ELSEVIER

Journal of Chromatography B, 688 (1997) 1–10

JOURNAL OF
CHROMATOGRAPHY B

Sensitive, selective gas chromatographic–mass spectrometric analysis with trifluoroacetyl derivatives and a stable isotope for studying tissue sorbitol-producing activity

Michihiko Kishimoto[a,*], Yoshitaka Kajimoto[a], Minoru Kubota[a], Takao Watarai[a], Yuichi Shiba[a], Ryuzo Kawamori[b], Tadanobu Inaba[c], Takenobu Kamada[a], Yoshimitsu Yamasaki[a]

[a]*First Department of Medicine, Osaka University School of Medicine, 2-2 Yamadaoka, Suita-City, Osaka 565, Japan*
[b]*Department of Medicine, Metabolism and Endocrinology, Juntendo University School of Medicine, Tokyo, Japan*
[c]*Department of Pharmacology, University of Toronto, Toronto Ont., Canada*

Received 8 March 1996; revised 28 May 1996; accepted 11 June 1996

Abstract

One of the major mechanisms involved in diabetic microangiopathy is considered to be an altered polyol pathway. However, clarifying the pathophysiology is difficult due to the lack of a sensitive method for measuring the reduction of glucose to sorbitol in tissue. Here we report a sensitive and selective method for polyol measurement using trifluoroacetyl (TFA) derivatives of polyols and stable isotope-labeled D-sorbitol (U-[^{13}C]sorbitol, $^{13}C_6H_{14}O_6$, 98.7%) as an internal standard. Gas chromatography–mass spectrometry (GC–MS) using an SE-30 capillary column gave elution of TFA derivatives of sugars, polyols and U-[^{13}C]sorbitol within 8 min, with clear separation of sorbitol. In the calibration study, the coefficients of correlation between the amount of sorbitol added and that determined in standard solutions containing 0.1–8.0 nmol sorbitol, erythrocyte mixture and liver cytosol mixture were r=0.999, r=0.997 and r=0.997, respectively. The precision of the GC–MS measurement of standard solution was C.V.=4.3%. Because glucose is used as a substrate, the method can clarify the polyol pathway under physiological conditions. With this method, K_m and V_{max} values of the reductase in erythrocytes were 115±19 mmol/l and 4.42±0.26 nmol/min/g of hemoglobin. In human liver, on the other hand, they were 755±132 mmol/l and 0.773±0.090 nmol/min/mg of protein, respectively. This difference of K_m values suggested that aldehyde reductase rather than aldose reductase is mainly responsible for reducing glucose to sorbitol in the liver. In conclusion, this newly developed method offers a highly sensitive and selective procedure for measuring low concentrations of sorbitol in various tissues and cells and should enable clarification of the kinetics of glucose reduction to sorbitol, which in turn can be used to evaluate the role of an altered polyol pathway in the pathophysiology of diabetic microangiopathy.

Keywords: Sorbitol; Polyol; Aldose reductase

1. Introduction

The late complications of diabetes are major determinants of morbidity and mortality. Possible causative factors include abnormal lipid profiles, platelet dysfunction, hypertension and hyperglycemia [1]. Widely accepted mechanisms underlying hyperglycemia-induced diabetic complications are non-

*Corresponding author.

0378-4347/97/17.00 Copyright © 1997 Elsevier Science B.V. All rights reserved
PII S0378-4347(96)00271-X

enzymatic glycation and increased metabolism of glucose via the polyol pathway [2–7].

To date, various studies have been performed to clarify the pathogenesis of increased polyol pathway metabolism [2,5–8]. Sorbitol, an alcohol produced by reduction from glucose, is well-known because of its pathophysiological involvement in some late diabetic complications [4]. However, accumulation of sorbitol is not the only cause of these complications. In tissues such as peripheral nerves, hyperglycemia increases the reduction of glucose to sorbitol as well as the oxidation of sorbitol to fructose and reduces the tissue myoinositol content and thereby alters phosphoinositide metabolism [6,7], decreases the availability of NADPH, increases the ratio of NADH/NAD [2] and impairs Na^+, K^+-ATPase activity [5,9]. These observations, revealing the involvement of the entire polyol pathway in various pathophysiological effects, suggest that research efforts should be focused on evaluating the overall activity of the polyol pathway. In this respect, it is important to measure the sorbitol-producing activity in tissues because the production of sorbitol is believed to be the rate-limiting step in the pathway.

Aldose reductase (EC 1.1.1.21), a well-known enzyme that facilitates the production of sorbitol from glucose, is a member of a family of monometric aldo-keto reductases that show broad substrate specificities for aldehydes and ketones [9–14]. Despite the general belief that aldose reductase is the key enzyme of the polyol pathway, the pathophysiological involvement of each member of the family is not actually known. Although sorbitol readily accumulates in the kidney cortex of diabetic animals, where it causes diabetic changes, only low levels of aldose reductase are present there [9,14], suggesting that some other aldo-keto reductases such as aldehyde reductase (EC 1.1.1.2) may contribute to polyol formation [12]. As more than one enzyme of the aldo-keto reductase family may be involved, the enzyme activity must be measured using only glucose as the substrate instead of any other aldehydes. This is because different enzymes will have different affinities for each substrate, and the results obtained using substrates other than glucose may not represent the real enzyme activities in vivo, where glucose is virtually the only substrate. In this respect, the most commonly used enzymatic method, in which sorbitol dehydrogenase is used for a coupled enzymatic reaction, can not be employed as it is often affected by other co-existing polyols and is also influenced by NADPH [15,16]. Gas chromatographic analysis and high-performance liquid chromatographic analysis use glucose as a substrate, but they also pose problems such as low sensitivity, multiple procedures including purification and lengthy analysis times [16–25].

To achieve accurate measurement of sorbitol-producing activity in vivo that allows reliable estimation of the polyol pathway activity, we used a new highly sensitive and selective gas chromatographic–mass spectrometric (GC–MS) analysis method with trifluoroacetyl derivatives and a stable isotope, which allows quantitative and specific determination of low amounts of sorbitol and thus enables detection of the polyol pathway activity in the liver, where it could not be detected by either of the two conventional methods. This method also makes possible the study of the enzymatic kinetics, enabling us to evaluate the relative pathophysiological significance of aldo-keto reductase in the development of late diabetic complications.

2. Experimental

2.1. Chemicals

N-Methyl-bis(trifluoroacetamide) (MBTFA) was obtained from Pierce (Rockford, IL, USA). Sorbitol (glucitol), glucose, mannose, xylitol, galactitol (dulcitol), ribitol, myoinositol, galactose, fructose, arabitol, mannitol and β-nicotinamide adenine dinucleotide phosphate (NADPH) were obtained from Sigma (St. Louis, MO, USA). Uniform ^{13}C-labeled D-glucose (U-[^{13}C]glucose, $^{13}C_6H_{12}O_6$; stable isotope-labeled D-glucose) was purchased from Cambridge Isotope Laboratories (Woburn, MA, USA). Sodium borohydride (extra pure reagent) was from Nacalai Tesque (Osaka, Japan). Other reagents were of HPLC grade and were from Yashima Pure Chemical (Osaka, Japan).

Uniform ^{13}C-labeled D-sorbitol (U-[^{13}C]sorbitol, $^{13}C_6H_{14}O_6$; stable isotope-labeled D-sorbitol) was

synthesized with sodium borohydride. First, 0.5 mmol U-[^{13}C]glucose was dissolved in 50 ml of methanol and 1.5 mmol of sodium borohydride was added. After the mixture was stirred for 60 min at room temperature, 4.5 mmol of hydrochloric acid were added to react with the excess sodium borohydride. Mixing of the solution was continued for another 15 min. As a result, 10 mmol/l of synthesized U-[^{13}C]sorbitol and 30 mmol/l of sodium chloride were present in the solution. GC–MS showed that the sorbitol compound contained 98.7% U-[^{13}C]sorbitol (M_r 188), 0.27% unlabeled sorbitol (M_r 182) and 0.99% U-[^{13}C]glucose (M_r 186). Other sugars accounted for less than 0.01%.

2.2. TFA derivatization and GC–MS procedure

Before analyses, sugars and polyols were changed to TFA derivatives based on the procedure of Sullivan and Schewe [27]. A 500-μl volume of a preparation was lyophilized and then 20 μl of MBTFA were added, followed by 20 μl of pyridine. The vial was capped and kept at 40°C for 60 min. Derivatization proceeded rapidly, with the reactions being completed in the reaction medium. For this procedure, six TFA groups were added to hexitol, such as sorbitol, and five TFA groups were added to hexose, such as glucose. After subsequent centrifugation at 2000 *g*, the supernatant was analyzed by gas chromatography.

A G-3000 gas chromatograph (Hitachi, Tokyo, Japan) combined with a G-2000 mass spectrometer (Hitachi) was used to analyze the samples. The gas chromatograph was equipped with a silicon SE-30 capillary column (30 m×0.25 mm I.D., Gasukuro Kogyo, Tokyo, Japan) and a split injector. A split ratio of 16:1 was used. Electron-impact ionization (EI) mass spectra were recorded at an ionizing energy of 70 eV and an ionization current of 300 μA. TFA derivatives of polyols and sugars were ionized to their fragment ions after loss of a trifluoroacetoxy radical. The injected volume of sample was 0.5 μl. Nitrogen was the carrier gas, at a flow-rate of 16 ml/min (1 ml/min in the column). The temperature of both the sample injection port and the detector port was 220°C. The column temperature was set at 85°C initially and held for 3 min, after which the temperature was programmed from 85 to 90°C at a rate of 1°C/min and from 90 to 220°C at a rate of 40°C/min.

2.3. Calibration and precision of the sorbitol assay

The sorbitol calibration standard consisted of 0, 1 or 3 μmol of glucose, 200 nmol of NADPH, 16 nmol of U-[^{13}C]sorbitol (as the internal standard) and 0–8 nmol of sorbitol to a total volume of 200 μl (standard solution). To examine whether sorbitol could be measured in existing erythrocyte preparations or in liver cytosol, other mixtures, which contained an additional 60 μl of the supernatant of the erythrocyte preparation or an additional 60 μl of the cytosol fraction from the liver preparation (described in Section 2.4 and Section 2.5) to a total volume of 200 μl, were prepared (erythrocyte mixture or cytosol mixture). After three volumes of ethanol (99.9%) had been added, the mixture was centrifuged for 20 min at 0°C at 10 000 *g*. Then, the supernatant fraction was taken to dryness. Recovery of 4 nmol of sorbitol from the different standard solutions, consisting of 0, 1 or 3 μmol of glucose and 200 nmol of NADPH, or erythrocyte mixture samples was determined. The precision of the GC–MS measurements was determined from three injections of standard solution or erythrocyte mixture, which contained from 0.02 to 8 nmol of sorbitol. Overall precision was determined from duplicate analyses of sorbitol in various erythrocyte mixtures.

2.4. Assay of enzyme activity in erythrocytes

To test the feasibility of this method, the activity of the polyol pathway in erythrocytes was measured. Blood was collected by venipuncture from five healthy volunteers (three females and two males), of mean age of 27.8±2.3 (±S.D.) years. The blood was put into polystyrene tubes containing heparin as an anticoagulant and was immediately centrifuged for 5 min at 4°C at 1600 *g*. After the plasma was removed, the erythrocytes were washed three times with ten volumes of cold isotonic saline. One volume of packed erythrocytes was lysed with 1.5 volumes of distilled water. The blood samples were then centrifuged for 20 min at 4°C at 10 000 *g*. The superna-

tant was used to prepare calibration curves, determine the precision of the sorbitol assay and measure the aldo-keto reductase activity for glucose.

To determine the reductase activity, a typical mixture consisted of glucose (0–100 μmol), NADPH (200 nmol), 60 μl of supernatant from the erythrocyte preparation and 50 mmol/l of sodium phosphate buffer (pH 7.4) in a final volume of 200 μl. The reaction was started by the addition of the cofactor, and the mixture was incubated at 37°C under aerobic conditions for 120 min. The reaction was stopped by rapidly cooling the test tube in ice. To the reaction samples, 16 nmol of U-[^{13}C]sorbitol were added as an internal standard. After three volumes of ethanol (99.9%) had been added, the mixture was centrifuged for 20 min at 0°C at 10 000 *g*. The supernatant fraction was evaporated completely under reduced pressure. Sorbitol and U-[^{13}C]sorbitol in the residue were then determined by GC–MS using TFA derivatization.

2.5. Assay of enzyme activity in human liver

Five livers from a kidney donor liver bank set up in the Department of Pharmacology, University of Toronto, were used in this study (two females and three males) [28]. The mean age was 29.4±13.9 years. None of the livers was from a subject with liver disease. Preparation of cytosol from the livers was performed by a previously reported method [28]. The 100 000 *g* supernatant fraction in 1.15% KCl (10 ml/g liver) was used to prepare calibration curves and to determine the aldo-keto reductase activity for glucose in liver cytosol.

Assay conditions were essentially the same as for the erythrocytes. The typical mixture consisted of glucose (0–100 μmol), NADPH (200 nmol), 60 μl of the liver cytosol fraction and 50 mmol/l of sodium phosphate buffer (pH 7.4) in a final volume of 200 μl. The mixture was incubated for 120 min and the reaction was stopped by rapidly cooling the test tube. After 16 nmol of U-[^{13}C]sorbitol and ethanol were added, the mixture was centrifuged for 20 min at 10 000 *g*. The supernatant fraction was evaporated under reduced pressure and sorbitol in the residue was determined with this method.

2.6. Data analysis and statistics

Experimental data represent means of duplicate incubations of each sample. The rates of glucose reduction in erythrocytes and human livers were expressed as nmol of sorbitol/min/g of hemoglobin and nmol of sorbitol/min/mg of cytosolic protein, respectively. The concentration of hemoglobin was determined by the spectrophotometric method with sodium lauryl sulfate (Wako Chemical, Osaka, Japan). Protein concentration was determined using a Protein Assay Kit (Bio-Rad, Hercules, CA, USA). The statistical significance of the differences between the groups was assessed using Student's *t*-test; $p<0.05$ or less was considered to be significant. Parameters based on linear or non-linear regression fitting were obtained by the computer program Enzfitter (Elsevier, Biosoft, Cambridge, UK).

3. Results

3.1. Mass chromatogram of TFA derivatives of sugar and polyol compounds

Table 1 shows the retention time and mass spectra of molecular ions and major fragment ions of TFA derivatives. Major fragment ions of TFA derivatives from pentitol, such as ribitol, arabitol and xylitol, were detected on selective ion chromatograms for mass/charge (m/z) 613, 519, 505, 404 and 379. That of TFA myoinositol was detected at m/z 643, 529, 414, 319 and 301. Those from hexitol, such as galactitol, sorbitol and mannitol, were detected at m/z 645, 631, 505, 379 and 303 (Fig. 1). Those from hexose, such as galactose, mannose, glucose and fructose, were detected at m/z 547, 413, 319 and 305.

Selective ion chromatograms for m/z 519, 547, 643 and 645 are shown in Fig. 2, obtained when TFA derivatives of the standard sample mixture, which consisted of the same amounts of sorbitol, glucose, mannose, xylitol, galactitol, ribitol, myoinositol, galactose, fructose, arabitol and mannitol, were applied to the capillary column. Peak identification was confirmed by the total coincidence between the peaks and those for each standard in

Table 1
Retention times and mass spectra of molecular ions and major fragment ions of TFA derivatives

	Retention time (min)	M_r	Molecular ion	Major fragment ions					
Ribitol	3.38	152	632	613	519	505	404	379	
Arabitol	3.78	152	632	613	519	505	404	379	
Xylitol	3.84	152	632	613	519	505	404	379	
Myoinositol	4.24	180	756	643	529	414	319	301	
Mannitol	5.38	182	758	645	631	505	379	303	
Sorbitol	5.48	182	758	645	631	505	379	303	
Galactitol	6.06	182	758	645	631	505	379	303	
Galactose	6.22	180	660	547	413	404	319	305	
Mannose	6.50	180	660	547	413	404	390	319	305
Glucose	6.96	180	660	547	413	404	390	319	305
Fructose	7.12	180	660	547	533	413	319	305	
U-[^{13}C]Sorbitol	5.48	186	764	651	636	383			

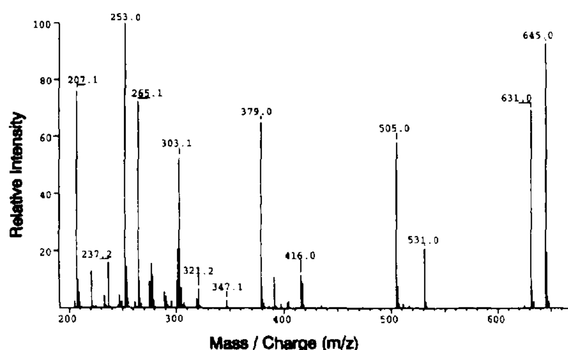

Fig. 1. Electron ionization mass spectrum of TFA sorbitol.

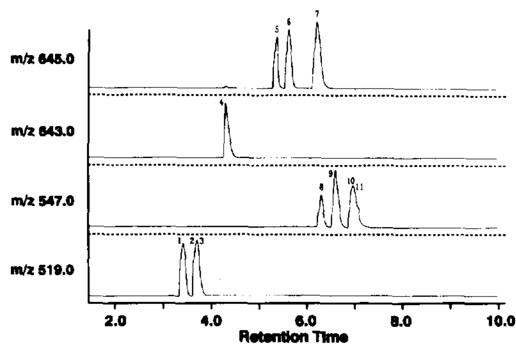

Fig. 2. Selective ion chromatograms for m/z 519, 547, 643 and 645, when TFA derivatives of ribitol (1), arabitol (2), xylitol (3), myo-inositol (4), mannitol (5), sorbitol (6), galactitol (7), galactose (8), mannose (9), glucose (10) and fructose (11) were applied in the same amounts to the capillary column.

terms of gas chromatography retention time and mass spectra obtained by mass spectrometry. All sugars and polyols were eluted within 8 min, and the peak of sorbitol was separated clearly from the other peaks of polyols and sugars. In this study, U-[^{13}C]sorbitol was chosen as the internal standard. The major fragment ion of TFA U-[^{13}C]sorbitol was detected by selective ion chromatography for m/z 651. The structure of the fragment ions of TFA U-[^{13}C]sorbitol and the retention time were essentially the same as those of TFA unlabeled sorbitol. We obtained the selective ion chromatograms for m/z 645 (sorbitol) and m/z 651 (U-[^{13}C]sorbitol) and calculated the amount of sorbitol based on the peak area of sorbitol in comparison with those of U-[^{13}C]sorbitol (Fig. 3).

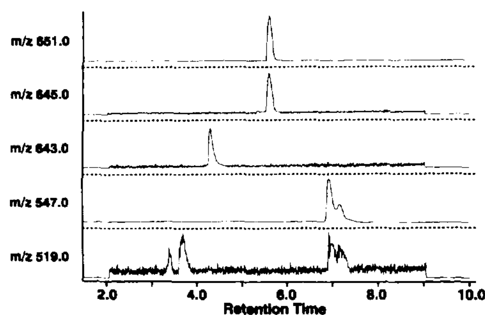

Fig. 3. Selective ion chromatograms of the derivatized extracts from an erythrocyte preparation with U-[^{13}C]sorbitol as the internal standard.

3.2. Calibration and precision of sorbitol assay

Calibration curves for sorbitol of the standard solution and the erythrocyte mixture are shown in Fig. 4 (curves A and B). Good correlation was achieved for the amounts of sorbitol added and determined. The coefficients of correlation between the amount of sorbitol added and the ratio of area under the peak obtained from the m/z 645 and m/z 651 chromatogram were r=0.999 (n=36) in standard solution and r=0.997 (n=36) in the erythrocyte mixture. The slopes of the two lines were almost identical. The differences in the 645/651 ratio between the calibration standard with and without the erythrocyte preparation, which shows the endogenous sorbitol level of the erythrocytes added, were quite consistent, in spite of the variable range of sorbitol (0–8 nmol). Calibration curves for sorbitol in the liver cytosol mixture were almost identical with that of the standard solution [r=0.997, n=6; Fig. 4 (curve C)]. The recovery level of sorbitol was not influenced by the co-existing glucose, the erythrocyte preparation or NADPH (Table 2). The precision of the gas chromatographic measurement esti-

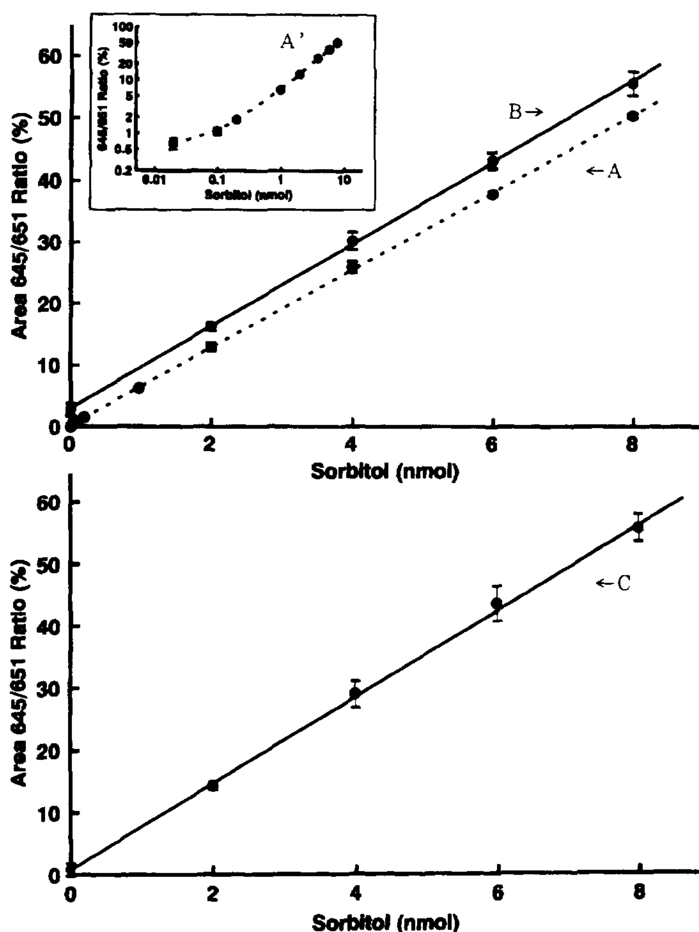

Fig. 4. Calibration curves for sorbitol of standard solution (A), erythrocyte mixture (B) and liver cytosol mixture (C). Calibration curve A′ is the data for standard solution A on a logarithmic scale. (Data show mean±S.D.). The coefficients of correlation between the amount of sorbitol added and the ratio of the area under the peak obtained by the m/z 645 and m/z 651 chromatogram were r=0.999 (A, A′: standard solution; n=32), r=0.997 (B: erythrocyte mixture; n=36) and r=0.997 (C: liver cytosol mixture; n=16). The differences in the 645–651 ratio between the standard solution and the erythrocyte mixture indicated the endogenous sorbitol level of the erythrocytes.

Table 2
Influence of high glucose concentration, the erythrocyte preparation and NADPH on the recovery level of sorbitol

	Sample	Area 645/651 ratio (%)
(A)	4 nmol Sorbitol	25.96±0.91
(B)	4 nmol Sorbitol, erythrocyte preparation	30.57±1.01
(C	4 nmol Sorbitol, erythrocyte preparation 1 μmol Glucose, 200 nmol NADPH	31.26±2.87
(D)	4 nmol Sorbitol, erythrocyte preparation 3 μmmol Glucose, 200 nmol NADPH	30.51±1.01

mated from multiple injections of standard solution and erythrocyte mixture was C.V.=4.3% (n=24) and C.V.=5.7% (n=19), respectively. The overall precision of the gas chromatogram measurement estimated from duplicate samples of the erythrocyte mixture was C.V.=8.6% (n=19).

3.3. Enzyme activity in erythrocytes

Initially, incubation/reaction time periods were changed up to 360 min and the relationship between the sample incubation time and the rate of reduction was studied. Glucose (5 or 15 mmol/l) was used as a substrate. The linear range for the time–activity study was from 0 to 240 min. The rates of 5 and 15 mmol/l glucose reduction to sorbitol within the initial 120 min in erythrocytes were 0.34±0.04 and 0.71±0.05 nmol/min/g of hemoglobin (Hb), respectively (Fig. 5). K_m values of the reductase in erythrocytes from five volunteers were found to be 115±19 (range, 90.5–153.0) mmol/l, which were comparable to the K_m values previously reported for aldose reductase. V_{max} of the reductase activity in erythrocytes was 4.42±0.26 (range, 2.68–5.60) nmol/min/g of Hb (Fig. 6).

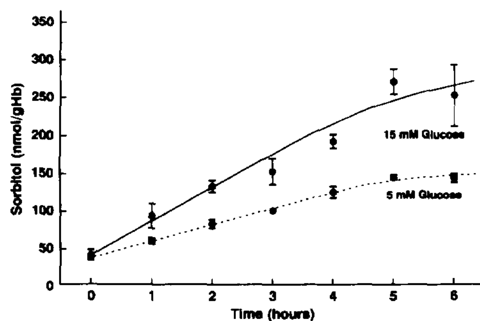

Fig. 5. Relationship between sample incubation time and the rate of reduction in erythrocytes. (Data show mean±S.D.). The rates of reduction of 5 or 15 mmol/l glucose to sorbitol on a linear range were 0.34±0.04 and 0.71±0.05 nmol/min/g of Hb, respectively.

3.4. Enzyme activity in human liver

The method presented here detected the activity of glucose reduction to sorbitol in all of the human livers examined. The rates of reduction were

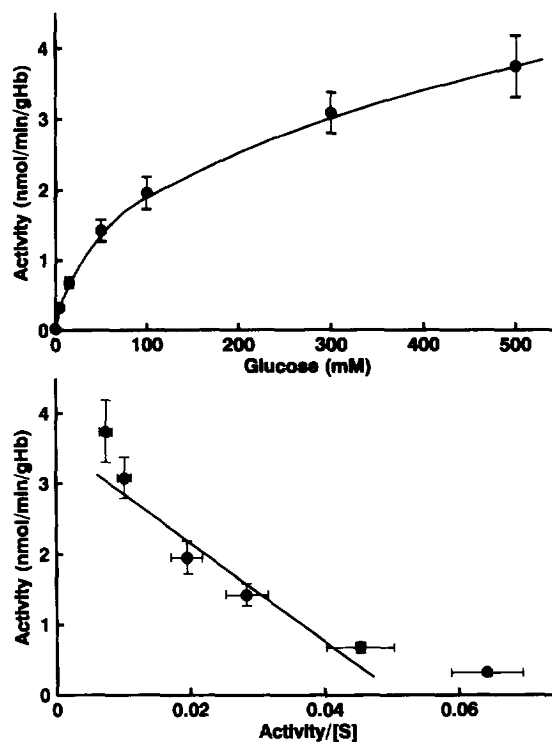

Fig. 6. Reductase activity in the erythrocytes of five volunteers. K_m and V_{max} values of the reductase were 115±19 mmol/l and 4.42±0.26 nmol/min/g of Hb, respectively. (Data show mean±S.D.).

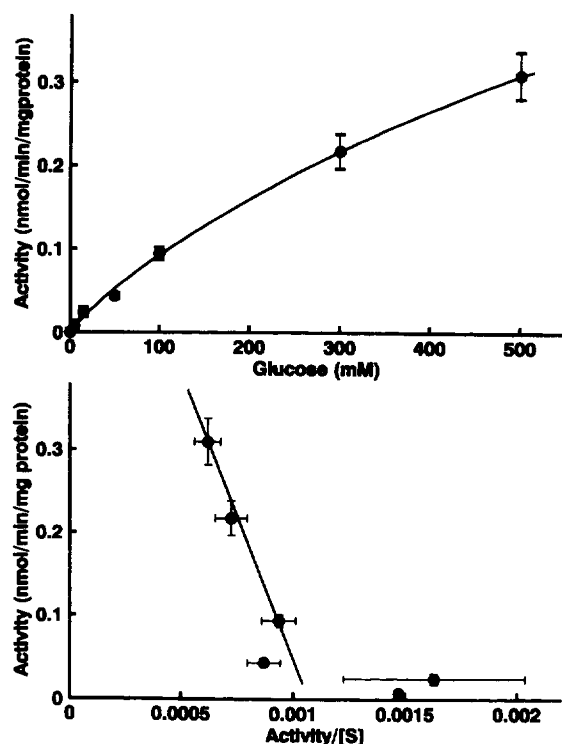

Fig. 7. Reductase activity in human livers from five kidney donors. K_m and V_{max} values of the reductase were 755±132 mmol/l and 0.773±0.090 nmol/min/mg of protein, respectively. (Data show mean±S.D.).

0.0084±0.012 and 0.026±0.012 nmol/min/mg of protein, when the substrate was 5 or 15 mmol/l glucose, respectively. K_m and V_{max} values of aldo-keto reductase in human liver were 755±132 (range, 342–1407) mmol/l and 0.773±0.090 (range, 0.43–1.29) nmol/min/mg of protein (Fig. 7).

4. Discussion

The methods presently used to measure sorbitol can be divided into three categories. In the first is the enzyme method that uses sorbitol dehydrogenase and measures the increase in induced NADH absorption. This is a highly sensitive method, offering a detection limit of as low as 100 nmol/l. However, in terms of specificity, it may be the least suitable as it detects non-sorbitol polyols such as ribitol and xylitol, whose total concentration may be almost as much as that of sorbitol [16]. Also, NADPH, which is added to be oxidized to NADP, has an absorbance peak at 340 nm as has NADH, which interferes with the measurement of NADH and thus reduces the sensitivity of the sorbitol measurement [16].

For more selective measurement of polyols and sugars, GC–MS was introduced, leading to a second group of methods. In these methods, substrates need to be acetylated or trimethylsilylated before being subjected to gas chromatography, in order to facilitate evaporation of the derivatives [23,24]. Polyol and sugar derivatives are separated efficiently according to their retention times and molecular masses, determined by mass spectrometry [22–25]. This allows high selectivity in sorbitol measurement. However, trimethylsilylated derivatives subjected to GC–MS tend to be degraded and yield less of the large fragment ions of the derivatives [24]. Therefore, the signal intensities are often too low to detect low concentrations of sorbitol. Also, a large amount of co-existing glucose can interfere with the gas chromatographic peak of sorbitol, and internal standards such as ribitol or galactitol are difficult to discriminate from those in tissue. Thus, despite their high selectivity, conventional GC–MS methods fall short because of their poor sensitivity.

The third group consists of relatively new methods that use high-performance liquid chromatographic analysis (HPLC) to separate polyol and sugar derivatives based on affinity for the HPLC column and the elution buffer [18–20]. However, co-existing glucose, added to be reduced to sorbitol, again tends to interfere with the sorbitol peak, thus decreasing the sensitivity. Another drawback is the relatively long time required for each run, which makes the HPLC method unsuitable for measuring large numbers of samples.

Our method uses GC–MS, but is a clear contrast to conventional GC–MS methods in that it uses TFA derivatives for gas chromatography and a stable isotope-labeled sorbitol, U-[^{13}C]sorbitol, as an internal standard. Mass spectrometry showed that the large fragment ions of TFA sorbitol, which has five TFA groups on a sorbitol, was obtained at m/z 651 and could be clearly discriminated from the other peaks of polyols and sugars (Fig. 2). Also, the peaks corresponding to the large fragment ions were rela-

tively high in TFA derivatives (Fig. 1) compared with those in trimethylsilylated- and acetylated derivatives, which generally gave low peaks [24,26]. In conventional chromatographic methods in which trimethylsilylated- and acetylated derivatives are used, derivatives of glucose eluted earlier than those of sorbitol [24,26]. Under such conditions, a large amount of glucose added to the reaction assay tends to interfere with sorbitol, which is eluted later. In contrast to that, TFA sorbitol is eluted earlier than TFA glucose. Our present method is not hampered by interference from derivatives of glucose and thus can offer better sensitivity and accuracy for sorbitol measurement.

Another advantage of our method arises from the use of U-[^{13}C]sorbitol as an internal standard. First, the absence of U-[^{13}C]sorbitol (less than 0.001%) from tissues allows a very low background. Second, the added U-[^{13}C]sorbitol prevents unlabeled sorbitol from being trapped non-specifically in the column. TFA derivatives of the isotope yield a m/z 651 peak, which can be detected as a discrete peak in the GS–MS experiment. In this assay, we used 16 nmol of this isotope, which contained 0.27% of unlabeled sorbitol (U-[^{12}C]sorbitol). The detection limit of sorbitol was revealed to be 20–100 pmol (Fig. 4), which is much lower than that with the conventional method. If purer U-[^{13}C]sorbitol is available in the future, our method should be able to detect even lower concentrations of sorbitol.

Still another positive factor of our method is the simplicity of its procedure. For TFA derivatization, all that is needed is the addition of MBTFA. With TFA sugars and polyols being eluted within 8 min, the average time needed for each run is approximately 10 min. This makes our GC–MS method with TFA derivatization suitable for measurement of large numbers of samples.

Our present method detected the activity of glucose reduction to sorbitol in all of the human livers examined, despite the contrasting results of a previous study in which an antibody was used against aldose reductase [29,30]. The K_m value for the liver samples was 755±132 mmol/l. The value was obviously higher than that of aldose reductase and close to that previously observed for aldehyde reductase, suggesting it to be responsible for most of the activity in the liver. Further studies are necessary to address the possible physiological roles of the glucose metabolism via the polyol pathway in the liver.

The precise determination of the K_m, made possible by our present method, reveals something interesting. When the K_m value of reductase in erythrocytes was recalculated based on a two-enzyme model, the K_m values of the high and low affinity sites were 28.7±3.9 and 713±199 mmol/l, respectively (Fig. 8). Although the V_{max} was higher for aldehyde reductase than for aldose reductase (5.19±0.54 vs. 1.67±0.18 nmol/min/g of Hb), the very high K_m of aldehyde reductase would not have allowed it to play a significant role in the metabolism of glucose in erythrocytes. However, this aldehyde reductase may have some physiological role in other tissues such as the kidney in which the aldehyde reductase is by far the most dominant enzyme in

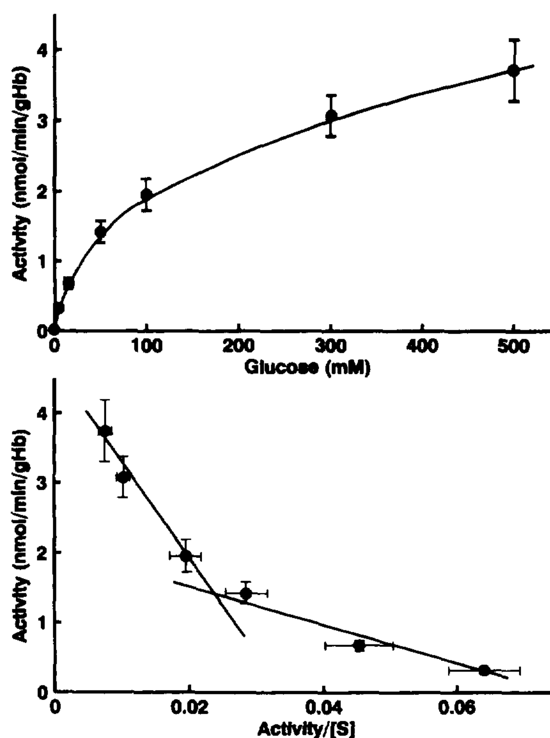

Fig. 8. Reductase activity in the erythrocytes of five volunteers (two-site model). The K_m values of the high and low affinity sites were 28.7±3.9 and 713±199 mmol/l, respectively. The V_{max} values of the high and low affinity sites were 1.67±0.18 and 5.19±0.54 nmol/min/g of Hb, respectively. (Data show mean±S.D.).

terms of protein amount. The new method developed in this study, allowing for highly sensitive and selective measurement, should provide a useful tool for studying the kinetics of glucose reduction to sorbitol by the aldo-keto reductase family. It should be worthwhile to examine which of the two enzymes plays a dominant role in reducing glucose to sorbitol in various tissues. This may be clinically important, because some aldo-keto reductase inhibitors seem to have a different effect for aldose reductase and aldehyde reductase.

Oxidative stress and reductive stress resulting from decreased availability of NADPH and an increased ratio of NADH–NAD may have some pathological effects on diabetic complications [2,5,7–9]). Although multiple factors may be involved in the change in the redox state observed in diabetes, the activation of the sorbitol pathway seems to play a significant role in it [2]. Our new method, a highly sensitive and selective assay using glucose as a substrate, may be the best tool to date for evaluating the overall activity of the polyol pathway and thus for investigating mechanisms underlying glucose toxicity through the polyol pathway.

References

[1] Y. Yamasaki, R. Kawamori, H. Matsushima, H. Nishizawa, M. Kodama, Y. Kajimoto, T. Morishima and T. Kamada, Diabetes, 43 (1994) 634.
[2] R.G. Tilton, K. Chang, J.R. Nyengaard, M.V. Enden, Y. Ido and J.R. Williamson, Diabetes, 44 (1995) 234.
[3] H. Kaneto, J. Fujii, H.G. Seo, K. Suzuki, T. Matsuoka, M. Nakamura, H. Tatsumi, Y. Yamasaki, T. Kamada and N. Taniguchi, Diabetes, 44 (1995) 733.
[4] J.H. Kinoshita and C. Nishimura, Diabetes. Metab. Rev., 4 (1988) 323.
[5] K.P. Mistry, A. Beyer-Mears and F.P.J. Diecke, Diabetes, 42 (1993) 1737.
[6] D.A. Greene, S. Lattimer, J. Ulbrecht and P. Carroll, Diabetes Care, 8 (1985) 290.
[7] D.A. Greene and S.A. Lattimer, J. Clin. Invest., 72 (1983) 1058.
[8] R.N. Frank, Diabetes, 43 (1994) 169.
[9] S. Sato, Am. J. Physiol., 263 (1992) F799.
[10] T.G. Flynn and N.C. Green, in H. Weiner (Editor), Enzymology and Molecular Biology of Carbonyl Metabolism, Vol. 4, Alan R. Liss, New York, 1993, p. 251.
[11] J.M. Petrash, I. Tarle, D.K. Wilson and F.A. Quiocho, Diabetes, 43 (1995) 955.
[12] K.M. Bohren, B. Bullock, B. Wermuth and K.H. Gabbay, J. Biol. Chem., 264 (1989) 9547.
[13] J.D.L. Vander and L.A. Hunsaker, in H. Weiner (Editor), Enzymology and Molecular Biology of Carbonyl Metabolism, Vol. 4, Alan R. Liss, New York, 1993, p. 279.
[14] B. Robinson, L.A. Hunsaker, L.A. Stangebye and D.L.V. Jagt, Biochem. Biophys. Acta, 1203 (1993) 260.
[15] T. Poli, A. Lapolla, A. Valerio and D. Fedele, Acta Diabetol. Lat., 22 (1985) 17.
[16] J.I. Malone and S. Lowitt, Metabolism, 41 (1992) 224.
[17] A. Kashiwagi, T. Obata, M. Suzuki, Y. Takagi, Y. Kida, T. Ogawa, Y. Tanaka, T. Asahina, M. Ikebuchi, Y. Saeki, R. Kikkawa and Y. Shigeta, Metabolism, 41 (1992) 1041.
[18] P. Lloyd and M.J.C. Crabbe, J. Chromatogr., 343 (1985) 402.
[19] J.M. Dethy, B. Callaert-Deveen, M. Janssens and A. Lenaers, Anal. Biochem., 143 (1984) 119.
[20] I. Miwa, M. Kanbara, H. Wakazono and J. Okuda, Anal. Biochem., 173 (1988) 39.
[21] S. Yoshioka, S. Saitoh, T. Fujisawa, A. Fujimori, Q. Takatani and M. Funabashi, Clin. Chem., 28 (1982) 1283.
[22] S. Yoshioka, K. Kameyama, M. Sanaka, I. Sekine, S. Kagimoto, S. Fujitsuka and S. Saitoh, Clin. Chem., 37 (1991) 686.
[23] C. Popp-Snijders, M.Z. Lomecky and A.P. de Jong, Clin. Chim. Acta, 132 (1983) 83.
[24] T. Niwa, N. Yamamoto, K. Maeda and K. Yamada, J. Chromatogr., 277 (1983) 25.
[25] E. Pitkanen, Scand. J. Clin. Lab. Invest., 42 (1982) 445.
[26] Z. Tamura and T. Imanari, Chem. Pharm. Bull., 15 (1967) 246.
[27] J.E. Suillivan and L.R. Schewe, J. Chromatogr. Sci., 15 (1977) 196.
[28] M. Kishimoto, T. Kawamori, T. Kamada and T. Inaba, Drug. Metab. Dispos., 22 (1994) 367.
[29] S.K. Srivastsva, N.H. Ansari, G.A. Hair and B. Das, Biochim. Biophys. Acta, 800 (1984) 220.
[30] H.P. Wirth and B. Wermuth, in H. Weiner (Editor), Enzymology of Carbonyl Metabolism, Vol. 2, Aldehyde Dehydrogenase, Aldo-Keto Reductase and Alcohol Dehydrogenase, Alan R. Liss, New York, 1985, p. 231.