ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
13 (1995) 1449 1452

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

# The effects of pasteurisation on albumin: an EPR binding assay for polymeric albumin

Bernard Gallez[a,*], Jean-Luc De Keyser[a], Rene Debuyst[b], Fernand Dejehet[b], Laurence Neuvens[a], Pierre Dumont[a]

[a]*Department of Pharmaceutical Sciences, Laboratory of Medicinal Chemistry, Catholic University of Louvain, Avenue Mounier 73.40, B-1200 Brussels, Belgium*
[b]*Department of Chemistry, Laboratory of Inorganic, Analytical and Nuclear Chemistry, Catholic University of Louvain, Chemin du cyclotron, B-1348 Louvain-la-Neuve, Belgium*

Received for review 18 March 1995

**Abstract**

The ability of a nitroxyl fatty acid (NFA) to bind specifically to albumin is abolished when, in the absence of stabilizers, a 4% solution of this protein is heated above a critical temperature of 60°C. This treatment leads to the formation of "albumin polymers" as classically evidenced by GPC. Since the bound fraction is evidenced in EPR spectroscopy by a large anisotropic component, the presence of this anisotropy can be used in the assessment of the quality of the pharmaceutical preparations of albumin, which are usually pasteurized in order to inactivate viruses. Moreover, in sharp contrast with the behavior of albumin dispersions, lyophilised albumin subjected to heat treatment at 70°C for 24 h left the protein untouched regarding its NFA binding and GPC profile.

*Keywords*: Spin labelling; Albumin; Nitroxyl; Fatty acid: Heating; Virus inactivation

## 1. Introduction

Different physical and chemical methods are commonly used in order to inactivate viruses (e.g. HIV, hepatitis B (HBV) and hepatitis C (HBC) viruses), and ensure the safety of blood derivatives [1–5]. Concerning the albumin dispersions, the most usual method is pasteurisation by heating for 10 h at 60°C. The efficiency of this method, well known since the end of the forties [6,7], was evidenced by the loss of the infectious activity of HBV contaminated albumin preparations. Owing to the lability of proteins, the quality control of blood derivatives must include, besides a viral contamination check, an assay aimed at checking the functional integrity of the protein, e.g. the ability of antihemophilic factors to exercise their procoagulant activity [8] or of the albumin to transport. In the case of albumin dispersions, it is known that the heat-treatment can induce reversible conformational changes of the protein [9], and that over-heating can induce the formation of a polymeric fraction [10]. Stabilizers such as caprylic acid are commonly used in order to avoid the formation of this fraction [11,12]. The pharmacopeias assess the quality of the heat treated albumin prepa-rations by checking by gel permeation chromatography (GPC) that the polymeric fraction is minimal [13]. Besides being slow and cumbersome, this technique is a quite indirect way to evaluate the functionnal integrity of the albumin and does not tell us anything about its ability to carry on specific tasks such as its transport function of natural

* Corresponding author. Dr Bernard Gallez is "chargé de recherches du Fonds National Belge de la Recherche Scientifique".


*SSDI* 0731-7085(95)01586-8

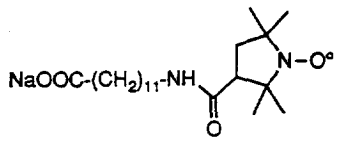


Fig. 1. Structure of NFA

and xenobiotic products. The purpose of this study is to provide a rapid test probing the binding property of albumin. The assay is based on the ability of albumin to bind fatty acids analogs: native albumin binds a spin labelled nitroxyl fatty acid derivative (NFA), leading to a large anisotropic component in the electron paramagnetic resonance (EPR) spectrum, while the polymeric albumin loses this ability, leading to an isotropic spectrum.

## 2. Materials and methods

The sodium salt of 12-(2,2,5,5-tetramethyl-1-pyrrolidinoxyl-3-carbonylamino)dodecanoic acid (Fig. 1) was prepared according to Ref. [14]. Human serum albumin (HSA) and bovine serum albumin (BSA), essentially fatty acids free grade, were purchased from Sigma. Aqueous dispersions of albumin (4%, corresponding to the plasmatic concentration) were prepared in water or Phosphate buffered saline (PBS) (0.1 M, pH 7.4). The dispersions were dispensed in 1 ml crimp-top glass vials (Chrompack) and heated for 2, 4, and 10 h at 40, 50, 52, 54, 56, 58, 60, and 70°C ( $\pm$ 0.1°C). The 60°C treatment was also performed for 15 and 30 min and with or without caprylic acid as stabilizer (15 mM final concentration). All samples were triplicated. After heating, the solutions were stored at 4°C before analysis by EPR spectroscopy or GPC analysis. The same treatment was operated on lyophilised albumin; before the analysis, the lyophilisate was dissolved in water to reach a concentration of 4%. A $10^{-2}$ M stock solution of NFA was prepared in a water/ethanol mixture (1:3, v/v). 20 $\mu$l of this stock solution was added to the albumin sample (volume 1 ml) before EPR analysis. The final concentration of ethanol in the medium never exceeded 2%. The samples, contained in a flat cell, were examined with a Bruker ER-200 tt spectrometer. The EPR spectra were recorded with incident 1 mW microwave power (frequency, 9.65 GHz), 100 G sweep width, and 2 G peak-to-peak modulation amplitude, center of the field 3447 G. Reference spectra of NFA were recorded in water and in albumin 4% (native) before the analysis of the heated products. The albumin dispersions were also analysed by GPC (High load Pharmacia system) on Sephadex G-200 (column size: 3 cm diameter; 1 m length), using water as eluent with UV detection at 280 nm. Commercial pharmaceutical albumin preparations (La Croix Rouge de Belgique, Belgium) were also analysed.

## 3. Results

Typical EPR spectra of NFA in heated dispersions are presented in Fig. 2. Fig. 2a is a spectrum representative of albumin preparations heated at 60°C without stabilizer for a time greater than 15 min and at 70°C. This isotropic spectrum is typical of free tumbling nitroxyl radicals. Above 70°C, precipitation of the protein is frequent.

Fig. 2b presents the typical EPR spectrum of NFA in buffered preparations of albumin (PBS, pH 7.4), when the dispersions are heated to below 60°C while Fig. 2c shows the EPR spectrum of NFA in a 4% unbuffered water dispersion of albumin. The analysis of these two last spectra reveals two components: three sharp lines from the freely moving radical spin label, and a more complex broad component due to the albumin-bound NFA. This anisotropic shape of the spectrum is a characteristic of all dispersions heated to below 60°C, or at 60°C in the presence of caprylic acid. Comparison of Fig. 2b and 2c shows that NFA is more strongly immobilized in non-buffered solutions (Fig. 2c) as in common pharmaceutical albumin preparations. Indeed, the distance between the two outer peaks of the broad component ($2A_{II}$) is higher in this latter case.

GPC analysis of the heated albumin preparations confirmed the presence of a majority of monomers in the dispersions heated to below 60°C, or at 60°C in the presence of caprylic acid while tougher treatment (60°C without stabilizer, or 70°C) yield a majority of polymeric fraction (Fig. 3).

In sharp contrast with the heating effect on albumin dispersion, we observed that dry stabilizer-free lyophilised albumin, redissolved for the analysis, remains unchanged up to 24 h after heating at 70 °C as evidenced by both GPC and NFA binding.

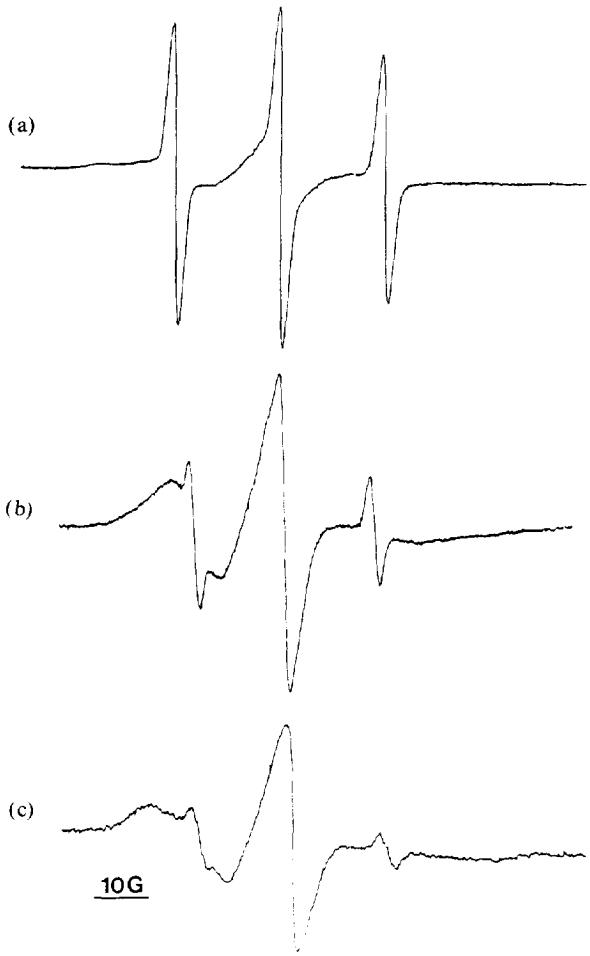


Fig. 2. Typical EPR spectra of NFA $2 \times 10^{-4}$ M in albumin solutions: a, over-heated albumin solutions (4%) (case shown: heating for 2 h at 60°C); b, albumin 4% in buffered solutions (PBS, pH 7.4) heated to below 60°C, or at 60°C in the presence of caprylic acid: c, albumin in water (20%, commercial solution). Note that the EPR spectrum a was registered with a receiver gain 5 times smaller than spectra b and c, because of the disparate broad component.

We did not observe a difference of behaviour between BSA and HSA with respect to their resistance to heating.

## 4. Discussion

Albumin is the most abundant protein in the blood, having an essential role in the preservation of the osmotic pressure and the transport of endogeneous and exogeneous substances. The binding of different types of ligands to albumin is most often the result of a specific interaction of the small molecule with a well defined site of the protein [15–18]. This interaction of albumin with fatty acids was also studied using spin labelled fatty acids such as doxyl-stearates [19–23], or NFA [14]. Molecular dynamics studies frequently use heating in order to observe conformational changes of macromolecules [14,19,24,25]. Studying the interaction of NFA with albumin over a large range of temperature [14], we observed an abrupt loss of the binding properties of albumin for NFA at 60°C. This change was irreversible. Because the temperature is critical in the heating of blood derivatives, we wanted to verify the ability of NFA to be used as a marker of albumin denaturation. Indeed, it is known that the albumin polymer fraction does not bind fatty acid analogues [26]. NFA as probe of albumin denaturation is preferable to doxyl-stearates, because its greater aqueous solubility avoids the formation of micelles and the consequent spin–spin exchange broadening in EPR spectra, and because of its greater sensitivity to this critical temperature [19].

This test can be used for the conditions described in this paper, or all conditions where the molar ratio albumin/NFA is greater than unity. Indeed, with these conditions, the bound fraction of NFA to albumin is greater than 90% [14], leading to the large anisotropic shape of the EPR spectrum.

The advantage of the test suggested here is its ease, its rapidity, and the ability to probe one of the functionalities of albumin such as the transport of fatty acids.

It is noteworthy that heating the albumin in its dry lyophilised state for 24 h at 70 °C has little, if any, detectable effect as evidenced by both GPC and NFA binding assay. The heat treatment of blood derivatives in the solid or lyophilised state is commonly used in order to inactivate viruses of the antihaemophilic factors [2,3,27,28] because the heating on solids does not lead to the denaturation of these factors. The same increase of stability in the lyophilised state was observed for the albumin in our study. Consequently, we suggest that the heating of albumin would also be preferable in the dry lyophilised state in order to avoid denaturation or loss of function of the protein. The time of heating of such preparations should of course be validated for the destruction of the virus inactivation because the efficiency of this inactivation also is lower when the heating is operated on solid preparations [2,3].

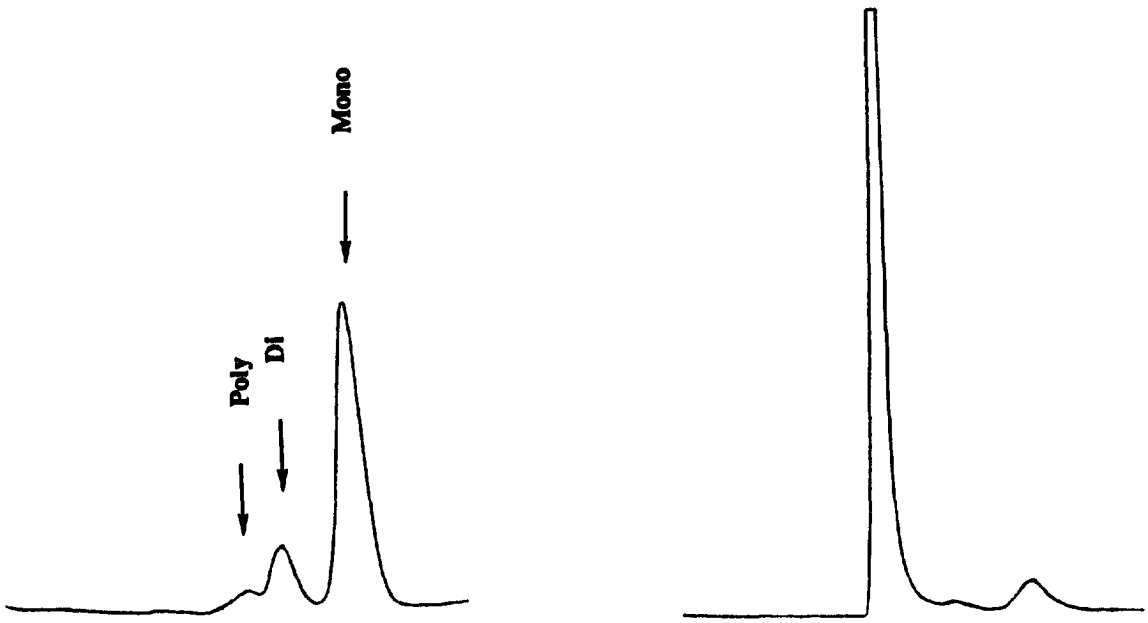


Fig. 3. GPC chromatograms of albumin solutions (4%). Left, albumin solution. The three fractions indicated by the arrows correspond to the albumin monomers, dimers, and polymers. Right, chromatogram representative of over-heated albumin solutions (case shown: heating for 10 h at 60°C). Note the presence of a majority of polymers.

## References

[1] N. Heimburger and H.E. Karges, in J.-J. Morgenthaler (Ed.), Virus inactivation in plasma products, Karger, Basel, 1989, pp. 23–33..
[2] B. Horowitz, Yale J. Biol. Med., 63 (1990) 361–369.
[3] A.M. Prince, B. Horowitz, S.M. Horowitz and E. Zang, Eur. J. Epidemiol., 3 (1987) 103–118.
[4] T. Burnouf, L. Martinache and M. Goudemand, Nouv. Rev. Fr. Hematol., 29 (1987) 93–96.
[5] R.J. Geretty and D.L. Aronson, Transfusion, 22 (1982) 347–351.
[6] S.S. Gellis, J.R. Neefe, J. Stokes, L.E. Strong, C.A. Janeway and G. Scatchard, J. Clin. Invest., 27 (1948) 239–244
[7] R. Murray and W.C.L. Diefendach, Proc. Soc. Exp. Biol. Med., 84 (1953) 230–231.
[8] P.K. Ng and M.B. Dobkin, Thromb. Res., 39 (1985) 439–447.
[9] K. Takeda, A. Wada, K. Yamamoto, Y. Moriyama and K. Aocki, J. Protein Chem., 8 (1989) 653–659.
[10] S.E. Barnes, D.A. Harris and J.D. Baum, Lancet, No. 7852, Vol. 1, 1994, pp. 313–314.
[11] M.E. Mackay and N.H. Martin, Biochem. J., 65 (1957) 284–288.
[12] S. Gumpen, P.O. Hegg and H. Martens, Biochim. Biophys. Acta, 574 (1979) 189–196.
[13] Pharmacopee Europenne, Maisonneuve S.A., Sainte Ruffine, France, 1987.
[14] B. Gallez, R. Debuyst, R. Demeure, F. Dejehet, C. Grandin, C., B. Van Beers, H. Taper, J. Pringot and P. Dumont, Magn. Reson. Med., 30 (1993) 592–599.
[15] A.A. Spector, J. Lipid Res., 16 (1975) 165–179.
[16] U. Kragh-Hansen, Pharm. Rev., 33 (1981) 17–53.
[17] K.J. Fehske, W.E. Muller, U. Wollert, Biochem. Pharmacol., 30 (1981) 687–692.
[18] M.A. Rothschild, M. Oratz, S.S. Schreiber, Hepatology, 8 (1988) 385–401.
[19] J.D. Morrissett, H.J. Pownall and A.M. Gotto, J. Biol. Chem., 250 (1975) 2487–2494.
[20] S.J. Rehfeld, D.J. Eathough and W.Z. Plachy, J. Lipid Res., 19 (1978) 841–848.
[21] R.C. Perkins, N. Abumrad, K. Balasubramanian, L.R. Dalton, A.H. Beth, J.H. Perk and C.R. Perk, Biochemistry, 21 (1982) 4059–4064.
[22] M. Ge, S.B. Rananavare and J.H. Freed, Biochim. Biophys. Acta, 1036 (1990) 228–236.
[23] T.G. Gantchev and M.B. Shopova, Biochim. Biophys. Acta, 1037 (1990) 422–434.
[24] B. Gallez, V. Lacour, R. Demeure, R. Debuyst, F. Dejehet, J.-L. De Keyser and P. Dumont, Magn. Reson. Imag., 12 (1994) 62–69.
[25] B. Gallez, R. Debuyst, F. Dejehet, J.-L. De Keyser, V. Lacour, R. Demeure and P. Dumont, MAGMA, 2 (1994) 61–68.
[26] Z. Gelis, L. Ghitescu and M. Simionescu, J. Cell Biol., 47 (1988) 358–365.
[27] D. Piszkiewicz, W. Thomas, M.Y. Lieu, D. Tse and L. Sarno, in J.-J. Morgenthaler (Ed.), Virus inactivation plasma products, Karger, Basel, 1989, pp. 44–54.
[28] L. Winkelman, P.A. Feldman and D.R. Evans, in J.-J. Morgenthaler (Ed.), Virus inactivation in plasma products, Karger, Basel, 1989, pp. 23–33.