

Bob Barrett:

This is the podcast from '*Clinical Chemistry*'. I am Bob Barrett. For many high volume Liquid Chromatography-Tandem Mass Spectrometry methods, the limits of chromatographic speed and mechanical front-end capabilities have been reached. This ends up curtailing the maximal achievable sample throughput.

However, a group of researchers from the Mayo Clinic in Rochester, Minnesota, have developed and validated a derivatization-based sample multiplex that increased sample throughput. This new assay reported in a brief communication published in the March 2011 issue of '*Clinical Chemistry*' by Dr. Stefan Grebe, found that multiplexing samples by differential mass tagging in LC-Tandem MS allows for reliable quantification with throughput increased over standard methods by the multiplexing factor.

Joining us in this podcast to explain how this new assay can help aid fellow clinicians is Dr. Grebe along with fellow researcher, Brian Netzel.

Dr. Grebe, your paper indicates that sample throughput was increased fivefold. Now, how is this possible and how did the described method affect other aspects of your LC-Tandem MS workflow?

Dr. Stefan Grebe:

As you will recall from the paper, the motivation for bringing this assay up was really the unreasonable increase in the number of vitamin D tests that most American labs has probably experienced over the last ten years and we were just pressed for space.

So by putting differential mass tags on different samples, we were able to pull, in this case, five samples, but as we would be indicated in the paper, it would be possible to pull more of this from modern instrumentation where the switching time of the MS Spectrometer is quicker. So 10 would be quite realistic with the latest generation type of instrumentation.

Besides increasing throughput of the instrumentation, the obvious savings and buying the new instrumentation and savings in floor space also provide a huge saving in solvent use.

So, on our floor we have not only the endocrine lab but the drug labs. So between the two labs, we have extremely high end unsaved solvent level because both use LC-MS/MS predominantly as analytical technique.

And for the mass spec test for vitamin D, which was our single but largest volume test at the end of 2009, close to a million samples in our little space. We were able to reduce

our solvent use by 80-90% which reduced the overall solvent use of the flow by about half.

In addition, because the assay has a complex workflow, it forced us really to automate the front-end and that automation of the front-end extraction and driving it with bar coding and so on, has really reduced our growth in FTE.

We don't need to have quite as many people during the same testing even as larger testing numbers than we used to.

There are, however, a few downtimes to this method too. One is, of course, because the batch size has now become very large, there is a delay in the first results going out.

So this type of methodology may not be suitable for a test which is time-critical. With this testing, vitamin D, which is not time-critical, we simply adjusted our starting times and people to come on shift a little later, and that works out fine. But if this was a time sensitive test, this could be a problem.

Host: Can sample multiplexing by differential mass tagging be applied to other analytes, and if so, what analytes are good candidates?

Dr. Stefan Grebe: I will pass you on to Brian to answer this question and he will elaborate a bit on that.

Brian Netzel: It's certainly possible to multiplex other analytes and in the realm of the endocrinology lab that we are in, we can give some specific examples.

The first would be, say, estrogens where you could use any sort of sulfonyl chloride like Dansyl or Dabsyl and then aldosterone can be done with alcohol moieties. Dihydrotestosterone can be done with Grignard reagents and really any neutral steroid with ketones or alcohol moieties or hydroxyl groups on them could potentially have reactions. But you need to be careful in those situations for potential multiple additions of the derivatizing reagent which could skew chromatography or potential ion-pairing if there isn't a complete reaction for those situations.

You can also do any amine containing compounds like catecholamines or metanephrines or any of the peptides or amino acids. And in specific situations, it might be worth mentioning that the iTRAQ reagents from applied biosystems are ones that have been used in the past for both shotgun proteomics and for immune containing compounds.

The major caveat that we should mention here is that in order to do specimen multiplexing, it's necessary to quench the derivatizing reagents completely prior to specimen combination. If there is any possibility of further reaction, it would immediately and drastically alter your CVs and your precision.

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Host: Could you multiplex analytes rather than samples?

Dr. Stefan Grebe: We could, of course, also apply this principle as you might guess from the answer to the last question to different analytes. As Brian mentioned, in either case, the quenching of the derivatizing reagent is important. But you could envision that for example, you have a sample where you have an order for dihydrotestosterone and for estrogens and you would derivatize aliquots of that sample that's either a Dansyl or a Dabsyl Chloride or a related moiety which is reactive to the estrogen, and then another aliquot of the same samples that would derive the reagents which would label the DHT.

That way, you could actually perform simultaneous analysis of DHT estrogen at high sensitivity.

Furthermore, if you use derivatization reagents which are analyte-specific and where you can add some form of R group like we did with the TAD reagent, you could theoretically expand this concept even further, because now what you could do is you could have an R group with very similar chemistry whereas the actual derivatization reagent is different for different analytes.

The R groups would be very similar and would raise the chromatography. So this would make it possible to actually even multiplex analytes which are normally not considered compatible because of the different chromatography.

You could then obviously expand that example to possibly do both multiplexing of analytes and of samples and the final end product of this chain of thought is really that you could come up with a random access mass spectrometer like an automated immunoassay machine where the instrument, depending on what is ordered on a patient sample or derivatize for the specific chemistry and then automatically inject.

Host: So Brian, are there any additional advantages to try azoline-dione derivatization?

Dr. Brian Netzel: Yeah, there is actually a couple that can be noted. The first, of course, is the ionization potential. In any mass spec

assay, really the sensitivity is going to be based on how well that molecule is going to hold on to its ionization. The three-membered nitrogen ring actually holds on to a charge differentially, but very strongly.

So it's a wonderful example of being able to increase the analyte's potential for ionization within the mass spec. Dr. Higashi and his colleagues were the first to really explore this with the triazoline-diones. And I believe in their original papers, they saw anywhere from ten to a thousand-fold sensitivity increases based on those derivatizations.

The second aspect that could be worth mentioning is that the structural changes that occur with these other reactions, the ring formation. It allows for an absolute and consistent fragmentation onto which really forms an ideal situation for carrying that positive charge of the derivatization, use that in the first place through to the second set of quadruples. So that really seemed to make an extreme difference.

That can be applied to what I suppose for things like estrogens where we derivatize with the sulfonyl chlorides. The same case applies there where we are actually carrying that derivatizing piece all the way through to the end product and there it actually hits the detector, so we see that same increase in that situation.

Bob Barrett: Doctor, in your article, you alluded to chromatographic challenges related to triazoline-dione derivatization, can you provide some more detail?

Dr. Stefan Grebe: Yeah, we alluded to this briefly in the article. So what actually happens is because the reaction happens in the structure of the vitamin D molecule where the cholesterol ring is broken and there has been a standard chain of carbon atoms that is two double bond and the triazoline-dione fits into this really like a key into a lock. But it can now either be flipped upwards or downwards in principle and hence there exists, really, an R and an S correlation of the derivatized molecule.

So depending on what chromatography you use, these two chiral enantiomers will migrate differentially. They may not, but they may. And we have chosen this case to optimize our chromatography to have them not separate and that had several reasons.

There is the option that you could separate them entirely, but what happens and in clinical practice, of course, is that each of the peaks, which, if separated, will have some analytical arrow on it, obviously. As you know, when you have two arrow terms, they are usually additive.

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So we reckoned, that was a strong possibility if you separated the peaks completely that our analytical precision would not be as good as it is if you fuse the peaks.

Furthermore, it probably would've meant a much more involved validation because the type of racemic mixture you are really observing here affects both the analyte and the internal standard whereas our assumption, of course, is that they affected in the same way. We would've had to prove that in detail if you had separated the peaks completely.

So for the purpose of this particular project, we chose chromatographic approach, which did not separate the two peaks that infused them. If necessary, you could do that and obviously, if, for example, you wanted to see the three epimers of Vitamin D, which do occur in very small children, sometimes and can give you a full vitamin D results, then you would be forced to separate the peaks and change your chromatography.

Bob Barrett: With the complexity of analyzing derivatized specimens, Brian, does this method affect column life?

Dr. Brian Netzel: It's a good question. The obvious stress of this is going to be the heightened matrix, given that we are injecting the matrix from five specimens rather than just one. In our hands, the column injection numbers actually haven't diminished, which immediately becomes an additional cost savings for the laboratory.

I should note that the pre-column filters that we put on that catch particular matter and the other junk if we can call it that that comes from these specimens are changed more frequently. But generally, those are about a 30th of the cost of the actual columns that we are using.

I'll probably note too that it eliminated the need for online specimen preparation; many labs are doing it in a two-dimensional chromatography where we are using a TurboFlow column, which is an additional cost, essentially doubles the column pricing, and because we are cleaning this up with the liquid extraction, that's no longer necessary.

So I should probably say that caveat is that it works for this situation specifically, the columns that we selected were through a long process of elimination, and not only do we factor in the chromatographic ability of the column, but also the column life.

So different stationary phases there is during our development validation did not fare quite as well as these columns did.

Bob Barrett: So finally, Dr. Grebe, were there any instances when you encountered cross-reactivity among derivatives?

Dr. Stefan Grebe: Yes, there were actually. So we looked or rather Brian, who was on appointment on this development validation, really looked at large number of TAD and the finalized we chose were the ones which had the most consistent derivatization and had no cross-reactivity. But there were some TADs which cross-reacted, and the ones which were problematic were those where the molecular weight of the TAD differed by either 12 or 18 atomic units. The reason for this is two-fold; 12 atomic units is, of course, the distance in molecular weight between vitamin D<sub>2</sub> and vitamin D<sub>3</sub>.

So in a case of a TAD, which differed from another one by 12, you could get confusion about the amount of vitamin D<sub>2</sub> versus vitamin D<sub>3</sub> or rather the vitamin D<sub>2</sub> and D<sub>3</sub> from different patients might not be distinguishable in a multiplex sample.

The other is an 8 atomic unit difference, which, of course, represents the water loss. Vitamin D particularly is very prone to water loss both in the first and the second quadruple and you can really not prevent it, almost.

So if you have a TAD which differs from another by a team, then you are setting yourself up for cross-reactivity where some patient samples may have water loss and others not and hence they will be mistaken for different samples.

So these where we found a, on first principles expected cross-reactivity, and then B, verified that indeed the very cross-reactivity experimentally, those be eliminated from our selection of TADs. But having said this we are using currently five TADs, but Brian has tested another seven TADs, which behaved quite well.

So potentially, there would be the possibility to do 12 different patients. As I alluded to in the first answer, that's probably not possible with the current instrumentation we're using, which is the API 4000 instrument. Their electronic switching time is not sufficient to give enough points across all these multiple peaks of more than five samples, don't forget we are measuring more analytes per sample the internal stems of D<sub>2</sub> and D<sub>3</sub>, plus D<sub>2</sub> and D<sub>3</sub>.

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But there is more modern instrumentation, we could easily get up to ten samples multiplex.

Bob Barrett:

Dr. Stefan Grebe and Brian Netzel are from the Laboratory Medicine and Pathology Department of the Mayo Clinic in Rochester, Minnesota. They have been our guests in this podcast from '*Clinical Chemistry*'. I'm Bob Barrett. Thanks for listening!

Total Duration: 15 Minutes