NMR spectroscopy for Structural Biology
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Lecture: 49
NMR in Drug metabolism II

Okay students, good morning. So, this week we are discussing how we can use NMR in drug discovery and last class I was discussing how we can use NMR in understanding the drug metabolism. Why it is important to understand the drug metabolism? As we discussed in the last lecture, that the drug starts its journey from mouth and when it reaches to the cell, where it has to act, it remains only say 15 to 20 percent or very less because it has to pass through first past metabolism and this can vary from person to person because the enzymes that degrades or metabolize these drugs, cytochrome P450 family, can be expressed differently in a different person. So, in a healthy person it can have one dose or one effective concentration or we call it bioavailable concentration, bioavailability, and in diseased person or different person it can be different. Therefore to understand the fate of the drug, how it travels from the site of administration to the site of action that comes under drug metabolism or pharmacokinetic.

That is very important to understand to define a dosage because one dose cannot fit for all and that will not treat the patient effectively. So NMR plays a significant role in understanding in a quantitative manner this drug metabolism or pharmacokinetic. How? That we are going to discuss now. Last class we looked at, there are some enhancer that probably enhances the bioavailability.

I showed you the example like if felodipin is taken like here, if this drug, which is used to treat blood pressure, if it is taken with the fruit juice it increases the bioavailability. You can see it here. And that is what we concluded and it actually helps in having higher plasma felodipin concentration. It also varies depending upon the cytochrome C450 CyP3A4 activity. So, in patient with increased activity, the bioavailable concentration in plasma is very less compared to healthy. Therefore, understanding this drug metabolism in a quantitative manner is of paramount importance. So, let us look at where NMR can help us.

So, as you know NMR is non-invasive technique, therefore it perturbs minimally and can be used for drug metabolism. What we can take from body for understanding the drug metabolism? Any bio-fluids, which can be urine, the plasma, serum, tears, sweat, anything that comes under bio-fluid. We can take our tissue extract or biopsy sample or even intact cell. These are the in vitro approach. We can take out these and record the NMR spectrum in vitro condition or even one can do in vivo condition, you can take whole animal model or a human subject that is a sister technique of NMR called MRI.

You can use these two techniques, spectroscopy plus imaging, to understand the drug metabolism in totality. So, that is a in vivo use of NMR spectroscopy to understand the drug metabolism and it has a unique ability because it permits both kind of study in vitro as well as in vivo. So like whole organism can be taken into the NMR magnet or MRI magnet and understand the drug metabolism and of course in vitro one can do with bio-fluids to tissue extract, so and so forth. So what it allows one to do? One can generate a metabolic signature of the drug.

What kind of metabolites comes out of a drug? One can understand this. It not only identify but it measures the metabolic flux like when drug goes through different cycle of different stage of metabolism. How the flux is changing, how flow of the drug is changing, NMR can monitor as well as measure it quantitatively. It also monitors the enzyme like some of the enzyme that we talked in the last class, what is the activity of enzyme. So once drug is administered, enzymes comes into play, how fast or how slow they are metabolizing, one can understand.

So one can actually decipher the pathway or kinetic pathway of drug metabolism and one can even monitor what kind of modification that happens in the metabolic path when drug is administered or get metabolized. Does drug make any adduct or it combines with some other molecules when it gets metabolized. So, essentially it can understand the effect of perturbants or toxins or any other things, when drug goes from the site of administration to the circulation. So one can study all these or excretion, one can study all of these.

So typically the fluids that are used for doing the in vitro study like a diagnostic fluids, plasma can be used, serum can be used, urine can be used, saliva or any other secretion even tears can be used for understanding drug metabolism using NMR spectroscopy in a non-invasive manner. One of the prominent one is a urine. Why? Because whatever we

take or consume as a drug finally it should be excreted and most of the things excreted out from urine. And a taking urine is a minimal painful.

Everybody urinates easily, one can take urine, identify the drug metabolism. For taking plasma or serum, you have to take blood. So people or patients may not like it. It is not a patient compliant technique. However, urine comes in enough quantity and that can be used critically to understand how the drug metabolism happens.

So everything that goes through kidney comes in urine and that can be used for detecting the drug metabolism. So other than these body fluids, if you want to do understand how drugs is reaching to different specialized location like a cerebral spinal location or thyroids or saliva, whether it is sublingual or paratoid or submaxillary or gastric location or even bile juices or pancreatic juices, lots of these can be done with a localized NMR spectroscopy, or even amniotic fluid, follicular or how drug is going into milk. You know milk contamination is one of the major one. So for babies mother's milk is very important.

So generally one want to know that what is the effect of drug on milk or even seminal vesicles how drug is affecting, whether it is going or prosthetic. Many of such things can be understood by NMR spectroscopy. The effect of a drug or the location of a drug metabolites can be identified by taking these body fluids and using the drug metabolites the effect can be understood in a quantitative manner. So what nuclei one can use for studying the drug metabolism? So see all the nuclei that we have studied can be exploited. The prominent one is a hydrogen. It is a half spin and it is natural abundance.

The chemical shift range is about 15 ppm. This is actually everywhere present. But there are some drawbacks with hydrogen. We will discuss that.

You require a minimal concentration say 0.01 mM. The other one is deuterium. Then come lithium, boron, carbon-13, carbon-13 can also be taken from the body fluids and can be exploited. Then say N-14, N-15, O-17, all these are NMR active nuclei. Like a fluorine, it is a precious nuclei that is used for understanding the drug metabolism. Similarly, 31P, it is a natural abundance found in nucleic acid, the bones and all those.

So these are some of the nuclei that can be exploited. So let us go little more detail and understand the pros and cons of each of these nuclei that can be used for understanding the drug metabolism. So as we know proton is present in mostly all drugs, and it has a highest sensitivity. The natural abundance for proton is about 99.98 %.

So it is a 100 percent natural abundance, you can consider this. So this is the most prominent nuclei that can be exploited. But there are some problem associated with the proton or hydrogen. It has a small chemical shift range of about 15 ppm. And then because of the J coupling, you see extensive multiplicity. So this actually makes the spectrum crowded and quantification of drug metabolites in those cases can be difficult.

So because of short chemical shift range and extensive multiplicity due to homonuclear coupling, the spectra is really crowded. So quantification or even distinguishing the drug metabolites can be difficult. Another big problem that whatever body fluid you take, water is huge. About 70% of all these will have will be dominated by water. Now you want to detect a very low concentration of drug from the body fluid where water is very high. Other than water, there are proteins or lipids that are there.

So before you start experiment you have to think about how we can get rid of proteins or lipid signal or reduce at least, if you cannot eliminate. So there are NMR based technique that you saw for proteins like you can do water suppression, presat or gradient-based suppression. You can use it or you can concentrate more, you can do freeze dry, or by using the T2 filters you can remove the signal of proteins or lipids. So these are some tricks that can be utilized for getting the signal of protons from the drug metabolites in presence of huge amount of water, protein and lipids. So these are some of the tricks that can be applied and then one can detect the protons. Not only in vitro even one can use proton in vivo but it comes with some problem and those are the problem, because it is a poor nuclei for in vivo monitoring of drug, because the tissue inhomogeneity is there and in vivo you cannot spin sample or they are not fast mobile.

So this averaging of anisotropic interaction is not that prominent therefore lines are bound to be broad due to tissue inhomogeneity, heterogeneity or restricted molecular mobility, because tissue cannot be spun faster or all those. Also, the tissue size can be little bigger than the whatever we have the homogeneous magnetic field. So magnetic field inhomogeneity can come. In this case relatively large sample volume and use of lower

magnetic field makes little bit difficult. You cannot put whole organism in a huge magnet of 20 tesla. You have to restrict to 3 tesla, 4 tesla.

In those cases, you do not want to perturb this ecosystem of an organism by exposing to high magnetic field. So, typically we do this experiment at a lower magnetic field and in those lower magnetic field the homogeneous space is limited however our tissue or the whole organism that we are scanning can be bigger. So, magnetic field inhomogeneity is invariably there and that basically broadens the signal. So in vivo signal line width are substantially broader than what we obtained in in vitro.

And those are the some of the limitation of using proton for in vivo drug metabolism experiment. Another important and beautiful nuclei is fluorine 19. So you know fluorine is interesting nuclei. First of all its nuclear spin is half, has a relatively narrow line shape. It is a 100 percent natural abundance and very high sensitive like about 83 percent of proton.

You can see it is sensitive, it's a natural abundance is also high and gives sharp lines. You do not need to do anything special because it is not a quadrupolar nuclei, it is a half integral nuclei, one spin is only half. Other than this, it has a large chemical shift range.

So peaks cannot be crowded. It is about 200 ppm range of chemical shift. So peaks will be separated. So you can identify this. And additional advantage which has, it has a short longitudinal relaxation time. So if T1 is short, that means you can afford to keep D1 also short.

So experiment can be performed on a fast scale. The only requirement is you need to have a probe that can detect fluorine. Once you have the NMR probe where you can tune your RF to F19 you can do these experiments in a much cleaner manner and much beautiful manner. So if you have this, one can do the rapid pulsing and you can increase the signal to noise ratio in per unit time, in a quicker time. Many drugs are actually fluorinated, a large number of drugs are fluorinated that are in clinical use. For those drugs NMR, F19 NMR offers a powerful methods for monitoring their pharmacokinetic and metabolism.

So remember, F19 is a beautiful nuclei to be used for understanding the drug metabolism and pharmacokinetic. So 13C you know for proteins it is widely used. Now, most of the time drugs cannot be isotopically labeled, so the only one has to rely on 1.1% of natural abundance of the carbon-13 in any drug, but it has a large chemical shift. If we can signal average to a large number of scan, it can produce actually a reliable chemical structure for identifying the drug molecule or one can isotopically label to enhance the signal to noise, but typically it cannot be done so easily because drugs cannot be isotopically labeled for all practical purpose.

However, for research purpose one can do it and then detect it. So mostly, we have to rely on a natural abundance that is 1.1 percent. So there are other tricks that one can apply. What are those tricks? Basically, what we can do is, we detect on carbon 13 but we transfer the polarization from proton in a HETCOR kind of experiment and then we can do this kind of detection. So we can improve the sensitivity by doing this polarization transfer experiment and then you can use this drug to understand the drug metabolism using HETCOR kind of experiment.

Now 13C can be used in vitro as well as in vivo. However, actually it is of very limited use because of its sensitivity, but yes it can be used. Other important nucleus for drug metabolism can be phosphorus-31. The good part is that it has 100 percent natural abundance. It's gyromagnetic ratio is about one-third of proton. So it is a significantly sensitive and phosphate is found in many drugs or even in the body.

So one can look at the phosphorus signal. So indigenous phosphate and derivatives or phosphodiester, many of these can interfere to the signal of phosphorylated drugs and metabolites. So one has to identify what is coming from the drug and what is coming from our system. But yes, it can be used and there are not too many phosphorus containing drugs. So they are fairly rare, but conceptually phosphorus-31 can also be used. Other than this, there are some other nuclei which can probably use, now lithium based drugs are used for bipolar disorder.

So lithium-7 can be used, boron-10, 11 can be used, then deuterium, tritium, O-17, N-15, N-14, platinum, platinum based drugs are basically used in cancer treatment. So actually one can use Pt195 for understanding the drug metabolism in case of cancerous tissue. So NMR offers a wide range of nuclei, a wide range of possibility to be used for drug

metabolism studies and few of the example we are going to discuss. But before we delve into few of the example, let us see what are the traditional techniques that are used for metabolic analysis. So these are low resolution, I would say not low resolution, it is also high resolution techniques called HPLC, high performance liquid chromatography, gas chromatography, the capillary electrophoresis, mass spectroscopy, all of these are typically used.

The good part of these is that they required minimum sample volume while NMR requires a significant volume. However, they may be getting perturbed with a column matrix or like a depending upon retention time or depending upon solvent use. So lots of possibility of modification can be there because of the nature of the experiment. Good part of NMR is that you are not perturbing any sample. However, it requires larger volume and it is less sensitive compared to these techniques HPLC, GC, CE, MS.

But, these are traditionally used in a quantitative manner to understand the pharmacokinetic. NMR is picking up now. What are the problems of these traditional methods? They require separation. You have to separate it and then identify.

So these coupled methodologies can be used. It requires optimization for separation. You cannot just take your sample, and go, and put in the HPLC, and get your data. That is not the case for NMR. So here you need to optimize by looking at the retention time, when your metabolite is coming. So some optimization is required, right? And many times you have to separate it like you have to separate a polar metabolites from non-polar metabolites, smaller from bigger, all these you have to do.

So typically it takes time and these are slow techniques. And basically you have to do lots of supervision, you require high skill, it is a tedious or manually intensive process in all these techniques HPLC, CE, GCMS. Now on the other hand NMR based drug metabolism study is a high throughput. You can use auto sampler put all the metabolites in one go 48, 24 whatever put it for overnight go and have a rest. Next day morning your results are there.

The good part is the high resolution. Minimal effort you need for sample preparation. You just have to take your sample, add D_2O for locking and then you are ready to go. So you require really minimal sample preparation. High throughput, high resolution and less effort.

Less effort on sample preparation and advantage is it is a quantitative. Looking at the peak intensity, you can integrate those peak and you can get the quantity of each of these metabolites that come out. Non-destructive, you can use same sample for doing even LCMS, GCMS. After doing experiment you can take same sample and use orthogonal technique to understand whatever we are getting in NMR is correct or not.

So you can be doubly sure by using these samples. So it is a non-destructive, same sample can be used and many metabolites can be detected simultaneously in a single experiment. So you do not need to separate it or isolate it, it can be used in a single analysis. It gives structural as well as quantitative information. So what kind of a structure of metabolites is coming can be inferred by looking at the chemical shift pattern, you can identify their structure and also looking at the intensity you can quantify and get the quantitative estimate of each of the metabolites. For few of the nuclei there is no or little spectral interference coming from the indigenous molecule like F19 or 31P or lithium or carbon-13 you have a very minimal interference.

However, for protons we have interference as we discussed from coming from water, coming from protein, coming from lipid, you can have interference from these nuclei. So how you go about sample preparation? Suppose you are doing experiment with one of the body fluid called plasma. You have to take about 300 µl plasma, add buffer 300 µl, you have 600 µl. So typically this is plasma, and here is buffer, where you have H_2O, TSP for internal reference, the sodium azide, so that bacterial growth does not happen and some buffer and then you need to have D_2O. So typically you can take some of these you do not need to centrifuge or do shaking, but because that can form bubble or foam. You just do this, once your experiment setup is ready, go and record the NMR.

So you can see minimal sample preparation. If you are doing with a urine, just take urine of about 90% and add D_2O, sodium azide, TSP for internal reference, sodium azide so that bacterial growth does not happen, and you are all set to record experiment. Again re-emphasizing the fact that we are doing minimal sample preparation while recording the spectrum. You do not need very high magnet, on a moderate magnet like a 500 MHz, 600

MHz one can do experiment. Typically experiments are very easy like 1D NOESY with pre-saturation of mixing time of 10 millisecond. For urine, you can even do CPMG. A CPMG delay of 200-300 millisecond and echo time of 200 millisecond one can use it for plasma.

Temperature, typically you can have 298 K, room temperature, and you do not need much scan, just 32, like within a minutes experiment will be done. Like here, what this is, because of water, and coming from those, so you have to get rid of water. So, you suppress the water or put a spectral region to 0 and you record few of the experiment like a 2D to have more dispersion like HSQC with some scans of 160 to get a better signal to noise. In one go, 1D or 2D whatever you record you are getting all the information from the body fluids. So typically what we get? We get a spectrum like this. So suppose we are taking human urine, you have lots of spectrum like this. Looking at the database, you can identify that these peaks are coming from glutamate, glutamine, betaine or glycine.

So these are typically dominated. Now we know from healthy patient that how the signature comes from the healthy urine. Now if we are administering drug and what extra peaks comes that you need to identify. So let us take some of the example of how we can do that. So one healthy patient was given this drug called Flurbiprofen, it is an anti-inflammatory drug. The spectra were recorded on 600 MHz, so you see you can exploit two nuclei here, at least two nuclei. One is proton that you can record the spectrum and you can find it out.

Another nuclei is beautifully located here, fluorine, it is a cleaner one. If you are detecting on fluorine, you have only signal coming from this drug and you can identify each metabolites, each path that it travels. However, for proton it will be little difficult because it is lots of indigenous signal will come from urine. So on 600 MHz, a sample of urine was collected after oral ingestion of 200 mg of this drug and then spectra were recorded.

So now the problem is lots of complication was coming. So one can actually couple the HPLC with NMR and you separate many of these whatever is coming depending upon the retention time and you can identify what happens after the drug is given. So what has happened, once drug was administered, the drug made some kind of conjugate with the β-D-glucuronic acid and that basically was also detected in the NMR spectrum. So depending upon how the metabolites went, what they found in the previous slide that two diastereomeric form of β-D-glucuronic acid conjugate of 40-hydroxyfluorobiprofen was

there and the resonance could be identified to resonance at 6.91 and 4.72 and other aromatic complex was coming at 7.19.

So using this signature one can identify that where these are coming. So for this drug the resonances came at the 5.49, you can see somewhere here the signals were coming very high.

So, somewhere here the signals were coming from the drug and the other signal come from the D-glucuronic acid were located between 3 to 6 ppm. So one can identify all these resonances and this paper that I was referring, they actually analyze all these and identify that how the drug travel, what adduct it made. The good part that this example emphasizes on that if we can couple HPLC with NMR, one can identify all sorts of modification happening. So first you purify using HPLC and then go and record NMR and that gives wealth of information in this case. Then another example that I want to show you is use of 13C NMR for drug metabolism study.

So these are the drugs, phenacetin and phenatidine. So basically these were administered and after one hour of administration, basically the carbon-13 NMR was recorded from the healthy sample and also from hepatitis B suffering patient. You just look at the spectrum that is there and TSP was used for internal reference. You can see lots of modification is happening, lots of extra peaks are coming and that helps us in identifying what is the fate, how the two person responds. So you can see, the peak differences that are coming here and additional peak that appeared at 66.9 ppm.

So one can quantify it and look at the effect of this drug on a normal person, on a person who is suffering from acute hepatitis. So these two examples demonstrate the application of NMR in understanding the drug metabolism in a quantitative manner. Next class I will be discussing how we can use NMR spectroscopy for in vivo detection of drug metabolism. Till then I think it will be good to have questions from you.

So looking forward for a healthy discussion over question and answer session. Thank you very much. Thank you.