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Study of the Heat-Treated Human Albumin Stabilization by Caprylate and Acetyltryptophanate

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ABSTRACT

Sodium salt of N-acetyl-DL-tryptophan (AT) and octanoic acid (caprylic acid CA) individually and in combination were studied as thermal stabilizer with different concentrations in heat treatment of human albumin solution. HPLC protein profiles before pasteurization of albumin showed less than 1% polymer. In free stabilizer albumin solution during pasteurization visible clot was formed. But by using variation of the above mentioned stabilizers, the amount of polymer was increased to about 4% during pasteurization (60îC, 10 h). By increasing the heat to the visible clot formation point, the most effective stabilizer against visible clot formation was found to be CA. However, in drug formulation it is important to choose the variety and concentration of stabilizers in accordance with regulations. *Iran. Biomed. J. 6 (4): 135-140, 2002*

Keywords: Octanoate, Acetyltryptophanate, Albumin stabilizer

INTRODUCTION

In the field of biopharmaceuticals, albumin has a longstanding record of safety and efficacy. There are a number of methods for the purification of human plasma albumin, including cold ethanol fractionation method, in which pasteurization is an essential step. Although high concentration of ethanol may have bactericidal and virucidal effects [1], all therapeutic albumin preparations, independent of source and process, are pasteurized at 60°C for 10 h to inactivate blood born viruses [2] such as hepatitis B, hepatitis C, and HIV. In order to avoid protein denaturation and albumin aggregation during pasteurization, stabilizers should be used.

In this respect, organic salts like NaCl and $CaCl_2$ were effective in inhibiting heat-induced aggregation in human serum albumin [3]. NaCl at low concentration is known to be a stabilizer of the albumin and probably other globular proteins, while $CaCl_2$ is known to be a salting-in agent capable of keeping destabilized molecules in solution [4, 5]. Saso *et al.* [6] have mentioned that non-steroidal anti-inflammatory drugs like diclofenac, ibuprofen and naproxen inhibited the aggregation of albumin at low concentrations.

Sodium salt of octanoic acid, (caprylic acid CA) and N-acetyl-DL-tryptophan (AT) are stabilizers that minimize changes in the protein during the pasteurization of human albumin solution [7]. AT and CA can be determined by UV spectrophotometry method [8] and gas chromatography [9], respectively. The mechanism of their stabilization action on albumin has not been fully elucidated, however it has been shown that the stability of proteins against heat stress can be increased by stabilizers that increase melting temperature of the proteins [10-12].

Based on heat treatment at constant temperature, such stabilizers decrease the equilibrium concentration of the unfolded state, thereby decreasing the rate of degradation from the unfolded state of the proteins. Alternatively stabilizers can increase the stability against heat stress of the proteins by increasing reversibility of minimizing the irreversible reaction following thermal unfolding [13-15]. Both octanoic acid and N-acetyl-DL-tryptophan bind to the human albumin. The stress of binding varies with the length of the carbon chain of the fatty acid and the steric configuration of long-chain fatty acids that bound to the region with a high affinity [16].

In this study, we added stabilizers, octanoate and tryptophanate separately and in combination to the human serum albumin with different proportion of

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stabilizers concentration. Then, after pasteurization at 60°C for 10 h, the percentage of albumin monomer, dimer and polymer was measured by HPLC.

MATERIALS AND METHODS

Reagents. All reagents were extra pure and obtained from Merck, Darmstadt, Germany. Ethanol (96%) was used.

Stabilizer. In order to make the stabilizers, sodium salt of octanoic acid and N-acetyl-DL tryptophan were prepared by the reaction of sodium hydroxide with octanoic acid and N-acetyl-DL-tryptophan and 1M stock solution of both stabilizers. Albumin was prepared by cold ethanol fractionation method. Two concentrations of 5% and 20% of albumin were prepared (pH 6.9) as stock solution of albumin without stabilizer.

The pH measurements of all protein solutions were taken after the dilution of protein solution to one percent W/V in order to avoid changes in the measurements. The pH was measured by Radiometer (PHM 82 instrument, Copenhagen, Denmark) at room temperature. Electrolyte measurements for Na^+ and K^+ were determined by Corning 480 flame photometer.

Determination of protein concentration. Scanning photometer (Philips PU 8750 UV/VIS, England) was used for the determination of protein concentration. Albumin concentration and total protein concentration were measured using bromcresol green reagent at 630 nm, and Biuret method at 540 nm, respectively [17]. Before heating experiments, three types of stabilizers were added to the formulated (total protein, albumin and Na^+ concentration were adjusted) 5% and 20% albumin solutions. These three types stabilizers were (a) variable concentration of sodium salt of octanoic acid, (b) variable concentration of sodium acetyl tryptophan and (c) variable concentration of sodium salt of octanoic acid plus sodium acetyl tryptophan. All solutions were filter sterilized. Heating experiment was carried out by a thermostated water bath. First, 5% and 20% albumin solution with different type and concentration of stabilizers were pasteurized at 60°C for 10 h and then the temperature was increased up to the point of visible clot formation.

HPLC. HPLC was carried out to determine the percentage of monomer, dimer and polymer of albumin after pasteurization. The Knauer HPLC system was equipped with a variable wavelength spectrophotometer. A TSK gel, SWXL guard (0.60 × 4 cm) and a TSK gel G 3000 WXL (0.78 × 30 cm) were connected in series. The column buffer (0.1 M Na_2 SO_4, 0.05% NaN_3 in 0.1M phosphate buffer, pH 6.7) was pumped at a flow rate of 0.5 ml/min, and the wavelength was set at 280 mm. For chromatography, 5% protein solutions (undiluted) and 20% protein solution (diluted 4-fold with the column buffer) injected into the column (5 µl via a 10 µl loop). All experiments were performed at room temperature.

RESULTS

Parameters such as concentration of total protein, albumin, electrolytes (Na^+, K^+) and pH in the presence of varying concentration of sodium salt of octanoic acid (CA) and sodium acetyl tryptophan (AT) were determined (Table 1). The stabilizer-free 5% albumin solution, 2 hours after heating at 60°C became opalescent and after about 5 hours, visible clot was observed. In stabilizer free albumin solution 20%, only after 10 minutes heating at 60°C, it became opalescent and after 20 minutes it started to coagulate.

HPLC protein profile before heating the 5% protein solution shows 99.33% monomer, 0.37% dimer and 0.30% polymer and for 20% protein solution shows 98.74% monomer, 0.58% dimer and 0.68% polymer (Fig. 1). HPLC protein profiles after heating 5% and 20% protein solutions with various levels of stabilizers, such as sodium salt of octanoic acid and sodium acetyltryptophan have been shown in Figure 2. The polymer formation of albumin after pasteurization at 60°C for 10 h in both 5% and 20%. solutions were determined by HPLC. The HPLC peaks at 11, 15, 17 minutes relate to polymer, dimer and monomer, respectively (Table 2).

In order to find out the most effective type and concentration of stabilizer against visible clot formation of albumin, after pasteurization the temperature was increased. After pasteurization, different albumin solutions of 5% were heated for another 240 minutes at 65°C, 120 minutes at 70°C and 150 minutes at 75°C. Then, the temperature continuously increased until the albumin visible clot of the most heat resistant solution was formed.

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Table 1. Determined parameters of albumin about 5% and 20% with different Stabilizers.

Total Protein (g%)	Albumin (g%)	Stabilizer		Na^+ (mmol/l)	K^+ (mmo/l)	pH
		Type	Concentration (mol/l)			
5.1	4.9	CA	0.010	104	0.04	7.01
5.1	4.9	CA	0.015	107	0.05	6.91
5.0	4.8	CA	0.020	110	0.04	6.82
5.0	4.8	AT	0.010	105	0.04	7.11
4.9	4.8	AT	0.015	109	0.04	7.13
5.0	4.8	AT	0.020	112	0.05	7.11
5.0	4.8	CA+AT	0.008+0.008	108	0.04	7.02
5.0	4.8	CA+AT	0.010+0.006	108	0.04	7.00
5.1	4.9	CA+AT	0.006+0.010	109	0.05	7.07
20.4	20.1	CA	0.020	82	0.05	7.12
20.4	20.1	CA	0.030	89	0.05	7.12
20.0	19.8	CA	0.040	98	0.05	7.14
20.1	19.8	AT	0.020	81	0.05	7.08
20.8	20.5	AT	0.030	88	0.04	7.08
20.1	19.8	AT	0.040	95	0.05	7.07
20.1	19.8	CA+AT	0.015+0.015	89	0.05	7.12
20.7	20.4	CA+AT	0.020+0.010	90	0.05	7.14
20.4	20.1	CA+AT	0.010+0.020	89	0.05	7.12

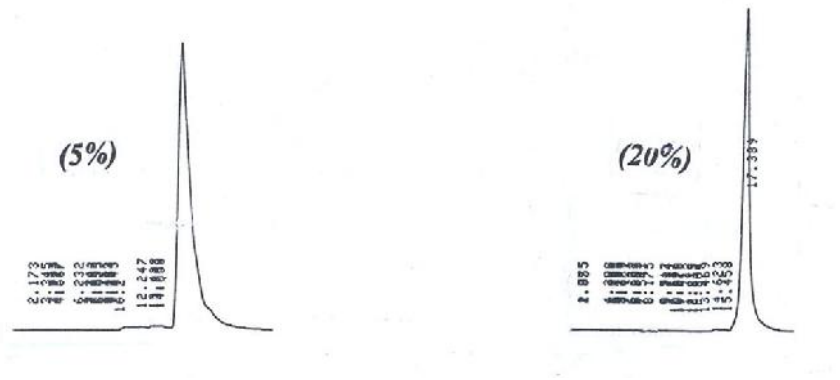

Fig. 1. HPLC protein profiles before heating the 5% and 20% protein solutions.

Table 2. Effects of CA and AT in terms of polymer formation resistant of albumin solution pasteurized at 60°C for 10 h.

Total Protein (g %)	Stabilizer		HPLC		
	Type	Concentration (mol/l)	Polymer (%)	Dimer (%)	Monomer (%)
5	CA	0.010	2.82	0.41	96.77
		0.015	3.73	0.42	95.85
		0.020	3.67	0.48	95.84
5	AT	0.010	3.90	0.40	95.69
		0.015	3.88	0.32	95.79
		0.020	3.78	0.30	95.92
5	CA+AT	0.008 + 0.008	3.68	0.45	95.87
		0.010 + 0.006	3.69	0.49	95.82
		0.006 + 0.010	3.33	0.44	96.24
20	CA	0.020	3.03	1.03	95.93
		0.030	2.95	0.98	96.07
		0.040	2.69	0.88	96.42
20	AT	0.020	1.65	0.87	97.49
		0.030	2.49	0.63	96.88
		0.040	2.78	0.55	96.68
20	CA+AT	0.015+0.015	2.58	0.65	96.77
		0.020+0.010	2.97	0.78	96.24
		0.010+0.020	2.63	0.65	96.72

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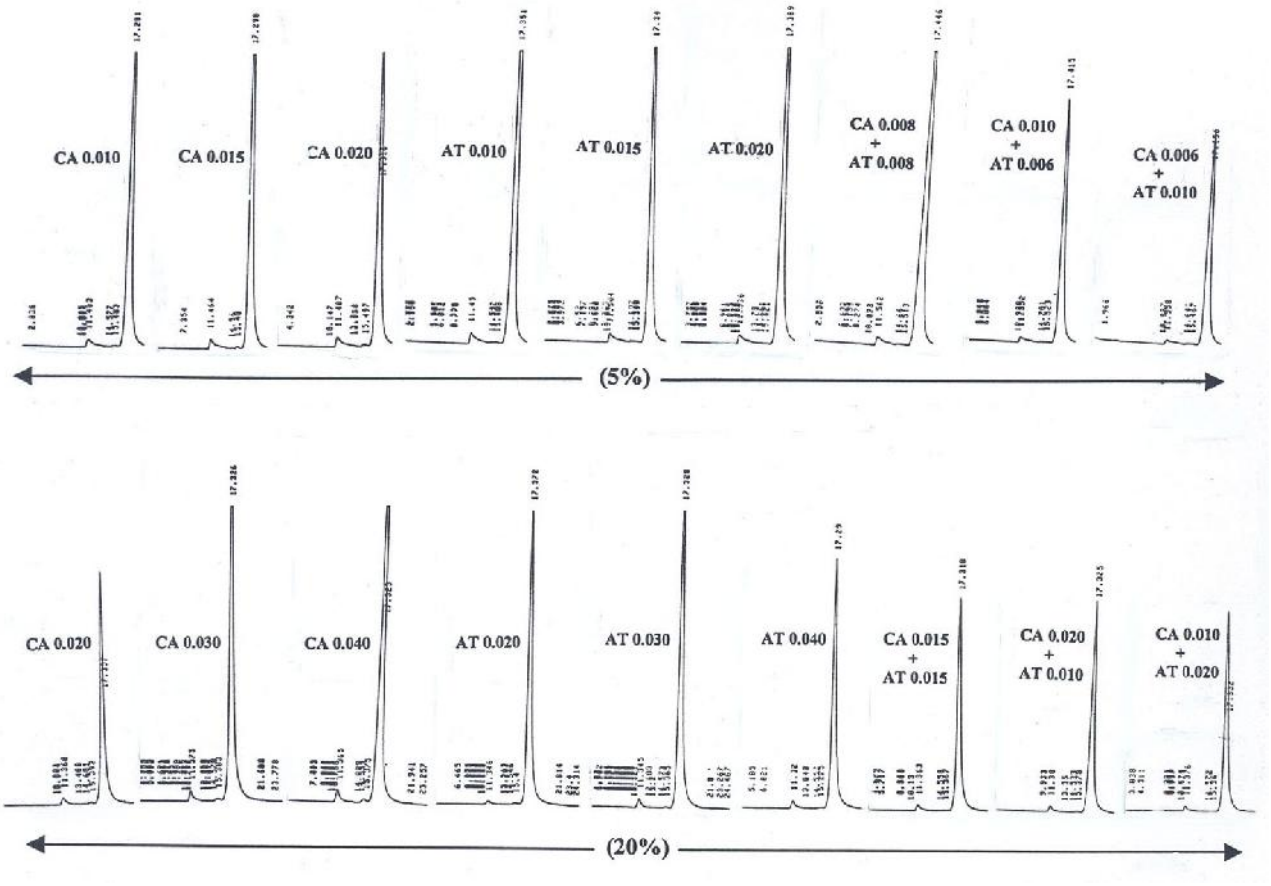

Fig. 2. HPLC protein profiles after pasteurization for the 5% and 20% protein solutions with various stabilizers (AT and CA concentrations are shown in the Figure).

For 20% albumin solution, after pasteurization, the temperature was increased and maintained at 65°C for 240 min, at 70°C for 160 min, and at 75°C for 60 min. Then, temperature was increased continuously until visible clot of the most heat resistant was formed. The results are presented in Table 3.

DISCUSSION

The present work studies the effect of sodium salt of octanoic acid, and N-acetyl-DL – tryptophan and combination of these two compounds as stabilizers against heat treatment in human albumin (5% and 20%). Before heating, HPLC protein profiles showed 0.30% polymer for albumin 5% and 0.68% for albumin 20%. Without using stabilizer during pasteurization, the amount of polymer will increase dramatically. In case of albumin solution 5%, two hours after pasteurization it became opalescent and after about 5 hours visible clot was formed, and in case of albumin 20% only about 10 minutes after pasteurization it became opalescent and after 20 minutes it started to clot. By using different stabilizers all albumin solutions could resist the temperature of 60°C for 10 h and the amount of polymers from less than 1% were increased to less than 4%. The effect of stabilizer concentration, and the order of stabilizing effectiveness were almost the same during the pasteurization. But, by increasing the temperature from 60°C up to the temperature of visible clot formation in different albumin solutions, some differences between the type and concentration of various stabilizers became apparent.

In this study, when 0.02 mol/l CA was used, the most heat resistant 5% albumin solution above pasteurization temperature was observed. While the visible clot was formed at 84°C. This result was almost similar when using combination of CA (0.010 mol/l) and AT (0.006 mol/l) as stabilizer where the 5% albumin solution resisted visible clot formation up to 80°C. In 20% albumin solution, the

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Table 3. Consideration of turbidity and coagulation in albumin solution 5% and 20% after pasteurization by further increase of temperature.

		Stabilizer Concentration (mol/l)								
		CA 0.010	CA 0.015	CA 0.020	AT 0.010	AT 0.015	AT 0.020	CA 0.008 AT 0.008	CA 0.010 AT 0.006	CA 0.006 AT 0.010
Albumin Solution 5%	Opalescent	65°C, 240 min 70°C, 120 min 75°C, 65 min	65°C, 240 min 70°C, 120 min 75°C, 150 min at 81°C	65°C, 240 min 70°C, 120 min 75°C, 150 min at 82.5°C	65°C, 240 min	65°C, 240 min 70°C, 5 min	65°C, 240 min 70°C, 10 min	65°C, 240 min 70°C, 120 min 75°C, 45 min	65°C, 240 min 70°C, 120 min 75°C, 80 min	65°C, 240 min 70°C, 120 min 75°C, 5 min
	visible clot formation	65°C, 240 min 70°C, 120 min 75°C, 150 min at 79°C	65°C, 240 min 70°C 120 min 75°C 150 min at 82°C	65°C, 240 min 70°C, 120 min 75°C, 150 min at 84°C	65°C, 240 min 70°C, 10 min	65°C, 240 min 70°C, 25 min	65°C, 240 min 70°C, 55 min	65i̇C, 240 min 70°C, 120 min 75°C, 150 min at 76°C	65°C, 240 min 70°C, 120 min 75°C, 150 min at 80°C	65i̇C, 240 min 70°C, 120 min 75°C, 25 min
		Stabilizer Concentration (mol/l)								
		CA 0.020	CA 0.030	CA 0.040	AT 0.020	AT 0.030	AT 0.040	CA 0.015 AT 0.015	CA 0.020 AT 0.010	CA 0.010 AT 0.020
Albumin Solution 20%	Opalescent	65°C, 240 min 70°C, 5 min	65°C, 240 min 70°C, 160 min at 75°C	65°C, 240 min 70°C, 160 min 75°C, 60 min at 80°C	65°C, 10 min	65°C, 60 min	65°C, 240 min at 69°C	65°C, 240 min 70°C, 10 min	65°C, 240 min 70°C, 60 min	65°C, 240 min 70°C, 5 min
	visible clot formation	65°C, 240 min 70°C, 15 min	65°C, 240 min 70°C, 160 min 75°C, 10 min	65°C, 240 min 70°C, 160 min 75°C, 60 min at 80.5°C	65°C, 20 min	65°C, 170 min	65°C, 240 min 70°C, 5 min	65°C, 240 min 70°C, 100 min	65°C, 240 min 70°C, 165 min	65°C, 240 min 70°C, 10 min

most heat resistant above pasteurization temperature was the one with 0.040 mol/l CA. In this case, the visible clot was formed at 80.5°C.

Our results correspond to the finding of other research groups such as Arakawa and Kita [7]. They used AT and CA to stabilize bovine serum albumin against heat treatment. They found that CA, as stabilizer has greater protection than AT. Also, CA is probably 5-10 fold more effective than AT in reducing aggregation during thermal unfolding of BSA. Yu and Finalyson [18] have studied the thermal stabilization of human serum albumin using CA and AT by monitoring the formation of albumin polymer. They have indicated that 16 mM AT was needed to equal the effect of 4 mM CA.

Finally, our study demonstrates that CA is an effective stabilizer against heat and its effect could be compared with the use of combination of CA and AT. However, in drug formulation it is important to choose different stabilizer in accordance with regulations. In this respect, combination of sodium salt of octanoic acid and sodium salt of N-acetyl-DL-tryptophan has been recommended.

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