EFFECT OF UREA ON WARFARIN-HUMAN SERUM ALBUMIN COMPLEX: FLUORSCENCE STUDY

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ABSTRACT

In order to evaluate the change in the drug binding ability of human serum albumin (HSA) with the alteration in its native conformation, effect of urea was investigated on the tertiary structure of HSA as well as on its interaction with warfarin, an anticoagulant drug, using fluorescence spectroscopy. Such studies are important from the physiological point of view as alteration in the drug binding capacity of HSA has been noted in several diseases. Both intrinsic fluorescence and tryptophan (Trp) fluorescence were used to monitor these urea-induced changes upon excitation at 280 and 295nm respectively. Fluorescence spectra of native HSA were characterized by the presence of an emission maximum around 339 and 343nm when excited at 280 and 295nm respectively. Incubation of protein with increasing urea concentrations for 12 hours produced a significant decrease in the fluorescence intensity and an initial small blue shift followed by a marked red shift in the emission maximum. In addition to these characteristics, peak splitting was also observed at higher urea concentrations upon excitation at 280nm, which was absent upon excitation at 295nm. Reducing the incubation time with urea from 12 hours to 60 minutes did not significantly affect these characteristics. Urea transition curve showed a two-step, three-state transition with accumulation of an intermediate around 5.2−5.6M urea upon excitation at 280nm against a single-step, two-state transition when monitored by Trp fluorescence, upon excitation at 295nm. Both decrease in the fluorescence intensity and the red shift in the emission maximum were suggestive of unfolding of the protein's tertiary structure at higher urea concentrations.

In warfarin binding experiments, both decrease in the fluorescence intensity and red shift in the emission maximum of HSA fluorescence spectra with increasing warfarin concentrations were suggestive of warfarin binding to HSA. These effects were more pronounced at lower drug concentrations (0−5μM) and sloped off at higher drug concentrations (10−40μM). Presence of urea in the incubation mixture affected both these signals in a proportionate manner, by showing lesser changes at higher urea concentrations. In other words, loss in drug binding to HSA was observed with increasing urea concentrations, being maximum loss at the highest urea concentration. These results were analyzed with the help of Stern-Volmer equation (Eftink and Ghiron, 1982) and Scatchard equation (Min *et al.,* 2004) to determine the quenching constant (K_{sv}) and apparent binding constant (K_b), respectively. Whereas decrease in the value of quenching constant with increasing urea concentrations suggested the increase in the distance between excited fluorophore (Trp-214) of HSA and the ligand (warfarin), loss in drug binding ability was reflected by the decrease in the binding constant in the presence of urea. Taken together, all these results suggested significant alteration in the three-dimensional structure of the protein at higher urea concentrations which affected its warfarin binding ability to a significant extent.

ABSTRAK

Dalam usaha untuk menilai perubahan dalam keupayaan pengikatan ubat pada albumin manusia serum (HSA) dengan perubahan dalam konformasi asli, kesan urea telah diselidiki pada struktur tertier HSA serta tindak balas dengan warfarin, ubat antikoagulan,menggunakan spektroskopi pendarfluor. Kajian sedemikian adalah penting dari sudut pandangan fisiologi sebagai perubahan dalam kapasiti pengikatan ubat pada HSA telah dicatatkan dalam beberapa penyakit. Kedua-dua intrinsik pendarfluor dan tryptophan (TRP) pendarfluor digunakan untuk memonitor perubahan-perubahan ini yang diaruhkan oleh urea ke atas pengujaan masing-masing pada 280 dan 295nm. Spektrum pendarfluor HSA asli yang dicirikan oleh kehadiran emisi maksimum sekitar 339 dan 343nm apabila diujakan masing-masing pada 280 dan 295nm. Pengeraman protein dengan peningkatkan kepekatan urea selama 12 jam telah menghasilkan penurunan yang ketara dalam keamatan pendarfluor dan permulaan kecil anjakan biru (blue shift) diikuti dengan anjakan merah (red shift) yang jelas dalam emisi maksimum. Tambahan pada ciri-ciri ini, belahan puncak juga dilihat pada kepekatan urea yang lebih tinggi apabila pengujaan pada 280nm, yang tidak hadir apabila pengujaan pada 295nm. Pengurangkan masa pengeraman dengan urea dari 12 jam hingga 60 minit tidak memberi kesan yang ketara kepada ciri-ciri ini. Lengkung transisi urea menunjukkan satu dua -langkah, transisi tiga-keadaan dengan pengumpulan satu keadaan antara pada kepekatan urea 5.2-5.6M apabila diujakan pada 280nm berlawanan terhadap tranisi satu langkah, dua transisi dua-keadaan apabila dimonitor oleh TRP pendarfluor, apabila penggujaan pada 295nm. Kedua-

dua pengurangan dalam keamatan pendarfluor dan anjakan merah (red shift) dalam emisi maksimum mensarankan berlakunya pembukaan (unfolding) struktur tertier protein pada kepekatan urea yang lebih tinggi.

Dalam uji kaji pengikatan warfarin, kedua-dua pengurangan dalam keamatan pendarfluor dan anjakan merah (red shift) dalam emisi maksimum spektrum pendarfluor HSA dengan peningkatan kepekatan warfarin mensarankan warfarin mengikat kepada HSA. Kesan ini adalah lebih ketara pada kepekatan ubat yang lebih rendah (0-5μM) dan menyelinap pergi pada kepekatan ubat yang lebih tinggi (10-40μM). Kehadiran urea di dalam campuran pengeraman memberi kesan kepada kedua-dua isyarat ini dengan cara yang seimbang, dengan menunjukkan perubahan yang kurang pada kepekatan urea yang lebih tinggi. Dalam erti kata lain, kehilangan dalam pengikatan ubat pada HSA telah diperhatikan dengan kepekatan urea yang semakin meningkat, dengan kerugian yang maksimum pada kepekatan urea tertinggi. Keputusan ini telah dianalisis dengan bantuan persamaan Stern-Volmer (Eftink dan Ghiron, 1982) dan persamaan Scatchard (Min et al, 2004) untuk menentukan 'quenching constant' (Ksv) dan apparent binding constant (Kb), masing-masing. Manakala pengurangan dalam 'quenching constant' dengan peningkatkan kepekatan urea mencadangkan peningkatan dalam jarak antara keterujaan fluorophore (TRP-214) HSA dan ligan (warfarin), kehilangan keupayaan pengikatan ubat dicerminkan oleh penurunan 'binding constant' dengan kehadiran urea. Diambil bersama-sama, semua keputusan ini mencadangkan perubahan ketara dalam struktur tiga dimensi protein pada

kepekatan urea yang lebih tinggi yang memberi kesan kepada keupayaan mengikat warfarin yang agak signifikan.

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TABLE OF CONTENTS

		Page
ABSTRACT		i
ABSTRAK		iii
ACKNOWLEDGEMENTS		vi
LIST OF FIGURES		ix
LIST OF TABLES		xv
LIST OF ABBREVIATIONS		xvi
CHAPTER 1.	**INTRODUCTION**	1
CHAPTER 2.	**LITERATURE REVIEW**	5
2.1.	GENERAL	5
2.2.	PHYSICO-CHEMICAL PROPERTIES OF HSA	6
2.3.	STRUCTURE OF HSA	7
2.3.1.	Amino acid composition	7
2.3.2.	Primary structure	7
2.3.3.	Three-dimensional structure	13
2.4.	DENATURATION	15
2.5.	LIGAND BINDING	16
2.5.1.	Drug binding	19
2.5.2.	Warfarin binding to HSA	20
CHAPTER 3.	**MATERIALS AND METHODS**	24
3.1.	MATERIALS	24
3.2.	METHODS	24
3.2.1.	pH measurements	24
3.2.2.	Analytical methods	25
3.2.3.	Absorption measurements	25

3.2.4. Fluorescence measurements 25
3.2.5. Denaturation experiments 25
3.2.6. Drug binding studies 26
3.2.7. Data analysis 27

CHAPTER 4. RESULTS AND DISCUSSION 28
4.1. EFFECT OF UREA ON THE FLUORESCENCE SPECTRUM OF HSA 28
4.1.1 Using excitation wavelength of 280nm 28
4.1.2 Using excitation wavelength of 295nm 32
4.2. WARFARIN-HSA INTERACTION 36
4.2.1 Warfarin binding to native HSA 36
4.2.2 Warfarin binding to HSA in the presence of urea 45

REFERENCES 83

LIST OF FIGURES

		Page
Figure 2.1	Illustration of the loop-link-loop pattern in the primary structure of serum albumin showing different domains and loops (L1-L9).	11
Figure 2.2	Amino acid sequence of human serum albumin.	12
Figure 2.3	Schematic drawing of the HSA molecule.	14
Figure 2.4	Summary of the ligand binding capacity of HSA as defined by crystallographic studies.	18
Figure 2.5	Chemical structure of warfarin	21
Figure 4.1	Fluorescence spectra of HSA (1.8μM) in the absence and presence of increasing urea concentrations in 0.06M sodium phosphate buffer, pH 7.0 at 25℃ incubated for 12 hours, upon excitation at 280nm.	29
Figure 4.2	Urea denaturation curves of HSA as studied by plotting (A) fluorescence intensity at 339nm upon excitation at 280nm and (B) emission maximum against urea concentration.	30
Figure 4.3	Urea denaturation curves of HSA (1.0μM) as studied by plotting (A) fluorescence intensity at 338nm upon excitation at 280nm and (B) emission maximum against urea concentration. Incubation time with urea was kept as 60 minutes.	33
Figure 4.4	Fluorescence spectra of HSA (1.8μM) in the absence and presence of increasing urea concentrations in 0.06M sodium phosphate buffer, pH 7.0 at 25℃ incubated for 12 hours, upon excitation at 295nm.	34

Figure 4.5 Urea denaturation curves of HSA as studied by plotting (A) fluorescence intensity at 343nm upon excitation at 295nm and (B) emission maximum against urea concentration. 35

Figure 4.6 Urea denaturation curves of HSA (1.0μM) as studied by plotting (A) fluorescence intensity at 342nm upon excitation at 295nm and (B) emission maximum against urea concentration. Incubation time with urea was kept as 60 minutes. 37

Figure 4.7 Fluorescence spectra of HSA (1.0μM) in the absence and presence of increasing warfarin concentrations in 0.06M sodium phosphate buffer, pH 7.0 at 25℃ incubated for 60 minutes, upon excitation at 280nm. 38

Figure 4.8 Plots showing change in the relative fluorescence intensity at 338nm upon excitation at 280nm (A) and the emission maximum (B) of HSA against warfarin concentration. 40

Figure 4.9 Fluorescence spectra of HSA (1.0μM) in the absence and presence of increasing warfarin concentrations in 0.06M sodium phosphate buffer, pH 7.0 at 25℃ incubated for 60 minutes, upon excitation at 295nm. 42

Figure 4.10 Plots showing change in the relative fluorescence intensity at 342nm upon excitation at 295nm (A) and the emission maximum (B) of HSA against warfarin concentration. 43

Figure 4.11 Fluorescence spectra of HSA (1.0μM) in the absence and presence of increasing warfarin concentrations but with 3.0M urea in 0.06M sodium phosphate buffer, pH 7.0 at 25℃ incubated for 60 minutes, upon excitation at 280nm. 46

Figure 4.12 Plots showing change in the relative fluorescence intensity at 337nm upon excitation at 280nm (A) and

the emission maximum (B) of HSA in 3.0M urea against warfarin concentration. 47

Figure 4.13 Fluorescence spectra of HSA (1.0μM) in the absence and presence of increasing warfarin concentrations but with 3.5M urea in 0.06M sodium phosphate buffer, pH 7.0 at 25℃ incubated for 60 minutes, upon excitation at 280nm. 48

Figure 4.14 Plots showing change in the relative fluorescence intensity at 335nm upon excitation at 280nm (A) and the emission maximum (B) of HSA in 3.5M urea against warfarin concentration. 49

Figure 4.15 Fluorescence spectra of HSA (1.8μM) in the absence and presence of increasing warfarin concentrations but with 4.0M urea in 0.06M sodium phosphate buffer, pH 7.0 at 25℃ incubated for 60 minutes, upon excitation at 280nm. 50

Figure 4.16 Plots showing change in the relative fluorescence intensity at 336nm upon excitation at 280nm (A) and the emission maximum (B) of HSA in 4.0M urea against warfarin concentration. 51

Figure 4.17 Fluorescence spectra of HSA (1.0μM) in the absence and presence of increasing warfarin concentrations but with 4.5M urea in 0.06M sodium phosphate buffer, pH 7.0 at 25℃ incubated for 60 minutes, upon excitation at 280nm. 52

Figure 4.18 Plots showing change in the relative fluorescence intensity at 336nm upon excitation at 280nm (A) and the emission maximum (B) of HSA in 4.5M urea against warfarin concentration. 53

Figure 4.19 Fluorescence spectra of HSA (1.0μM) in the absence and presence of increasing warfarin concentrations but with 5.3M urea in 0.06M sodium phosphate

buffer, pH 7.0 at 25℃ incubated for 60 minutes, upon excitation at 280nm. 54

Figure 4.20 Plots showing change in the relative fluorescence intensity at 335nm upon excitation at 280nm (A) and the emission maximum (B) of HSA in 5.3M urea against warfarin concentration. 55

Figure 4.21 Fluorescence spectra of HSA (1.0μM) in the absence and presence of increasing warfarin concentrations but with 6.5M urea in 0.06M sodium phosphate buffer, pH 7.0 at 25℃ incubated for 60 minutes, upon excitation at 280nm. 56

Figure 4.22 Plots showing change in the relative fluorescence intensity at 340nm upon excitation at 280nm (A) and the emission maximum (B) of HSA in 6.5M urea against warfarin concentration. 57

Figure 4.23 Fluorescence spectra of HSA (1.0μM) in the absence and presence of increasing warfarin concentrations but with 8.0M urea in 0.06M sodium phosphate buffer, pH 7.0 at 25℃ incubated for 60 minutes, upon excitation at 280nm. 58

Figure 4.24 Plots showing change in the relative fluorescence intensity at 342nm upon excitation at 280nm (A) and the emission maximum (B) of HSA in 8.0M urea against warfarin concentration. 59

Figure 4.25 Plots showing change in the relative fluorescence intensity of HSA at the emission maximum with increasing warfarin concentrations at different urea concentrations when excited at (A) 280nm and (B) 295nm. 60

Figure 4.26 Plots showing change in the emission maximum of HSA with increasing warfarin concentrations at different urea concentrations when excited at (A)

280nm and (B) 295nm. 61

Figure 4.27 Fluorescence spectra of HSA (1.0μM) in the absence and presence of increasing warfarin concentrations but with 3.0M urea in 0.06M sodium phosphate buffer, pH 7.0 at 25℃ incubated for 60 minutes, upon excitation at 295nm. 64

Figure 4.28 Plots showing change in the relative fluorescence intensity at 342nm upon excitation at 295nm (A) and the emission maximum (B) of HSA in 3.0M urea against warfarin concentration. 65

Figure 4.29 Fluorescence spectra of HSA (1.0μM) in the absence and presence of increasing warfarin concentrations but with 3.5M urea in 0.06M sodium phosphate buffer, pH 7.0 at 25℃ incubated for 60 minutes, upon excitation at 295nm. 66

Figure 4.30 Plots showing change in the relative fluorescence intensity at 341nm upon excitation at 295nm (A) and the emission maximum (B) of HSA in 3.5M urea against warfarin concentration. 67

Figure 4.31 Fluorescence spectra of HSA (1.8μM) in the absence and presence of increasing warfarin concentrations but with 4.0M urea in 0.06M sodium phosphate buffer, pH 7.0 at 25℃ incubated for 60 minutes, upon excitation at 295nm. 68

Figure 4.32 Plots showing change in the relative fluorescence intensity at 341nm upon excitation at 295nm (A) and the emission maximum (B) of HSA in 4.0M urea against warfarin concentration. 69

Figure 4.33 Fluorescence spectra of HSA (1.0μM) in the absence and presence of increasing warfarin concentrations but with 4.5M urea in 0.06M sodium phosphate buffer, pH 7.0 at 25℃ incubated for 60 minutes,

upon excitation at 295nm. 70

Figure 4.34 Plots showing change in the relative fluorescence intensity at 341nm upon excitation at 295nm (A) and the emission maximum (B) of HSA in 4.5M urea against warfarin concentration. 71

Figure 4.35 Fluorescence spectra of HSA (1.0μM) in the absence and presence of increasing warfarin concentrations but with 5.3M urea in 0.06M sodium phosphate buffer, pH 7.0 at 25℃ incubated for 60 minutes, upon excitation at 295nm. 72

Figure 4.36 Plots showing change in the relative fluorescence intensity at 343nm upon excitation at 295nm (A) and the emission maximum (B) of HSA in 5.3M urea against warfarin concentration. 73

Figure 4.37 Fluorescence spectra of HSA (1.0μM) in the absence and presence of increasing warfarin concentrations but with 6.5M urea in 0.06M sodium phosphate buffer, pH 7.0 at 25℃ incubated for 60 minutes, upon excitation at 295nm. 74

Figure 4.38 Plots showing change in the relative fluorescence intensity at 347nm upon excitation at 295nm (A) and the emission maximum (B) of HSA in 6.5M urea against warfarin concentration. 75

Figure 4.39 Fluorescence spectra of HSA (1.8μM) in the absence and presence of increasing warfarin concentrations but with 8.0M urea in 0.06M sodium phosphate buffer, pH 7.0 at 25℃ incubated for 60 minutes, upon excitation at 295nm. 76

Figure 4.40 Plots showing change in the relative fluorescence intensity at 350nm upon excitation at 295nm (A) and the emission maximum (B) of HSA in 8.0M urea against warfarin concentration. 77

Figure 4.41 Stern-Volmer plots for tryptophan fluorescence quenching of HSA at increasing warfarin concentrations in the absence and presence of different urea concentrations. 78

Figure 4.42 Plots of Log [(F0-F)/ F] versus Log [Warfarin] in the absence and presence of different urea concentrations. 81

LIST OF TABLES

		Page
Table 2.1	Some physico-chemical properties of human serum albumin.	8
Table 2.2	Amino acid composition of human serum albumin.	10
Table 4.1	Values of the Stern-Volmer constant (Ksv) for tryptophan fluorescence quenching of HSA upon warfarin binding in the absence and presence of different urea concentrations.	79
Table 4.2	Values of the binding constant (K_b) for the warfarin-HSA complex in the absence and presence of different urea concentrations.	82

LIST OF ABBREVIATIONS

AFP	α-fetoprotein
Ala	Alanine
ANS	1-Anilinonaphthalene-8-sulfonic acid
Arg	Arginine
℃	Degrees Celsius
CD	Circular dichroism
cm	Centimeter
$E_{279nm}^{1\%}$	Specific absorption coefficient
F_0	Fluorescence intensity in the absence of drug
F	Fluorescence intensity in the presence of drug
Gc	Group specific component
GdnHCl	Guanidine hydrochloride
His	Histidine
HSA	Human serum albumin
Ile	Isoleucine
K_{SV}	Stern-Volmer constant
K_b	Binding constant
Leu	Leucine
M	Molar
mg	Milligram
ml	Millilitre
µl	Microlitre
µM	Micromolar
n	Number of binding sites
nm	Nanometer
No.	Number
Phe	Phenylalanine
Q	Molar concentration of the drug
R^2	Correlation coefficient
Ser	Serine
Trp	Tryptophan
Tyr	Tyrosine
Val	Valine
UV	Ultraviolet
%	Percentage