

SPECTROPHOTOMETRIC INVESTIGATIONS OF THE BINDING OF EBSELEN TO HUMAN SERUM ALBUMIN

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Abstract

The interaction of the novel antioxidant drug ebselen, 2-phenyl-1,2 benzoselenazol-3 (2H)-one, with human serum albumin (HSA) was investigated by spectrophotometry. 6.7 mM, pH 7.4 of phosphate buffer containing 0.4 % (v / v) dimethylformamide (DMF) was used as the solvent. Various amounts of ebselen and HSA mixtures were used. Incubations of the mixtures were performed for 15 minutes at 25°C and 30 minutes at 37°C. An irregular change and the lack of an isobestic point in the final spectra of the mixtures indicated that the formation of mixed albumin selenodisulfides. It was also determined that the structures of the final products formed from the interactions of the drug and HSA, were depended on both drug and HSA concentrations.

Keywords: Ebselen (PZ 51), human serum albumin, spectrophotometry.

Ebselenin İnsan Serum Albüminine Bağlanmasının Spektrofotometrik olarak İncelenmesi

Antioksidan özellikte yeni bir ilaç maddesi olan ebselen'in (2-phenyl-1,2 benzoselenazol-3 (2H)-one) insan serum albümini (HSA) ile etkileşimi spektrofotometrik olarak incelendi. Çözücü olarak 0.4 % (v / v) dimetilformamid (DMF) içeren 6.7 mM, pH 7.4 fosfat tamponu ve farklı miktarlarda ebselen-HSA karışımları kullanıldı. Karışım inkübasyonları 25°C de 15 dakika ve 37°C de 30 dakikada gerçekleştirildi. Sonuç spektrumlarındaki düzensiz bir değişim ve bu spektrumlarda izobestik bir noktanın bulunmaması karışık albümin selenodisülfürlerin oluştuğunu göstermiştir. Ayrıca ebselen ile HSA etkileşimi sonucunda oluşan ürün yapılarının ilaç ve HSA'nın her ikisinin de konsantrasyonlarına bağlı olduğu saptanmıştır.

Anahtar kelimeler: Ebselen (PZ51), insan serum albümini, spektrofotometri.

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INTRODUCTION

Ebselen, (PZ 51), (2-phenyl-1, 2 benzisoselenazol-3 (2H)-one) was described as a novel biologically active selenoorganic compound. The chemical structure of the drug is shown in Figure 1.

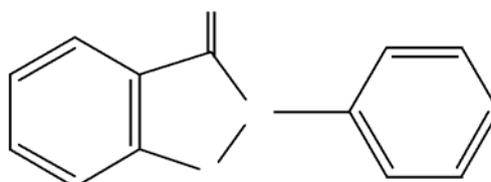


Figure 1. The structure of ebselen

It exhibits glutathione peroxidase like activity *in vitro*, reducing hydroperoxides in the presence of a thiol in a catalytic reaction (1-3). The *in-vivo* anti-inflammatory effect was its first attracted property (4). Recently many pharmacological effects have also been reported (5-7). It has just been introduced to the market in Japan. In contrast to many selenium compounds, ebselen has extremely low toxicity because the metabolism of the compound doesn't liberate the selenium moiety, which remains within the ring structure (8-9). Despite the realization of a wide variety of *in-vitro* and *in-vivo* studies of understanding unusual pharmacokinetic behaviour of ebselen, there are still unclear points of its pharmacology. The selenium atom, as a center of nucleophilic attack in the ebselen molecule, has an unusual redox chemistry. The detected metabolites of ebselen have the same characteristics in opening of the isoselenazole ring by the cleavage of the Se-N bond (10).

There is no reported *in-vitro* study about the binding reaction to ebselen up to date. However, in model studies using bovine serum albumin, rapid binding of ebselen to albumin was observed (11). It is reported that the radioactivity was detected on human plasma from labelled ebselen only bound to albumin and negligible amounts in association with the globulin fractions upon gel electrophoresis (12-13). It is just assumed that ebselen binds to the reactive thiol group at cysteine 34 in albumin, the location identified for albumin-bound cysteine or glutathione (14).

As it is known, in blood, albumin is the general transport protein that contributes significantly to the distribution and metabolism of hydrophobic ligands (14). It is suggested that the transport of ebselen is likely to be via ebselen-bounded proteins using their sulfhydryl groups and there is an interchange with low molecular thiols within cells and tissues (15-16). It is reported that the diselenide and selenol of ebselen, which are formed in excess of thiol groups of glutathione, are responsible for the glutathione (GSH) peroxidase like activity of the drug (17-18). On the other hand, ebselen is able to interact with sulfhydryl groups of membrane associated proteins *in-vitro* (19).

The effect of interference of ebselen with sulfhydryl groups in proteins is only partly understood, therefore the investigation of the interaction of ebselen with HSA has a crucial importance on understanding of its metabolism.

In the present study, the *in vitro* binding reaction of HSA to the drug was followed by spectrophotometry, since the absorption spectra of ebselen is very sensitive to chemical changes at the selenium atom (20).

EXPERIMENTAL

Apparatus and chemicals

Ebselen was kindly provided by Rhone-Poulenc Pharma, as a gift. Human serum albumin (A – 1187 % 96), essentially fatty acid free, prepared from fraction V, was obtained from Sigma. $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, K_2HPO_4 and HCl (37 %) were of all analytical reagent grades and all were provided from Merck. Dimethylformamide (DMF) was provided from Lab-Scan. All glassware used are brown coloured. Shimadzu 2100 spectrophotometer assembled a kinetic kid and Gilson automatic pipets were used. All the glassware and pipets were calibrated.

Experimental design

0.4 % DMF containing 6.7 mM phosphate buffer of pH 7.4 was used as the solvent. All the solutions were kept one week at 4 °C and away from daylight. All the experiments were done in triplicate.

The interaction of ebselen and HSA was investigated in seven different concentrations of HSA (113, 400, 480, 530, 650, 780, 1200 μM). Appropriate amounts of HSA were added to 54.70 μM ebselen solution. The mol ratios of the HSA / drug The mol ratios of the HSA / drug were 2-22 in the final mixtures. Appropriate blanks containing the same amounts of HSA as in the samples were prepared in all cases. The flasks containing ebselen and HSA were incubated for 15 minutes at 25°C. The resulting absorption spectra were recorded versus the appropriate blanks at 25°C (Figure 2 and Figure 3). The other different concentrations of HSA (8.10 - 70.00 μM) were added to each 27.36, 54.70, and 81.90 μM ebselen solution and incubated for 30 minutes at 37°C. The resulting absorption spectra were recorded versus the appropriate blanks at 37°C (Fig. 4, Fig. 5 and Figure 6)

The saturation of HSA with ebselen versus time was investigated in two set of mixtures. The mixtures were 1200 μM HSA and 54.70 μM ebselen; 600 μM HSA and 27.36 μM ebselen. Incubation time was 15 minutes. The absorption spectra were recorded versus the appropriate blanks at 25°C. The absorbance values versus time at 302 nm are given in Graph 1.

1.0, 0.8 and 0.3 ml were taken from the mixture of 54.70 μM ebselen and 1200 μM HSA and diluted to 1.6 ml with the phosphate buffer. HSA and ebselen concentrations were 750, 600, 225 μM and 34.20, 27.36, 10.26 μM respectively, after each dilution (Figure 7 b, c and d)

RESULTS AND DISCUSSION

The spectrum of ebselen shows three maxima at 237, 256 and 323 nm. (Figure 2e) The absorption at 256 and 323 nm can be attributed to the aromatic and isoselenazol rings respectively. Many drugs in aqueous solution containing a protein, show bathochromic shifts due to their binding to the protein. In a previous study after incubation of ebselen with glutathione 7-7, an enzyme concentration dependent change in the UV spectrum was observed. The maximum/minimum ratio A_{323}/A_{293} decreased. This finding was attributed to the opening of the selenazol ring (21,17).

The maximum / minimum ratio A_{323}/A_{293} , about 1.89 (without albumin) decreased to 1.13 in the presence of 400 μM HSA in correlation with the glutathione 7-7 (21) and

glutathione (17) (Figure 2 b) and this finding indicates reductively opening of isoselenozol ring by thiols of HSA and thus forming albumin-selenenyl sulfide. (Fig. 2 e, a, b).

The patterns of the absorption spectra of ebselen itself and the mixtures with 113 μM and 400 μM HSA were similar, whereas a drastic change was investigated when the HSA / drug mol ratio was about 9 (HSA concentration 480 μM). There was no specific peak belonging to ebselen itself between 230-290 nm and total amount of the drug was bound to HSA in these conditions. In the presence of excess thiols, above 480 μM HSA (albumin thiols in this case), albumin-selenenyl sulfide was very likely converted into the diselenide of ebselen (a benzanilide derivative of the drug) and very likely bound to HSA via two different regions and formed HSA selenodisulfides. One region is attributed to the maximum at around 302 nm, and the other is attributed to the shoulder at 320 nm (Figure 2 c). This situation is in harmony with the diselenid and selenol forming of ebselen in excess of thiol groups of glutathione (17). It was also verified that the benzanilide derivatives of ebselen gives an absorption between 270 - 300 nm depending on the thiol compound (16).

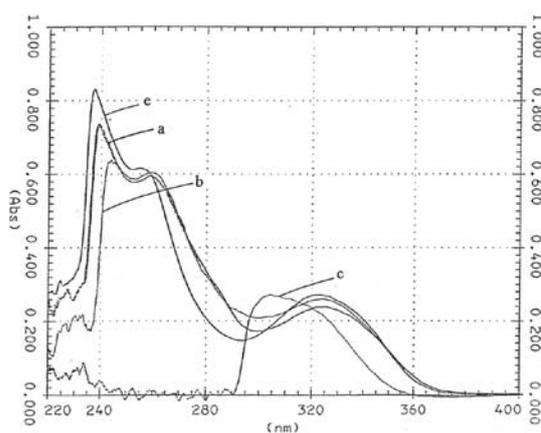


Figure 2. e: The absorption spectrum of 54.70 μM ebselen solution in 6.7 mM phosphate buffer of pH 7.4 containing 0.4 % DMF. a: The absorption spectra of the mixture of (e) and 113 μM HSA, b: 400 μM HSA, c: 480 μM HSA

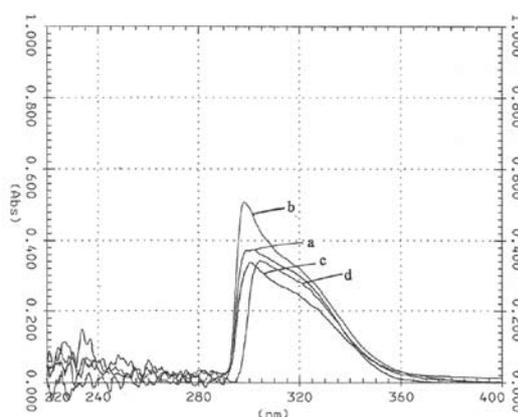


Figure 3. a: The absorption spectrum of the mixture of (e) and 530 μM HSA, b: 650 μM HSA, c: 780 μM HSA, d: 1200 μM HSA

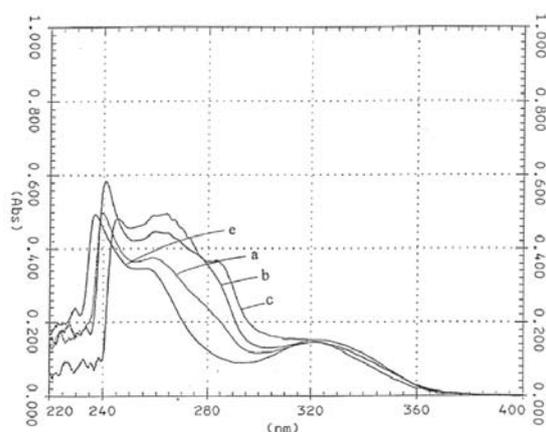


Figure 4. e: The absorption spectrum of 27.36 μM ebselen solution in 6.7 mM phosphate buffer of pH 7.4 containing 0.4 % DMF. a: The absorption spectra of the mixture of (e) and 19.85 μM HSA, b: 24.70 μM HSA, c: 69.12 μM HSA.

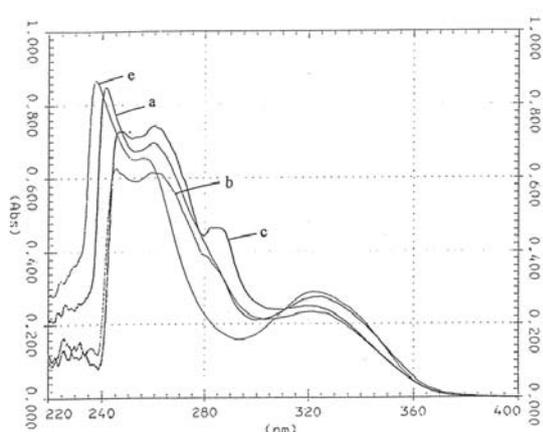


Figure 5. e: The absorption spectrum of 54.70 μM ebselen solution in 6.7 mM phosphate buffer of pH 7.4 containing % 0.4 DMF. a: The spectra of the mixture of (e) and 22.80 μM HSA, b: 56.60 μM HSA, c: 70.60 μM HSA.

The general pattern of the spectrum didn't change above 480 μM HSA, however the absorbance around at 302 nm increased when the HSA concentration was 530 μM (Figure 3 a) and had the maximum value of 0.505 when HSA concentration was 650 μM and the shoulder at around 320 nm was more clear. (Figure 3 b) It decreased to 0.337 when HSA concentration was 780 μM (Figure 3 c) and kept the same value along with the shift to the longer wavelength when the HSA concentration was 1200 μM (Figure 3 d). This irregular change of the spectra could be attributed to the forming of the mixed selenodisulfides when the excess HSA was used. The lack of an isobestic point of the spectra supports this assumption (Figure 3).

To investigate the effect of the drug concentrations and HSA on the binding reaction, the experiments were performed with lower amounts of HSA (8.10 - 70.00 μM) with 27.36 μM , 54.70 μM and 81.90 μM of ebselen. The same shift to the longer wavelengths was also observed with both 27.36 μM and 54.70 μM of ebselen. The opening of isoselenazol ring can be followed from the change of A_{323} / A_{293} in Figure 4 a and Figure 5 a as in Figure 2 a, b. The appearance of the shoulder at around 280 nm was observed when the HSA/drug mol ratio was about 1/1 in both cases. (Figure 4 b: HSA/drug mol ratio was 24.70 μM / 27.36 μM ; Figure 5 b: HSA/drug mol ratio was 56.60 μM / 54.70 μM). This was attributed to the binding of selenenyl sulfide to HSA. When the amount of HSA was increased, the shoulder was more clear at around 285 nm (Figure 4 c and Figure 5 c) The absorption patterns of all intermediate products of drug-albumin compound, appearing from the mixtures of 27.36 μM and 54.70 μM ebselen and 8.10 – 70.00 μM HSA were all similar (Figure 4 and Figure 5). Whereas, when the ebselen concentration was higher (81.90 μM) and the mol ratio was 0.68, the pattern totally changed especially between 270-280 nm, with another shoulder appeared at around 310 nm (Figure 6 c and d). From these

findings it can be concluded that the binding reaction is dependent on not only the HSA but also the drug concentration.

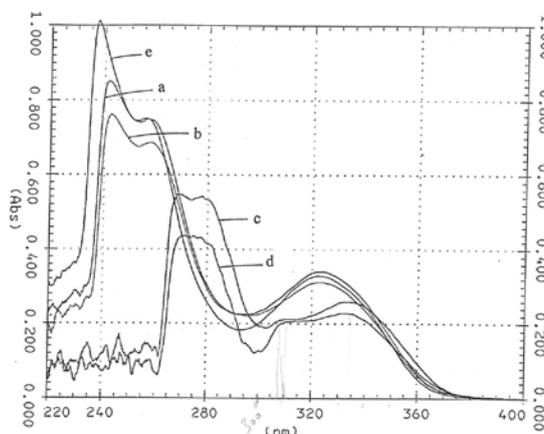
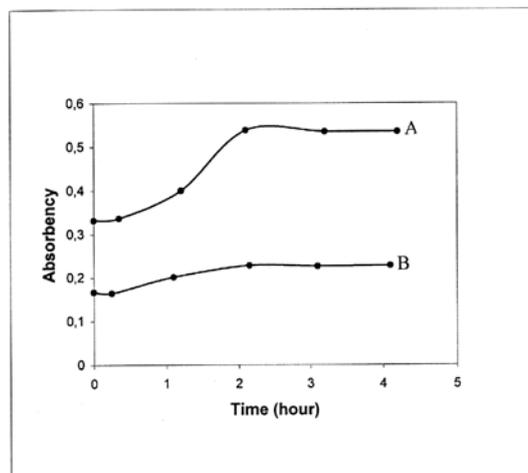


Figure 6. e: The absorption spectrum of 81.90 μM ebselen solution in 6.7 mM phosphate buffer of pH 7.4 containing % 0.4 DMF. a: The spectra of the mixture of (e) and 19.85 μM HSA b: 24.70 μM HSA, c: 55.88 μM HSA, d: 69.85 μM HSA.

As a result, three bonding sites on the albumin molecule depending on the HSA concentration can be seen from the related spectra. One is at around 280 nm, the others are at 302 and 320 nm as a shoulder. Three binding sites of bovine serum albumin has been proposed by Ullrich, et al., one is GSH-sensitive, another exchanges slowly and is independent of added GSH, the third might be tightly bound to the ebselen derivative (16). Our results are in correlation with these findings. However it is difficult to make a decision whether the bound is covalent or noncovalent. The location of the bonding sites is also under question.

The saturation of the HSA with drug completed in 130 minutes (Graph. 1). The initial absorbances doubled at 302 nm with the absorbance values of 0.167 (1200 μM HSA and 54.70 μM ebselen) and 0.332 (600 μM HSA and 27.36 μM ebselen) depending on the initial concentrations.



Graph 1. The changes of the maximum absorbance at 302 nm versus time in HSA / ebselen product of A: 1200 μM HSA and 54.70 μM ebselen; B: 600 μM HSA and 27.36 μM ebselen.

The spectra after dilution: The solution containing 54.70 μM drug and 1200 μM HSA was diluted with phosphate buffer. The HSA concentrations were 750, 600 and 225 μM and the appropriate drug concentrations were 34.2, 27.36 and 10.26 respectively after each dilution. The maximum absorbance value at 302 nm increased along with the shift to the shorter wavelength (Figure 7 b. c.). This increase was in correlation with the decrease at 302 nm along with the shift to the longer wavelength when 750 and 600 μM of HSA was added to 54.70 μM ebselen solution (Figure 7 b: 750 μM HSA and Figure 3 c: 780 μM HSA; the highest values are: Figure 7 c: 600 μM HSA Figure 3 b: 650 μM HSA). If there is just one species of albumin selenodisulfide there wouldn't be any shift at the maxima after dilution when the excess HSA was added. The existence of mixed albumin selenodisulfides was also verified by this way.

The pattern of the spectrum completely changed in the final dilution. It was the same as HSA itself with a shift to the longer wavelength (Figure 7d).

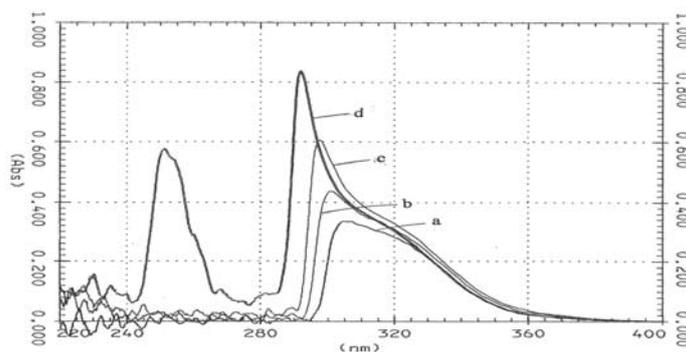


Figure 7. a: The spectrum of 54.70 μM ebselen and 1200 μM HSA. The spectra of this mixture after appropriate dilutions. HSA concentrations after dilutions: b: 750 μM , c: 600 μM , d: 225 μM .

In conclusion, the in-vitro interaction of ebselen with HSA was studied in various aspects in the present study. Our findings show that the interaction between ebselen and HSA depends on both drug and HSA concentrations. The existence of the mixed albumin selenodisulfides was shown by the irregular change and the lack of an isobestic point of the spectra of the final mixtures. However, the exact molecular structure of ebselen moiety transferred between proteins hasn't yet been identified and the reason of surprising low toxicity also hasn't been yet clarified. It is just proved that ebselen is transferred via albumin complex to some low-molecular weight thiols (22) and membrane proteins (12). The formation of the protein selenodisulfides and the transportation role of albumin in relation to this, is an evident hint that HSA is being interfered to the metabolism process of ebselen. This study would be helpful especially for understanding the mechanism of action of ebselen and probably its toxicity.

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