

# Quantifying Drug-Protein Binding *in vivo*

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## QUANTIFYING DRUG-PROTEIN BINDING *IN VIVO*

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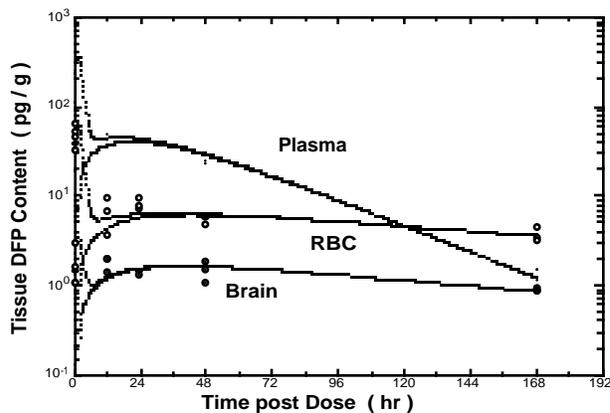
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*Keywords:* Accelerator mass spectrometry, AMS, energy-loss, quantitation, protein

### INTRODUCTION

Accelerator mass spectrometry (AMS) provides precise quantitation of isotope labeled compounds that are bound to biological macromolecules such as DNA or proteins [1,2,3]. The sensitivity is high enough to allow for sub-pharmacological (“micro-”) dosing to determine macromolecular targets without inducing toxicities or altering the system under study, whether it is healthy or diseased. We demonstrated an application of AMS in quantifying the physiologic effects of one dosed chemical compound upon the binding level of another compound *in vivo* at sub-toxic doses [4]. We are using tissues left from this study to develop protocols for quantifying specific binding to isolated and identified proteins. We also developed a new technique to quantify nanogram to milligram amounts of isolated protein at precisions that are comparable to those for quantifying the bound compound by AMS.

### DFP-PROTEIN BINDING

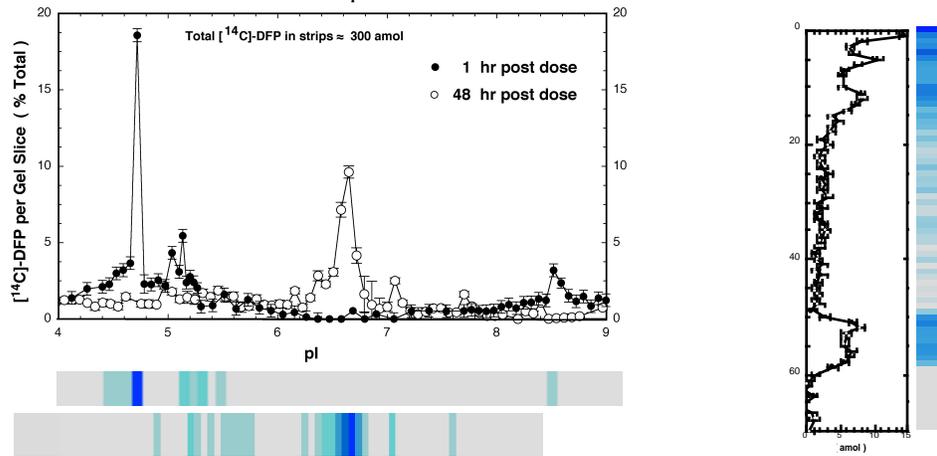


**Figure 1.** Kinetic profile of [<sup>14</sup>C]-DFP in murine tissue.

Di-isopropyl-fluorophosphate (DFP) is a miotic that acts through long term inhibition of cholinesterases. It is also a well known functional probe of serine hydrolase activity. Its covalent bond to hydrolyzed serine or to cystein thiols makes it a convenient reporter compound that can be introduced into a biological system to trace movement or changes in hydrolase enzymes. As an anti-actylcholinesterase, it is a toxic nerve agent with an oral murine LD-50 of 2 mg/kg. We dosed mice at 1 µg/kg with [<sup>14</sup>C]-DFP to quantify changes in brain bound concentrations due to pre-exposures with pesticides parathion and permethrin and the temporary esterase inhibitor, pyridostigmine bromide [4].

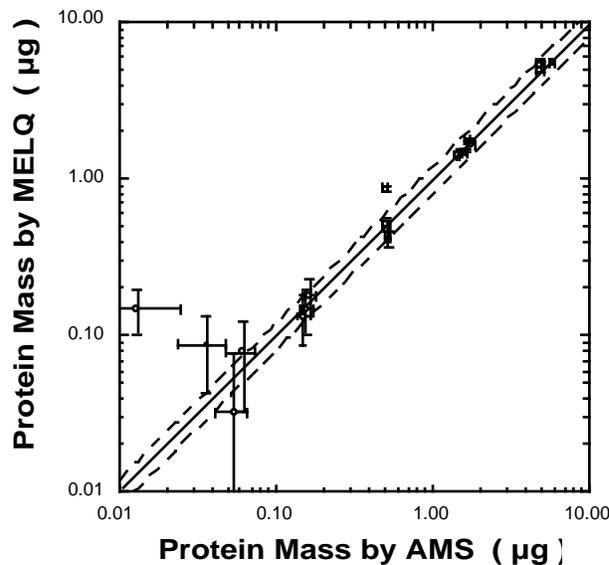
Figure 1. shows the kinetic response of plasma, red blood cells (RBC), and homogenized brain tissue to DFP up to 1 week post dose. Only protein-bound DFP is present after 12 hours, and the bound fraction is removed by proteolysis with mean turnover times of 2 days in plasma and > 7 days in RBC and brain. Plasma proteins were isolated from samples at 1 hr and 48 hrs post dose along isoelectric focussing strips without removal of the albumin. The gel strips were cut into 1 mm long pieces and individually quantified by AMS for <sup>14</sup>C content, as shown by the plot and “gel mimic” image in Figure 2A. The mass of the gel pieces included  $0.93 \pm 0.15$  mg carbon, allowing an absolute determination of the amount of [<sup>14</sup>C]-DFP in each piece from the AMS isotope ratio. Error bars, when visible, represent 1-2 amol of bound DFP. Figure 2B shows the molecular PAGE gel quantified by AMS of the protein eluted from the 4.7 - 4.8 pI peak of the 1 hour IEF separation. Much of the <sup>14</sup>C migrated with the dye front, indicating binding to very low molecular weight, probably the cysteines in glutathione. Two peaks at 80 and 120 kDa coming from the 4.75 pI peak are consistent with a lipoprotein hydrolase: paraoxonase. Both the paraoxonase and glutathione clear quickly from plasma in their detoxification roles. PAGE analysis of the 48 hr peak is

not yet available, but the pI agrees well with the expected plasma targets of carboxylesterase and butyrylcholinesterase, both proteins having turnover times consistent with the plasma kinetic data..



**Figure 2** . AMS quantified [<sup>14</sup>C]-DFP bound to proteins separated along IEF strips (left) and along an MW PAGE lane after elution from the pI= 4.75 peak of the 1 hr IEF separation (right).

The final isolated “paraoxonase” peaks in Figure 2B represent only 25 amol of protein , or about 2.5 picograms protein. We could obtain the specific binding to this protein if we could measure the amount of protein after elution from these bands followed by AMS quantitation of bound DFP. Precise and sensitive mass determination of samples for AMS quantitation has been a problem for over a decade. We found that measuring the energy loss of accelerated ions as they pass through a protein sample on a thin and very uniform silicon nitride support provides picogram mass measurements [5]. These same substrates are compatible with MALDI MS protein identification, opening the way to quantitation, identification, and binding specificity measures on exactly the same isolated protein aliquot. Unfortunately, the source of energetic ions is even larger than our AMS spectrometer. We developed a bench top version of our Mass by Energy Loss Quantitation (MELQ) using <sup>210</sup>Po as a source of energetic alpha particles that can measure sample masses from 100 ng to 100 µg with better than ±10% precision [6].



**Figure 3**. Aliquots of [<sup>14</sup>C]-hemoglobin were quantified both by MELQ and by AMS, using the specific activity to convert from <sup>14</sup>C content to mass. Except for an outlier due to known causes, the masses agree well from 100 ng to 100 µg.

## CONCLUSION

We developed the tools for separating small amounts of identifiable proteins from tissues and fluids of animal (or human) subjects that are dosed with very low, sub-toxic, levels of chemical compounds to determine the protein targets of these compounds *in vivo* and to quantify their absolute binding specificity relative to other proteins in the same sample.

## ACKNOWLEDGEMENTS

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