

Host: This is the podcast from *Clinical Chemistry*. I am Bob Barrett.

Oral fluid, a promising alternative metrics for drug monitoring in clinical and forensic investigations offers noninvasive sample collection under direct observation. Cannabinoid distribution into oral fluid is complex, and incompletely characterized due to lack of controlled drug administration studies.

The August issue of *Clinical Chemistry* reported that measurement of carboxy-THC, in oral fluid, not only identifies cannabis exposure, but also minimizes the possibility of exposure solely by passive inhalation and may be a better analyte for detection of cannabis use.

Dr. Marilyn Huestis is the Chief of Chemistry and Drug Metabolism Intramural Research Program, National Institute of Drug Abuse at the National Institutes of Health. And David Schwobe is a Doctoral Fellow at the National Institute on Drug Abuse. They are both authors of the report and our guests today in this podcast.

Dr. Huestis, the currently proposed Substance Abuse in Mental Health Services Administration Guidelines for cannabinoid oral fluid testing suggest a 4 microgram per liter cutoff for THC in oral fluid. Now, you suggest that the metabolite carboxy-THC may offer a better solution by removing the possibility of passive oral cavity contamination. Do you believe that federal approval of this approach is likely, and if it is approved, do you believe laboratories will embrace this approval?

Dr. Marilyn Huestis: Well, this is a very interesting topic of oral fluid testing for monitoring drug use. There is tremendous potential for this new technology in laboratory testing of workplace drug use, of driving under the influence of drugs, and emergency room as well. In fact, pain management is looking very seriously at oral fluid testing. So it's an important new technology.

The reason the guidelines were written for 4 microgram per liter cutoff for the parent compound, the major psychoactive compound in cannabis, Δ -9-tetrahydrocannabinol or THC is that, that was all we were able to detect until a few years ago.

Then with GC mass spec, tandem mass spec, we were able to find low nanogram per liter concentration of the metabolite carboxy-THC. The reason this is so important, Bob, is because we know that there is THC, the parent compound, in cannabis smoke, and individuals that are in an

environment where there passive smoke could be around could possibly contaminate the oral cavity.

So when SAMHSA developed their guidelines, we had been trying to get oral fluid, sweat, and hair testing approved since as early as 1994, when they came out with these proposed guidelines, they were very concerned about the possibility of passive inhalation.

So what they said is that you must collect a urine sample along with an oral fluid sample in order to detect cannabinoid. So that if the oral fluid screened positive, that then you could go back and test the urine.

Well, this in essence killed the approval and the use of oral fluid testing in federally mandated guidelines, because of the fact of who wants to collect two specimens and then have to analyze the second specimen.

But now that we have been able to identify carboxy-THC, the metabolite that is not present in cannabis smoke, we have the opportunity of perhaps making this the target analyte and eliminating that concern.

In this work, and in our controlled Drug Administration Study that provided the samples for this validation, we were able to monitor low nanogram per liter concentrations of carboxy-THC in oral fluid, and lo and behold, found a much higher percentages of samples that were positive for the carboxy-THC.

Now, additional research is needed, and we have those studies underway now. But when we finish that work, I think we will have a good body of data to help drive scientific base drug policy. And I do believe both the manufacturers and the laboratories would embrace this new analyte, because it would mean more assuredness that there wasn't passive exposure, which means focusing on a single analyte, the carboxy, rather than the THC, and it can be accomplished with instrumentation that are currently available in most laboratories.

Host: Tell us about the way you developed and validated your methods for carboxy-THC, and could that serve as a model for amino assay validations in general?

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Dr. Marilyn Huestis: I believe this is a very good validation of an ELISA methodology. Very complete. It was part of our workup with our research that we are currently doing on cannabinoids, and it evaluated many different aspects of the ELISA assay performance, including, especially rigorous evaluation of, not only the cannabinoid metabolites that might be present

in oral fluids, but also we did a very large evaluation of other potential interference that have not been looked at in most assays that are commonly used. So coffee, mouthwash, orange juice, toothpaste, and other compounds that could interfere were evaluated.

The other aspect that we did that I think was really important is that, we did find that this ELISA assay could provide good semiquantitative data over a very large linear range. That's important, because laboratories would like to be able to predict the concentration range that might occur in their confirmation assays. So the fact that this assay was semiquantitative will save money and time for the laboratories.

Another aspect that we tested was to ensure the variability that might occur and effects of stability on reading different plates, both between plates over time, to make sure that individuals then knew that whether or not they had to read the plate immediately or whether they could work it in to their schedule over the next short period of time. And I think that was important for the actual usability of the kit.

We also evaluated, because we used authentic oral fluid samples collected in our research, we also were able to identify multiple cutoffs, and we found the most efficient cutoff when using two-dimensional GC-MS as the confirmation. I think this is very important and most laboratories don't evaluate this rigorously to do that. And it certainly provides information to policymakers on appropriate cutoffs as well.

We also were able to test, not only THC and its primary metabolite, the 11-hydroxy-THC and carboxy-THC, but we also evaluated Phase II cannabinoid metabolites, the glucuronides, which may be important even in oral fluid. We know that they are very important in urine and blood, but there has been one report of measurement of the glucuronides also in oral fluid.

And then, besides evaluating the controls that were provided by the manufacturer, we also prepared in-house controls, multiple in-house controls to evaluate performance of the assay around the cutoff and other potential cutoffs that we tested as well.

So I think it's a very nice comprehensive immunoassay validation that will serve as a model for other people to do evaluations of many different other assays. So I think it's very complete and rigorous.

Host: Well, what makes it particularly rigorous? What is it about this validation that does that?

Dr. Marilyn Huestis: Well, most of the things that I just spoke about. I think it was very complete. I think we looked at performance around multiple cutoffs. We included controls above and below each of those cutoffs, and we also gave ideas about how people could test different interferences in oral fluid as well.

So it is very complete in looking at all aspects. We even tested things like the hook effect to make sure that really high concentrations of drugs in the oral fluid would not affect the assay performance. We also evaluated the performance of many different cross-reactives and other components that are present for instance in cannabinoids, like cannabidiol and cannabinol. I think that's really key that people fully validate an assay before they put it into routine use.

One other aspect is that we evaluated oral fluid that was present from a multitude of different people to see the effects on performance, and I think that's really key, because oral fluid is a new matrix, and we are just learning about potential interferences and specific things in different oral fluid that could affect performance.

Host: Okay. Now, David, do you think that immunoassays are more time and cost-effective than instrumental techniques such as GC-MS or LC tandem MS screening? And what are some of the advantages of each?

David Schwope: Well, Bob, I think that's a good question. I think a lot of it depends on the specific laboratory that you are talking about. One of the most important things I think to consider is the positive sample rate that you see in your laboratory. Certain laboratories that come across many negative samples, such as workplace drug testing, are probably more amendable to ELISA than say something like labs that have a higher positive rates such as pain management testing.

I think when you have a lower positive sample rate, it's better to have something that's less time consuming, in terms of the data analysis, and ELISA gives you that through simple assaying procedures.

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And I think part of the issue is that, when you have higher positive rates, such as in pain management testing, it's more efficient to screen by instrumentation sometimes, as you can have semiquantitative results upfront.

And then I think another thing to consider is the data analysis time that it takes, especially when you are talking

about negative results. For ELISA, you can typically have negative results much more quickly than something like GC-MS screening, because you are only looking at an absorbance value for specimens instead of a full chromatogram and mass spectrum. This is important I think for situations like workplace drug testing, where the timeliness of the result reporting is a factor.

Another thing I think you have to consider is unconfirmed positives by immunoassay, and I think a lot of times the cross-reactivity that you have in immunoassays can lead to a higher rate of unconfirmed positives than what you would see by instrumental screening, and this can lead to further confirmations that are needed by instrumental screening later on.

I think that another thing that you have to consider is the sample size that's required. Immunoassay typically only needs something like 25 to 50 microliters, whereas for certain analytes, you might be required to use as much as 1 milliliter of sample, and if sample size is something that you need to consider, it may be more efficient to screen by immunoassay.

Host: Okay. With that in mind, Dr Huestis, you said you tested a wide range of potential interferences, including mouthwash, coffee, and orange juice in the current validation, were you surprised to see no effect? Do you think other substances such as say soda, herbs, and spices, and vitamins could lead to potential interferences, and if so, does this mean that these substances won't be problematic in other immunoassays.

Dr. Marilyn Huestis: Well, I think the key factor is to test these things in your individual ELISA or immunoassay that you are validating, and we can't just predict, we need to actually test and determine whether there are problems. But I think this is a very nice sign that probably we won't have interferences with many of these components, and I think a lot of that is the fact that you use such a small amount of the actual oral fluid itself. Remember, the oral fluid is collected with a device and then diluted with the buffer. And the buffer serves several purposes. It removes the drugs from the collection pad, it also stabilizes the drugs and provides an appropriate pH to test the drugs.

So in general, you are diluting out any potential interference and using a small amount, and then it's added to all of the other components of the testing device. So we weren't surprised to see no effect but, I think it's important that as we go forward we look for these things. And this is generally has been what's happened with immunoassay validations,

and then of course when someone does find an interference, it gets highly publicized, so that the field is aware of this.

We did a good job of testing these substances, because we looked both immediately after use, as well as about 30 minutes after exposure, and that was very important.

There is the possibility that an interference might actually prevent drug absorption on the collection pad of the collection device. We have not found that yet, but it's something to keep in mind and consider.

Also, if you, for instance, had something like coffee and you were looking at caffeine assay, of course then you might actually really have some interference, because coffee contains caffeine, and if that was the analyte you were looking at, you might find some interference there, but that would be pretty easy to predict.

So I think that as we move forward, these are good signs that we might not have interferences, but of course we will learn over time with additional research

Host: Okay. David, you indicated that all controls were matrix-matched in this ELISA validation. Do you believe it is important to matrix-match controls during immunoassay screening and is it possible to use synthetic oral fluid for controls?

Dr. David Schwoppe: Well, I think matrix-matching is an important thing to consider for determination of interferences. A lot of times the different matrices will have different indigenous interferences present that will be present in one matrix but not necessarily present in another. Not to mention purely physical differences in sample viscosity and pH, etcetera.

I think part of the problem with using authentic oral fluids is simply the feasibility of doing such. It's not really that difficult in a research setting, such as the one that we are in here, but I think it's more problematic when testing hundreds or thousands of samples a week, trying to come up with authentic oral fluid that you can use for calibrators and controls, may be a little bit more difficult.

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I think synthetic oral fluid use is possible. I think it depends on the specific application. I think it's important to test synthetic oral fluid against maybe a small number of authentic oral fluid specimens. If you don't see any differences, you may be able to proceed cautiously.

I think if you do see a difference, you can determine how much it differs, whether or not it affects the overall assay, and now you have that kind of characterization that you can use to guide your interpretation of future results.

Really I think it's one of these things that also needs a lot of further research to kind of guide decisions about whether or not use is possible, but I think right now I would say, if you are able to validate the use of that synthetic oral fluid, then I don't think that it's problematic.

Host: You defined intraplate drift as variations in absorbance as a function of physical location on the 96-well plate. In your validation you observed statistically significant drift for the controls at 2 and 8 micrograms per liter. What does this drift mean for general assay uses? Do you feel this is something that should concern clinical laboratories?

Dr. David Schwobe: Well, I think the drift is observed in many immunoassays, I think it's certainly more prevalent when you are using single channel pipettes, or if you are doing manual pipetting as opposed to automated, I think this gives more time for say wells at the beginning of the plate, it gives more time for them to react, and wells at the end of the plate.

I think that what we saw was statistically significant drift, I think however it had no effect really on the qualitative positive or negative determinations. I guess a good way to say this is, while it was statistically significant in our findings, we didn't really feel that it had any clinical significance.

I think that this drift is also manufacturer and assay dependent. Really, as part of the validation, I think it's important to test the drift prior to implementing use of the plate. Again, just so that if you do observe it, you can kind of characterize and determine whether or not it's going to affect your qualitative results.

I think it's possible that you could help mitigate this drift by running controls at both ends of the plate, and by that I mean by bracketing the samples with controls. You can then use the mean absorbances of the controls on both sides of the plate and you can also ensure that the entire plate is functioning correctly by having controls on both ends of your samples.

But I think the important thing to keep in mind is that the number of controls that you are running should conform to SAMHSA rules regarding controls, and that is that 10% of the plate should be controls and calibrators to ensure that the plate is properly functioning.

Host: Okay. So this immunoassay has high sensitivity or 100%, however, specificity is lower, at 89.5%. Doctor, you stated in the article that this reduction in specificity may result from cross-reactivity with another cannabinoids, such as metabolites. Is cross-reactivity a problem in immunoassays in general, and if so, what are the consequences of higher rates of cross-reactivity for the clinical laboratory?

Dr. Marilyn Huestis: Well, cross-reactivity is a key to an immunoassay. There are advantages and disadvantages. The advantages are that the purpose of an immunoassay is to screen large numbers of samples and identify those that might then be confirmed positive by a chromatographic assay.

So you want high cross-reactivity, so that you don't have any false-negative tests. That's key. With the immunoassay portion of a laboratory test, you want to have the highest sensitivity possible. With the confirmation assay, you want to have the highest specificity possible.

So it's a good thing that the cross-reactivity is high for this cannabinoid assay. And what is really important is that you are able to identify all these possible positives.

Now, the negative of this is the fact that more samples might not be confirmed by the chromatographic assay and that means added time to get a result out and additional labor cost to do the more highly intensive confirmation.

So I think it's very positive. In this particular case, we had good specificity, but we had one individual who was responsible for the majority of the unconfirmed positive immunoassay tests, which brings up a very interesting question. This individual in their oral fluid had high concentrations of the carboxy metabolite, and so it's possible there was something different about this individual with their metabolism that was producing these results.

That is something that would be very good as we go forward and look at carboxy-THC as a possible targeting analyte rather than the parent. And of course cross-reactivity is extremely important to evaluate in each and every different kit, because the immunoassays are based on the production of antibodies, and each immunoassay that varies may vary from manufacturer to manufacturer, is based on different antibodies that are formed, and that's the key to your cross-reactivity.

So absolutely, it should be evaluated. In general, you would like to have a broad cross-reactivity for a class of drugs.

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A very good example would be benzodiazepines. There are many, literally hundreds of benzodiazepines available, and a laboratory would like to know that their immunoassay cross-reacted with as broad a spectrum as possible. However, that's not always achieved because, for instance, with benzodiazepines, you have a wide variety of potencies in the medications.

So cross-reactivity is absolutely key for an individual laboratory to understand. And when they are communicating with their end users, they have to make it very clear what they may detect and what they may miss based on that different cross-reactivity.

So I think that's probably key, is that for any screening assay, you would like high cross-reactivity, understanding the limitations that, that may bring on with additional confirmation assays.

Host: Now, you stated that most cross-reactivity and unconfirmed positives were absorbed in samples from that single individual during your clinical study. David, do you believe this user was atypical, and if so, what could cause this?

Dr. David Schwope: Well, I think there's certainly a possibility that he could have been atypical. I think based on the testing that we did, we didn't really notice anything that was atypical about this user. There were no substantial demographic differences; age, race, sex, Body Mass Index were all consistent with other participants.

And also, based solely on the metabolite ratios for 11-hydroxy-THC and the carboxy-THC, we didn't really see substantial differences in metabolism based on the ratios of those to the parent THC compound.

I think the frequency and chronicity of use for this individual is a factor. This individual I think was a very heavy user, self-reported intake of greater than eight blunts per day, which is, I am not sure of the exact ratio of blunts to joints. I think a blunt is rated at about four joints.

Host: He could be a roadie for the Grateful Dead is what you are saying?

Dr. David Schwope: Yeah. I mean certainly. Also, based on the blood concentrations that we saw, it was likely that this individual had smoked just prior to entry into the study, and we saw carboxy levels that were the highest of any other user that we had in our study, and these high carboxy concentrations

in oral fluid could have potentially cross-reacted with the plate and then contributed to the unconfirmed positives.

It's also possible that there was some 11-hydroxy contribution as well. I mean, our limits of quantification by GC-MS were 0.5 micrograms per liter; it's possible that there was 11-hydroxy present in the oral fluid below this concentration that we didn't detect by GC-MS, but were there in enough quantity to cross-react with the plate as well.

Bob Barrett: Okay. Well, finally, Dr. Huestis, are there specific areas regarding oral fluid immunoassay screening that would benefit for additional research?

Dr. Marilyn Huestis: Well, any new technology, and that's exactly what we have here is a new technology, oral fluid testing for detecting drug use, whenever we have a new technology, there is a great deal of research that's needed to provide the underpinnings and scientific database for not only performing that testing, but also in interpreting the testing results.

So I would say one area that we have identified already today is the importance of evaluating the performance of synthetic oral fluid and its use for the preparation of calibrators and controls.

I think that's absolutely key, because it's one thing for us; we are a research organization, to be able to actually collect authentic oral fluid and do all of our testing and all of our preparation of calibrators and controls in authentic oral fluid. That's a different story for laboratories that have strong demands for rapid turnaround time and large sample volumes that they need to identify. So it's key that we evaluate this synthetic oral fluid.

It's interesting that there has been a report that the synthetic oral fluid can affect LC-MS-MS chromatographic analysis, and that's been suggested that it may have a different performance in chromatographic assays. So that's very important. But we need to know that the immunoassays will perform as well with the synthetic as with authentic. Otherwise, you are going to have a major bias in your assay. So I would say that's probably one very important area.

Another issue that is important I think from a cost perspective and availability perspective is, currently oral fluid collection devices are pretty matched to the immunoassays that are available. In other words, if you collect with a Quantisal collector, then you would like to analyze in the Quantisal immunoassay and the same for

other manufacturers. They prefer and match their collection device buffers with their immunoassay.

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I think in the future additional research is needed to be able to see whether expectorant alone could be used without a collection device in these assays. Does that perform well? It would be very nice to know that you were not tied into using a specific kit, that you could collect with whatever collector was the best for you, and then you could use whatever immunoassay was available, and that would give laboratories more flexibility. I think we still need research in that particular area to understand limitations that, that might produce for us.

Another issue that we know from the interpretation point of view is we know that the pH of oral fluid is very important for the concentration of the drugs that will be measuring in it, and this is similar to what we find with urine testing. If you have a very high or low pH urine, it will really affect the amount of amphetamines that are excreted in the urine.

We don't fully understand the changes in oral fluid pH that might affect our ability to detect drugs in oral fluid. We know that oral fluid generally has a lower pH than blood and it facilitates movement of drugs into oral fluid that are basic drugs and that can crossover and become iron trapped in the lower pH of the oral fluid, and that's very helpful for improving detection of basic drugs like opiates and cocaine in oral fluid.

But when you stimulate the production of oral fluid, it raises the pH, and that can affect the amount of drug we can measure. We certainly need more research in that particular area as well. And now we know that you should do non-stimulating collections to get the best results.

We also learned, unfortunately, we learned from our mistakes that it's very critical to have buffer to improve recovery of drugs off of the collection device. So we have learned that as well.

I think also besides this particular article that really characterize the cross-reactivity well of cannabinoids in this assay, in general, we don't know as much as we need to know about metabolite cross-reactivity, and I think that's an area we need to go forward with as well.

And then of course there are not immunoassays available for many of the drugs that are important to us and we need to create new immunoassays and broader cross-reactivity, so that we can do a better job of screening with

immunoassay rather than requiring the very expensive and the need for highly trained people to do screening with chromatographic, usually tandem LC-MS.

And as Dave was speaking to earlier, it really does depend on what your target population is. So immunoassay will continue to play a very important role, especially in workplace drug testing and drug treatment programs, but perhaps in programs that are focused on looking at a wide variety of different drugs and metabolites, like driving under the influence of drugs or pain management, perhaps their screening with chromatographic assays would be more important, and we need to do the research to establish all of this.

Bob Barrett:

Dr. Marilyn Huestis is the Chief of Chemistry and Drug Metabolism Intramural Research Program, National Institute on Drug Abuse at the National Institutes of Health. David Schwoppe is a Doctoral Fellow at the National Institute on Drug Abuse. They have been our guests in this podcast.

I am Bob Barrett. Thanks for listening.

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