Available online at www.sciencedirect.com
ScienceDirect
International Journal of Pharmaceutics 329 (2007) 19–24
INTERNATIONAL JOURNAL OF PHARMACEUTICS
www.elsevier.com/locate/ijpharm

The role of *N*-acetyl-methioninate as a new stabilizer for albumin products

Makoto Anraku [a], Yousuke Kouno [a], Toshiya Kai [a,b], Yasufumi Tsurusaki [a], Keishi Yamasaki [a], Masaki Otagiri [a,*]

[a] *Department of Biopharmaceutics, Graduate School of Pharmaceutical Sciences, Kumamoto University, 5-1 Oe-honmachi, Kumamoto 862-0973, Japan*
[b] *Pharmaceutical Research Center, Nipro Corporation, 3023 Nojicho, Kusatsu, Shiga 525-0055, Japan*

Received 13 April 2006; received in revised form 5 August 2006; accepted 12 August 2006
Available online 17 August 2006

Abstract

Sodium octanoate (Oct) and *N*-acetyl-L-tryptophanate (*N*-AcTrp) are widely used as stabilizers during the pasteurization of albumin products. However, *N*-AcTrp has a possible side effect of intracerebral disease. To provide safe and risk-free albumin products, we validated *N*-acetyl-methioninate (*N*-AcMet) as a new stabilizer for albumin products. The effect of *N*-AcMet on oxidation was examined using 2,2′-azobis(2-amidino-propane) dihydrochloride (AAPH) as an oxidizing agent. Carbonyl content in the presence of *N*-AcMet, as well as that in the presence of *N*-AcTrp after 24 h (Anraku et al., 2004), was significantly decreased. The effect of AAPH on the oxidative status of 34-Cys on human serum albumin was also studied by HPLC. It was found that *N*-AcMet as well as *N*-AcTrp, has a large protective effect on the sulfhydryl group after 1 h. Further, *N*-AcMet was found to be a superior radical scavenger to *N*-AcTrp using 1,1′-diphenyl-2-picrylhydrazyl (DPPH) radicals. The thermal stabilizing role of *N*-AcMet manifested as an increase in denaturation temperature and calorimetric enthalpy, as determined by differential scanning calorimetry (DSC). In the present study, we suggest that use of *N*-AcMet in albumin preparation is safe and free of risk of side effects.

Keywords: Albumin; Sulfhydryl groups; Calorimetry (DSC); Antioxidant activity; *N*-Acetyl-L-methioninate; Oxidation

1. Introduction

Human serum albumin (HSA) is the most abundant protein in plasma, and in addition to being the primary colloid, it serves as an important transport and depot protein (Peters, 1996). Large amounts of albumin are used clinically during surgery and to treat shock trauma. As the only source of HSA for clinical application is donated human blood, the risk of transmitting pathogenic viruses, such as those causing hepatitis, HIV, and as yet unidentified diseases, exists. Pasteurization of HSA is carried out by heating at 60 °C for several hours with sodium octanoate (Oct) and *N*-acetyl-L-tryptophanate (*N*-AcTrp) as commonly used stabilizers (Shrake et al., 1984; Ross et al., 1984), a process that usually destroys the viruses present. These commonly used additives effectively protect HSA by increasing the melting temperature as determined by differential scanning calorimetry (DSC) and decreasing the formation of aggregates after heating (Arakawa and Kita, 2000). We have previously shown that Oct has the greatest stabilizing effect against heat, while *N*-AcTrp diminishes oxidation of HSA (Anraku et al., 2004). However, *N*-AcTrp has a possible side effect of intracerebral disease (Aguilera et al., 2001). In Trp metabolism, 3-hydroxykynurenine is known to have particularly strong neurotoxic properties (Topczewska-Bruns et al., 2003), and the accumulation of Trp metabolites in nervous tissue due to HSA product administration may be involved in pathogenesis of several neurological disorders in uremia. To provide safe and risk-free albumin preparations, it is important to find new stabilizing reagents instead of *N*-AcTrp.

All amino acid residues of proteins are susceptible to oxidative modification by one or more forms of reactive oxygen species (ROS) (Vogt, 1995; Brot and Weissbach, 1983). The oxidative modifications of sulfur-containing amino acids such as cysteine and methionine (Met) could serve as antioxidants via their cyclic oxidation and reduction. In particular, Met residues of proteins are susceptible to oxidation by almost all forms of ROS (Vogt, 1995). On the other hand, no effect on oxidation was found for *N*-acetyl-cysteinate in our previous studies, although cysteine (Cys) residues of proteins are susceptible to oxidation (Anraku et al., 2004). Thus, we focused on a sulfur-containing

* Corresponding author. Tel.: +81 96 370 4150; fax: +81 96 362 7690.
E-mail address: otagirim@gpo.kumamoto-u.ac.jp (M. Otagiri).

0378-5173/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2006.08.014

amino acid having mercapto groups, *N*-acetyl-methioninate (*N*-AcMet).

In the present study, we investigated the protective effect of *N*-AcMet on the oxidation of albumin. In addition, we investigated the stabilizing effect of *N*-AcMet by differential scanning calorimetry (DSC). We suggest that the co-use of *N*-AcMet and Oct produces an excellent stabilizing effect on albumin and depresses agglomeration safely and without any risk of side effects.

2. Materials and methods

2.1. Materials

HSA donated by Chemo-Sera-Therapeutic Research Institute (Kumamoto, Japan) and genetically recombinant human serum albumin (rHSA) donated by Nipro Corporation (Shiga, Japan) were defatted using charcoal treatment as described by Chen (1967). After dialysis against distilled water, the protein fraction was freeze-dried and stored at −20 °C until use. While HSA was used for all the experiments, rHSA was used only for the expected examination for the clinical application. *N*-Acetyl-L-methioninate (*N*-AcMet) and *N*-acetyl-L-tryptophanate (*N*-AcTrp), were purchased from Nacalai Tesque (Kyoto, Japan). Fluoresceinamine (isomer II) and sodium octanoate (Oct) were purchased from Sigma Chemical Co. (St. Louis, MO). 2,2′-Azobis(2-amidino-propane)dihydrochloride (AAPH) and 1,1′-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Nacalai Tesque (Kyoto, Japan).

2.2. Methods

2.2.1. Effect of oxidation on HSA in the presence and absence of ligands

HSA (50 μM), with and without (250 μM) additives, was oxidized by exposure to AAPH (10 mM) in 67 mM sodium phosphate buffer (pH 7.4, 37 °C), as described by Niki (1987). After incubation for 1 or 24 h, oxidation was stopped by the addition of acetone. Protein carbonyl content was determined using the method of Climent et al. (1989). The carbonyl groups were derivatized with fluoresceinamine and their numbers were calculated from the absorbancy of the complexes at 490 nm (Jasco Ubest-35 UV–vis spectrophotometer). Mercaptalbumin (HMA; reduced form) and nonmercaptalbumin (HNA-1 and -2; oxidized forms) were separated by application to an HPLC-column packed with *N*-methylpyridinium polymer cross-linked with ethylene glycol dimethacrylate, prepared as described previously (Sugii et al., 1989; Narazaki et al., 1997). From the HPLC profiles of HSA, the values of each albumin fractions (f(HMA), f(HNA-1), and f(HNA-2)) were estimated by dividing the area of each fraction by the total area corresponding to HSA.

2.2.2. Scavenging of DPPH (1,1′-diphenyl-2-picrylhydrazyl) radicals in solution

Radical scavenging activity of *N*-AcMet and *N*-AcTrp was tested in ethanolic solution (10 ml of ethanol, 10 ml of 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (pH 5.5) and 5 ml of 0.5 mM DPPH in ethanol) with an albumin concentration of 50 μM and a ligand concentration of 250 μM. Radical scavenging was estimated from the decrease in absorbance of DPPH radicals at 517 nm due to scavenging of an unpaired electron from stable DPPH radicals by ligands (Kogure et al., 1999).

2.2.3. Effect of heating on HSA in the presence and absence of ligands

Differential scanning calorimetry (DSC) was carried out on HSA with a protein concentration of 0.1 mM in 67 mM sodium phosphate buffer, pH 7.4 using a MicroCal MC-2 ultrasensitive DSC (MicroCal Inc., Northampton, MA) with a heating rate of 1 K/min. The calorimetric reversibility of the thermally induced transition was checked by reheating the cooled protein solution from the first run in the calorimetric cell, which was flushed with nitrogen. The results showed, as was also observed by Picó (1997), that heating to or above 85 °C causes irreversible denaturation. The data obtained from DSC were applied to non-linear fitting algorithms to calculate thermodynamic parameters of thermal denaturation temperature (T_m), calorimetric enthalpy (ΔH_{cal}) and van't Hoff enthalpy (ΔH_v), and analyzed by Using Origin™ scientific plotting software to determine C_p from the temperature dependence of excess molar heat capacity. Each sample was recorded before heating and after heating to 60 °C for 30 min.

2.2.4. Thermal stabilities on HSAs in the presence and absence of N-AcMet for clinical application

Aqueous solutions (25%, w/v) were prepared by dissolving HSA and rHSA in 500 ml physiological saline. Then, stabilizers Oct (1662 mg) and *N*-AcMet (1912.5 mg) were added and dissolved, and 50-ml aliquots of HSA or rHSA solutions were hermetically sealed in 50-ml vials. The samples were subjected to heat treatment under pasteurization conditions of 60 °C for 30 min, and the generation of contaminants was observed.

2.2.5. Statistics

Statistical significance was evaluated using ANOVA followed by the Newman–Keuls method for comparisons of more than two means. A value of $p < 0.05$ was regarded as statistically significant. Results are reported as mean ± S.D.

3. Results

3.1. Effect of oxidation on HSA in the presence and absence of ligands

HSA exposed to AAPH results in the formation of carbonyl groups. The carbonyl content of HSA, which has not been exposed to AAPH, was 0.037 ± 0.002 mol/mol protein for all the samples ($n = 3$). It is evident from Fig. 1 that the carbonylation increased with incubation time. The presence of Oct has no inhibiting effect on carbonyl formation after 1 hr exposure to AAPH, while *N*-AcMet had a protective effect against prolonged exposure to the oxidant. Similar results were also observed for