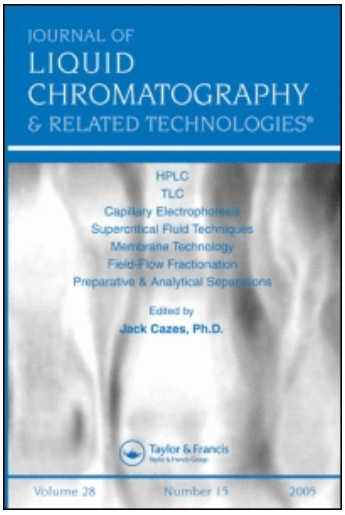

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Some Advances in Application of TLC to Diagnostic Toxicology

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SOME ADVANCES IN APPLICATION OF TLC TO DIAGNOSTIC TOXICOLOGY

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Introduction

During this last year we have progressed in our abilities to make diagnostic toxicological analyses by TLC. Two which I will present are the analysis for Lincomycin and Slaframine.[1] The former, a drug which is very bad for horses, and the latter, a naturally occurring mycotoxin which has had a very large effect on animals this last year.[2] In addition to cases involving closer pastures, this last year alfalfa pasture and hay and grasses have been involved.

The second part of my discussion will have reference to TLC as a reactive system to do chemistry. Our speakers have discussed substrate (absorbent) and optimizing solvents for separation and Dr. Touchstone has discussed application of the advances to actual situations.

Reagents & Apparatus

Finnigan GC/MS 4000; TLC plates, .25mm; silica gel normal phase (E. Merck, Brinkmann, Chicago, IL) (Whatman, Clifton, NJ) and C^{18} reverse phase (Whatman, Clifton, NJ); solvents: ethanol, acetic acid, chloroform, methanol, toluene, ethyl acetate all Nanograde^R or equivalent (Mallinckrodt, St. Louis); Millipore^R Q water, (Millipore Corporation, Cambridge, MA); vanillin, ACS reagent, (Fisher, Pittsburgh, PA); sulfuric acid and ammonium hydroxide - ACS reagent (Fisher); TLC equipment, tanks (Brinkmann Instruments, Chicago, IL); short base TLC PMD developing system (Regis Chemical, Chicago, IL); micropets (Fisher Scientific); UV light (Ultra Violet Products, Inc., San Grabriel, CA); 800 scanner (Kontes, Vineland, NJ); quantitative and volumetric glassware (Kontes, Vineland, NJ).

Experimental

A. Lincomycin

1. standard was obtained from U.S. Pharmocapedia, Rockbridge, MD. Samples of feed were spiked with

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microgram quantities, extracted with methanol, defatted with pet ether after 10% water was added. (50g/100 ml methanol).

2. the pH is adjusted to 2 with 2NHCl and the volume is made 200 ml with water and the aqueous layer is extracted 2 times with 100 ml CH_2Cl_2.

3. The pH is adjusted to about 12 with 1 ml 60% KOH (NaOH) and the aqueous layer is extracted 2 times with 100 ml CH_2Cl_2.

4. The CH_2Cl_2 layer is concentrated to dryness.

5. The extract is redissolved in 90/10 $CHCl_3$/methanol an aliquot equal to 0.1, 0.5, 1 gram of sample is spotted on TLC. Normal feed samples contain 800 ppm as a feed additive.

6. The plate is developed in 80/20 $CHCl_3$/methanol. Multiple developments or the short base PMD developing chamber may be used to separate bands in difficult samples.

7. After developing the plate is sprayed with 5% vanillin in methanol and 20% sulfuric acid in methanol (wt%). The plate is gently heated to observe Lincomycin (yellow spots).

8. To quantitate samples the plates are heated until red brown spots develop which are stable and amenable to TLC direct analysis.

B. Slaframine

1. Slaframine standards may be obtained from H. P. Broquist, Vanderbilt University. The amine is derivatized[1] with pyridine and acetic anhydride if the underivatized standard is obtained. (If supplied as the picrate acidification and partition into CH_2Cl_2 is necessary before conversion to the acetylated product).

2. 50 grams of sample are extracted as in the Lincomycin procedure. Spikes are done as above also.

3. Thin layer analysis is done as above in the Lincomycin procedure. Except visualization is done by Iodoplatinate or iodine fumes.

4. Sulfuric acid methanol has been used to visualize the spots, but so far quantitation is done by GLC.

Results

Canary yellow spots are produced on TLC by the vanillin sulfuric acid spray and gentle heating. The spots turn brown with continued heating and become stable for densitometeric quantitative analysis. Tenths of micrograms are visible by this procedure and TLC.

The Rfs of the Lincomycin is 0.5 in 80/20 chloroform methanol and .8 in 65/30/1 reverse phase solvent[1] (for C^{18} Whatman plates). The Rf in 60/40/1 (methanol/water/NH_4OH) is 0.5. Microgram quantities may be detected. The Rf of acetylated Slaframine is .5 in 6/2/1 - toluene/ethyl acetate/acetone.

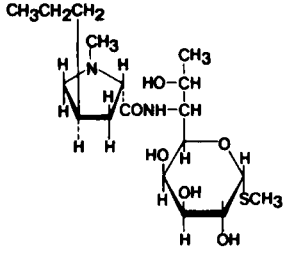
Microgram quantities of Slaframine may also be visualized by iodine and 10 nanograms by fluorescence quenching.

Discussion & Conclusions

Levels of detection for mycotoxins in tissues have been greatly improved by the use of reverse phase and normal phase as reaction surfaces to allow resolution of interfering bands from the bands of interest. Clean up of samples with preliminary steps using the combination of cleanup steps has been successful in allowing analysis of tissues for poisoning levels of fluorescent mycotoxins. These specific techniques will be featured in later papers in complete form. However, the real challenge of TLC has been in analyzing compounds w/o fluorescence or "real handles". (Figure 1) Lincomycin has no uv absorption or fluorescence and is very polar. Reverse phase systems require a basic solvent media. Partition using the very polar basic functional group allows cleanup. Visualization by vanillin allows qualitative and quantitative analyses by thin layer chromatography.

Analysis for Slaframine has a similar difficulty. It is even more easily decomposed. (Figure 2) Acetylation of this compound greatly improves its stability and allows detection by TLC and iodometry. Mass spectrometeric analysis may be used for confirmation. So far mass spectrometric confirmation of Lincomycin is still being developed. A desorption technique may be required.

It is important to recall when applying TLC methods and optimizing solvents that in the real world samples probably won't



Lincomycin mol. wt. 406.56

$C_{18}H_{34}N_2O_6S$

Figure 1. Lincomycin structure

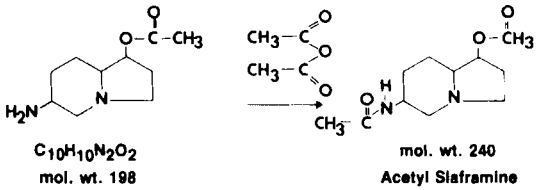


Figure 2. Slaframine derivatization

perform reversibly and predictions from pure standards and solvent systems are just that - predictions.

Multiple developments in a linear or a two dimensional fashion allow bands to separate which otherwise might be inhibited by "junk" which co-partitions and forms a matrix for chromatography. The short bed PMD system of Regis Chemical Company also allows experimental separations of difficult to observe bands by optimizing solvent to absorbent and junk to analyte. Once qualitative knowledge of the analyte is available, methods using multiple linear developments and/or optimization of a cleanup can be used to make quantitative analysis possible.

Conclusions

Thin layer chromatography can be used as an experimental reaction system to detect and optimize the analysis of any analyte. The advent of predictable (high performance) normal and reverse phase and fluorescent quenching has made such techniques widely used. The use of cleanup procedures, functional groups, and chemical reaction will make thin layer analysis of any analyte possible.

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