AppliedPhotophysics
Ultrasensitive Spectroscopy for the Life Sciences

CHIRASCAN SERIES APPLICATION NOTE

The use of Chirascan™ to study the competitive binding of diazepam and ibuprofen to human serum albumin (HSA)

Abstract *This study describes the application of the Chirascan™ spectrometer to examine the binding interactions of two well-known small molecule drugs; ibuprofen and diazepam, to the drug binding protein, human serum albumin (HSA). It is demonstrated that protein-ligand interactions can be monitored using Circular Dichroism (CD) spectroscopy by observing changes in both the protein and drug molecule CD signal. The technique is also applied to study a protein-ligand binding interaction in a system where there is ordinarily no observable change in CD signal. This is achieved by coupling the interaction to a protein-ligand system where there is an observable CD signal change.*

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INTRODUCTION

Many drug molecules bind reversibly to plasma proteins and often circulate in the body as this bound form with a small population free in solution. Two major proteins that regularly bind small molecule drugs are human serum albumin (HSA) and α1-acid glycoprotein. The binding of drug molecules to these proteins has a large impact on the pharmacokinetics of the drug, and changes in free plasma concentrations of a drug have significant bearing on the pharmacological activity as well as the rate of breakdown and excretion.

Particular drugs, metabolites and other molecules have high affinities for certain binding sites on plasma proteins. The different affinities of different molecules for specific binding sites can result in a drug being displaced from the protein by another molecule. These complex interactions can significantly change the pharmacokinetics of a drug and is one of the mechanisms by which multi-drug interactions occur.

Monitoring changes in circular dichroism (CD) spectra of either the protein or the ligand is a very specific signal for structural changes induced in either the protein or the ligand. The ligand does not need to be chiral to produce a CD signal when bound to a protein.

In this application note, the binding interactions of two well known and characterised small molecule drugs, ibuprofen and diazepam[1, 2], to HSA are studied using CD spectral changes recorded on a Chirascan™ spectrometer.

KEYWORDS

- Circular Dichroism
- Chirascan
- Competitive Binding
- Ibuprofen
- Antibody
- Spectroscopy
- Titration
- Diazepam

EXPERIMENTAL CONDITIONS AND INSTRUMENT SETUP

A Chirascan™ CD spectrometer fitted with a CS/TT titration accessory was used for all the equilibrium titration experiments. The HSA protein was kept at a concentration of 0.5mg/mL for all equilibrium experiments, in a pH7.0 buffer (50mM Sodium phosphate). Protein/buffer solution in a 1cm cuvette was titrated with a solution of buffer containing a high concentration of the ligand, by automated subtractions and additions of volumes out of the cell. The cuvette was stirred and allowed to equilibrate for 1 minute after additions before spectra were recorded.

All stopped-flow CD kinetic data was collected on a Chirascan™ fitted with the CS/SF stopped-flow accessory, and a 150W xenon-mercury lamp as the light source. The monochromator bandpass was 4nm. The cell pathlength was 2mm.

DIAZEPAM BINDING TO HSA

Diazepam was titrated into HSA in the concentration range of 0 to 0.8mM in 20 concentration steps. The diazepam binding to HSA resulted in the CD spectral changes in the near-UV shown in Figure 1.

At 320nm (a region that has no contribution from protein CD) there is a significant induction of CD corresponding to the interaction of the achiral diazepam molecule with the chiral protein binding site. The majority of the CD changes observed are due to the induced chirality in the bound diazepam molecule.

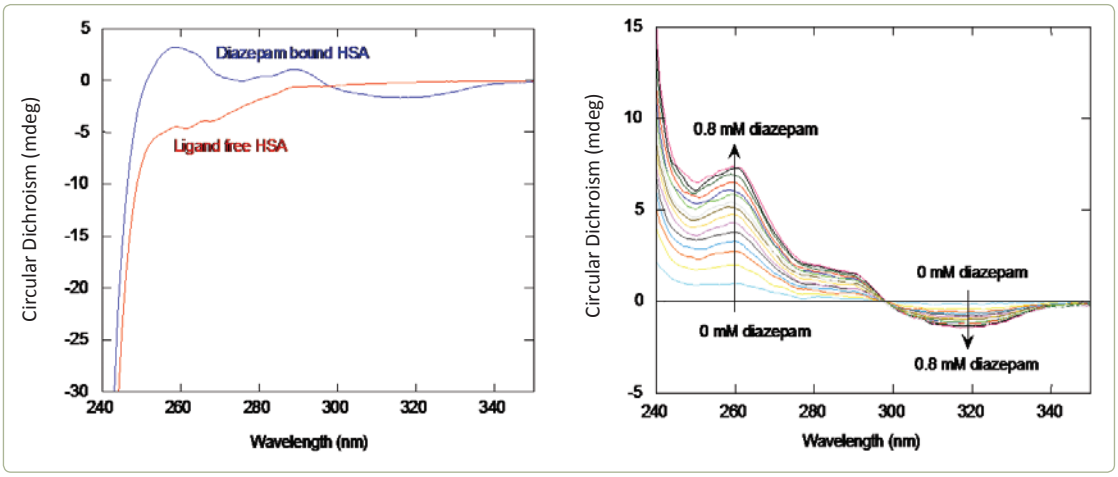

Figure 1. Binding of diazepam to HSA results in an observable change in CD signal. Left: near-UV CD spectra of free (red) and diazepam bound (blue) HSA. Right: change in the near-UV CD spectrum with increasing concentration of diazepam.

The data doesn't fit easily to a single binding site model, as diazepam is known to bind at two binding sites on the protein[1, 2]. The complex nature of the binding of diazepam to HSA is also displayed in stopped-flow CD of diazepam binding to HSA at 260 and 320nm (Figure 2). The reaction required a two exponential function to satisfactorily model all the reaction traces.

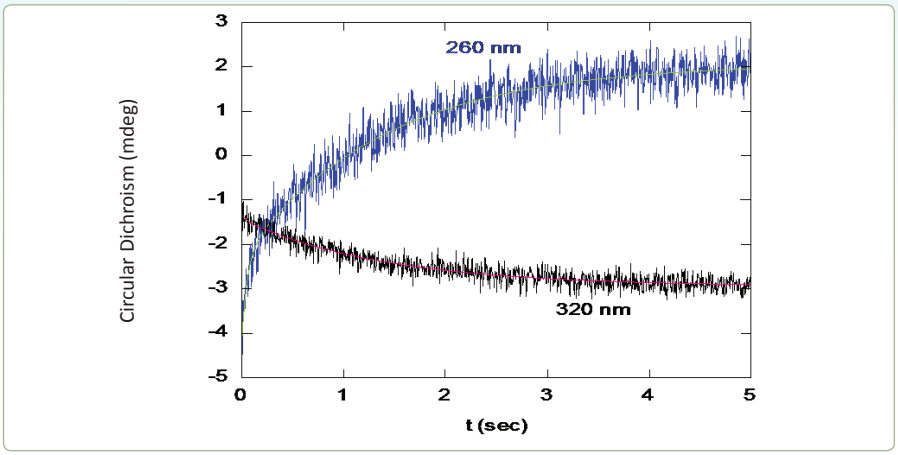

Figure 2. The pre-steady state kinetics of binding of diazepam (0.5mM) to HSA (2.5mg/mL). Stopped-flow CD measured using the CS/SF accessory for the Chirascan™. Both wavelengths (320nm and 260nm) could be fitted successfully with a simultaneous double exponential function, the fitted rate constants were of k_1= 19 and k_2=0.72 s^{-1}.

IBUPROFEN BINDING TO HSA

Ibuprofen was titrated into HSA in the concentration range of 0 to 1.4mM in 21 concentration steps. There was little observable change in the CD spectra upon binding ibuprofen (Figure 3).

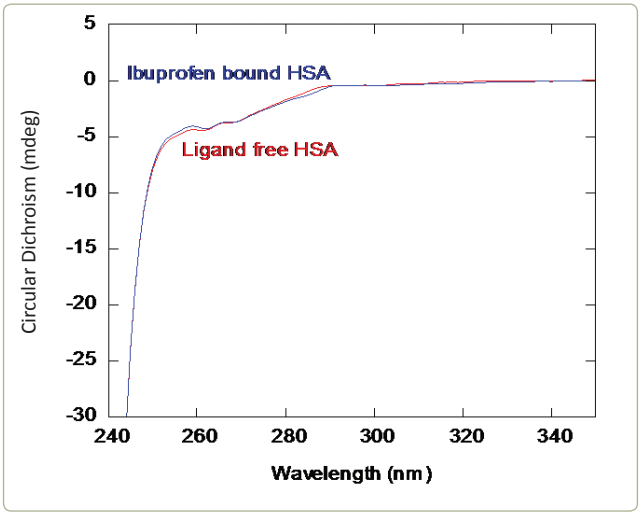

Figure 3. Near UV CD spectra of ligand free (red) and ibuprofen bound (blue, 1.4mM ibuprofen) HSA. There is little change in CD spectra for the protein or the ligand when ibuprofen binds.

The absence of a change doesn't mean there is no binding of ibuprofen to the protein; it may just demonstrate that there is no significant change in the structure of the protein or the ligand upon binding the molecule.

HSA with bound diazepam (0.5mM) was then titrated with ibuprofen from 0 to 1.5mM concentration. The change in CD signal (Figure 4) shows a change from the diazepam-bound spectrum, to the ibuprofen-bound spectrum shown in Figure 3. This is due to the diazepam being replaced with ibuprofen.

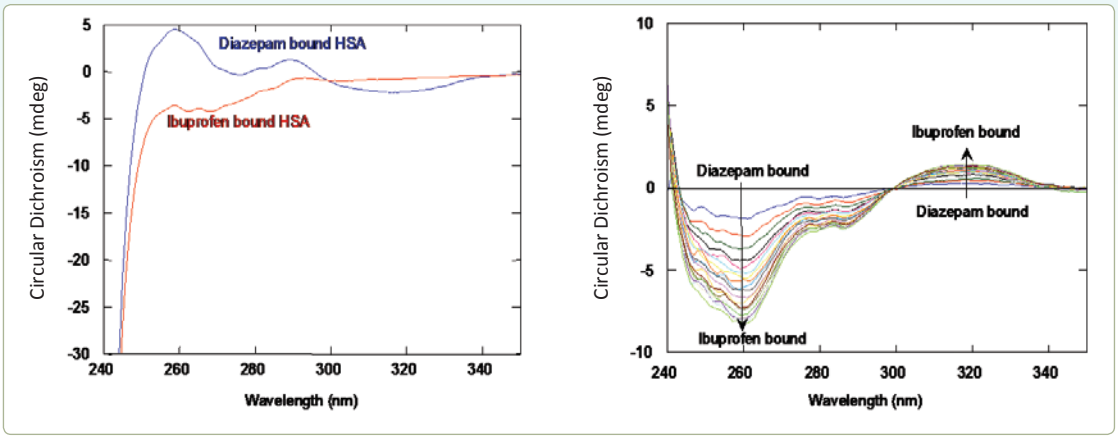

Figure 4. Displacement of bound diazepam by ibuprofen on HSA monitored by changes in near-UV CD. Left: near-UV CD spectra of diazepam (0.5mM) bound (blue) and ibuprofen bound (1.5mM) HSA (red) after displacement of diazepam. Right: change in the near-UV CD spectrum of bound diazepam with increasing concentration of ibuprofen.

These observations demonstrate that ibuprofen does bind to HSA and will competitively displace the diazepam ligand from the protein. There is no induced CD change on ibuprofen binding to HSA, but the displacement of another ligand with an induced CD signal can be used to study the protein-ligand interaction of this molecule.

Pre-steady-state stopped-flow CD kinetics of the displacement of diazepam by ibuprofen was measured. The displacement of the diazepam by ibuprofen could be modelled by a single-step process (Figure 5).

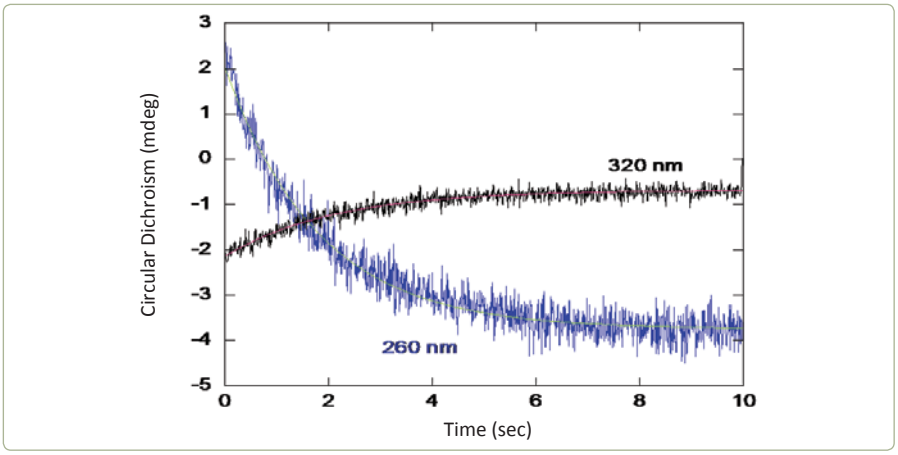

Figure 5. The pre-steady-state kinetics of displacement of diazepam (0.5mM) bound HSA (2.5mg/mL) by ibuprofen (2mM). Stopped-flow CD measured using the CS/SF accessory for the Chirascan™. Both wavelengths (320 and 260nm) could be fitted with a single exponential model, with an apparent rate constant of k= 0.5s^{-1}.

CONCLUSION

Monitoring induced chirality with Chirascan™ is a very useful method for monitoring ligand and protein interactions. Changes in CD spectra are specific to the chiral protein site changing or creating a chiral environment of the ligand. In this example the interaction of two drugs, ibuprofen and diazepam, with HSA has been used to demonstrate the principle. Studies of this type will improve understanding of the pharmacokinetics of drugs and drug interactions.

For further reading see Zsila *et al*.[3], a review paper outlining the use of monitoring induced chirality by CD for protein binding studies.

REFERENCES

[1] R. Brodersen, T. Sjodin and I Sjoholm *J. Biol. Chem*. 1977, **252**, 5067-5072.

[2] U. Kragh-Hansen *Biolchem*. J. 1983, **209**, 135-142.

[3] F. Zsila, Z. Bikadi, I Fitos, M. Simonyi, *Current drug Discovery Technologies*, 2004, 1, 133-153.

NEW APPLICATIONS FOR CD SPECTROSCOPY

Optimising biotherapeutic formulations

Used as a label free stability-indicating assay, Chirascan™-plus automated circular dichroism (ACD) can identify good formulation candidates earlier for further downstream processing. By culling formulations that are likely to fail early and focusing on those that are more viable for real time and accelerated stability studies, users can make savings in both time and money.

Combining the label-free and information-rich technique of dynamic multimode spectroscopy (DMS) with the productivity of automation gives a whole new approach to establishing conformational stability under different formulation conditions. The conformational integrity of biotherapeutics as a function of more than one stress condition (e.g. Temperature, pH, ionic strength) is readily determined in unattended operation.

Statistical comparison of similar proteins (Biosimilarity)

Research into biosimilar pharmaceutical products has grown exponentially over the last few years turning it into a multi-billion pound business. Structure, biological activity and stability are just a few of the complex studies required and, traditionally, these use multiple techniques which are very time consuming and labour intensive.

Automation lends itself to measuring samples repeatedly and thus to generating statistical comparisons. To answer the question: ‘Are these two CD spectra the same?’ is no longer a matter of guesswork – a statistical significance can be associated with the measurements and a quantitative judgment about similarity or otherwise can be made.

Drug Discovery

Fast determination of protein characteristics is key to any drug discovery department in pharmaceutical research. Applied Photophysics offers a unique solution providing simultaneous circular dichroism, absorbance and fluorescence measurements in a single, easy to use, automated experiment.

The Chirascan™-plus ACD spectrometer can provide structural, functional, thermodynamic and aggregation data. By automating our system we provide unparalleled productivity, low sample volumes and no human error reducing the pressure on analytical labs and enabling them to focus on discovery.

Protein Engineering

Monoclonal antibodies, antibody-like proteins, and other biotherapeutics represent a large and growing number of molecular entities entering human clinical trials in virtually all disease indications. The long-term stability of these potential therapeutics is of crucial importance for their development to drug products.

The Chirascan™-plus ACD spectrometer provides rapid, accurate, and easy to perform measurement of the thermal melting (T_m) points, which has proven to be an exceptionally good indicator of the relative stability of engineered proteins.

AppliedPhotophysics

Ultrasensitive Spectroscopy for the Life Sciences

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Applied Photophysics was established in 1971 by
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