Carbohydrate Research, 215 (1991) 117–125
Elsevier Science Publishers B.V., Amsterdam

Determination of furosine in biomedical samples employing an improved hydrolysis and high-performance liquid chromatographic technique*

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(Received September 7th, 1990; accepted for publication in revised form December 10th, 1990)

ABSTRACT

Traditionally, the most sensitive and specific determination of non-enzymatic protein glycation has involved an 18–24-h acid hydrolysis in order to generate the compound furosine, which has been detected employing reversed-phase h.p.l.c. In this study, we have reported that significant quantities of furosine can be generated with much shorter hydrolysis times employing a 90-min vapor-phase acid hydrolysis procedure. The furosine generated by vapor-phase hydrolysis is then quantitated by pulsed amperometric detection using anion-exchange high-performance liquid chromatography. Employing this method, we were able to show that furosine generated from acid hydrolysis of purified hepatic membranes in a diabetic and non-diabetic animal model agreed with traditional methods assessing total glycated protein (*i.e.*, boronate affinity methods).

INTRODUCTION

The pathogenesis of tissue complications secondary to the diabetic condition has been strongly linked to clinical control of the blood glucose[1–4]. One postulated mechanism implicating hyperglycemia in this process involves the non-enzymatic glycosylation of serum and tissue proteins (*i.e.*, glycation)[3–5]. The glycation reaction begins in all cases with D-glucose reacting with protein amino groups with formation of a Schiff's base. The Schiff's base subsequently rearranges, and accumulation of a stable Amadori

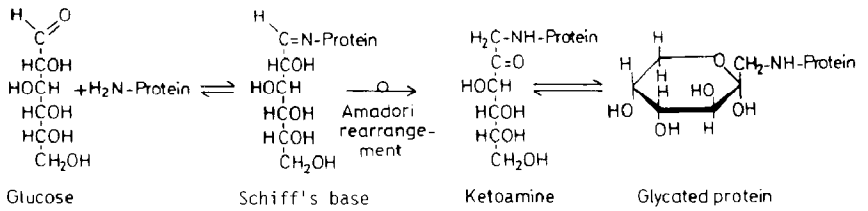

Fig. 1. Pathway for non-enzymatic glycation of proteins. (From Schleicher and Wieland[21]).

* Presented at the XVth International Carbohydrate Symposium Yokohama, Japan, August, 12–17, 1990.
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0008-6215/91/$03.50

product occurs (Fig. 1, from Schleicher *et al.*[2]). Over weeks, equilibrium is obtained such that tissue proteins, whose half-lives are greater than the time required to reach steady-state levels, accumulate advanced glycated end-products (*i.e.*, products of protein cross-linking)[3,4]. The formation of these advanced products is postulated to contribute greatly to the tissue dysfunction prevalent in the diabetic state[6,7]. In order to study the efficacy of clinical interventions proposed to reduce tissue glycation, sensitive and specific methods to detect these tissue products need to be validated. The most sensitive and specific method devised thus far employs detection of furosine [N^{ϵ}-(2-furoyl-methyl)-L-lysine], an acid hydrolysis product of glycated proteins that involves the amino acid, L-lysine[8,9]. The measurement of furosine traditionally has involved an 18–24-h acid hydrolysis step, followed by reversed-phase h.p.l.c. detection[8,9].

We have designed a modified approach in the detection of furosine that employs a rapid hydrolysis step (90 min), followed by high-pressure anion-exchange chromatography with pulsed amperometric detection (p.a.d.). To evaluate our method, we performed studies designed to compare our method of determining specifically non-enzymatically attached glucose (*i.e.*, furosine) to traditional methods assessing total glycation (*i.e.*, affinity methods) in a diabetic and non-diabetic animal model.

METHODS

Sample preparation. — Sprague-Dawley rats (26 animals) were used for study, and the diabetic state was induced with streptozotocin (65 mg/kg of body weight i.p.) in thirteen of the animals. After confirmation of the diabetic state, animals were divided into control (non-diabetic, 13 animals) and diabetic groups (13 animals). All animals were given water *ad libitum* and were fed standard lab chow. Maintenance of the diabetic state was assessed weekly by tail-stick glucose analysis. After eight weeks, the animals were sacrificed, blood was obtained, and hepatic tissue was obtained for membrane purification. Membranes were prepared according to the method of Maeda *et al.*[10], as we have previously performed[11]. Protein content on the purified membranes was determined as per the method of Bradford[12]. The membrane nature of the protein was confirmed by employing a membrane marker, 5′-nucleotidase, which showed a 4–6 fold enhancement in activity in the membrane preparation compared with the total homogenate[13].

Blood from the study animals, obtained at study completion, was assayed for glycated serum albumin (interassay c.v.* = 4.52%, intra-assay c.v. = 3.43%) using boronate affinity chromatography[14,15] (Pierce Chemical Co., Rockford, IL) and for total glycated hemoglobin employing automated affinity high-performance liquid chromatography (interassay c.v. = 1.2%, intra-assay c.v. = 2.1%) as performed on a Primus CLC-330 h.p.l.c. instrument (Primus Corporation, Kansas City, MO). Total glycated hepatic membrane protein was also determined by employing boronate affinity

* c.v.% = standard deviation/mean of runs (n = 10) × 100.

chromatography as previously described[11] (interassay c.v. = 4.5%, intra-assay c.v. = 4%). Serum glucose, at study completion, was determined on a Cobas Mira Chemistry Analyzer (Roche Diagnostics, Inc., Nutley, NJ).

In order to detect the furosine content of purified hepatic membranes, vapor-phase acid hydrolysis was performed as follows: An aliquot containing purified hepatic membrane protein (10–50 μg) in 6 × 50 mm tubes was dried in a vacuum centrifuge. The tubes containing the dried membrane protein were then placed in reaction vials with vacuum ports (Waters Corporation, Cary, NC) containing glass beads (1 cm) and 6N HCl (1 mL) at the bottom of the reaction vial. The reaction vials were sealed under vacuum with nitrogen and placed in an oven at 110° for the specified hydrolysis time. With the exception of the time-course experiments (hydrolysis times of 1.5, 6, and 24 h), all subsequent hydrolysis times for furosine determination were 90 min. Once hydrolysis was completed, the vials were cooled, the tubes containing the membrane protein were removed from the reaction vials, excess HCl was removed from the outside of the tubes, and the hydrolyzed protein was redried under vacuum to insure a completely desiccated state. The hydrolyzed protein was resuspended in distilled H_2O (1 mL) and filtered through a Millipore Ultrafree MC filter unit (0.45 μm) (Millipore, Bedford, MA). An aliquot (20 μL) was then subjected to h.p.l.c. analysis for furosine determination.

H.p.l.c. analysis. — The system, employing high-pressure anion-exchange chromatography with pulsed amperometric detection (p.a.d.), consisted of a Dionex Bio-LC Series 5000 instrument, (Dionex Corp, Sunnyvale, CA), a Dionex Bio LC gradient pump, a CarboPac PA-1 column (4.6 × 250 mm), and a Model PAD-2 detector. The following were the p.a.d. operating parameters: $E_1 = +0.05$V ($T_1 = 5$ msec); $E_2 = +0.65$ V ($T_2 = 3$ msec); $E_3 = -0.95$ V ($T_3 = 1$ msec). The mobile phase consisted of an isocratic run of 150mM NaOH at 1 mL/min. The eluants were prepared by suitable dilution of 50% NaOH solution with 18-megohm deionized water (Pure Flow, Inc., Mebane, NC). The Dionex Eluant Degas Module was employed to saturate the eluants with helium to degas and minimize absorption of CO_2. Samples were injected manually with a Hamilton microliter syringe (Hamilton Co., Reno, NV) fitted with a 22-S gauge needle *via* a Dionex Microinjection Valve equipped with a 20 μL sample loop. The resulting chromatographic data was integrated and plotted using Dionex AI-450 software.

For specific furosine determination, the hydrolysate (20 μL) obtained from the 90-min vapor-phase hydrolysis of the purified hepatic membrane protein was injected and plotted. The peak areas under the curve were used for quantitation of chromatograms after calibration against standard furosine chromatograms. Furosine has been identified in this system after comparison with the authentic furosine standard. Authentic furosine standard was obtained from Nestec, Ltd., Nestlé Research Centre, Lausanne, Switzerland. The structure of the furosine standard was confirmed by desorption chemical ionization mass spectrometry. Quantitative levels of furosine in the hepatic membrane protein were expressed as ng furosine/μg protein. In this system, the minimal detectable level of furosine was ~0.5 μg/mL. The precision of the instrument was determined with both low (6 μg/mL) and elevated concentrations (200 μg/mL) of

furosine. The coefficient of variation (c.v.%) was determined to be in the range 0.9–1.3%. Data was analyzed with Student's *t*-test where appropriate using ANOVA statistical software.

RESULTS

Fig. 2 demonstrates the chromatogram obtained from injection of the known furosine standard, whose structure was confirmed by mass spectrometry (data not shown). As shown, the furosine standard in this system was found to have a retention

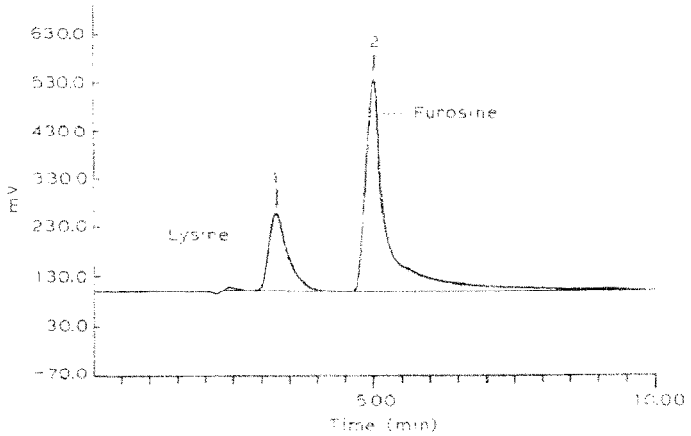

Fig. 2. H.p.l.c. detection of furosine and L-lysine in a standard preparation: lysine (peak 1, 3.18 min) and furosine (peak 2, 5.02 min).

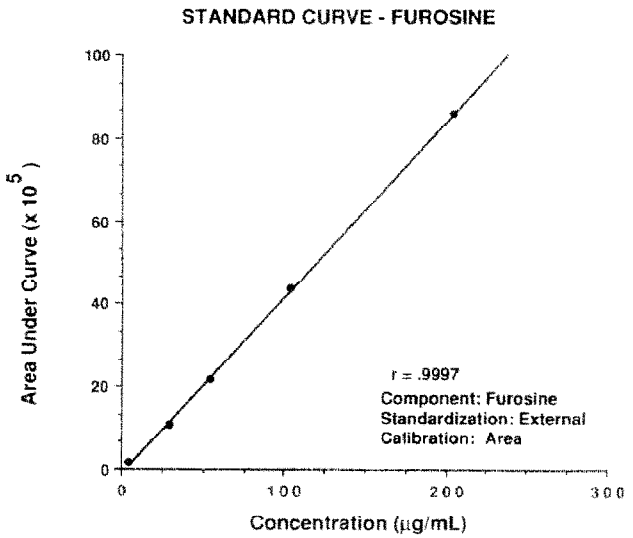

Fig. 3. A five-point standard curve of authentic furosine standard at concentrations 0.5–200 µg/mL from peak areas by h.p.l.c. analysis.

time of 5.02 min, and L-lysine was found to have a retention time of 3.18 min. Fig. 3 demonstrates the standard curve for furosine employing concentrations up to 200 μg/mL in the h.p.l.c. system calibrated for the areas under the curve. In addition, Fig. 4 represents the chromatogram obtained from injection of the vapor-phase acid hydrolysis product of the hepatic membrane. The peak noted at 5.02 min (peak no. 6) has been determined to correspond with the known furosine standard.

Fig. 5 demonstrates the time-course generation of furosine by vapor-phase hydrolysis for 1.5, 6, and 24 h at 110°. As shown, vapor-phase hydrolysis of 50 μg of hepatic membrane protein demonstrates significant quantities of furosine produced employing hydrolysis times of 1.5 and 6 h when compared to the 24-h time.

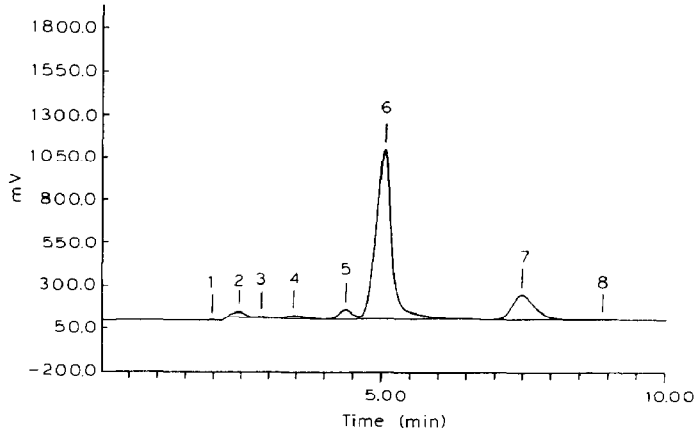

Fig. 4. H.p.l.c. analysis of products obtained from vapor-phase hydrolysis of purified hepatic membrane preparations at 90 min. Peak 6 had a retention time of 5.02 min and was found to co-migrate with our authentic furosine standard.

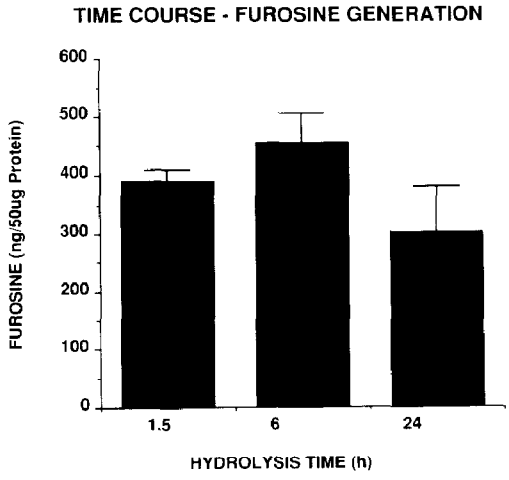

Fig. 5. Demonstrates quantitative levels of furosine generated from vapor-phase hydrolysis of purified membrane preparation at 1.5, 6, and 24 h. As shown, significant quantities of furosine are detected at both 1.5 and 6 h, in comparison to the 24-h time point.

Fig. 6 demonstrates the differences in the measured level of glycated serum albumin, glycated serum hemoglobin, glycated hepatic membrane *via* affinity methods, and glycated membrane as assessed with furosine generation between the control and diabetic animals. As shown, there was a significant difference between the control and diabetic animals using all the above mentioned methods ($p < 0.001$ for each method). In addition, serum glucose determined at study completion differed markedly in the diabetic animals *vs.* the control animals (30 ± 6 *vs.* 9.3 ± 2mM, resp., $p. < 0.001$) (data not shown).

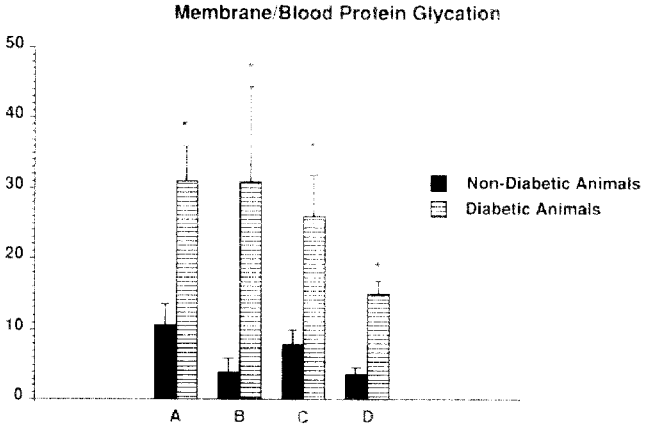

Fig. 6. Demonstrates the differences in glycated membrane and glycated blood proteins detected in our diabetic and non-diabetic animals.
A = Percent glycated membrane determined by boronate affinity chromatography (× 10). B = Glycated membrane determined by quantifying furosine generated after vapor-phase hydrolysis and detection (ng./μg protein ÷ 10). C = Percent glycated serum albumin determined by boronate affinity chromatography at study completion (× 10). D = Percent glycated hemoglobin determined by automated affinity h.p.l.c. at study completion (× 10). * $p < 0.001$.

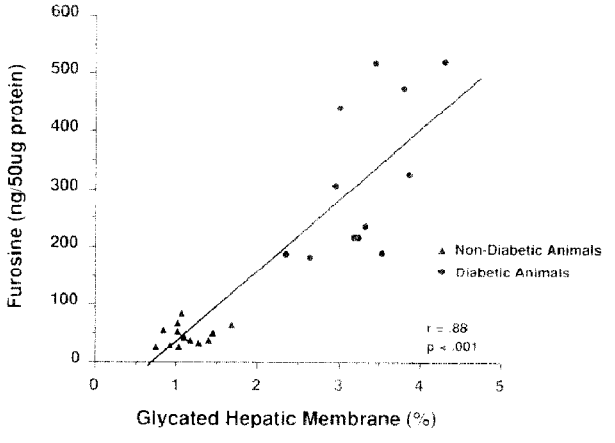

Fig. 7. Correlation between boronate affinity measurement of percent glycated membrane protein and the amount of furosine generated and detected in both diabetic and non-diabetic hepatic membrane preparations.

Fig. 7 demonstrates the correlation between percent glycation as assessed with boronate affinity methods and quantitative furosine generation in both diabetic and non-diabetic hepatic membrane preparations. As seen, significant correlation existed between the two methods ($r = 0.88$, $p < 0.001$).

DISCUSSION

This study demonstrates a novel method for detecting furosine generation, that being vapor-phase hydrolysis at 110° for 90 min, followed by h.p.l.c. separation employing pulsed amperometric detection (p.a.d.). The results demonstrate that the furosine generated by this method agrees quite well with traditional boronate affinity methods of evaluating total glycated proteins as a significant correlation existed between the methods ($r = 0.88$, $p < 0.001$). As measurement of furosine traditionally has employed a 24-h hydrolysis step, this study demonstrates that vapor-phase hydrolysis at 110° for 90 min, followed by h.p.l.c. separation employing p.a.d., can significantly shorten this process and potentially make the furosine method less time consuming for laboratory analysis.

The study of non-enzymatic glycation is achieving much interest recently in explaining the mechanisms by which chronic clinical hyperglycemia contributes to the development of diabetic complications such as retinopathy, nephropathy, and neuropathy[3–7]. On a clinical level, it has been well established that use of glycated protein measurements provides an objective parameter in assessing glycemic control depending on the half-life of the major protein measured. As such, obtaining a glycated hemoglobin level will correlate with average blood glucose over the preceding 2–3 months due to the half-life of the hemoglobin ranging from 60–120 days[16,17]. In contrast, measurement of glycated albumin, due to the half-life reported as ranging from 14–20 days, provides a valid index over the preceding 2–3 weeks[14,15,18]. However, it is believed that tissue proteins with much longer half-lives, once glycated, proceed to form advanced products (*i.e.*, products of protein cross-linking), and it is the presence of these advanced products that is believed to greatly contribute to diabetic tissue dysfunction[4–7]. Therefore, it becomes increasingly more important to evaluate those methods that can specifically detect tissue accumulation of D-glucose.

Traditionally, measurement of non-enzymatic glycation has employed many methods. Over the recent years, there has been interest in using colorimetric methods for the detection of total glycated serum proteins. As this method relies on the ability of ketoamines to reduce the dye nitro blue tetrazolium [298-83-9] under alkaline conditions, it is achieving wide spread clinical use and correlates well with antecedent D-glucose control[19,20]. However, the method is not amenable for the study of tissue protein. Other methods that have been used to detect non-enzymatic glycation employs boronate affinity chromatography or the 5-hydroxymethylfurfural method[21]. Again, neither of these methods are specific for measuring total non-enzymatic glycated protein. However, the furosine method has been assessed as being the most sensitive and specific measure of total non-enzymatically glycated proteins and is specific for L-lysine-bound ketoamine derivatives[8,9].

To generate furosine, acid hydrolysis needs to be performed. Acid hydrolysis of the stable ketoamine derivative (1-amino-1-deoxy-D-fructose) leads to the formation of furosine and pyridosine with the yield for furosine reported to approach 30% (ref. 8). However, due to the long hydrolysis time (reported to be up to 24 h), this has been the major limiting factor in the use of this particular method. However, we have demonstrated that significant furosine generation is achieved in as short as 90 min by employing vapor-phase acid hydrolysis at 110°. That significant furosine generation is accomplished by employing shorter hydrolysis times has been suggested, as Heyns *et al.*[22] has reported *N*-(2-furoylmethyl)amino acid generation in as little as 2 h, and Schleicher and Wieland[23], although traditionally employing acid hydrolysis at 95°, have stated that furosine can be detected in as short as 90 min by employing increased reaction temperature.

It is clear from this experiment that our animals were clearly diabetic in comparison to controls. Not only was the serum D-glucose markedly elevated in these animals at study completion compared to the control animals, but two objective glycemic parameters, glycated serum albumin and glycated hemoglobin, were significantly elevated in the blood of the diabetic animals, compared to the control animals, demonstrating the presence of a hyperglycemic state during the course of the study. In addition, boronate affinity methods to detect total glycated protein in the hepatic membranes showed a significant difference between diabetic and control animals. Employing the furosine method, we were able to show significant differences in membrane accumulation of specific non-enzymatically attached D-glucose in the diabetic and non-diabetic animals, which agreed with the boronate affinity method in this regard.

The h.p.l.c. system employed demonstrated a high degree of precision in the detection of furosine. As mentioned above, we used a concentration of 0.5 μg/mL as our minimum detectable level in our standard curve. In addition, we calculated the coefficient of variation between runs at both a low furosine concentration (6 μg/mL) and a relatively high value (200 μg/mL). We report a range for c.v. of 0.9–1.3% which demonstrates a high degree of precision.

In conclusion, we report on a rapid h.p.l.c. technique to detect the Amadori product accumulation in biomedical samples. The validated technique requires only a 90-min vapor-phase acid hydrolysis step to generate furosine compared to traditional methods requiring up to 24 h. This method was able to detect differences in furosine generated from hydrolysis of tissue membrane between diabetic and non-diabetic animals and correlated well with total glycated membrane protein as determined by traditional affinity methods. Therefore, this method appears to offer a more rapid analysis of furosine than has been traditionally employed.

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