PHARMACOGNOSY AND PHYTOCHEMISTRY

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Week 11

Lecture 51

Quantitative Evaluation of Herbal Drugs Using Spectroscopic Methods

Hello everyone, and welcome to week 11 of the NPTEL course in pharmacognosy and phytochemistry. This week, we are studying various quality control methods for the evaluation of herbal drugs. To recap, in week 10, we covered organoleptic methods of quality control, macroscopic and microscopic methods of quality control, physical methods of quality control, and we began with chemical methods of quality control.

In chemical methods, we also saw that they could be qualitative, telling us what type of compounds are present, or quantitative, indicating specifically how much of the compounds are present. telling us specifically how much the compounds are present. So, let us recap: we covered organoleptic, macroscopic, microscopic, and physical methods, and now we are focusing on chemical methods for drug evaluation.

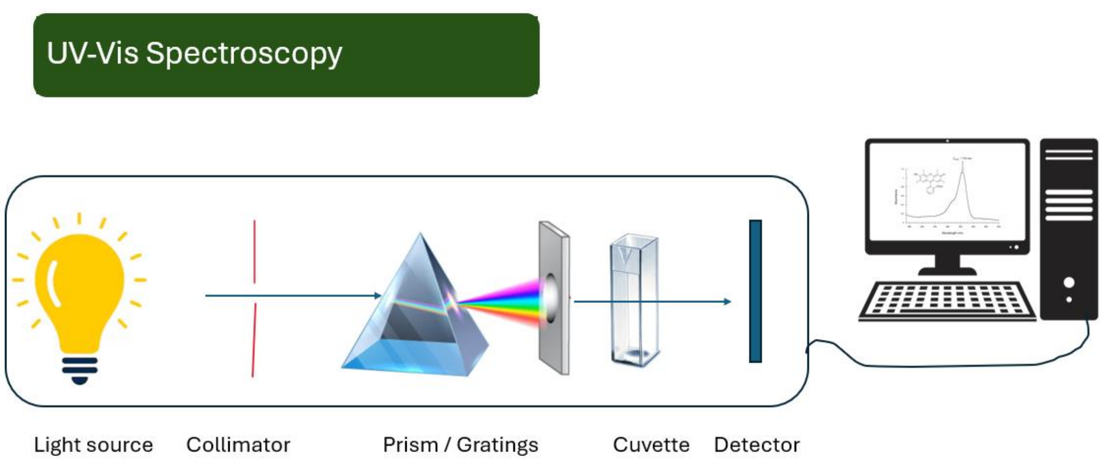
More specifically, we have completed the qualitative method of evaluation. Now, we are moving on to the quantitative method of drug evaluation. In quantitative methods, I want my answers in numbers: how pure is my drug? Is it 90% pure? How much gallic acid do my galls contain?

So, it will be a number, a numerical value that will indicate the quality of your drug. There are different methods for this. When it comes to chemical methods of evaluation, we have already covered qualitative methods such as phytochemical tests. We have finished the microchemical test.

We have done a limit test, especially with respect to heavy metals, as well as thin-layer chromatography to check whether the standard or the marker compound is present or not. In this session, we will focus more on quantitative tests. When we are doing the quantitative test, we are going to assess different spectroscopic as well as chromatographic methods to quantify the drugs.

Especially the phytochemicals. This includes UV-Vis, spectrofluorometry, HPLC, HPTLC, UPLC, GC, and so on. Chemical methods have also been used to quantify toxins such as pesticides, aflatoxins, as well as residual solvents. So let's delve more into spectroscopic methods in this session.

So, what is the spectroscopic method? Let's try to understand with a simple example of UV-Vis spectroscopy. Now, in UV-Vis spectroscopy, you have lamp one corresponding to UV, which is your deuterium lamp, and one corresponding to visible light, which is your halogen lamp. So both of these lamps cover a broad spectrum.



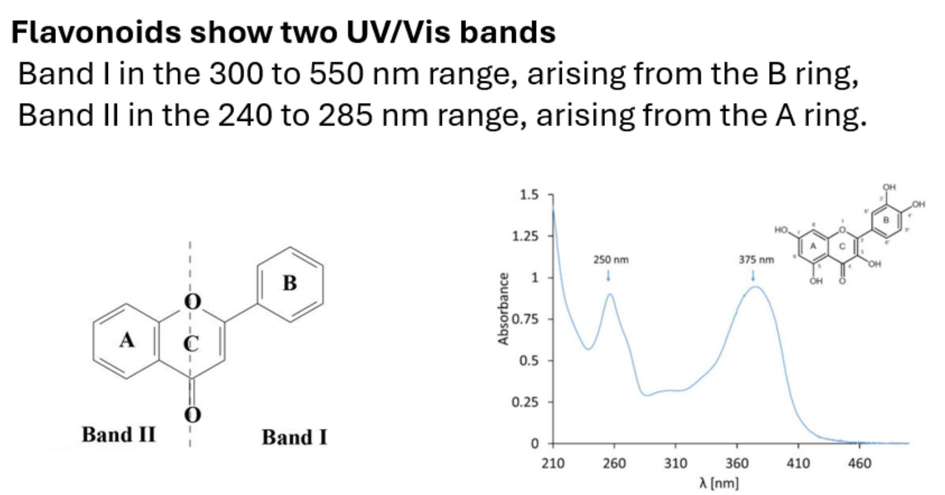
Of light, that is from 200 to almost 800, in some cases even 900 nanometers. Now, what happens is this lamp then passes through a collimator where a parallel beam of light is taken, and then it passes through a prism or grating. Now, this white light splits into a spectrum after it passes through a prism or grating.

Now depending upon my analyte at which wavelength I want to analyze I will set it as using a grating. Once that is done the selected wavelength of light will pass through my sample If my sample absorbs. So if it is called the absorbance value. So per mole how much it absorbs is kind of called molar absorptivity.

So I will put a specific content or a specific concentration of my sample. Say 100 microgram per ml. And I will check its absorbance value. Now when I do that maybe as output what I will see here is. You will see something like this.

That is, you will see a UV spectrum and you will see an absorbance reading. Now, this absorbance reading will tell me how much quantity of my substance is present. So this is in brief what we do in our spectroscopic methods. And let's now understand how we apply the spectroscopic methods to analysis, especially quantitative analysis of phytoconstituent.

Now, take for example, we will start discussing UV, about absorbance values with respect to a very simple compound such as flavonoids. Now, we say flavonoids typically contain a flavonoid nucleus, which is a phenylbenzogamma-pyrone nucleus. And when you saw the flavonoids chapter, if you remember, we placed ring A here. Ring B and later on, rings A and B fuse to form ring C.



Now, if I split this molecule from here, what I observe is what is called a typical cinnamoyl moiety. Now, this cinnamoyl moiety represents your phenylpropanoid moiety from biosynthesis. So this is like your one So in addition to your phenyl, you have a C3 addition, and that's why we call it your cinnamoyl moiety. Now, on this side, if you see carefully, you just have a plain phenyl or a benzene ring.

Now, this phenyl ring and a cinnamyl ring or conjugation—when I compare in UV—what is important is the wavelength of a substance or a compound depends on the nature of single as well as double bonds. So single bonds mostly absorb in the UV region, whereas if you add conjugation, as you saw in carotenoids, the more the conjugation, the more colored they become.

So in a similar manner, there is a rule or certain rules which can be applied to the compounds, and these are like your Woodward-Fisher rules. So when you do that kind of addition, you can predict at what wavelength your compound will absorb. Now if you see carefully if I break this into two you will see that the number of carbons and the number of double bonds on this side is higher, and

especially more important are alternating or conjugated double bonds. So you can see them very nicely in cinnamyl, which has about 3, 4, 5 double bonds, and all of them are conjugated. Then you compare it with A, which has 3 conjugated double bonds. So what you see here is the spectra of flavonoids split basically into two.

This part is called your Band 1, and this is attributed to the cinnamoyl moiety. This part is called your Band 2. And this is attributed more to your phenyl moiety. So you will see most of Band 1 is because of your B ring and partially due to the C ring. Whereas your Band 2 arises from your A ring. Now if you see carefully, you will see most flavonoids showing this pattern.

So depending upon what UV maxima your Band 1 and Band 2 have, remember your Band 1 is always going to be at a longer wavelength. So Band 1 of anthocyanins goes as long as your colored range, and that is the reason you will see most anthocyanin pigments are colored, like your flower petals. And your Band 2 still stays in your UV region.

Your aurones are about 380 to 430. Chalcones, 340 to 390. There is definitely some overlap but not a complete overlap if you see it carefully. Flavin 3-hydroxy substituted or 3-alls where the 3-hydroxy is substituted by a glucosyl. In that case, you will see it shows a band 1 at 328 nanometers and 360 nanometers or between this range.

But when the 3-hydroxy is free, like you saw here, in this case, the 3-hydroxy is free. In the case of routine, what happens is your sugar is attached. So when the sugar is attached, you get somewhere between 328 to 360. When it is free, you get a slightly higher wavelength out there. But your band 2 remains the same because that side is not involved clearly in any bond formation.

Similarly, you can have your flavones, which show a band 1 between 330 to 350. Flavonones between your... 300 to 330 nanometers isoflavones. Now, isoflavones show a slightly what is called a shoulder peak. Now, what is a shoulder peak here is if I say my flavonoid absorbs something like this.

In the case of isoflavones, what is going to happen is my spectra—now this is the absorbance, this is the lambda. So I am just putting. In that case, what is going to happen is this is definitely going to come down in the same way and rise at about 250 nanometers. But my band 1 is going to be a tiny thing. It's not going to rise up.

And that's the reason this is like as good as a shoulder and it is called as your shoulder peaks. So for isoflavones your peak especially your band 1 is converted into a shoulder peak. Now how do you use this data for quantification? Now this quantification I can use or I can set my UV spectrophotometer at a UV maxima corresponding to that particular flavonoid band 1 and band 2 and I can measure the concentration.

Say, for example, I say my quercetin is giving me an absorbance value at 0.8 for 100 microgram. If my sample is also giving the 0.8 value, that means in my sample also quercetin is 100 microgram. But this is not as simple. Why? Because your flavonoids don't occur singly.

In plants they occur with different different other constituents and many of this constituent also absorb in a similar range that is 320 to 380. So even saying that your band 2 especially this one and band 1. very peculiar of flavonoids that doesn't assist me when I am doing quantification from the plant extracts if I am doing the quantification of pure actives pure quercetin definitely I can directly use this but when I am using plant extracts because other compounds are interfering I will have to create some colored complex so what do I do I create colored complex with metals now imagine some metals like your aluminium okay Imagine some metals like your aluminum. So in this case, what is going to happen is your Al will try to chelate itself. Now, Al is like a 3 positive, right? So it will try to chelate with a negative.

So it forms a kind of complex, and this complex then polymerizes. See, all the three valences are not satisfied. So what is going to happen? It is going to take another hydroxy also. Probably this hydroxy of the next flavor.

And it gives a nice structural attribute to that, which results in the formation of a nice blue color complex. And that blue color complex, I can quantify it. So initially, I had my band 1. Here at 360, I had my band 2 at 250 nanometers. But the moment I add my aluminum chloride to it, what do you see?

What happens to the wavelength is called a bathochromic shift. That means it now absorbs at a slightly longer wavelength. So now it is absorbing at 425 nanometers instead of 630 nanometers. What does that do? Now that moves my wavelength from a UV or near visible to almost a good colored wavelength and

the reason why I will get more accuracy when I measure it in this because all my compounds are not going to absorb in this region. The majority of them absorb between 200 to 350 nanometers. So when that major chunk is taken care of, I can selectively get my absorbance of flavonoids at 425 nanometers. Similarly, I can use other metals.

I can use iron. I can use tin. I can use zinc and other metals and so on. So it forms complexes with most of the metals and gives you colored complexes. The color with aluminum chloride is more predominant and lasting.

So how do you do this? In this particular test, when I have to quantify or carry out a total assay—assay means quantification of flavonoids—I can do it by preparing my sample extract. Now, when I am preparing the sample extract, the most important concern for me is which solvent to choose. Now, when we did a chemical test for

flavonoids, if you recollect, a very good non-specific solvent for extraction of flavonoids, both aglycones as well as glycosides. So glycones as well as the aglycones can be that is the glycosides can be extracted from using ethanol as a solvent. So I'll take one gram of drug and I'll probably boil it with about 100 ml of sample or so and I'll start preparing a stock or a sample stock.

So I'll take this sample stock and the sample stock I'll take about points, you know, approximately in this range. Now, this range is taken for this particular assay that I've used. But depending upon the flavonoid content of your drug, this value may vary. Say, for example, when you say Sophora, which contains 20 percent flavonoids. You know that whenever you have to do quantification by UV, you have to have the reading less than 1 absorbance.

And it is said that less than 1 absorbance perfectly aligns with the Beer-Lambert's law where absorbance is proportional to concentration. But when you move on the higher side, that is absorbance value more than 1, the Beer-Lambert law deviations start. So we don't want that to happen and depending upon the drug this value is optimized. So you just take about 0.5 ml of a given value that you can decide depending upon the flavonoid content.

Then some amount of alcohol to dilute it and then you add your reagent. Your reagent is required in a very small quantity that is just 0.2 ml of 10% aluminium chloride and about 0.2 ml of your buffer that is your potassium acetate. You dilute it and let it react. Now, what is going to happen is when you let it react for 30 minutes, you will see that slowly, the solution is turning

bluish in coloration. In some cases, it is even kept in the dark to eliminate any effects of the atmosphere on it. So, once that is done, you compare it with your standard. Now, similarly, as you prepared your extract, prepare the solutions with quercetin standard so that each test tube contains 2 micrograms or even 15 micrograms.

So, somewhere between 2 to 10 to 15 micrograms per ml. You will prepare a stock and then react it with alcohol, 0.2 ml of 10% aluminum chloride, and 0.2 ml of 1 molar potassium acetate. So, when this happens, what is going to occur is it will show you an absorbance value, and we are going to record it. So, when you are recording it, say, for example, you have your 2, 4—this is just me putting it as micrograms per ml—or 2, 4, 6, 8, 10 micrograms per ml.

And this is the absorbance value. The maximum value I can go up to is 1. So, maybe I am just putting here as 0.5. 0.25 and 0.75. So, I will start preparing what is called a standard curve.

So, when I do a standard curve of quercetin, I may get some linearity, meaning my absorbance increases as the concentration increases. So, I might find my spots or that is five spots like this so when I draw a line through this this might be almost very linear now what happens is I will check the absorbance of the unknown that is my plant extract and say for example

this is the absorbance of my unknown so what I do here this absorbance value I'll just extrapolate it back And wherever it falls, say for example 9, then 9 microgram per ml is the concentration of quercetin or flavonoid in terms of quercetin in the given extract. So that is how I used to quantify. Now if you use any dilution, you multiply it by your dilution factor.

So this is how total assay of compounds, especially flavonoids is carried out. Now, moving on to the next one, you can also do the same for your tannins or polyphenolic compounds. Now, when I say polyphenolic, polyphenolic includes anything that has dihydroxy and a phenyl group. So, this can be a gallic acid or any other compound which contains your polyphenol. So what happens in the folin-Ciocalteu method?

Now folin-Ciocalteu method is a specific method for polyphenols and this is based on its reaction with what is called as phosphomolydic and phosphotungstic acid. Again if you just check plain absorbance of gallic acid you will get 225 and 265. Like I said most of the compounds absorb well and they will absorb below 350 nanometers

and that's why this region becomes very crowded as far as the plant extracts. Now if by some reagents we are able to move the wavelength to a higher side that is the detection wavelength that becomes a good method of quantification. So in this folin-Ciocalteu method, we add a base. Now, this base is generally sodium hydroxide, or if you want a milder base, you can use sodium carbonate.

The intention here is to bring the pH value to about 10. So, pH 10 is what we are looking for. And at pH 10, what is going to happen? These groups are going to become ionized. So that they will react with Na and become -ONa

So, they go on becoming ionized, and eventually, what is going to happen is when they are ionized, they tend to react with phosphomolybdic and phosphotungstic acid. So, when they react with phosphomolybdic acid and phosphotungstic acid, there is a slight change in aromatization. You will see all these oils becoming ions.

So, you will be seeing a lot of quinones. You will be seeing a lot of negative charge, and what is going to happen is it is going to release the electron. This electron will convert your phosphomolybdic acid into I am just drawing it roughly. I am not putting a quantitative balance, but just for your understanding.

You know, this 12 I can just split into 4, and this, so 4 plus 12, eventually it will make it 12 over 40. So what is going to happen here is it's partially oxidizing so this will remain plus 6 this will remain plus 5. So partial oxidization happens this electrons go here change in and this gives what is the blue coloration. So this happens in an alkaline pH and that is why it is so important. Now once this reagent is changed to a partially you know like a

converted compound. In that case, you will see that this reagent will now absorb at 750 nanometers. Now, this is what correlates with the absorbance maxima and this is what we are going to use for quantification of your polyphenols, especially tannins and flavonoids using your folien-Ciocalteu method.

Again, if you draw a curve of absorbance versus concentration, It will find a good correlation and definitely you will be able to spot the concentration of the unknown. Now the next method which is used again is a colorimetric method and that is for determination of steroids. We can also determine and this is the same test. You remember your Liberman-Burchard test.

This is the same test what you are going to do is but you are going to be little selective. So you're going to take your steroid containing drug. You're going to extract it in chloroform.

The reason is if you see your lipophilic compounds, your lipophilic compounds, especially your steroids or triterpenoids, they will be more soluble in your chloroform.

In some cases where you have to determine the saponins, in that case it is advisable to do the hydrolysis first so that the sugars are cleaved and thereafter you are going to extract it in chloroform. In that case, the aglycone part of saponin gets extracted in your chloroform layer. Now you are going to take this chloroform layer and dehydrate it. The reason why we dehydrate it because the presence of moisture kind of retards the color as well as the reaction.

So you will treat it with about you know acetic anhydride. First we'll check in case if it is insoluble it is always advisable to dissolve it with little amount of glacial acetic acid. But if it is nicely soluble in chloroform no need to do it. Now you're going to add acetic anhydride that is almost 19 volumes and then sulfuric acid 1 volume. So in that ratio when you add it it is said that your compound is

gets converted into acetate and then sulfate and later on what is called as the sulfonic acid so it kind of dehydrates and you know releases or causes lot of unsaturation this unsaturation later on kind of aromatize now in addition to that what you will get is also a sulfonic acid derivative So SO_3H okay so in this case when you get a sulfonic acid derivative now what happens here is This sulfonic acid derivative is or the sulfated products or sulfonated products give you greenish coloration

and I can quantify this greenish coloration at 625 nanometers. So the amount of steroid present I do a standard curve and again I will be able to quantify how much amount of steroids are present in your given sample. Having used chloroform as an extracting solvent, I will be very specific and as a result other molecules will not form a sulfonic acid derivatives.

Now, the next one is, in some cases, there are molecules which produce fluorescence. A good example of that is quinine sulfate. So, quinine per se doesn't have much fluorescent property, but if you convert quinine into quinine sulfate, it produces fluorescence. Now, what is fluorescence?

Fluorescence is when the compound absorbs one wavelength. That is, you can see it is absorbing 317 and 349 nanometers, but it is emitting at a much higher wavelength. So, in this case, when it is emitting radiation, it is emitting at about 481 nanometers. So, in this case, you have to change the UV. In the UV, we saw that there is a source, which is the lamp.

Then, you have your collimators, then you have your prism, and from that, you are going to choose a monochromatic light through your prism or grating, and that is going to fall on the sample. Now, that sample will absorb, and whatever doesn't absorb reaches the detector. That was your UV principle.

Now, in the case of fluorescence, because this is going to be one wavelength. This is going to be, say, for example, 317 nanometers. My detector is not going to be set at 317 nanometers. Interestingly for fluorescence I will not measure it in this manner. For fluorescence you know my compound is emitting.

So I can measure it more perpendicular to the incident layer of light so that whatever this 317 is passing which is not being used by the compound let it go this side. So I am not going to put my detector here. I am going to put my detector here and as a result only the wavelength that is emitted due to the glow that is emitted due to the fluorescence will reach my detector and that is my

481 nanometers. So I will set my spectrofluorimeter which is modified UV to a emission wavelength and excitation wavelength. The excitation wavelength is the wavelength which will cause the electrons to excite and this is your absorbance wavelength that is say for example 370. And then emission wavelength is what the molecule is emitting.

In this case it is 481. So again I can do a curve but in this case what I will do is I will check the fluorescence instead of absorbance. And this is going to be a concentration. So as the concentration increases the fluorescence also increases. Okay.

And then I can quantify by unknown by saying that my unknown shows this amount of fluorescence. So, as a result, its concentration is going to be this much. Okay. So here are a few references to different spectroscopic methods for the analysis of the compound. So there are huge numbers.

But yes, apart from that, your pharmacopoeias and your quality control books from WHO also provide this method. And thank you, everyone, for your patient listening. Thank you.