Journal of Chromatography, 573 (1992) 201–206
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6125

Effect of glycation on the heterogeneity of human serum albumin analysed by reversed-phase high-performance liquid chromatography in a solvent containing formic acid

P. Vidal[☆,*], E. Nielsen and B. S. Welinder

Department of Protein Chemistry, Hagedorn Research Laboratory, 6 Niels Steensensvej, DK-2820 Gentofte (Denmark)

(First received February 12th, 1991; revised manuscript received August 20th, 1991)

ABSTRACT

Non-enzymic glycation of human serum albumin (HSA) induces a change in its charge heterogeneity that may account for its particular renal clearance in patients with early diabetic nephropathy. A new high-performance liquid chromatographic analysis for the study of HSA heterogeneity is described based on a high content of formic acid in the mobile phase combined with a concave gradient of isopropanol. Under these conditions, native HSA was separated into three individual components (I, II and III). When glycated HSA was analysed, it was found that although the present method is not suitable for the separation of glycated from non-glycated HSA, it shows the effect of glycation in producing changes in HSA heterogeneity that are different from those reported on surface change. This finding suggests an additional factor (probably conformational changes) that is contributing to the heterogeneity of glycated HSA.

INTRODUCTION

Four major reasons for the heterogeneity of human serum albumin (HSA) in plasma are possible: (a) polymerization; (b) differences in thiol content; (c) the presence of bound compounds; and (d) the possibility of mutants or/and post-synthetic modifications of the protein (see refs. 1–3 for review). Small amounts of dimers and polymers of HSA are normally present in plasma, probably arising as a result of disulphide linkages [4]. The thiol content in HSA has led to the classic distinction between two major subgroups: the "mercaptalbumins" (where a free thiol group is conserved and has the capacity of forming dimers *in vitro* through reaction with mercuric ions) and the so-called "non-mercaptalbumins" (where the thiol group is blocked by cysteine or by glutathione [1,5]). Many different compounds can also bind HSA and contribute to its heterogeneity (including fatty acids, bilirubin, tryptophan, various metal ions, some hormones and numerous drugs) [2,6]. Finally, different mutants of HSA have been reported to affect the heterogeneity of HSA [7], and several post-translational modifications (including deamidation, acetylation, etc.) have been also suggested to increase the heterogeneity of HSA [1].

On the other hand, it has been reported that formic acid (probably owing to its dissolving power) is a very useful solvent for the reversed-phase high-performance liquid chromatographic (RP-HPLC) separation of highly hydrophobic proteins [8,9] and peptides [10]. The separation of albumin derivatives containing different modifications at cysteinyl residues has been achieved using this principle, and found to be mainly based on their differences in hydrophobicity and intrinsic charge [8], which might be of interest for the study of HSA heterogeneity.

We have recently demonstrated that the post-synthetic reaction of protein glycation induces an

☆ Address for correspondence: Department of Endocrinology, Hospital Sta Creu I S. Pau, Universidad Autonoma Barcelona, Av. Am. Claret 167, E-08025 Barcelona, Spain.

0378-4347/92/$05.00 © 1992 Elsevier Science Publishers B.V. All rights reserved

anionic heterogeneity of HSA [11,12], which may help to explain its particular clearance in early diabetic nephropathy [13]. We report here an RP-HPLC method, using formic acid in the mobile phase, which is able to separate up to three major fractions from native HSA, as well as the effect of non-enzymic glycation on its heterogeneity as analysed using this method.

EXPERIMENTAL

In vitro glycation of human serum albumin

Fast-reacting contaminants from [U-^{14}C]-D-(+)glucose (Amersham, Buckinghamshire, UK) were removed by pre-incubation with albumin as described earlier [9]. Monomeric HSA was obtained by gel permeation chromatography of crystalline albumin (Behringwerke, Marburg, Germany) on a 100 cm × 5 cm I.D. column of Sephacryl HR-200 (LKB, Bromma, Sweden); 40 mg/ml monomer was incubated in 0.1 *M* phosphate buffer (pH 7.4) containing 0.15 *M* NaCl, 0.02% NaN_3 and 50 m*M* [^{14}C]glucose (specific activity 6 μCi/mg) during 0–15 days in a shaking water-bath (30 cycles/min) at 37°C. Salts, free glucose and non-covalently bound glucose were separated from the HSA using a 95 cm × 2.5 cm I.D. Sephadex G-50 column (Pharmacia, Bromma, Sweden) eluted with 0.1 *M* NH_4HCO_3 (pH 8.0) at 28 ml/h. Incubation of HSA for 0, 5, 10 and 15 days resulted in a specific activity of 0.9, 19.9, 47.5 and 70.3 · 10^3 dpm/mg of protein, respectively.

Affinity chromatography on Glycogel B

Prepacked microcolumns of 3-aminophenylboronic acid immobilized to agarose (Glycogel B, Pierce, Rockford, IL, USA) were used for the separation of glycated and non-glycated albumins. The columns were equilibrated with 0.25 *M* ammonium acetate (pH 8.0) containing 0.05 *M* $MgCl_2$. After application of 2 mg of HSA, 20 ml of the same buffer were passed through the column, and the bound (glycated) albumins were eluted with 5 ml of 0.2 *M* sodium citrate (pH 4.5).

RP-HPLC of human serum albumin in formic acid-containing solvent

RP-HPLC was performed on Aquapore RP-300 (200 mm × 4.6 mm I.D.; Brownlee Labs., Santa Clara, CA, USA) equilibrated with 50–70% formic acid containing from 0–20% isopropanol. The best resolution was obtained using two columns in series after equilibration with 60% formic acid containing 6.5% isopropanol. Elution was achieved with a concave gradient (up to 10% isopropanol) using 60% formic acid as buffer A and 60% formic acid containing 10% isopropanol as buffer B with the following programme: 0 min, 65% B; 5 min, 75% B; 20 min, 77% B; 25 min, 80% B; 30 min, 100% B; 40 min, 100% B; 40.1 min, 65% B. An SP 8700 chromatograph (Spectra-Physics, Santa Clara, CA, USA) equipped with a U6K injector (Millipore Waters, Milford, MA, USA) were used through-

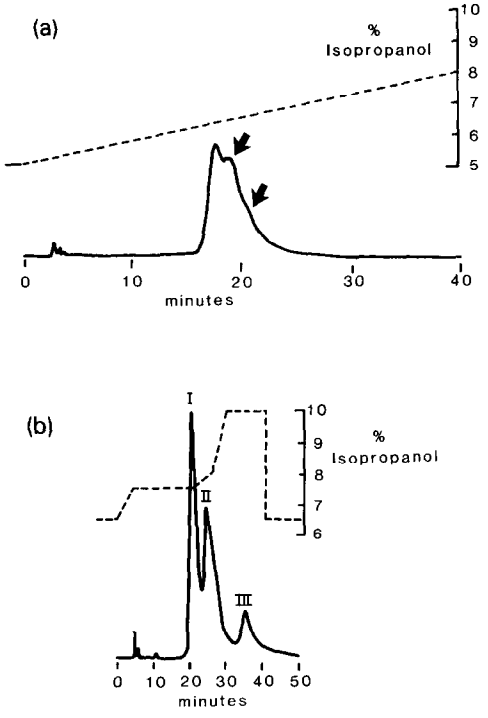

Fig. 1. RP-HPLC separation of 1 mg of native HSA. (a) Aquapore RP-300, 60% formic acid in buffer A, 60% formic acid plus 20% isopropanol in buffer B; linear gradient from 5 to 8% isopropanol in 40 min (0 min, 25% buffer B; 40 min, 40% buffer B; 40.1 min, 25% buffer B); flow-rate, 1 ml/min; detection wavelength, 280 nm (a.u.f.s. 0.05). (b) Aquapore RP-300 (two columns in series), 60% formic acid in buffer A, 60% formic acid plus 10% isopropanol in buffer B; concave gradient from 6.5 to 10% isopropanol in 30 min (see text); flow-rate, 0.8 ml/min; detection wavelength, 280 nm (0.05 a.u.f.s.). Note fractions I, II and III.

out. The flow-rate was 0.8 ml/min, and the absorption at 280 nm was monitored using a UV–VIS 200 detector (Linear Instruments, Reno, NV, USA). When ^{14}C-glycated -HSA was analysed, fractions of 500 ml were collected, the solvent was eliminated by simple overnight evaporation, and the residue was finally dissolved in 2.5 ml of Optiphase "HiSafe" (LKB) for analysis of radioactivity in a Tri-Carb 460 liquid scintillation counter (Packard, Downers Grove, IL, USA).

Isoelectric focusing

High-resolution isoelectric focusing in the pH range 4–6.5 was performed using a Phast System (Pharmacia) as described previously [9]. Phast-Gel Silver Kit (Pharmacia, Cat. No. 17-0617-01) was used for staining according to the instructions from the manufacturer.

RESULTS

RP-HPLC of native HSA

After RP-HPLC of albumin, using two Aquapore RP-300 columns in series and 60% formic acid containing 6.5% isopropanol in a concave gradient up to 10%, three major fractions (I, II and III) could be demonstrated (Fig. 1).

RP-HPLC of glycated HSA

Albumin after 10–15 days of *in vitro* glycation showed a second chromatographic peak (II) with a tendency to coelute with peak I (Fig. 1). In addition, the heterogeneity in the peak III region was found to be noticeably increased (Fig. 2). Nevertheless, when fractions I, II and III were rechromatographed, no differences in glucose content (as judged by the UV-to-radioactivity ratio) were observed (Fig. 3). A value of 2.35 mol glucose per mol HSA was estimated.

RP-HPLC analysis after phenyl boronate chromatography

Glycated HSA obtained after 10 days of incubation was separated from non-glycated HSA using phenyl boronate affinity chromatography, and both "bound" and "unbound" fractions (normally considered to be glycated and non-glycated protein, respectively) were further analysed by RP-HPLC. A reduced retention time of the second peak obtained from the "bound" fraction (glycated) was again evident (Fig. 4).

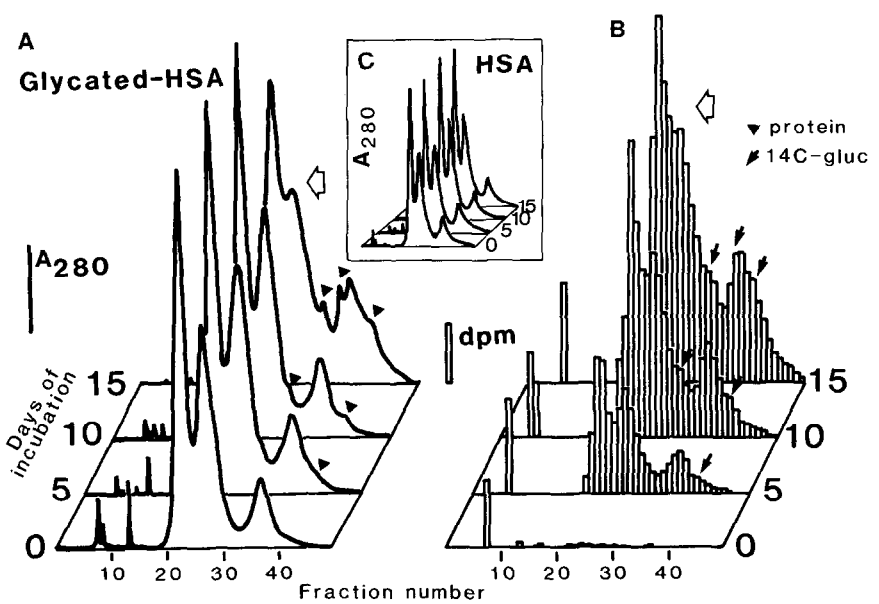

Fig. 2. RP-HPLC separation of ^{14}C-glycated HSA. (A) A_{280} profiles of HSA incubated for 0–15 days with glucose, showing a reduced retention time of peak II after 15 days glycation and an increased heterogeneity of peak III. Chromatographic conditions as in Fig. 1B (except a.u.f.s. 0.1). (B) Radioactive profiles of the same samples. (C) A_{280} profiles of HSA incubated for 0–15 days without glucose.

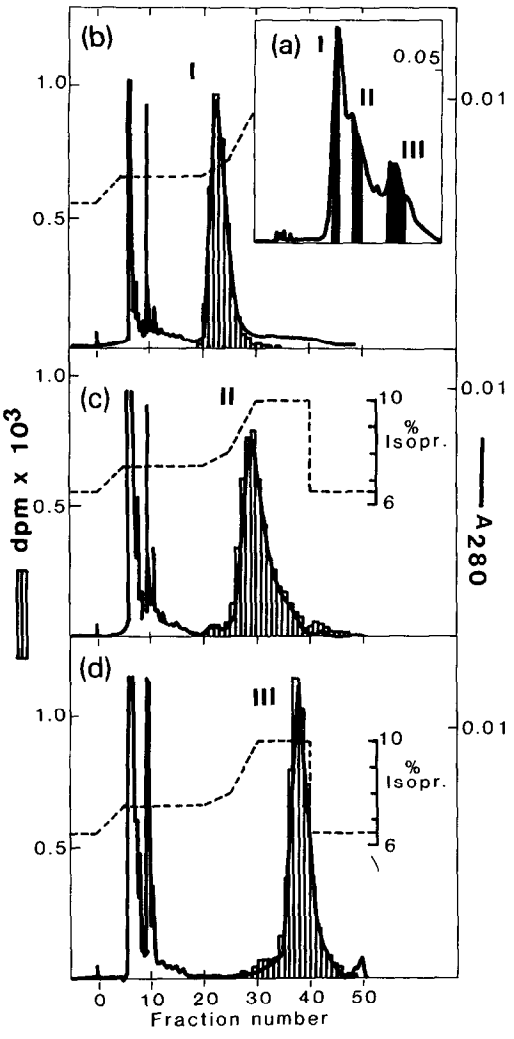

Fig. 3. Rechromatography of fractions I, II and III. Chromatographic conditions as in Fig. 1B (except that in B, C and D the a.u.f.s. was 0.02). (a) A_{280} profiles of HSA after 15 days glycation with [^{14}C] glucose, showing fractions I, II and III. (b) Rechromatography of fraction I. (c) Rechromatography of fraction II. (d) Rechromatography of fraction III. Note the similar UV-to-radioactivity ratio in all three fractions.

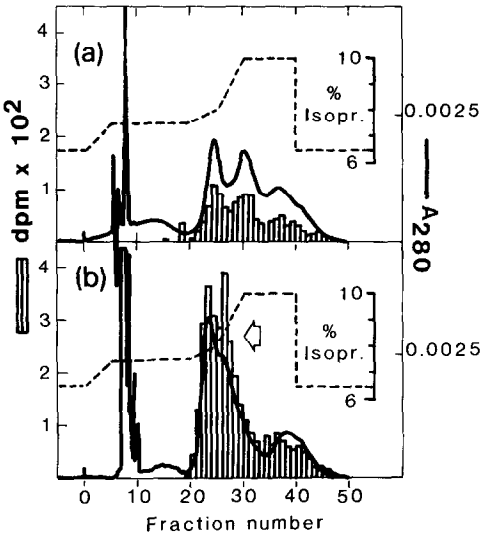

Fig. 4. RP-HPLC analysis of phenyl boronate fractions. HSA after 10 days glycation with [^{14}C]glucose after affinity chromatography: (a) non-glycated ("unbound") fraction; (b) glycated ("bound") fraction.

Isoelectric focusing

When the charge heterogeneity of each of the RP-HPLC fractions (I, II and III) was analysed by isoelectric focusing, no major differences were observed (Fig. 5).

DISCUSSION

The use of high concentrations of formic acid in the mobile phase and a linear gradient of isopropanol has been reported to be successful for the separation of several derivatives of bovine serum albumin (BSA) by RP-HPLC [8]. Under these conditions, all the modified BSA derivatives eluted earlier than the completely reduced BSA, suggesting a relationship between the elution pattern and the conservation of disulphide bridges. The change of their elution behaviour observed after modification of thiol groups indicates that the charge and hydrophobicity of BSA influenced the interaction with the reversed-phase matrix.

In order to study differences in charge after non-enzymic glycation of HSA, we applied the principle of RP-HPLC using formic acid in the mobile phase to the analysis of *in vitro* glycated HSA. With 60% formic acid and a concave gradient from 6.5 to 10% isopropanol, we have been able to separate up to three different compounds (I, II and III) from native HSA. After 10–15 days of *in vitro* glycation of HSA, fraction II tended to elute faster during the HPLC separation. This was consistent with results obtained after enrichment of the sample in glycated HSA using affinity chromatography on phenyl boronate, although it is known that this matrix shows a certain level of non-specificity to separate glycated from non-glycated HSA, depending on the glucose content of the molecule and is conformational state [12].

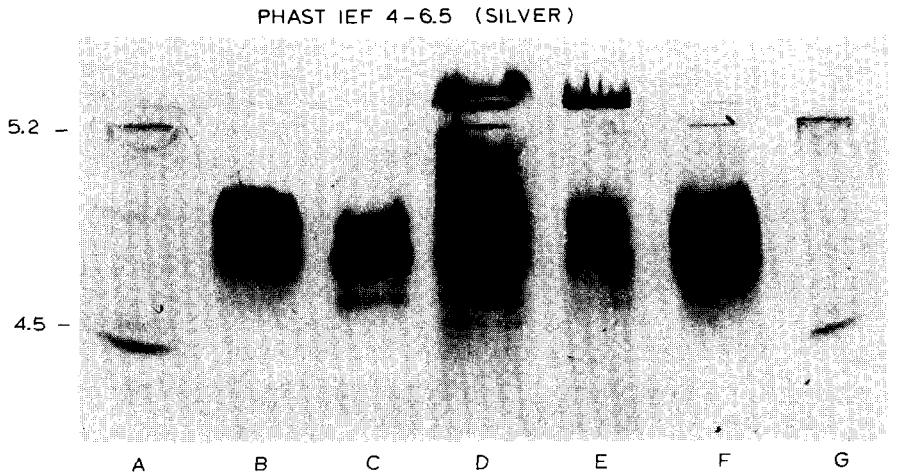

Fig. 5. Isoelectric focusing (pH 4–6.5) of the isolated RP-HPLC fractions. Lines A and G, p*I* standards; line B, non-glycated HSA [showing three major fractions differing in charge (4.9, 4.8 and 4.65)]; line C, HSA after 15 days glycation, showing an increase of fraction 4.65 at the expense of fraction 4.9; lines D, E and F, the RP-HPLC fractions I, II and III, respectively, obtained from a sample after 15 days glycation. Precipitated protein that does not enter the gel appears in the sample application area (upper part).

In the present experiments, the retention time for fraction II obtained by RP-HPLC of glycated HSA was markedly decreased compared with that obtained from the non-glycated protein. Glucose incorporation during *in vitro* glycation (calculated from the incorporation of labelled glucose) progressively increased in all three fractions as the glycation time increased. Nevertheless, the glucose content in the RP-HPLC fractions (as judged by the UV-to-radioactivity ratio) was found to be virtually the same in all three fractions, suggesting that the rate of glycation was the same but the hydrophobicity of fraction II is particularly affected. In addition, after 10–15 days of glycation, peak III progressively showed increased heterogeneity in contrast to controls incubated without glucose.

Non-enzymic glycation of HSA has been demonstrated to produce a major change in its isoelectric focusing pattern, mainly consisting of an increase of the p*I* 4.8 and 4.65 fractions at the expense of the p*I* 4.9 fraction [9]. When the RP-HPLC fractions I, II and III were analysed using isoelectric focusing under the same conditions, no major difference in p*I* heterogeneity was found. Therefore, the lack of difference in charge heterogeneity observed by isoelectric focusing analysis of the RP-HPLC fractions I, II and III corresponds to the lack of differences in the extent of glycation.

The protein structure in these RP-HPLC fractions is unknown. However, one possibility might be that these fractions contain HSA differing in thiol content (probably a major fraction of mercaptalbumin and two others of cysteine–non-mercaptalbumin and glutathione–non-mercaptalbumin) [5,16] with no relation to the glucose content. It is well known that glucose incorporation into HSA via non-enzymic glycation takes place mainly at the third domain of the protein and, to a lesser extent, at the second domain [14,15], whereas the free thiol group of HSA is located at the first domain [2]. This might explain the lack of major differences in glucose content observed in the RP-HPLC fractions, assuming that the present separation derives from properties of HSA mainly related to its first domain.

CONCLUSION

An RP-HPLC analysis for the study of HSA heterogeneity, based on a high content of formic acid in the mobile phase combined with a concave gradient of isopropanol, has been developed. Under these conditions, native HSA was separated into three individual components (I, II and III). Although this method is not suitable for the separation of glycated from non-glycated HSA, it shows that the effect of glycation in producing changes in HSA heterogeneity (decreased

retention time of fraction II and increased heterogeneity of fraction III) is different from the reported effect on the p*I* [11,12]. These small changes might be explained by minimal differences in conformation and/or by charge heterogeneity in regions of the HSA molecule that are not exposed under mild conditions (isoelectric focusing) but become exposed during RP-HPLC in a mobile phase containing the strongly denaturing formic acid.

REFERENCES

1 J. F. Foster, in V. M. Rosenoer, M. Oratz and M. A. Rothschild, (Editors), *Albumin, Structure, Function and Uses*, Pergamon Press, Oxford, 1977, p. 53.
2 T. Peters, *Adv. Protein Chem.*, 35 (1985) 161.
3 C. Le Q. Darcel, *Int. J. Biochem.*, 19 (1987) 295.
4 G. Franglen, in A. C. Allison (Editor), *Structure and Function of Plasma Proteins*, Plenum Press, London, 1974.
5 M. Sogami, S. Nagaoka, S. Era, M. Honda and K. Noguchi, *Int. J. Peptide Protein Res.*, 24 (1984) 96.
6 U. Kragh-Hansen, *Pharmacol. Rev.*, 33 (1981) 17.
7 K. Huss, J. Madison, N. Ishioka, N. Takahashi, K. Arai and F. W. Putnam, *Proc. Natl. Acad. Sci. U.S.A.*, 85 (1988) 6692.
8 J. Heukeshoven and R. Dernick, *J. Chromatogr.*, 252 (1982) 241.
9 D. D. Sheumack and R. W. Burley, *Anal. Biochem.*, 174 (1988) 548.
10 D. J. Poll and D. R. K. Harding, *J. Chromatogr.*, 469 (1989) 231.
11 P. Vidal, B. S. Welinder, T. Deckert and B. Hansen, in C. Schafer-Nielsen (Editor), *Proceedings of the VIth Meeting of the International Electrophoresis Society, Copenhagen, July 1988*, VCH, Weinheim, 1988, p. 468.
12 P. Vidal, T. Deckert, B. Hansen and B. S. Welinder. *J. Chromatogr.*, 476 (1989) 467.
13 A. Kverneland, B. Feldt-Rasmussen, P. Vidal, B. S. Welinder, L. Bent-Hansen, U. Soegard and T. Deckert, *Diabetologia*, 29 (1986) 634.
14 R. L. Garlick and S. Mazer, *J. Biol. Chem.*, 258 (1983) 6142.
15 N. Iberg and R. Fluckiger, *J. Biol. Chem.*, 261 (1986) 13 542.
16 K. Nishimura, H. Harada, S. Masuda and A. Sugii, *J. Chromatogr.*, 525 (1990) 176.