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## **A MODIFIED FOLIN-DENIS OVERSPRAY FOR THE DETECTION OF PHENOLIC ALKALOIDAL DRUGS \***

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**Abstract**

Investigation of a modified Folin-Denis thin-layer chromatographic overspray for detection of phenolic alkaloidal drugs was undertaken. The spray, employing molybdosilicic acid in place of molybdophosphoric acid proved capable of detecting 5-15 nanograms of these drugs on a thin-layer plate. Plate background following the overspray was very light in colour.

Also investigated was the use of heptane sulphonic acid for "period-ion" extraction of phenolic alkaloids.

**Introduction**

Thin-layer chromatography (TLC) is the most widely used drug detection technique in equine drug testing laboratories. The method is relatively simple, sensitive, and detects a broad range of drugs and other organics (Stahl, 1969). TLC can be highly specific, de-

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pending upon the nature of the detection technique (i.e. U.V. quenching, colourmetric oversprays, fluorescence, densitometry *et cetera*). For example, reserpine, oxidized to a fluorescent product, is readily detected in equine blood extracts for several days, when analyzed by TLC (Sams and Huffman, 1978). Numerous excellent overspray procedures yield highly visible spots, with minimum background colour, enabling the analyst to detect many drugs on the chromatographic plate (Clark, 1969; Zweig and Sherma, 1972).

In the United States, thin-layer procedures are being employed for pre-race blood testing as well as for post-race urine and blood testing (Maylin, 1977; Woodward, 1977). The advent of high performance thin-layer plates and associated techniques has improved the minimum detectable quantities of many drugs, so that low nanogram, or even picogram, quantities of substances may be analyzed by TLC. This increased sensitivity is further enhanced by excellent spot resolution and R<sub>f</sub> reproducibility (Vitek and Kent, 1978).

In our laboratory at the University of Kentucky, drug screening tests must be capable of detecting not only prohibited drugs, but concurrently, large numbers of "permitted" drugs in equine biological fluids.<sup>1</sup> It is imperative then that the tests adequately detect the "permitted" substances yet be sensitive enough for prohibited drugs.

The success of blood testing by thin-layer chromatography in New York, Pennsylvania and Ohio, has encouraged our laboratory to develop similar procedures. The ability to detect drugs by the use of "mini" thin-layer procedures at plate concentrations of 100 nanograms or less is exciting and has enabled the analyst to provide a more comprehensive screening service, often with less sample. "Mini" TLC procedures are now being used in our laboratory for blood screening. Studies

would suggest that even for urine analyses "mini" techniques may result in enhanced detection of drugs. "Mini" thin-layer procedures may be defined as the use of standard or high performance TL plates, 5 x 10 cm, with or without a fluorescent indicator. Solvent development is for a distance of 5 or 6 cm, rather than the 10-12 cm commonly employed. The initial spots at the origin are a maximum of 1.5 mm in diameter.

Since the sample size of the biological fluid used is small, 2-7 millilitres, the detection method for TLC must be sensitive. Plate background must be light in colour relative to the detected spot. These requirements are met by a modified Folin-Denis overspray which was investigated for use with alkaloidal drugs and will be described in this paper. The modified Folin-Denis overspray was first reported by Todd for plant alkaloid detection (Todd and Biftu, 1976).

Since "mini" TLC procedures were to be used primarily for blood analyses, maximum use of a limited sample was necessary. One such approach was a "paired-ion" extraction, which would permit both acidic and basic drugs to be partitioned in one extract (Karger and Persson, 1974). I-heptane sulphonic acid showed early promise as a counter-ion. "Paired-ion" extraction of morphine with heptane sulphonic acid was chosen as the model system.

#### *Solvents, Reagents and Supplies*

Solvents and supplies used throughout these procedures were obtained from Fisher Scientific Company, Louisville, KY, unless otherwise noted. Organic solvents were spectroanalyzed for quality. Extractions were performed in screw-top, glass culture tubes (Kimble), 16 x 125 mm with polypropylene caps. The spotting of thin-layer plates was done with Pasteur pipettes drawn into fine spotting capillaries.

Thin-layer plates were Macherey-Nagel, Sil G-25, 0.25 mm silica gel, with fluorescent indicator. Plates were obtained from Brinkmann Instruments, Inc., Des Plaines, Illinois. 12-

<sup>1</sup> Kentucky State Racing Commission rules permit the use, during performance, of non-steroidal anti-inflammatory and cortico- and anabolic steroid drugs, as well as diuretics, salicylates and muscle relaxing drugs. In the calendar year 1977, the laboratory detected 4888 such drugs in official samples.

molybdosillicic acid was purchased from Pressure Chemicals Co., Pittsburgh, Pennsylvania. Drugs used were obtained from Pharmacy Central Supply, University Hospital, University of Kentucky, Lexington, Kentucky. I-heptane sulphonic acid (0.01 M) with glacial acetic acid was a commercial preparation sold by Waters Associates, Milford, Massachusetts, under the trade name Reagent B-7<sup>®</sup>.

#### *Preparation of the Modified Folin-Denis Reagent*

The Folin-Denis reagent overspray was prepared by mixing 10 grams sodium tungstate, 2 grams 12-molybdosillicic acid, 5 millilitres of concentrated phosphoric acid and 50 millilitres of distilled or deionized water. This mixture was refluxed for 2 hours. At the completion of reflux, the mixture was diluted to 100 millilitres total volume with additional water. The overspray should be stored at 0-5° C and is stable for long periods. After spraying the plates, ammonia vapour greatly enhances colour development.

#### *Procedure for Thin-Layer Analysis*

5.0 ml of an aqueous standard or biological fluid, i.e. blood or urine, were adjusted to pH 8.5. Adjustment may be made with concentrated NH<sub>4</sub>OH or a saturated solution of sodium tetraborate, depending upon whether the sample was previously hydrolyzed. The pH of the fluid, contained in a 125 ml culture tube, was monitored by a "pencil" electrode and a Fisher, Model 230 pH meter. Liquid-liquid extraction of the drug(s) was into 5 ml of a dichloromethane (DCM)-isopropanol mixture

(8:1 V/V). Extraction was carried out by rotating the capped culture tube on a Fisher "Rotorack" for five minutes.

Following extraction and centrifugation, the organic phase was isolated and transferred to a clean tube. The organic phase was evaporated in a water bath at 60° C, preferably under a stream of nitrogen.

Residue in the tube was recovered in 50 microlitres of DCM for spotting on a silica gel TL plate (5 x 10 cm). Spotting was done with a long capillary drawn from a 22.9 cm Pasteur pipette. The solvent was gently spotted at the origin with a slow flow of hot air over the plate surface to aid in rapid solvent evaporation. It was imperative that the original spot be no greater than 1.0-1.5 mm in diameter, in order to enhance resolution and minimize spot diffusion during development.

Plate development was conducted in 2 different systems. System A, methanol-NH<sub>4</sub>OH (100:1.5) provides maximum resolution; System B, chloroform-methanol (90:100) permits maximum sensitivity. Table I presents the R<sub>f</sub> data of several phenolic alkaloids in these two development systems.

Following development, which takes about 15 minutes for the solvent to traverse a 6 cm distance, the plates were carefully dried in a forced air flow. After drying, the plates were sprayed with the modified Folin-Denis reagent. Exposure of alkaloid spots to fumes of ammonium hydroxide maximized the blue-green colour reaction.

In an attempt to simplify the extraction procedure and maximize sample use, morphine was used as a model for "paired-ion" extraction. "Paired-ion" extraction was performed by adding 1.0 ml of a 0.01 M solution of heptane sulphonic acid (HSA) to 2.0 ml of aqueous solution containing morphine at varying concentrations. HSA was obtained as the commercially prepared reagent, B-7<sup>®</sup> (Waters Associates). The pH of the above mixture was 3.2. Extraction was into 5 ml of an 8:1 (V/V) chloroform-isopropanol mixture as noted in the preceding procedure. Following extraction for five minutes on a Fisher

Solvent System	CH <sub>3</sub> OH-NH <sub>4</sub> OH (100:1.5)	CHCl <sub>3</sub> -CH <sub>3</sub> OH (90:10)
	R <sub>f</sub>	
Morphine	0.42	0.05
Apomorphine	0.90	0.03
Pentazocine	0.73	0.20
Nalorphine	0.82	0.23
Hydromorphone	0.25	0.06

Table 1—R<sub>f</sub> data of selected phenolic alkaloids. M-N SIO plates. Six cm development distance.

**ALKALOIDS**  
**Morphine Nalorphine**  
**Hydromorphone Pentazocine**

Fig. 1—Phenolic alkaloids sprayed with modified Folin-Denis. NOTE the light-coloured background. M-N SIO, plates. CH<sub>3</sub>OH:NH<sub>4</sub>OH (100:1.2) 6 cm development distance.

"Rotorack" in 125 mm culture tubes, the organic phase was transferred to a clean tube and evaporated.

Extraction yield of the ion-paired morphine was determined at pH 3.2 relative to the extraction yield of free morphine at pH 8.5. PFPA derivatives of the various concentrations were prepared for quantitation of the recoveries (Blake and Tobin, 1976).

Chromatographic quantitation of the resulting derivatives was determined on a Varian 2740 equipped with scandium tritide electron capture detectors. The column was a 3-foot, 3% OV-101 on Chromosorb WHP, 2 mm I.D. and 0.25 in O.D.

#### Results and Discussion

The modified Folin-Denis overspray proved to be an excellent detection spray for phenolic alkaloids providing high sensitivity with

**MORPHINE (ng)**  
**900, 450, 225, 112,**  
**56, 28**

Fig. 2—Morphine extracts sprayed with modified Folin-Denis. Concentrations (nanograms) from left to right are: 900, 450, 225, 112, 56, 28. M-N SIO, plates. CH<sub>3</sub>OH:NH<sub>4</sub>OH (100:1.2) 6 cm development distance.

negligible background colour on the thin-layer plate. Minimum detectable quantities of alkaloids on standard TLC plates were in the range 5-15 nanograms. It is anticipated that the use of high performance TLC plates would enable the analyst to detect even lower quantities of drugs. Control plasma extracts showed no interfering spots on the thin-layer plates in the R<sub>f</sub> ranges of interest.

Figure 1 illustrates the light background colour on the thin-layer plates following a modified Folin-Denis overspray. The spray can be used in conjunction with other sprays with minimal interference. For example, our laboratory presently uses the sequence: acetic acid fumes (reserpine), FeCl<sub>3</sub>-H<sub>2</sub>SO<sub>4</sub> (phenothiazines) and the modified Folin-Denis reagent (phenolic alkaloids) in a blood plasma analytical procedure.

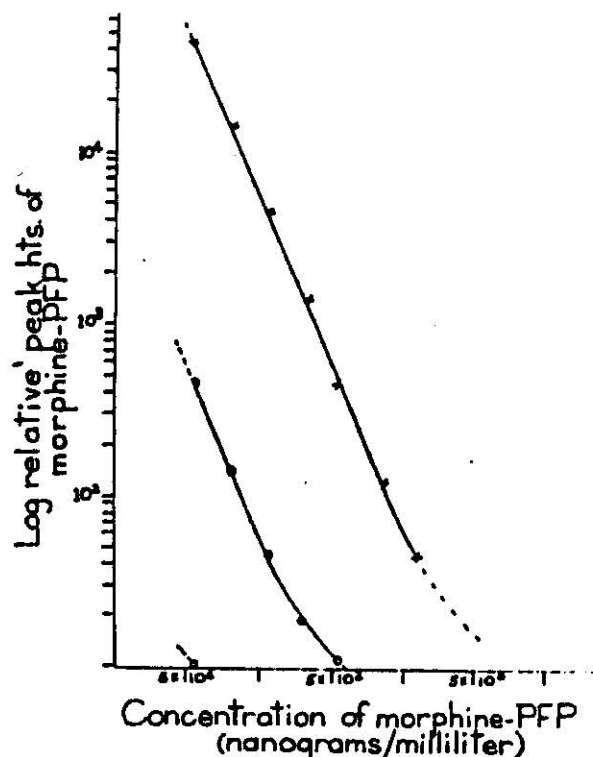


Table 2—Morphine recovery following extraction:

- X pH 8.5, free morphine.
- O pH 3.2, with heptane sulphonic acid as a "counter ion".
- pH 3.2, no "counter ion".

The sea green-blue spot is a reduction product of silicomolybdic acid and is probably due to hydrogen atoms extracted from phenolic hydroxyl groups. Four such hydrogen atoms may become attached to the four  $\text{Mo}_3\text{O}_4$  groups symmetrically located about the central  $\text{SiO}_4$  tetrahedron of the acid structure. It has been suggested that a royal blue colour may be produced by further reduction of the molybdosilicic acid with stannous chloride (Strickland, 1952).

Phenolic alkaloids may be detected at very low concentrations on the thin-layer plates by the modified Folin-Denis overspray. Five to 15 nanograms of drugs from this group may be visualized on standard thin-layer plates. Figure 2, showing morphine concentrations on a standard plate in the range 28-900 nanograms, again illustrates the very light background colour.

The use of a commercial heptane sulphonic

acid preparation which would permit the simultaneous extraction of acidic and basic drugs was not deemed adequate for recovery of drugs in the low nanogram per millilitre range. Although ion-pairing did enhance the extraction of the drug morphine at pH 3.2 relative to free morphine at pH 3.2, the extraction yield was poor (Table 2). Therefore, the use of heptane sulphonic acid as a counter ion was not adequate for routine extraction of morphine. Table 2 indicates that the paired-ion extraction yield of morphine at pH 3.2 is about 1% of the same concentration of free morphine extracted at pH 8.5. It is possible that other counter ions not yet tested may improve this extraction yield appreciably.

The modified Folin-Denis overspray is being used routinely in blood plasma analytical procedures for the phenolic alkaloids. A change in the formulation of the overspray is presently being investigated to permit a general nitrogenous drug overspray, such as Ludy Tenger's, to be incorporated into the spray sequence without losing drug detection sensitivity.

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