Journal of Pharmaceutical & Biomedical Analysis
Vol. 9, No. 9, pp. 781–784, 1991
Printed in Great Britain

0731–7085/91 $3.00 + 0.00
Pergamon Press plc

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Observations of conformational changes in human serum albumin following removal of fatty acid by charcoal

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Sir:

It has already been reported that serum albumin preparations contain variable amounts of impurities [1, 2]. These impurities appear to consist of fatty acids, globulin, bilirubin and other lipid impurities. The exact composition of impurities varies from sample to sample. According to Foster [3], it is not clear whether the fatty acids have any influence on the physical properties of the proteins. Treatment of these samples with charcoal at low pH results in the virtually complete removal of fatty acids [4].

In the present communication, it is suggested that there is a conformational change in human serum albumin during removal of the fatty acid by charcoal. Four different types of human serum albumin (HSA) were investigated by circular dichroism, fluorescence and equilibrium dialysis techniques. These commercial albumin samples were: (1) HSA, crystallized, fatty acid free (A3782, Lot No. 114F-9310); (2) HSA, crystallized containing fatty acid (A8763, Lot No. 55F-9326); (3) HSA, Fraction V containing fatty acid (A1653, Lot No. 35F-9442) and (4) HSA, Fraction V, fatty acid free (A1887, Lot No. 124F-9440) (all lots from Sigma Chemical Co., St Louis, MO, USA).

Samples Nos 1 and 4, containing charcoal-treated defatted HSA (both Fraction V and crystallized) were used as such, but for HSA containing fatty acid (both Fraction V and crystallized), the molar ratio of fatty acid to albumin was first reduced from 1.9 to 0.8 by passing the albumin solutions through mixed-bed ion-exchange resins, several times [5].

The ion-exchange resins, Dowex 50W-X8 and Dowex 1-X8 were obtained from the Baker Chemical Company (Philadelphia, PA, USA). The resins were converted to the hydroxide and acidic forms and washed with deionized water until a constant pH was obtained. This water also shows no UV spectrum after passing over the resins. The characteristics of these resin-treated deionized samples of HSA were then compared with those of the charcoal-treated defatted HSAs, both having similar fatty acids contents. According to the supplier (Sigma) caprylic acid was added to the crystallized albumins as a stabilizer during the purification process. Some of this added material should be removed during the crystallization process and brought to a minimum by the charcoal treatment or the deionization process. The characteristics of the deionized samples were then compared with the charcoal-treated albumins of similar fatty acid content. The molar ratio of fatty acid to albumin in different samples was measured by the method described by Chen [4], however a Thymol Blue solution was used instead of the Nile Blue A reagent. The indicator solution

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was made by diluting a 0.1% (v/v) aqueous solution of the dye, 10-fold with absolute ethanol. The acidity of this solution required approximately 10 μl of titration alkali (0.02 N) for neutralization [6]. In the case of defatted albumin, the calculated amount of sodium oleate was added to keep the fatty acid concentration constant at a ratio of 0.8 in all four samples of albumin.

The fluorescence of warfarin has been shown to be greatly enhanced in the presence of HSA, the results being interpreted in terms of a binding constant rather than a conformational change. A number of potential antagonists were also investigated [7]. In this report tolmetin was used as a potential antagonist for warfarin binding to the four HSA samples. Fluorescent titrations were carried out with a Perkin–Elmer LS5 Fluorescence Spectrophotometer (Perkin–Elmer, Norwalk, CT, USA) with excitation at 335 nm and emission at 378 nm. Constant HSA concentrations of 2.8×10^{-6} M in 0.1 M and pH 7.4 phosphate buffers at 25°C were used. Constant fatty acid to HSA ratio of 0.8 and tolmetin to HSA ratio of 1.7 were used for the warfarin titrations. It was observed (Fig. 1) that for deionized, crystalline and Fraction V HSAs, tolmetin decreased the fluorescent emission due to the warfarin–HSA interaction whereas tolmetin increased the signal following interaction of warfarin with the charcoal treated HSAs. Addition of bilirubin to these bilirubin free HSA samples caused no changes in the above observations. The decreased signal with the deionized albumin has been related to decreased warfarin binding in a previous study [8], the increased signal with the charcoal treated may be due to increased binding and an increase in the B form of albumin [9].

The circular dichroic (CD) spectra of solutions of the albumins should give insight into the secondary structure of the albumins. The CD spectra were measured using a JASCO J-500 spectropolarimeter (Japan Spectroscopic Co. Ltd, Tokyo, Japan). All measurements were made using a 0.1 M sodium phosphate buffer of pH 7.4 with a path length of 0.5 cm

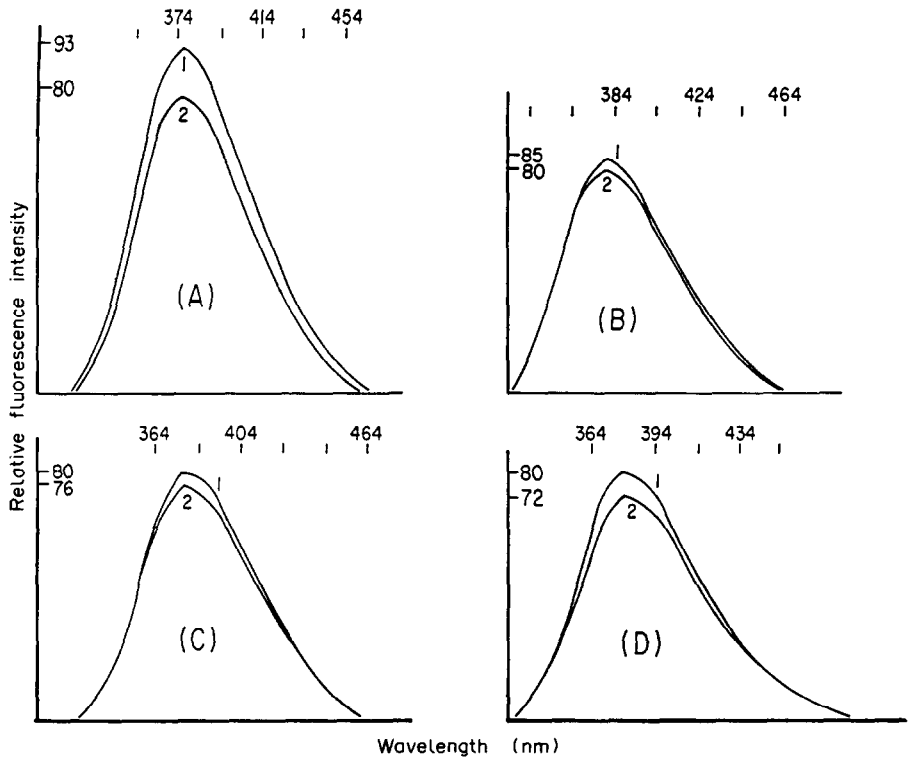

Figure 1
Effect of tolmetin on the binding of warfarin to HSA as observed by fluorescence. A pH 7.4, 0.1 M phosphate buffer was used. Excitation was 335 nm and slit width 3.5 nm, emission 378 nm and slit width 4 nm. (HSA: 2.8×10^{-6} M, warfarin: 9.6×10^{-6} M and tolmetin: 5×10^{-6} M.) (A) Fraction V, (A1653); 1, without tolmetin; 2, with tolmetin. (B) Crystalline (A8763); 1, without tolmetin; 2, with tolmetin. (C) Fraction V, fatty acid free, (A1887); 1, with tolmetin; 2, without tolmetin. (D) Crystalline, fatty acid free, (A3782); 1, with tolmetin; 2, without tolmetin.

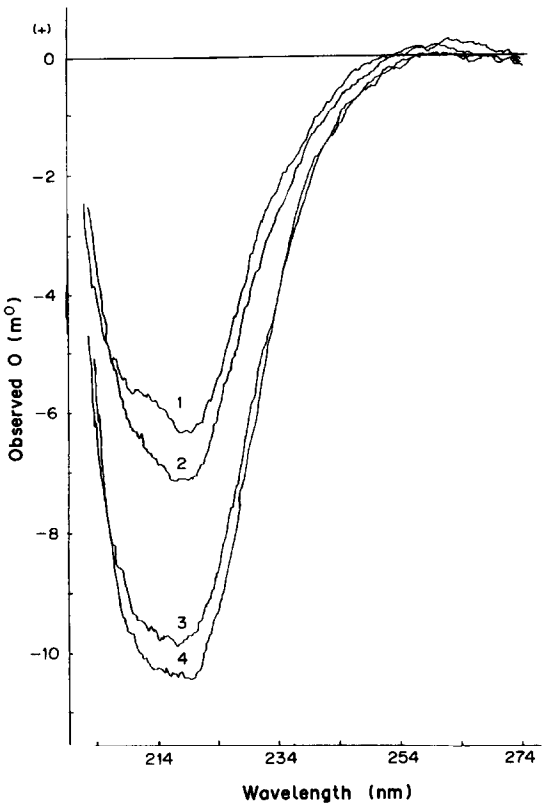

Figure 2
CD spectra of the four albumin samples. (HSA: 1.44×10^{-6} M in pH 7.4; 0.1 M phosphate; path-length = 0.5 cm.) 1, Crystalline, fatty acid free (A3782); 2, Fraction V, fatty acid free (A1887); 3, Fraction V (A1653); 4, Crystalline (A8763).

and an albumin concentration of 1.44×10^{-6} M at 25°C. All four HSA samples have curves of almost identical shape (Fig. 2) having a peak near 220 nm; however, both fatty acid free HSAs showed greatly reduced CD intensity. This finding together with the fluorescence observations strongly suggest that there had been denaturation of the protein during removal of the fatty acid by charcoal. This is in good agreement with previously published observations [10]. At this stage, however, it seems that this difference in the intrinsic optical activity of albumin samples should not only be attributed to the impurities present in the albumins, but more likely that secondary structure of the protein is changed by the charcoal treatment. A computer analysis [11] of the data showed that in the charcoal-treated defatted sample, 50% α-helicity is lost compared with the deionized HSA containing fatty acids and that there is α-helix–β-sheet transformation taking place during charcoal adsorption.

Previous equilibrium dialysis work [8] has shown that addition of tolmetin to HSA ratios of 1.5–2.5 to warfarin deionized fraction V HSA with added fatty acid caused large increases in the free concentrations of warfarin. Additional experiments at tolmetin–HSA ratios of 2.3 and warfarin–HSA ratios of 1.0 confirm these observations but when charcoal-defatted HSA with added fatty acid was substituted little change was found in the free concentrations of warfarin as determined by liquid chromatography (Table 1). These results confirm the observations made by fluorescence, and suggest that the small increase in fluorescence on interaction of the tolmetin with the charcoal-treated albumin, causes a small shift towards the B form in the neutral–base transition [9] of HSA.

The question arises as to what causes these differences in warfarin binding. Three possibilities arise, namely: resin treatment, stabilizers added to the albumins and charcoal treatment. The fluorescent behaviour of the tolmetin–warfarin system was found to be the same for samples 2 and 3, (i.e. containing fatty acid, pre- and post-resin treatment). This suggested that resin treatment did not induce the discrepancies. As for the addition of stabilizers like *N*-acetyl-tryptophan and caprylic acid [12, 13] to the albumin samples the supplier has informed us that caprylic acid was added to the crystallized forms. In other ways all albumins were treated similarly, and as such should contain similar quantities of dimers. The defatting and deionization techniques can be

Table 1
Effect of tolmetin on the binding of warfarin to HSA at 25°C in 0.1 M phosphate buffers. HSA and warfarin concentrations were 2.5×10^{-4} M and the tolmetin concentration was 5.75×10^{-4} M

Drug	HSA variety Fraction V	Free conc. of warfarin (10^{-4} M) $n = 6$	% Change
Warfarin alone	Fraction V with fatty acid	0.627 ± 0.022	—
Warfarin with tolmetin	Fraction V with fatty acid	1.372 ± 0.044	1118.8
Warfarin alone	Fraction V without fatty acid	0.487 ± 0.008	—
Warfarin with tolmetin	Fraction V without fatty acid	0.493 ± 0.018	1.2

expected to bring the caprylic acid concentration to a minimum. Therefore it seems that the charcoal treatment may have been responsible for the changes in the binding of warfarin to the albumin dispite the claims by Chen [4] that the charcoal treatment is a gentle (non-denaturing) procedure. A conformational change in the HSA, induced by the charcoal treatment, which altered the geometry of the warfarin binding site is feasible. This is in agreement with the recent report by Honore and Pedersen [14], who have investigated fatty acid-induced conformational changes in human serum albumin by fluorescence energy transfer.

In general for drug–albumin investigations some careful standardization of the albumin is necessary. The binding of the drug tolmetin to albumin seems to be especially sensitive to pretreatment of the albumin. There are differences in the secondary structures and in the sensitivity of the N → B transition to drugs in the deionized and charcoal-treated albumins. The deionizing technique seems to be less harsh and is recommended.

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[Received for review 15 May 1991; revised manuscript received 2 July 1991]