Watson's Notes

Innovative Solutions of difficult problems

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ISI INVESTIGATIVE SCIENCE INCORPORATED

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INSIDE

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"You have recently been in Afghanistan, I perceive"

With these words was born the most famous team in detective fiction; Sherlock Holmes and his trusted comrade and biographer, Dr. John H. Watson.

In the spirit of Watson, who chronicled the exploits of Holmes, we have created this newsletter named "Watson's Notes".

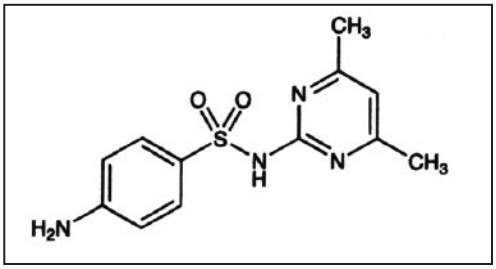
In the pages of "Watson's Notes", modern day scribes document the discoveries, unusual cases and other news of Investigative Science Incorporated, our scientific consulting firm in Burlington, Ontario, Canada.

Like Holmes, ISI is retained to conduct investigations into a range of seemingly intractable scientific problems. Also like Holmes, the ISI staff are first class scientists and masters of deduction. We hope you enjoy reading of our exploits through our own "Watson's Notes".

Please contact us if you have comments, and please read on.

NEW CFIA SULFAMETHAZINE ASSAY NOW ON-LINE AT ISI

Sulfamethazine is a synthetic antibiotic used widely in animal feeds to treat infections.



Sulfamethazine is a synthetic antibiotic

A trace of the drug in the meat of animals, easily detected by quick test kits used by inspectors, is enough to cause the animals to be rejected, meaning lost income for the farmer.

Occurrences such as these generally kick-off a follow-up investigation into the source of the drug. Feeds are sampled and analyzed to determine where the drug originated. In such cases, the laboratory is looking for traces of the drug where it is not supposed to be. We call this trace level or residue analysis.

Manufacturers of medicated feeds, including pharmaceutical firms, feed mills and farmers, periodically need to assure themselves that the correct level of sulfamethazine is present in their feed products. Typically, the feed or premix should contain within ± 20% of the expected amount, according to current Canadian Food Inspection

ELEMENTARY MY DEAR WATSON

Sherlock Holmes and Dr. Watson go on a camping trip, set up their tent, and fall asleep. Some hours later, Holmes wakes his faithful friend.

"Watson, look up at the sky and tell me what you see."

Watson replies, "I see millions of stars."

"What does that tell you?"

Watson ponders a minute. "Astronomically speaking, it tells me that there are millions of galaxies and potentially billions of planets. Astrologically, it tells me that Saturn is in Leo. Timewise, it appears to be approximately a quarter past three. Theologically, it's evident the Lord is all powerful and we are small and insignificant. Meteorologically, it seems we will have a beautiful day tomorrow. What does it tell you?"

Holmes is silent for a moment, then speaks. "Watson, you idiot, someone has stolen our tent."

test is working properly, no sulfamethazine should be detected in the blank.

How do Test Kits Compare?

Quick test kits are available today for testing a range of veterinary drugs, including sulfamethazine. In this section we answer some commonly-asked questions concerning these kits.

How quickly can they be done?

Generally, the test kits are based on an antibody reaction and give a quick "yes-or-no" answer. They can usually be done the same day. Although HPLC tests can often be done the same day, they generally need 2-5 days.

How sure can I be that the test is actually measuring the right drug?

This is an important question. With HPLC analyses, there is generally little doubt about the identity of the drug. To be detected by the CFIA Method, the drug must elute from the HPLC at the expected time and react with the post column reagent. There aren't many other molecules that will do that. In trace level work, though, a positive HPLC result can easily be cross-checked with a test kit or checked using a different HPLC test. There are also many other ways to confirm the identity by HPLC.

The test kits, if used alone, can be fooled by other molecules. According to Agriculture Canada no false negatives have occurred with the test kits, but false positives can occur. Generally, positive test kit results should be confirmed by another type of test, like HPLC.

Can I use test kits to measure the amount of drug in my feed?

No. Test kits generally do not reliably measure the amount of drug, only if it is present or absent. They are referred to as qualitative tests.

HPLC tests do tell you the amount of drug, normally to within ± 10% or better. They are referred to as quantitative tests.

How sensitive are the tests?

The test kit for sulfamethazine can detect down to 0.1 to 1.0 ppm in feed. The new CFIA test as reported in the method can only detect down to 5 ppm. Modifications are required to detect less than 5 ppm. We use another HPLC method to detect down to 0.1 ppm for trace level work.

How much do test kits cost?

The sulfamethazine test kit can be done by most labs for $20-25 per test. The HPLC test will cost $100-250 per test depending on the laboratory and how many samples you send at one time.

How can I best use the two kinds of tests?

We recommend that you use HPLC to measure the amount of drug in your feed. By all means use the test kits to screen for trace levels, but confirm positives by HPLC.

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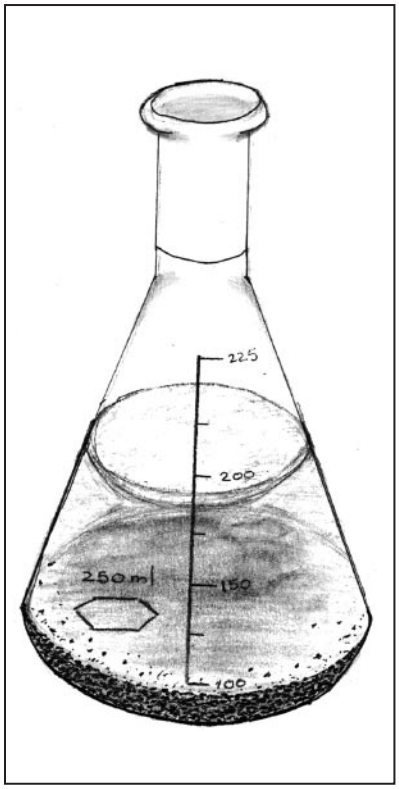
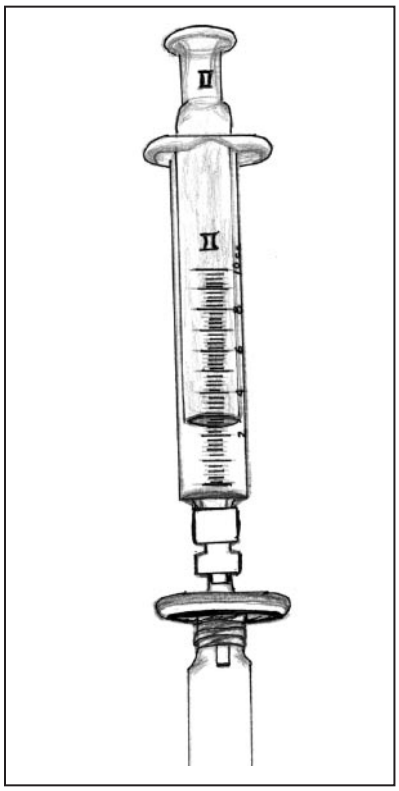


After mixing and settling, there will be clear liquid at top and grains at bottom



The settled extract is now filtered

Agency (CFIA) guidelines. In our lab we refer to this type of test as a verification analysis.

In late 1999 the CFIA issued their new assay for sulfamethazine in feed products. The CFIA refer to this method as FD-DRUGS-SQN. The method was submitted by the CFIA to the Association of Official Analytical Chemists (AOAC), in order to test the new method in a range of laboratories. After performing well in this comparison test, the new method was adopted by the AOAC as official method 999.16. As AOAC official methods, validated by inter-lab studies, are recognized internationally and are favoured by the CFIA, we geared up to provide this method to our feed customers.

In this article, we discuss the new sulfamethazine assay and compare it to the other approaches used to measure the drug.

First: How does it work?

Preparing the Sample

We start by grinding the sample so that we can take a sub-sample (usually 5 grams) for analyses.

We use a rotary grain grinder and mortar and pestle to transform the entire sample into a fine powder. An analytical balance is then used to weigh 5g of sample into a 250 mL Erlenmeyer flask. The sub-sample is now ready to be extracted.

Extracting the Sample

An extraction is how we separate the drug from the sample.

First, an internal standard is added to all samples. This is a chemical very similar to sulfamethazine, called sulfamerazine. Because of its chemical similarity, the internal standard behaves in the same fashion as sulfamethazine. It serves as an indicator of extraction efficiency and will correct the results should the extraction of sulfamethazine be poor.

Next, we add a mixture of methanol, water, diethylamine and hydrochloric acid. This solvent will extract the drug. The solution is put on a mechanical shaker for one hour and allowed to settle for 10 minutes. After settling, there will be a clear liquid at the top and grains at the bottom.

We carefully pour the supernatant into a 10 mL glass syringe which is connected to a syringe filter. The filtrate, which contains the sulfamethazine from your sample along with the internal standard, is collected in a small vial. The filtered extract is now ready for analysis.

Analysis by HPLC

A small sample of the extract is now injected into a high pressure liquid chromatograph (HPLC). HPLC is an analytical instrument which causes the components of the sample to be separated and detected. The separation happens by a distribution of the sample components between two different types of material called phases.

One phase is a set of specially coated particles held in a steel column. The other phase is a solvent that is pumped at high pressure through the column. In this case, the solvent or mobile phase, is a mixture of acetonitrile and water containing acetic acid (much like vinegar).

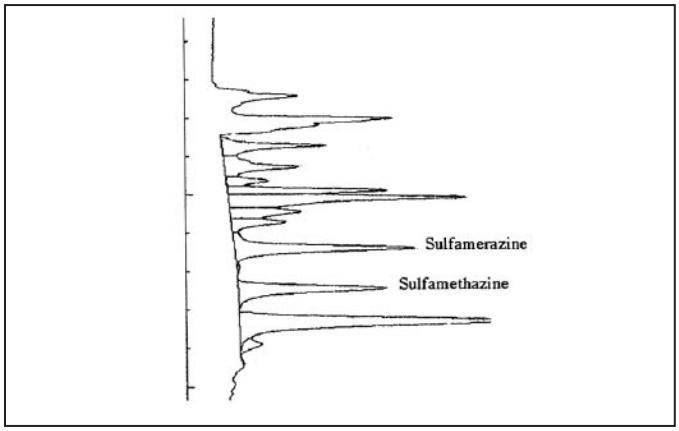
As the sample extract travels through the column, the components will distribute differently between the liquid

and the solid phases according to their chemical characteristics. For example, sulfamethazine and the internal standard sulfamerazine differ by only one carbon atom. Sulfamerazine is the smaller of the two. On the analytical column we use, sulfamerazine comes out first, followed soon after by sulfamethazine.

How are the drugs detected?

The new CFIA method employs a neat trick to detect the drug. It's called post-column reaction. Here's how it works:

As we discussed above, the drug, internal standard and other components of the sample are separated on the HPLC column. Once they come through the column they are exposed to a coloured chemical that reacts with sulfamethazine, sulfamerazine and other related sulfa drugs such as sulfathiazole. Generally, most other components of the sample don't react and are invisible to the detector.



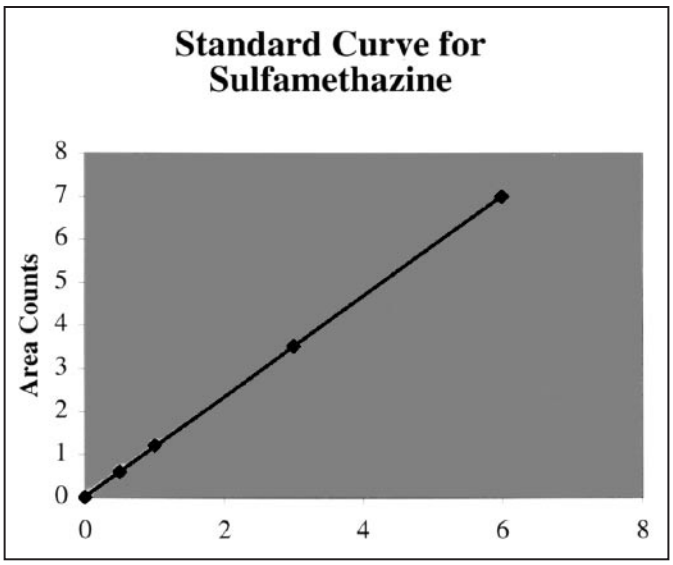
The printer records the data as peaks

The coloured material passes through an ultraviolet detector. The detector shines light having a wavelength of 450 nm (blue in colour) through a photocell, through which the sample passes. Any material that has reacted with the coloured material will absorb the light. Photodiodes register the loss of light and convert it into an electrical signal. These changes in light intensity are recorded as a series of peaks called a chromatogram.

Connected to the detector are an integrator and a printer. The printer records the chromatogram. The integrator calculates the area under each peak and the time each component leaves the HPLC column (called the retention time).

How do we know which peak is sulfamethazine?

We use pure standards, purchased from a reputable chemical supplier, which we dilute at different concentrations. These standards are injected into the HPLC for comparison with the actual samples.



We use a calibration graph to determine how much sulfamethazine is in the sample.

How do we know how much sulfamethazine is in your sample?

Using the pure standards, we perform a calibration to convert from peak area to the actual amount of sulfamethazine in your sample. From the standards, a graph of concentration versus area is produced.

By comparing the ratio of drug peak area to internal standard peak area in your sample to the ratio for the standards used to prepare the calibration graph, we can calculate how much drug is in your sample.

How good is the test?

The new CFIA method can detect as low as 5 ppm in feed and up to high percent levels in premixes. We use another HPLC method to see below 5 ppm for trace level analysis.

The result obtained by the CFIA method is usually within ± 5% of the true value. We check this each time we analyze a batch of samples by including a feed sample which contains a known amount of sulfamethazine.

We also include a feed sample which doesn't contain the drug, called a blank. If the