PHARMACOGNOSY AND PHYTOCHEMISTRY

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Week 11

Lecture 53

Quantitative Evaluation of Herbal Drugs Using Chromatographic Methods (Part 2)

Hello everyone, and welcome back to the NPTEL course in pharmacognosy and phytochemistry. We are studying quality control methods of herbal drugs, and so far, we have covered the chemical methods of drug analysis. Now, if you recall, the chemical methods of drug evaluation includes qualitative methods which we have discussed that includes

microchemical, limit tests, and TLC. As for quantitative methods, we have procedures for determining different constituents using simple chemical tests as well as chromatographic techniques. In previous sessions, we covered one of the chromatographic techniques, namely HPTLC. In this session, we will focus on HPLC as a method for evaluating herbal drugs.

Not only is HPLC used as a chromatographic method for quantification, you will see along with HPTLC, HPTLC, these chromatographic methods are also used for determining pesticidal residues, aflatoxins, and residual solvents. However, for residual solvents, gas chromatography is more preferred. But for determining pesticidal residues and aflatoxins, both HPTLC

HPLC, as well as UPLC methods, can be used for quantification and determination. So, let us explore what HPLC is and what the HPLC methods entail. HPLC stands for high-pressure liquid chromatography, also called high-performance liquid chromatography. It is very similar to HPTLC. If you remember in your HPTLC, we had a thin layer of

what is called the stationary phase. Now, this stationary phase was silica many times, or it could be alumina or cellulose. Now imagine this stationary phase—instead of putting it on a plate, if we fill it in a capillary or a tube. What is going to happen is exactly the same phenomenon, but rather than running in a planar manner, it would run in a three-dimensional manner.

Not only that, if you take this silica and further reduce the size of the particles, or the resolution of the bands—or the resolution of the substances depending on their polarity—will also enhance. But at the same time, what happens is As the particle size decreases, what happens is there is more resistance. So your mobile phase will not flow naturally, but it has to be pushed through.

This pushing is what requires pressure. And hence, this technique is called high-pressure liquid chromatography. So, let's learn about this chromatographic technique. Again, when I have to analyze my herbal drug material or herbal formulation, polyherbal formulation, or any other formulation—say, for example, your shampoos, your toothpaste containing

all these herbal materials—I will take those and again do a sample preparation. This sample preparation will extract the molecules to be determined from these compounds, and then I will subsequently inject or insert this compound into a chromatographic column now how this is done is you can see here this takes help of your mobile phase this is the same mobile phase that was used in tlc but because i said the particle size is fine now

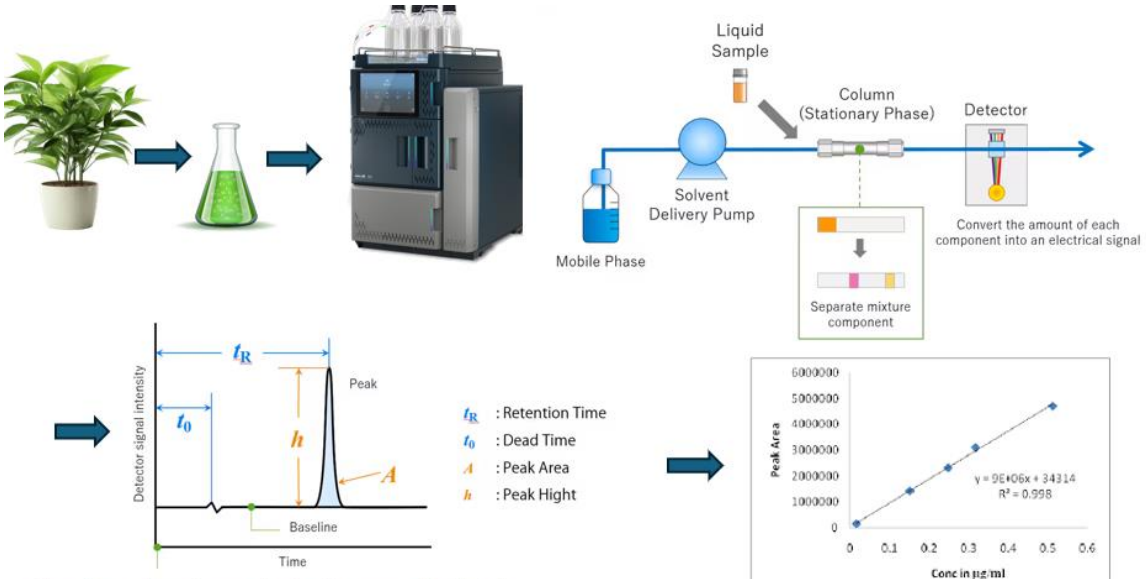
it requires a pump to push in so this is what you call it as a solvent delivery pump and this is actually your plate but this is your plate in a three-dimensional form so it's actually a pipe filled it with your what is called as stationary phase, which could be a silica or sometimes depending upon if it is a reverse phase, it is octadecyl coated silica and so on. So here, now if you remember in your TLC, we used to spot the sample either manually or with help of applicator.

In this case, the sample is actually injected in a pipe. And this injection is done manually or it could be done with the help of device called as auto sampler. Now, once inserted in this pipe or once inserted in this mobile phase, it mixes with this mobile phase and starts

traveling through the column. Now, as it is traveling through the column, what happens is a very same phenomenon that we observed in TLC.

As it moves, it goes on resolving. So, you can see at the entry point, many of the compounds are together. But as it is moving through the column because of its affinity towards the stationary phase or towards the mobile phase, Depending upon its polarity, it is going to partition between your stationary and mobile phase and eventually elute out. In TLC we stopped at a point say about 90% of the plate and then we measured retention factor.

Imagine if we don't stop the plate what is going to happen slowly slowly each of this band is going to run forward. Now we allow in HPLC this bands to move forward so much so that we allow it to run out of column. Okay, so now once it runs out of column, it will come back to the tube. Say for example, this orange is your mixture. It has separated into two components, pink and yellow bands as you can see.



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Now this pink and yellow band are traveling through this and what happens is your yellow band will run into your mobile phase again. Now it has come out of column. Now this will travel with the mobile phase and reach the detector where it is quantified. So it will give you a signal or it will give you a peak based on its UV maxima. Again because after this

there is solvent again there will be a decrease there will be no UV absorption till your second compounds come in.

Then again you will see some UV absorbance. So say for example I have one compound or this pink compound eluting out. So this pink compound elutes out. Now what is this is it is seen or it can be visually represented by a chromatogram. So this HPLC chromatogram have been taken from the website of Shimazu.

So what is done is the output is seen something like this on your computer on basis the software. So you have something called as time. So how much time is required for this peak to come out and reach the detector? So, how do you do this? So, this is the point where you actually injected the sample.

Remember this. So, this is the time when your actual runtime, your computer starts calculating. The moment you inject, it's going to sense that you injected and start counting the time. So, this is something called your injection time. And then you will see that.

There are certain solvents or residues which you use in your sample preparation. Those do not retain on this column. So, those will flow directly. So, from your injection, say for example, you prepared your sample in methanol. So, this methanol will not be restricted.

It will come out. Now, even methanol will be slightly detected by the detector. So, this is what is called your solvent peak or often referred to as T0. T0 is the time taken from your injection time till a point your solvent mostly the solvents which do not retain come out. After that, you will get again a plain baseline because nothing is coming out.

Your baseline is basically a mobile phase coming out as it is unchanged till then you see your substance coming out. Now, depending upon that, if you have a UV detector, this substance is absorbing and slowly, slowly you will see the substance coming out and it will be a nice peak effect. Now this peak will have an intensity depending upon how much it absorbs, depending upon its concentration as well as you can measure the peak area.

So there are two components, there is peak height and there is peak area. Both of them are measured and then what you do is for quantification purpose, you plot a graph. You plot a

graph of peak area here. versus concentration in ml. So when you plot this you might get a linear curve and based on that you might want to plot your sample

and you will get your unknown concentration. So this is in brief or in summary how your HPLC works. So there are few parameters we need to see in a manner very similar to your HPTLC. The first one is sample preparation. Now, the sample preparation for your HPLC also remains almost the similar.

So, you have to consider the same parameters. What is the concentration of my phyto constituent in the plant? Accordingly, I will have to extract my raw material. What is the solubility of my phyto constituent in the extracting solvent? What is the stability?

Is it going to be stable? Is it going to be completely extractable or not if it's not going to be completely extractable if it has a little less solubility like I said in the previous session, you could use a Soxhlet extractor to ensure more complete extraction. After that, you should see that other impurities.

Now, these other impurities may be very related substances. So if you see cinchona and I want to check quinine. You could have your quinidine, cinchonine, and cinchonidine coming out. So these related substances or related alkaloids. Do they have the same solubility?

Is there any way I can get rid of those to selectively quantify only quinine? These are some things. So if I know my quinine is soluble in one particular solvent in which these other compounds are not. I would prefer such solvent for selectivity. But at the same time, I should ensure that the extraction is complete because my aim is to quantify here.

So one more thing which here differs from your HPTLC is in this case, you're going to run the sample. So we just saw if you remember this, we just saw we are going to inject this. the sample in this. Now, say for example, my mobile phase is water. I'm just giving you a random example.

My mobile phase is water, but I have extracted my sample in alcohol and my compound, say for example, or routine quercetin are not soluble in alcohol, are not soluble in water. So, in that case, what happens is the moment I inject dissolved quercetin, in methanol and

I inject it to a mobile phase, which is just water, it is going to precipitate out because your injection is just going to be like 5 microliters to 20 microliters, but your mobile phase is in ml quantities.

So this, because of its insolubility, will precipitate out. Worst case, it's very close to the column. So all this precipitate or particles will go and clog the column. That should not happen. And for that reason, during sample preparation,

especially for HPLC, sample before its injection is kind of solubilized in the mobile phase. So even if my mobile phase is acetonitrile-water, 90-10. I will take my alcoholic extract and first solubilize it. Then I will filter it so that nothing remains.

Now here, I have to ensure that complete solubility is achieved because if I filter it and all my analyte stays on top of the filter paper, that will not be an accurate analysis. So I need to take into account the solubility, especially the solubility with respect to the mobile phase. In some cases, buffers are also added to mobile phases, such as phosphate buffer, to enhance the solubility of the substances.

After that, despite that, we still don't want any particles to clog our column. So we use what are called syringe filters. So once that is done, you take the extract and pass it through a syringe filter. You can see there's a cartridge in this. There's a filter film or a filter paper-like membrane.

And then what comes out is actually a clear filtrate. Now you do this, and that is what is injected here. So I'll give you one example where your sample preparation is used very effectively, as you saw with your alkaloids. In your alkaloids, we said that the free bases are more soluble in organic solvents, whereas the salts are more soluble in water.

So if my mobile phase is water, In this case, it is better for me to extract my alkaloids in acid because this acid will convert alkaloids into alkaloidal salts, and these alkaloidal salts will easily dissolve in my mobile phase. Now imagine if I basify and then extract my alkaloids, my alkaloids will be as free bases. Free bases are water-insoluble.

The moment I inject it into my HPLC, my alkaloids are going to be So due care must be taken, and many times in that case, we use what is called a guard column to protect our

analytical column. So what is this guard column, and what is this analytical column? Let us see the different columns that are used.

Now, there are two types of columns principally used in HPLC. One is what is called an analytical column, the column which actually resolves the substances into bands or different peaks, as we saw, and guard columns are a kind of We just saw that due to some incompatibilities between the sample and the mobile phase,

there are chances that your substance might precipitate. In that case, if you see the particle size, the particle size of the analytical column is very fine. So if you see it, just 2.5 to 10 microns. So there is a very high chance that I will clog it. What will happen as it is, I am pumping it with high pressure.

Imagine pumping a column that is already clogged. The pipeline would kind of push it to the extent where the machine starts leaking. So, to prevent that, what I can do is put a guard column, which is a little bigger in particle size. So if you see here, your guard column has a particle size of about 40 microns. That is like a good filtration media, and it will block most of your particles without clogging your analytical column.

So guard columns are very tiny, about 2 centimeters wide. in length and just about 4.6 mm internal diameter. But this are kind of saviors when it comes to your herbal extract, especially when you have a poly herbal extract you don't know what is going to precipitate out when it's mixed with your mobile phase. So guard columns do protect it.

And then this is subsequently followed by analytical columns. Analytical columns are the ones that actually resolve your substances. Depending upon their stationary phase, they are classified into different classes. This includes reverse-phase columns where your silica is coated with something like a C18 chain or a C8 chain. So that is very lipophilic.

Phenyl pentafluorophenyl—this is the column base, or this is a stationary phase, which is mostly used for the determination of pesticidal residues. Then you have amino and cyano columns. Amino and cyano columns are generally used for polar compounds. This includes your carbohydrates and water-soluble compounds.

Your column length would be anywhere—especially the analytical columns. You can see here. They are anywhere between 10 centimeters to 25 centimeters. Whereas you can see here, these are your tiny guard columns. This could be somewhere between 1 to 2 centimeters.

Now, most of your phytoconstituents, if you go to see, can be detected by your C8 to C18. The majority of the assays, especially if you see the pharmacopoeial methods, are all done by C18 columns. So now, going up from the stationary phase to the mobile phase, what mobile phase do you use in HPLC? So if I am talking about the normal phase, in the normal phase, my stationary phase—that is, my column—is polar. That means my mobile phase has to be nonpolar.

In that case, your solvents such as hexane, heptane, isopropanol, ethyl acetate, or even tetrahydrofuran. So you'll be using more of a lipophilic solvent. Whereas if my column is a reverse-phase column, that means something like your C18, it's extensively lipophilic. In that case, my mobile phase will be polar. So this includes solvents which are very polar, that is water, methanol, and certain types of buffers.

To enhance the solubility, we can add phosphoric acid or formic acid. Acetonitrile is also used in case you want to make it more non-polar. So These are the different mobile phases. Now you can have a mobile phase, and HPLC has a slight advantage here: when

you did your HPTLC, you had a fixed mobile phase. You could not change it, and the whole plate runs like that. In your HPLC, you can change your mobile phase with respect to time. So, if your mobile phase is fixed—say, for example, methanol 90 or acetonitrile 90, water 10—and throughout your analysis, if that is the mobile phase running, I call it an isocratic mobile phase.

But at the same time, if I want to change it, Now, this is generally done for faster elution and sharper peaks. In that case, what is done is after every minute I will change it. So maybe initially I started with acetonitrile water 90-10 but after say for example 1 minute I change it to 80-20. After maybe 5 minutes, I change it to 65-35 and that goes on.

So what happens is I am gradually changing the polarity of my mobile face. Initially, acetonitrile was very high. That means it was little non-polar. But later on, the acetonitrile came down to 65 and water increased to 35. So from non-polar, I went to a little polar mobile face.

So I can do that and that helps me achieve a shorter run time and much, much sharper peaks. So, yes, you can use or you can play with your mobile face to do that. Also, the pharmacopoeia methods of especially your amla also uses what is called as gradient elution as per the Indian pharmacopoeia. Now going to the detection.

Now this compounds are coming out. How do you detect it? There are different type of detectors. The most simple detectors which were there in your colorimetry which were there in your HPTLC as well as which are there in your HPLC includes your UV-Vis detectors.

So the moment your compound comes out, there is a particular wavelength being measured. Same as your UV spectrophotometer. And this will measure everything that comes out with respect to time. So you can see here, for example, when I inject a cinchona extract, you will see that this is the solvent peak.

T0, and then what happens is, slowly, compounds start eluting out. These compounds range from polar to extensively nonpolar if it is a C18 column. So first, at 5 minutes, your cinchonidine comes out. So I set my wavelength, for example, at about 223 nanometers. So I see one peak—that's cinchonidine.

After some time, your cinchonine comes out. After some time, your quinidine comes out at 7.5 minutes. At 8.3 minutes, your quinine comes out; at 9.7 minutes, So you will see that, based on their UV absorbance, your compounds become visible. Now, in some cases, it happens that the compounds you want to analyze absorb at a different wavelength.

For example, quinine might absorb at 223 nanometers, but an anthocyanin like cyanidin may absorb at 500 nanometers. In that case, I can use what is called a photodiode array detector. A photodiode array detector measures all the wavelengths. So I can quantify at 500 as well as at 200 simultaneously and still estimate both of them.

Now there are certain compounds like quinine sulfate which we measure by spectrofluorimetry. This can be measured, or this quinine can even be converted to quinine sulfate and checked for fluorescence using fluorescence detectors. In some cases, when you want to detect the compounds, a good technique is to analyze their mass. So this LC is given in a hyphenated form.

Output to a mass spectrometer or a mass spectrophotometer, and in that case, what you will get is the mass, which will help you identify each of these peaks. In some cases, you use what is called an electrochemical detector, which measures the current. And conductivity detectors, which measure the conductivity. Now, in some cases, what you will observe is that the UV properties

as well as fluorescence properties are not very good for a given substance. So in that case, you can just use a simple RI detector. They are very primitive but still, I mean, not as sensitive as your current detectors such as ELSD. They do the work of RI detection and can quantify compounds even those which are not UV-absorbing. Then you have your evaporative light scattering detectors,

wherein the mobile phase is evaporated, and then whatever residue is left, the light is scattered through that. So you will see that depending upon what the residue is, you will get the light scattering, and it is the most sensitive detector as far as HPLC is concerned. Now, let us see some examples. Here is a method of estimation of digitoxin using the British Pharmacopoeia method.

And you can see it's a very simple method which uses a reverse-phase column C18 and digitoxin. A mobile phase is what you call it as isocratic. It's not changing. Fixed mobile phase, water acetonitrile 72 is to 28 because it's a glycoside. So I need a more polar mobile phase with UV detection at 220 nanometers.

So when I inject it, what I'll get is you can see digitoxin somewhere here. Elutes at your 5.3. So you can see it in the standard, you can see it in the sample as well. So I can use that for quantification if I know the area under the curve as well as the concentration of the standard. Now in some cases, not only just quantification, it is also like your HPTLC where you can develop a chromatogram.

So if you remember, we just discussed quinine. So what is here is a typical fingerprint of cinchona. I can use this fingerprint and then I can analyze another species of cinchona, legeriana, saxirubra, officinalis, and I can check whether the amount of these alkaloids is the same. The area and the curve of this is same or is it different?

In some cases, even if there are unidentified substances, I can still see the RT of peak. And I should say that if it is Synchona officinalis, that unidentified peak should be seen at this particular retention time. So it helps me in identification of plants. by a fingerprint and understanding the species variation it helps me in quantification validation and standardization of raw material because once I quantify I can easily understand

if there is any seasonal geographical or any variation brought about not only that I can see the foreign peaks which should not be there in my sample and I can tell if there is adulteration Again, I know the fingerprint. So if there is some synthetic drug which is provided to me, say for example, some other lipid which has been a synthetic lipid, the HPLC-RT, that is the retention time will change.

Synthetic colors because they will absorb at a different wavelength and other plant materials. Not only that, if you use your fluorescence detector, you can even quantify your aflatoxins. This is a very good method in terms of its sensitivity. It outperforms your HPTLC as well and hence can be used for detection of even small nanogram or picogram quantities of your analyte in your herbal formulations.

Now we will move on to the next part and that is the determination of pesticides. Now pesticides is a big issue especially with herbal drugs because most of the herbal drugs become infested by pests. And in order to prevent it, your farmers often spray it with numerous pesticides. But an important quality criteria when we are manufacturing herbal medicines is this pesticide residue should stay within the limits and should be safe for consumption if it is not possible to completely eliminate it.

So in some cases you will see that limits of certain pesticides have been specified by the pharmacopoeia itself. Say for example for ethion it is 2 mg per kg. It should not exceed that. For methoxychlor it is still minute that is 0.05 mg per kg of body weight. But there are certain substances which are new.

We don't know. In that cases, your WHO or most of the pharmacopoeias give you a formula of limit of pesticide, which is your acceptable daily intake. That is how much you can consume safely. per day in terms of mg per kg this values are often given by WHO into your body weight mostly it is taken as 60 kilos

and this is divided by what is called as daily dose of herbal drug so if that is a formulation how much of that drug you are going to consume per day multiplied by 100. So this is how you kind of estimate it but now your pharmacopoeias majority of them the limits have been laid down for newer pesticides

this is the one what you are using. Now, this determination of pesticides, how do I carry out? Now, this determination of pesticide has a very interesting sample preparation methods which have been standardized. It is called as your QuEChERS method. Now, QuEChERS refers for quick, easy, cheap, effective, rugged, but safe method for sample preparation.

Now, this has not only been used in pharmaceuticals, but also in foods and many other industries where the determination of pesticidal residues is vital. So, QuEChERS method, I have just taken it from their site. You take about 10 grams of sample. This could be a fruit, vegetable, herbal drug, raw material, or a formulation.

Now, to that, you add about 10 ml of acetonitrile. Now, what happens is when you take fresh fruits and vegetables, they naturally have some water in them. So, you need not add water. But in cases where your raw material or herbal drug material is dry, your acetonitrile often is not able to soak in properly.

So, for 10 grams of sample, you first macerate it with 10 ml of water and then with 10 ml of acetonitrile. But if it is a moist or wet drug, like a vegetable or fruit, in that case, direct acetonitrile is also good. In some cases, you can add an internal standard to check. If I have added 1 mg of internal standard, can I quantify that back? So, if it comes back as 1 mg even after analysis, that means I have extracted my compound completely.

Otherwise, it's an incomplete extraction. So, I'll deliberately add an internal standard to check if I'm extracting it properly. Then I will kind of pulverize properly. Or grind it. Make the sample into fine paste to facilitate extraction.

And then what I do is to that I will go on adding salt. Now you know as water becomes saturated. Water and acetonitrile initially are very nicely miscible. But as water becomes saturated with your salt. Especially NaCl and magnesium sulfate.

You will see the acetonitrile phase separating out. We want that. So that phase separation happens and it can be fastened by centrifugation. So you can do a centrifugation and take that acetonitrile layer. This acetonitrile layer still contains lot of other impurities including your compounds.

So take for example, if you extracted turmeric, it will contain curcuminoids plus pesticides. So in some cases, a sample cleanup is recommended and So this sample cleanup is done by a solid phase cartridge and the solid phase cartridge is the one which is kind of loaded. with the help of a mobile phase in your HPLC itself or sometime it is done separately on a different pump and then you change the mobile phase to cause unloading so this solid phase will take up most of your impurities

and leave only or leach out only the pesticides so majority of your cleaning up happens there in some cases sorbets such as PSA is used and then Eventually what is eluted out is a very clear solution containing pesticides. Now once this pesticide containing solution is ready with you, then I just need to inject it in my HPLC and I need to analyze it. So here are a few examples wherein your HPLC has been used for analysis.

So this is a column called as newcrome BH by SELEC. Now here what they have done is they have used acetonitrile water and for creating that little pH gradient they have used H2SO4. So it's a gradient mobile phase you can see here initially. My acetonitrile is 10 water is 90 and sulfuric acid is 0.05

but after 5 minutes I am going to change acetonitrile from 10 to 50 water from 90 to 50 and sulfuric acid from 0.05 to 0.02. When I do this and I just keep it for a hold for another five minutes, I can see my most of the pesticides being separate out. So you can see here there's

a list of pesticides, the numbers of which which are placed here. So you can see your monocryptophores, you can see your metribuzin and all of this depending upon their mobile phase will have a slight different illusion and slightly different what is called as area under curve.

So I can use different different mobile phase, optimize it depending upon the nature of pesticide that I want to determine. Not only that, the industries now have started taking into account your machine learning algorithms. So this machine learning algorithms, if I couple my output with a mass spectrophotometer, my machine learning algorithm with a pre-saved library.

Will tell me what exactly the pesticide is. So I need not have my pesticide standards. The library search, along with ML algorithms, will rightly tell me that this is my drug. This is the content of my actives, and this is the amount of pesticide that it contains. The same method or same technique is also applied for the detection of aflatoxins.

So, yes, HPLC is a very important tool as far as quality control is concerned. It is going to help me in quantification and detection of adulterants. Species identification, standardization of my formulation, not only that HPLC also helps me in understanding there is any aflatoxin present, if there is any pesticide present, and nowadays, even in the detection of newer analytes. So here are a few references if you wish to go through more of this content, and thank you, everyone, for your patient listening.