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Journal of Chromatography B, 667 (1995) 328–332

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Short communication

Reliable determination of furosine in human serum and dialysate proteins by high-performance liquid chromatography

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First received 28 September 1994; revised manuscript received 6 January 1995; accepted 16 January 1995

Abstract

Furosine, formed by hydrolysis of 1-deoxy-fructosyl-lysine (fructose–lysine), is a product of the Amadori rearrangement of glucose and ϵ-NH_2-lysine. Fructose–lysine can react further with tissue and circulating proteins to produce advanced glycation end-products (AGEs). Peritoneal dialysate used in the treatment of patients with end-stage renal failure contains high concentrations of glucose which may lead to intraperitoneal formation of AGEs. To quantitate the kinetics of formation and peritoneal clearance of glycated peritoneal dialysate proteins, we developed an effective approach to the measurement of furosine in clinical samples of serum and peritoneal dialysate.

1. Introduction

Glucose reacts nonenzymatically with primary amino groups on proteins to form glycated residues, called Amadori products. With time, Amadori products undergo a series of dehydration and fragmentation reactions to form the stable covalent adducts called advanced glycosylation end-products (AGEs). Accumulation of AGEs in plasma and in tissue has been associated with perturbations of a broad range of cellular functions, especially of endothelial cells and macrophages [1,2]. In renal failure patients, the loss of the normal renal clearance of AGEs results in high circulating and tissue levels of AGEs [3]. Since dialysate used in the treatment of patients with end-stage renal failure contains high concentrations of glucose, we reasoned that peritoneal dialysis would result in an increased rate of formation of glycated proteins in peritoneal fluid, due to the in vivo incubation of glucose and peritoneal proteins. To investigate this hypothesis, we developed a method, based on furosine analysis, to quantitate the kinetics of formation and peritoneal clearance of Amadori product in peritoneal and serum proteins during routine peritoneal dialysis (see Fig. 1).

Although methods to quantitate furosine have been previously reported for cow milk [4], rat serum [5] and human serum albumin [6], quantitation of furosine in peritoneal proteins using established protocols has been fraught with problems due to the fact that proteins from uremic patients are extensively modified by AGEs. We

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0378-4347/95/$09.50
SSDI 0378-4347(95)00038-0

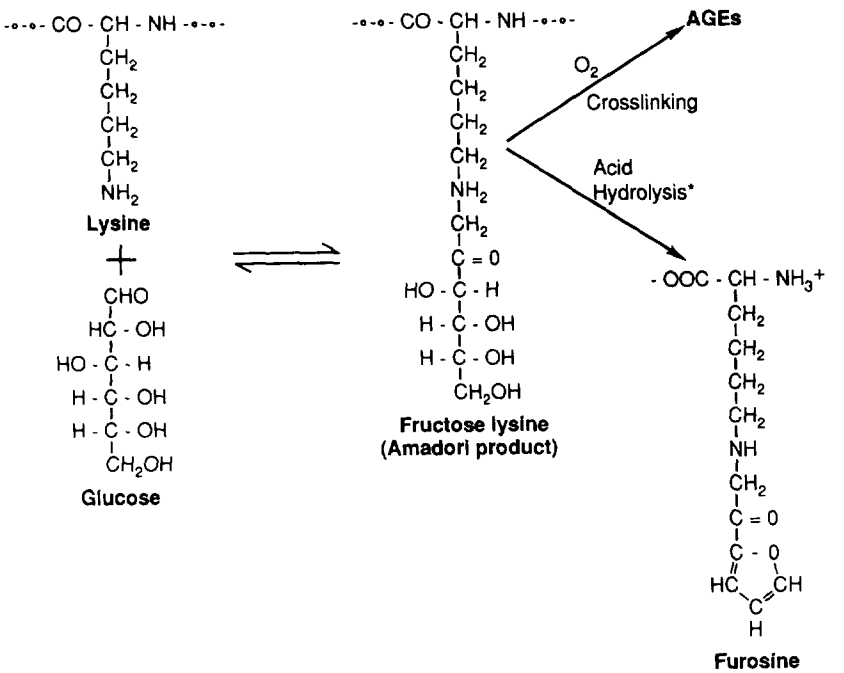

Fig. 1. The first step in the Maillard reaction results in the non-enzymatic formation of an Amadori adduct to a protein amino group (lysine in this illustration). Compounds such as fructose–lysine and other Amadori adducts undergo further reactions to yield advanced glycosylation end-products such as carboxymethyl-lysine and pentosidine (not shown). [*When subjected to acid hydrolysis, glycated proteins (Amadori product) yield approximately 30% of the reaction product furosine, 50% free lysine and 20% other products (Ref. [8]); -o-o- represents an unspecified protein structure.]

report the development of an HPLC protocol for reliable furosine determination in human serum and dialysate proteins in such patients.

2. Experimental

2.1. Development of negative control

Each sample was assayed with a negative control from which all Amadori product had been removed by the addition of sodium borohydrate ($NaBH_4$). To find the optimal concentration of sodium borohydrate for use in samples derived from human serum and peritoneal dialysate, initial experiments were performed incubating 1 mg/ml of glycated bovine serum albumin (BSA) for three incubation time periods (30, 120, 240 min) and at two concentrations of sodium borohydrate (0.029 and 0.29 *M*) (see Table 1). Glycated BSA was made by incubation of 100 mg/ml BSA with 1 *M* glucose in sterile aqueous solution pH 7.4 for 30 days at 37°C. The higher concentration and 4-h incubation reduced the furosine concentration by 100% as shown in Table 1. For fully glycated BSA, at approximately 59 lysine residues per molecule, this concentration represents a molar ratio of 333:1 (33:1 for the lower concentration of $NaBH_4$).

Table 1
Reduction of furosine by $NaBH_4$

Incubation time (min)	Furosine (pmol/125 μg protein)	
	0.029 *M* $NaBH_4$	0.29 *M* $NaBH_4$
30 min	89.49	33.88
120 min	70.73	26.91
240 min	64.49	0.00

2.2. Sample preparation

The concentration of protein in the serum or dialysate samples was measured in a micro-assay modification of the Bradford method using Coomassie brilliant blue G250 (Biorad Laboratories, Melville, NY, USA). The protein concentration in the samples was then adjusted to 1 mg/ml in phosphate buffered saline (PBS) pH 7.4. Protein samples were subjected to acid hydrolysis after incubation with or without borohydrate (0.29 *M*) for 10 min at room temperature followed by 4 h on ice. Protein (250 μg) was precipitated on ice with 40 μl of 50% trichloroacetic acid (TCA, Fisher, Pittsburgh, PA, USA). To avoid problems with deterioration of sample or standard, as outlined by Hartkoph and Erbersdobler [7], all samples were kept on ice and at low pH during processing. The pellets of precipitated protein were washed twice with 5% cold TCA and acid hydrolyzed in 1 ml of 6 *M* HCl for 14 h at 110°C in borosilicate tubes with PTFE-coated screw caps. Acid was removed by vacuum centrifugation (Savant, Farmington, NY, USA). The hydrolyzed pellet of precipitated protein was then resuspended with 400 μl of 0.4% acetic acid and filtered using a 0.45-μm nylon microfilterfuge tube (Rainin, Woburn, MA, USA). Based on the method of Resmini et al. [4], an additional set of hydrolyzed pellets were resuspended in 0.5 ml of 3 *M* HCl and filtered before loading onto a Sep-Pak tC_{18} cartridge (Millipore, Bedford, MA, USA) prewetted with 3 *M* HCl. The cartridge was then eluted with 3 ml of 3 *M* HCl. The eluent was collected and dried by vacuum centrifugation. The dried protein pellet was then prepared as described above for injection (Table 2).

2.3. HPLC analysis

Furosine standard was purchased from Neosystem laboratories (Strasbourg, France). A 200-μl volume of filtered hydrolysate (half of the original amount of protein precipitated on ice with TCA and equivalent to 125 μg of protein) was injected onto the HPLC system (Model 600E, Waters, Marlborough, MA, USA). A Guard-Pak in-line filter (Waters) was used to filter the injected sample in the precolumn holder. The column used was a furosine-dedicated C_8 column (250 × 4.6 mm I.D., Alltech, Milano, Italy). The solvents used were 0.4% acetic acid (solvent A) and 0.27% potassium chloride in solvent A (solvent B). The elution gradient used was as follows (proportion of solvent B): 0–12.5 min, 0%; 12.5–22 min, 50%; 22–32 min, 0%. The flow-rate is 1.2 ml/min. Furosine elutes at ~29 min as monitored by a UV detector (Waters, LC Spectrophotometer 481) set at 280 nm, 0.01 AUFS. Peak areas were integrated by a computer program called Baseline 810 (Waters).

Table 2
Recovery of furosine with and without solid-phase extraction (SPE)

Condition	Furosine (pmol/125 μg protein)	
	With solid-phase extraction	Without solid-phase extraction
Pooled dialysate	163	155
Reduced pooled dialysate	20	9
Reduced pooled dialysate + 54 pmol furosine	72	70
Normal serum	74	73
Reduced serum	6	6
Reduced serum + 54 pmol furosine	60	57

3. Results and discussion

The correlation between the amount of furosine assayed by HPLC and the recorded peak area was linear. Sodium borohydrate was used to reduce Amadori products in the glycated proteins of samples before hydrolysis, preventing furosine formation. By comparing chromatograms with these borohydrate reduced and non-reduced standards and samples, we were able to identify the furosine peak at a retention time of around 29 min (Fig. 2A, 54 pmol standard). Fig. 2B,C shows the furosine peak and the effect of

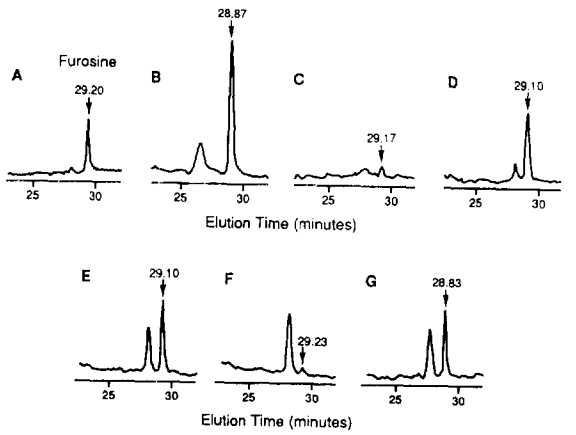

Fig. 2. Determination of furosine in proteins derived from patient samples: (A) furosine standard (54 pmol); (B) protein from pooled spent dialysate; (C) reduced dialysate; (D) reduced protein from dialysate with furosine standard added; (E) normal human serum; (F) reduced serum; (G) reduced serum with furosine standard added. All the injected samples contained 125 μg of protein. Values at arrows indicate retention time in min.

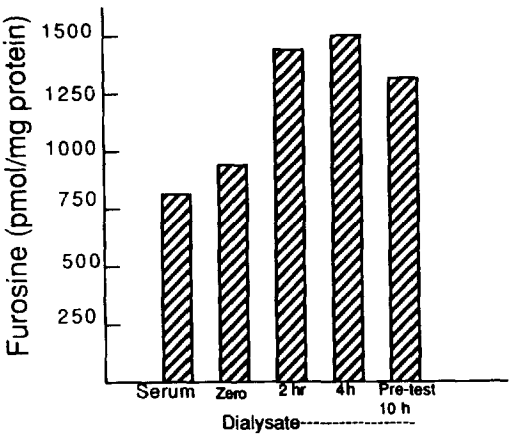

Fig. 3. Kinetic studies of furosine in peritoneal dialysate. A 125-μg amount of protein was injected onto the HPLC system for each condition with the exception of the time 0 point. Because of the low concentration of protein present in dialysate at this time, 76 μg was injected.

borohydrate reduction in a pooled sample of spent peritoneal dialysate obtained from 20 patients. Fig. 2D shows the recovery of the 54 pmol standard, added to the pooled peritoneal dialysate. The pattern in normal human serum is similar. The furosine peak is present at 29 min (Fig. 2E), is abolished with addition of $NaBH_4$ (Fig. 2F), and the addition of furosine gave a full recovery (Fig. 2G).

We attempted to use Sep-Pak tC_{18} cartridges for solid-phase extraction (SPE) to minimize contamination of the column. However to avoid loss of the intensity of the furosine signal diluted in 3 ml as described by Resmini et al. [4], we modified the SPE method (see above). As shown in Table 2, the results obtained after SPE do not differ from those demonstrated in Fig. 2. Although the baseline after SPE has fewer extraneous peaks, the separation of furosine still can be discerned without SPE (Fig. 2). Because SPE takes an additional day for extraction and drying, and because SPE did not further enhance resolution in plasma and dialysate, we omitted the SPE step for further sample processing.

To investigate the formation and degradation of furosine during peritoneal dialysis we quantitated furosine in serum and dialysate from peritoneal dialysis patients. Fig. 3 shows the results of a representative series of chromatograms from a peritoneal equilibration test (PET) performed on a non-diabetic patient with end-stage renal failure. Dialysate is drained from the abdomen, usually 8–12 h after the last exchange (pre-test furosine level, Fig. 3). For the PET, fresh dialysate (with a glucose content of 2.5%) is infused and mixes with any undrained fluid remaining in the abdominal cavity. After infusion a sample is immediately drawn from the peritoneal cavity for testing ("zero"), samples of dialysate are obtained at 2 h and 4 h of dwell time (Fig. 3). In the individual PET test demonstrated, the serum furosine level (Fig. 3) is lower than that in the dialysate. In addition, the data show increasing levels of furosine with increasing dwell-time of dialysate in the peritoneum.

In summary, the adaptation of the method of Resmini et al. [4] previously developed to quantitate furosine in food products, for furosine determination in biological and clinical samples is a major improvement compared to previous methods in which poor furosine retention by C_{18} columns was associated with errors. We also

demonstrate preliminary evidence for the in vivo formation of this product in peritoneal dialysate from patients on peritoneal dialysis.

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