A simple chlorination-heat procedure for the detection of hydroxamic acids and amides on paper chromatograms*

During the course of studies now underway concerning the physiologic disposition of short-chain, aliphatic hydroxamic acids in the mouse[1], it became necessary to utilize staining reactions for amides and hydroxamates after chromatography on paper. Hydroxamates were easily detected at the microgram level with the classic ferric chloride reaction (10% $FeCl_3$ + 1% HCl in absolute ethanol) which gave red-purple spots on a yellow background[2,3]. The amides, however, proved much more difficult to detect with corresponding sensitivity, and several procedures were tried without particular success:

Iodine vapor exposure[4] and ninhydrin spray reagent[5] were ineffective in detecting short-chain amides, and attempts to adapt the mercuric chloride–bromothymol blue solution-reagent of TROFIMENKO AND SEASE[6] to a chromatographic spray reagent were unsatisfactory due to autooxidation and background precipitation. Conversion of the chromatographed amides to the corresponding hydroxamic acids by spraying with alkaline hydroxylamine followed by heat and then ferric chloride treatment[7] was satisfactory for detection of 10 μg levels of the one to four carbon amides. The only difficulties encountered were spot migration and loss of 10–30% of chromatographed hydroxamic acids during the hydroxylamine treatment, which rendered exact localization and quantitation somewhat difficult.

The active hydrogens in unsubstituted and mono-substituted amides can be replaced by chlorine on exposure to chlorine gas, after which detection is provided by a starch–iodine spray[8] or by a tolidine–iodide spray for chloride ions[9]. The first of these procedures was found to have low sensitivity for the detection of short-chain amides, while the second, although sensitive at the 1 μg level, was subject to rapid fading. With both procedures the corresponding hydroxamates stained much more intensely, indicating that the α-hydrogens in these compounds are more readily substituted than are those of the amides.

While evaluating these procedures, the chlorinated chromatograms were heated to speed the evaporation of Cl_2–HCl vapors, and browning reactions were noted at the sites of amides and hydroxamates. Development of the color reaction was simple, reproducible, sensitive, and proportional in intensity to compound quantity (as determined by densitometry), and eliminated the problems of fading and spot migration.

The paper chromatogram was suspended in a mason jar containing a fresh mixture of 20 ml of 1.5% potassium permanganate and 20 ml *N* HCl. After a 15 min exposure, the chromatogram was placed in an oven at 180–200° for 1 min. A browning reaction occurred in regions containing (hydroxy) amido compounds. On heating at higher temperatures or for longer periods of time, the entire paper charred and the discrimination of reactive compounds from background was reduced. Pure chlorine gas, whether water-washed or not, also caused the entire chromatogram to brown upon heating, so that the "home-made" preparation of Cl_2 is actually preferable, in this instance, to compressed cylinder gas.

* This work was supported in part by Grant AM 10960-01 from the National Institutes of Health, and in part by a research contract, Project No. 3A025601A822, from the Medical Research and Development Command, U.S. Army, Washington, D.C.

Browning did not occur on thin-layer silica gel chromatograms, indicating that some reaction of the N-chloro compound with cellulose is probably involved. Reaction of amino acids, upon heating, with the aldehydic groups derived from short-chain celluloses present in paper to give browning or fluorescence is a well-known phenomenon[10,11]. In the case of amides and hydroxamates, however, browning did not occur in the absence of prior chlorination.

The chlorination–heat treatment of paper chromatograms gave intense browning at the site of 0.1 μmole acetamide, hydroxyurea, dihydroxyurea, urea, formohydroxamic acid, acetohydroxamic acid, propionohydroxamic acid and isobutyrohydroxamic acid. A mild to moderate color reaction was obtained with the same molar quantity of propionamide, isobutyramide, hexaneamide, and uric acid. No visible coloration occurred with 0.1 μmole creatinine, formamide, N,N-dimethylpropionamide, dimethyl formamide, oxalic acid, sodium succinate and glucose.

Amines, ammonium salts, and similar compounds (β-alanine, indole, diethylamine, ammonium chloride, hydroxylamine hydrochloride) gave browning reactions on heating without prior chlorination. The same chromatogram could then be chlorinated and re-heated to locate hydroxamates and amides. A single heating, therefore, does not destroy the reactive cellulose groups in the paper, nor the chromatographed amido compounds. No browning reaction occurred when the filter paper was first chlorinated and heated, then spotted with amide and reheated. Chlorination of cellulose substituents therefore does not contribute to the browning, and it may be inferred that halo-amide formation is the prerequisite chemical reaction.

The sensitivity of the test for amides and hydroxamates decreased as the alkyl chain length increased and, for a given chain length, was much greater for the corresponding hydroxamate. The lack of a positive test for formamide is believed due to sublimation of the compound from the paper rather than to unreactivity. This conclusion is based on a similar lack of reaction with the hydroxylamine–ferric chloride procedure[7] on paper chromatograms, despite a positive reaction in aqueous solutions with a sensitivity equivalent to that of longer chain amides. The reason for a negative test with creatinine is unclear at present.

The procedure described provides a simple method for detecting amido and amino compounds on paper chromatograms. Although inapplicable to silica gel thin-layer chromatography, it is quite likely that it would also be suitable for TLC procedures employing cellulose.

Biochemistry Branch, Armed Forces Institute of Pathology, Washington, D.C. 20305 (U.S.A.)

CHARLES L. STREETER
WILLIAM N. FISHBEIN

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Received August 4th, 1967