amniotic fluid samples. The procedure of Allen *et al.* (2) was modified. To 1 ml of amniotic fluid, 5 nmol of D_2 -galactitol were added and proteins were precipitated by the addition of 200 μl of 4% sulfosalicylic acid. Following centrifugation, the supernatant amniotic fluid was transferred to a Pasteur pipette filled with 4 cm of Dowex AG 2-X8, 200–400 mesh (OH⁻ form, freshly prepared) at neutral pH with a top layer of 2 cm of Dowex AG 50W-X4, 100–200 mesh (H⁺ form) at neutral pH. The column was eluted with 6 ml of deionized, distilled water, and the eluate was lyophilized. To the dried residue was added 200 μl of pyridine and 200 μl of acetic anhydride, and the sample was heated for 60 min at 80°C for form the hexaacetyl derivatives. Excess reagent was removed under nitrogen and the dry residue was dissolved in 100 μl ethyl acetate.

Conditions of GCMS analysis. Analysis by GCMS was done using a Finnigan 4021 quadrupole mass spectrometer coupled with a direct transfer line to a Finnigan gas chromatograph and to the INCOS data system for data processing. The GC column was 2 mm ID by 180 cm 3% SP-2340 (Supelco, Bellefonte, PA), and the column temperature was 220°C isothermal. Helium was the carrier gas, and the flow was 20 ml/min. The injector temperature was 255°C, the direct transfer line was 280°C, and the ion source was 220°C. Five μl of the samples or standards were injected, corresponding to 0.25 nmol of D_2 -galactitol hexaacetate and 0.0125 to 25 nmol of the hexitol hexaacetates. Similar results were obtained with the injection of 5 or 10 μl . Ammonia was used as the reagent gas for CI. The ammonia pressure in the source was 9 pascals, and the ionizing voltage was 70 eV. The following CI pseudomolecular ions, $\text{M}^+ + 18$ ($\text{M} + \text{NH}_4$)⁺ were chosen for selected ion monitoring: m/z 454 for D_2 -galactitol hexaacetate, 452 for galactitol hexaacetate, mannitol hexaacetate, and sorbitol hexaacetate, and 450 for inositol hexaacetate. The dwell time was 100 msec at each m/z . Peak areas were calculated from the selected ion chromatograms using the computer after the operator visually selected the base line points.

Calibration curves were developed for galactitol, mannitol,

sorbitol, and inositol. Aqueous standard solutions containing 5 nmol of D_2 -galactitol and variable amounts of galactitol (0.25–20 nmol), mannitol (0.25–20 nmol), sorbitol (10–500 nmol), and inositol (10–500 nmol) were prepared and carried through the identical procedure as the amniotic fluids. The peak areas at m/z 454 for the D_2 -galactitol hexaacetate internal standard were corrected by subtraction of 5% of the area at m/z 452 for galactitol hexaacetate. This removed the contribution of the isotope cluster ion at m/z 454 of galactitol hexaacetate. Calibration curves were plotted as the ratio of the SIM peak areas of the pseudomolecular ions of the compounds to the corrected SIM peak areas of the pseudomolecular ion of the internal standard D_2 -galactitol hexaacetate versus the molar ratios of the compounds to D_2 -galactitol and the lines were calculated by linear regression analysis. The amount of galactitol and the other hexitols in the amniotic fluid samples were calculated from the ratios of the SIM areas of the pseudomolecular ions of the compounds to the corrected SIM areas of the D_2 -galactitol hexaacetate internal standard using the equation of the standard calibration curve obtained for each compound.

The measurement of low levels of galactitol in amniotic fluid is complicated by the relatively large amounts of glucose present. On the 3% SP-2340 GC column, the separation of the hexaacetates of mannitol, galactitol, sorbitol, and inositol is excellent, but glucose pentaacetate coelutes with galactitol hexaacetate. The glucose pentaacetate with a pseudomolecular ion of m/z 408 has no interfering fragment or cluster ion which would confound the SIM analysis of the hexaacetates of galactitol, mannitol, sorbitol, or inositol; its high concentration causes a fluctuation of the ammonia pressure and therewith an instability of the SIM analysis of galactitol hexaacetate. It was for this reason that the Dowex 2-X8 (OH⁻ form) column was employed to retard glucose sufficiently to obtain in almost complete (98%) separation from the faster eluting galactitol (2). The method was modified to collect not only the first but the second 3 ml of eluate. Galactitol and inositol were not detectable in the second portion but 25% of the total mannitol and 5% of the total sorbitol were eluted with the second 3 ml. The recoveries of galactitol and D_2 -galactitol were 80%, which is comparable to the 84% reported by Allen *et al.* (2). Recovery of mannitol was nearly 75% and sorbitol 70%, while the recovery of inositol was close to 100%. Recoveries were assessed at concentrations between 1 and 100 nmol and did not vary with the actual concentration of these compounds. The recovery of D_2 -galactitol from the amniotic fluid samples was 80%, similar to the recovery of 80% with the aqueous standards. Since galactitol and D_2 -galactitol had the same recoveries from aqueous standard solutions, the standard curves which are based on the ratios of galactitol to D_2 -galactitol are valid for the amniotic fluid samples. Electron impact ionization of galactitol hexaacetate was found to produce a considerable fragmentation of the molecule. A pseudomolecular ion could not be seen, and in the upper mass range there were no abundant characteristic ions. The base peak was m/z 115 which comprised only 10% of the total ions. For mannitol, sorbitol, and inositol hexaacetates, the situation was similar. Therefore, chemical ionization was chosen.

RESULTS

Figure 1 illustrates the CI mass spectra for galactitol hexaacetate and its deuterated analog. The relatively mild chemical ionization leads to a small amount of fragmentation and a mass spectrum containing a high abundance of the pseudomolecular ion of $(\text{M} + 18)^+$. The base peak m/z 452 for galactitol hexaacetate amounted to 55% of the total ions. A similar magnitude was found for m/z 454 in the case of D_2 -galactitol hexaacetate. SIM analyses of galactitol hexaacetate and the internal standard were therefore carried out at m/z 452 and 454, respectively. For galactitol hexaacetate, there is an isotope cluster at m/z 452, 453, and 454, in which 454 comprises about 5% of the base peak at

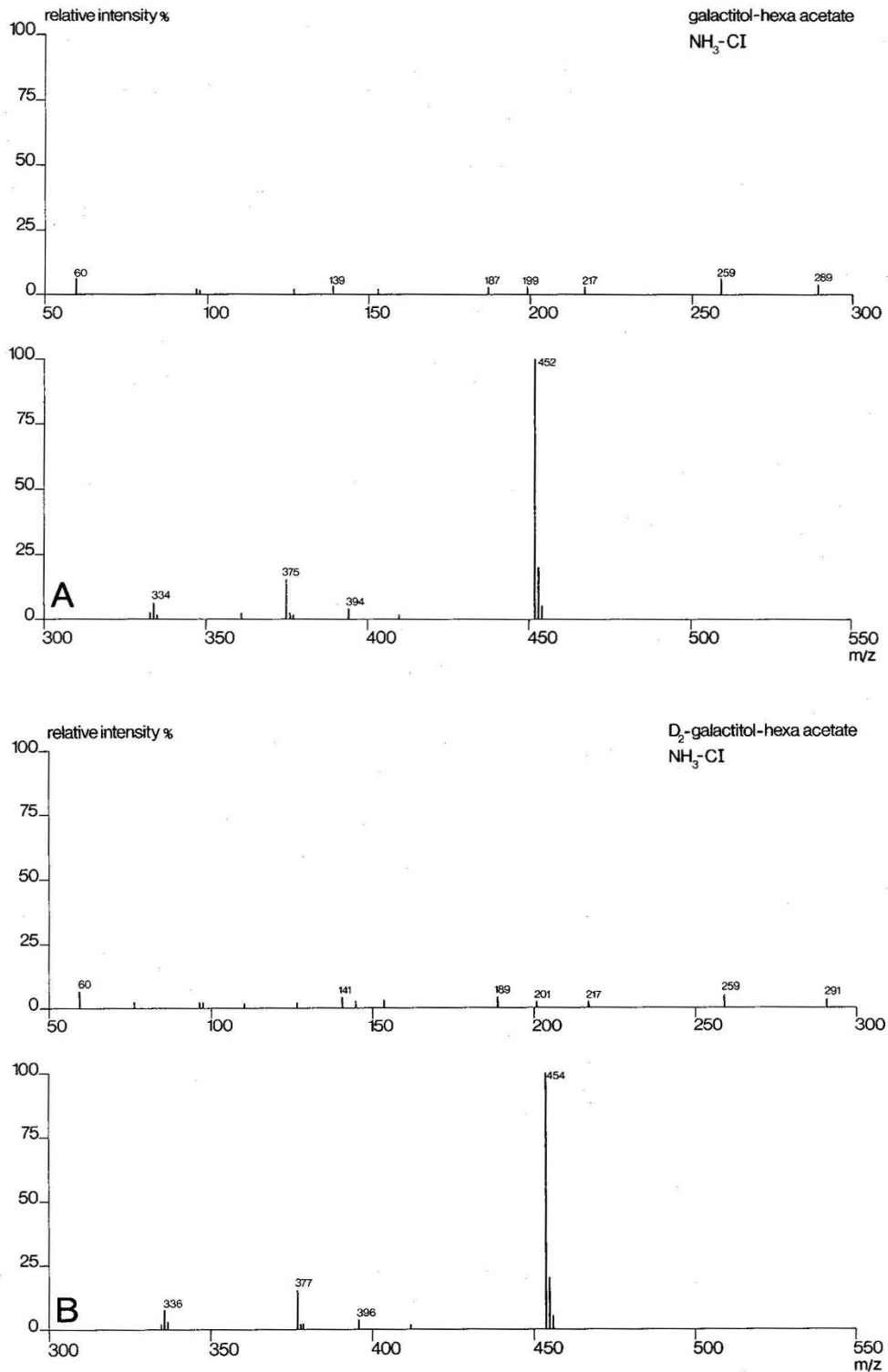


Fig. 1. *A*, Ammonia chemical ionization mass spectrum of galactitol hexaacetate. The base peak is at m/z 452; the pseudomolecular ion is $(M + 18)^+$. *B*, Ammonia chemical ionization mass spectrum of D₂-galactitol hexaacetate. The base peak is at m/z 454; the pseudomolecular ion is $(M + 18)^+$.

m/z 452. The same isotope cluster was found for mannitol and sorbitol hexaacetates and the base peaks at m/z 452 were chosen for SIM. For inositol hexaacetate, the isotope cluster is m/z 450, 451, and 452 and the base peak at m/z 450 was chosen for SIM.

Figure 2 presents typical SIM chromatograms of a normal amniotic fluid. The SIM chromatograms at the m/z of the pseudomolecular ions were not normalized in order to show the small peaks of galactitol hexaacetate (*peak A*) at m/z 452, D₂-galactitol hexaacetate (*peak B*) at m/z 454, and mannitol hexaa-

cetate (*peak C*) at m/z 452 in the presence of the much larger peaks of sorbitol hexaacetate (*peak D*) at m/z 452 and myoinositol hexaacetate (*peak E*) at m/z 450. The relative intensities of the isotopic ions with an m/z two higher than the pseudomolecular ions were about 5%. With the large amounts of sorbitol hexaacetate, this gave a large peak at m/z 454 as well as m/z 452. Similarly, the large amount of myoinositol hexaacetate gave a large peak at m/z 452 as well as at m/z 450. The concentration of galactitol in this amniotic fluid sample was 0.91 $\mu\text{mol/liter}$.

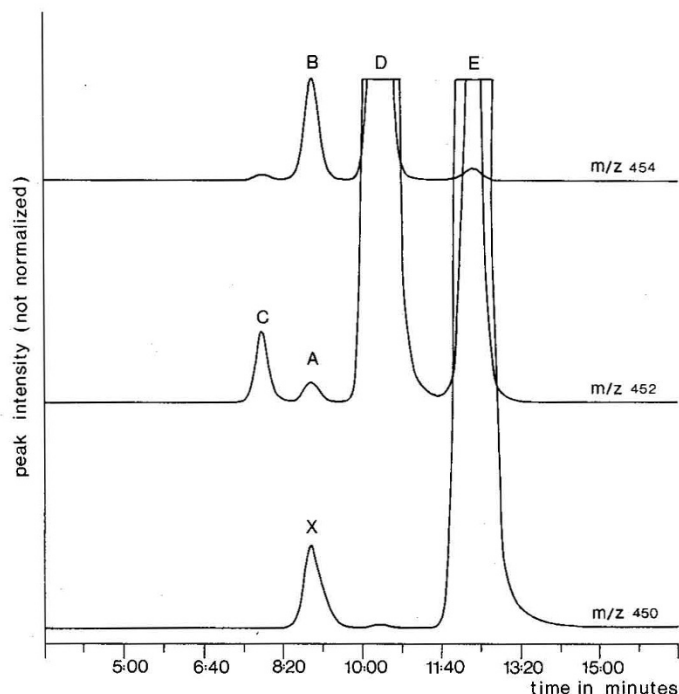


Fig. 2. Selected ion monitoring ammonia chemical ionization gas chromatograms of the hexaacetate derivatives from normal amniotic fluid. *Peak A*, galactitol hexaacetate pseudomolecular ion at m/z 452; *peak B*, D_2 -galactitol hexaacetate pseudomolecular ion at m/z 454; *peak C*, mannitol hexaacetate pseudomolecular ion at m/z 452; *peak D*, sorbitol hexaacetate pseudomolecular ion at m/z 452 and isotopic ion at m/z 454; *peak E*, myoinositol hexaacetate pseudomolecular ion at m/z 454 and isotopic ion at m/z 452; and *peak X*, unknown ion at m/z 450.

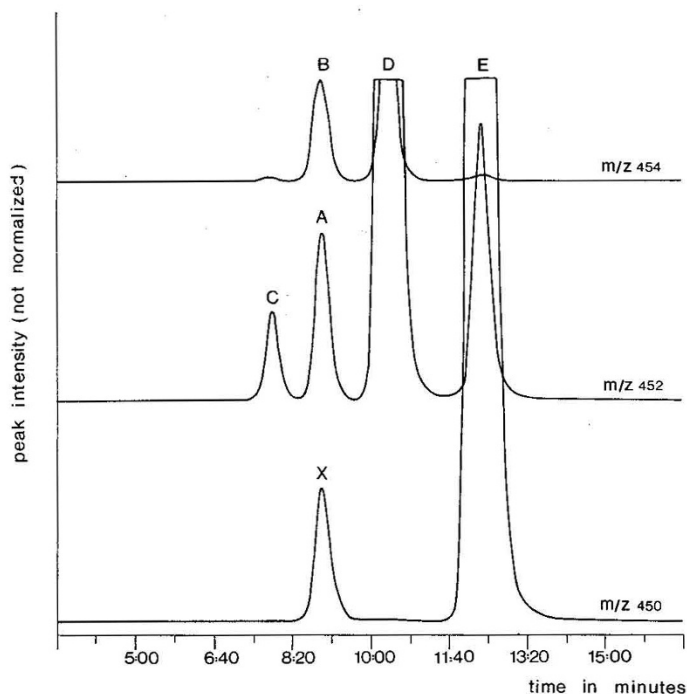


Fig. 3. Selected ion monitoring ammonia chemical ionization gas chromatograms of the hexaacetate derivatives from amniotic fluid of a fetus with galactosemia. *Peak A*, galactitol hexaacetate pseudomolecular ion at m/z 452; *peak B*, D_2 -galactitol hexaacetate pseudomolecular ion at m/z 454 with a 6.8% contribution of the isotopic ion of galactitol hexaacetate at m/z 454; *peak C*, mannitol hexaacetate pseudomolecular ion at m/z 452; *peak D*, sorbitol hexaacetate pseudomolecular ion at m/z 452 and isotopic ion at m/z 454; *peak E*, myoinositol hexaacetate pseudomolecular ion at m/z 454 and isotopic ion at m/z 452; and *peak X*, unknown ion at m/z 450.

In Figure 3, the SIM chromatograms are those of the amniotic fluid of a pregnant woman carrying a fetus affected with galactosemia. The concentration of galactitol in this amniotic fluid sample was $7.96 \mu\text{mol/liter}$. The distinct positions of the hexaacetates of galactitol (*peak A*), mannitol (*peak C*), sorbitol (*peak D*), and inositol (*peak E*) are shown in Figures 2 and 3. In addition, there was an unknown peak (*peak X*) on the SIM trace of m/z 450, eluting with galactitol hexaacetate. Because of the isotope cluster, there is a large contribution from sorbitol hexaacetate to the SIM trace of m/z 454 and from inositol hexaacetate to the SIM trace of m/z 452. There is also a contribution of approximately 5% from galactitol hexaacetate to the area of D_2 -galactitol hexaacetate, which is corrected for this contribution.

In the standard calibration curve (Fig. 4) there was good linearity of the ratio of the areas at m/z 452 to 454 (corrected for the contribution of galactitol) versus the ratio of the variable amounts of galactitol (0.25–20 nmol) to the constant amount of D_2 -galactitol (5 nmol). The correlation coefficient was >0.999 . The calibration curve for mannitol using D_2 -galactitol as internal standard also displayed very good linearity with a correlation coefficient of >0.999 . The calibration curves for sorbitol and inositol were satisfactorily linear in the lower region (0.25–20 nmol), but the extended curves (10–500 nmol) were less linear, and therefore the amounts in amniotic fluid calculated from the calibration curves were less accurate.

The concentrations of galactitol, mannitol, sorbitol, and inositol found in normal amniotic fluid and that surrounding a fetus with galactosemia are summarized in Table 1. The mean normal concentration of galactitol was 0.70 ± 0.18 (1 SD) $\mu\text{mol/liter}$. The range was 0.44 to 0.91 $\mu\text{mol/liter}$. The level of galactitol in the amniotic fluid of the fetus with galactosemia was $7.96 \mu\text{mol/}$

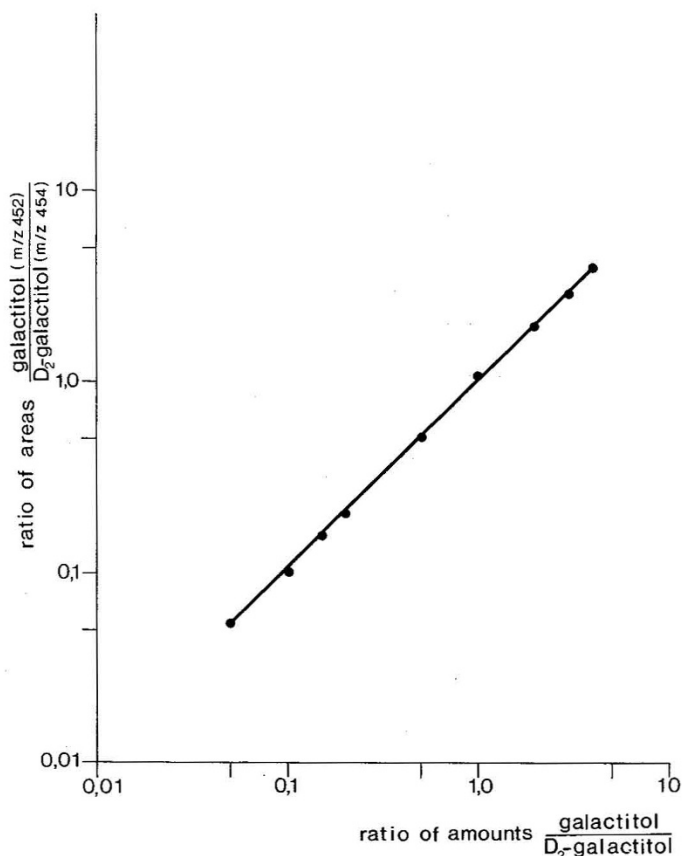


Fig. 4. Standard curve for selected ion monitoring ammonia chemical ionization gas chromatography-mass spectrometry of galactitol. The ratios of peak areas for galactitol hexaacetate (m/z 452) to the peak areas of the internal standard, D_2 -galactitol hexaacetate (m/z 454) (corrected for the isotopic contribution of galactitol hexaacetate) are plotted against the mole ratio of galactitol to the internal standard D_2 -galactitol (5 nmol).

Table 1. Concentrations of galactitol, mannitol, sorbitol, and inositol in amniotic fluid of controls and a fetus with galactosemia.

Gestational age	Subject	Concentration ($\mu\text{Mol/l}$)			
		Galactitol	Mannitol	Sorbitol	Inositol
Normal	1	0.80	3.24	102.90	79.47
	2	0.91	4.18	176.09	63.12
	3	0.72	1.84	90.26	43.83
	4	0.44	8.92	64.32	15.19
	5	0.64	1.73	188.55	92.50
16-18 weeks	(n=5) \bar{X}	0.70 \pm 0.18	3.78 \pm 2.94	124.41 \pm 54.41	58.83 \pm 30.40
17 weeks	Galactosemia	7.96	5.03	58.09	35.09

liter, which was 10 times the control mean and 40 SD above the normal mean. Mannitol was found to be a normal constituent of amniotic fluid. Data are also provided on the broad range of concentrations of sorbitol and inositol in amniotic fluid.

DISCUSSION

Stable isotope dilution assay for galactitol in amniotic fluid provides a rapid, sensitive, and accurate method for prenatal diagnosis. The deuterium-labeled galactitol serves as a carrier during preparation of the sample for GCMS, and measurement of the ratio of nonlabeled to labeled compound corrects for any losses during the ion exchange, derivatization and GC procedures. Measurement of the internal standard verifies that the preparation of the sample was satisfactory and enhances confidence in the results. The use of D_2 -galactitol, a nonphysiological compound, as internal standard is clearly preferable to use of mannitol, which has been used in reported procedures (2, 3, 9) but has been criticized because this compound is present endogenously (8).

The use of D_2 -galactitol as an internal standard has also permitted us to obtain accurate quantitative data on the concentration of mannitol in amniotic fluid and semiquantitative data on the concentrations of sorbitol and inositol. The coeluting compound which has a retention time close to that of galactitol hexaacetate on the SIM trace of m/z 450 would not be distinguished in a simple GC analysis. This might be the explanation for the 2-fold higher normal value of 1.40 $\mu\text{mol/liter}$ for galactitol in amniotic fluid reported by Allen *et al.* (2) compared to the value of 0.70 $\mu\text{mol/liter}$ which has been found by stable isotope dilution. This compound has not been identified. It could be an isomer of inositol. As many as nine isomers of inositol are known of which myoinositol is the major compound occurring physiologically.

The measurement of galactitol in amniotic fluid by stable isotope dilution should avoid the need for culturing amniotic fluid cells. This is not only time consuming, but cells may fail to grow, or contamination and overgrowth by cells of maternal origin may give a false negative result (4). Nevertheless, until more experience is gained, it would be prudent to monitor diagnosis by direct analysis of galactitol with analysis of enzyme activity in cultured cells. With experience, it should be practical to utilize this method alone. The method could be extended to any other body fluid in which the measurement of a very low concentration of galactitol is of interest. The possibility that the measurement of concentrations of galactitol in maternal blood or urine could provide for prenatal diagnosis of galactosemia has not been explored.

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