Journal of Chromatography, 138 (1977) 220–222

CHROM. 10,101

Note

Benzidine substitute visualizing agents for iron porphyrins

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(Received March 28th, 1977)

The best visualizing agents in chromatography will use the species studied as a catalyst in a color-producing reaction. Iron porphyrins can act in a peroxidase-like manner to catalyze the oxidation of various aromatic amines and phenolic compounds to produce color.

Benzidine has long been used to detect occult blood in urine or feces with this reaction[1], and has been used to visualize iron porphyrins in chromatography[2]. However, since benzidine is classified as a possible carcinogen[3], it would be desirable to find less hazardous substitutes. The purpose of this work is to examine a large number of compounds which might be used as benzidine substitutes in order to determine their relative sensitivities.

Several compounds have been suggested as benzidine substitutes. 3,5,3′,5′-Tetramethylbenzidine has been shown to be non-tumorigenic in rats[4]; and non-mutagenic in the microsome–salmonella tests of Ames and co-workers[5]. 4-Aminodiphenyl amine is also non-mutagenic[5], and diphenylamine has been suggested as a substitute since it is not known to induce tumors[6]. Gum guaiac has been used in occult blood detection for more than a century[7]. Others suggested for blood detection include: chlorpromazine[8], 4-dimethylaminoantipyrine[9], 3-aminophthalic acid hydrazide[10], phenolphthalin[11], and leuco malachite green[12]. Syringaldazine was developed in this laboratory as a reagent for determining chlorine in water[13], and leuco crystal violet has been used for the same purpose[14].

EXPERIMENTAL

Chemicals used were reagent grade, and were used without further purification. Iron(III) mesoporphyrin IX was obtained from Porphyrin Products (Logan, Utah, U.S.A.). Chlorpromazine was a gift from Centerchem (New York, N.Y., U.S.A.). Gum guaiac was obtained from Harleco (Philadelphia, Pa., U.S.A.). From Aldrich (Milwaukee, Wisc., U.S.A.) were obtained: syringaldazine, tetramethylbenzidine, dimethylaminoantipyrine and 3-aminophthalic acid hydrazide. From Eastman-Kodak (Rochester, N.Y., U.S.A.) were obtained: 4-aminodiphenylamine, diphenylamine, phenolphthalin, leuco crystal violet, leuco malachite green.

For these tests, 1 mg iron(III) mesoporphyrin IX was dissolved in 10 ml 1.5 M ammonia. This solution was diluted 10:1 and 100:1 in the dilute ammonia, and 10 μl of each solution was spotted in subdued light on Whatman No. 1 paper. The solvent was evaporated in a stream of air and then the spots were sprayed to visualize.

To avoid premature, uncatalyzed oxidation of the chromagen, a dual spray technique was used, with the chromagen sprayed first in a methanol solution (approx. 10^{-2} *M*). The second spray was sprayed immediately; it contained 1% hydrogen peroxide, 5% pyridine, and 0.1 *M* phosphate buffer, pH 7. For chlorpromazine and dimethylaminoantipyrine, 10% acetic acid was the only ingredient with the hydrogen peroxide. For 3-aminophthalic acid hydrazide and phenolphthalin, 1.5 *M* ammonia and 5% pyridine were the only ingredients in the second spray with the hydrogen peroxide.

RESULTS AND DISCUSSION

The results of this test are shown in Table I. Colors were observed in bright light, except for 3-aminophthalic acid hydrazide, which involves a chemiluminescent reaction and must be observed in complete darkness.

TABLE I

LEVELS OF IRON(III) MESOPORPHYRIN IX DETECTED WITH BENZIDINE SUBSTITUTES

Chromagen	*Color*	*Amount of iron porphyrin detected (μg)*
Tetramethylbenzidine	greenish blue	0.01
Syringaldazine	purple	0.01
Leuco crystal violet	purple	0.01
Gum guaiac	blue	0.01
Aminodiphenylamine	blue	0.01
Diphenylamine	brown	0.1
Leuco malachite green	bluish green	0.1
Chlorpromazine	pink	0.1
Phenolphthalin	pink	1
Dimethylaminoantipyrine	pink	1
3-Aminophthalic acid hydrazide	[chemiluminescent]	1

Since the detection of as little as $3 \cdot 10^{-4}$ μg of hemin has been reported using benzidine[15], it seems likely that several of these compounds will have detection limits significantly lower. A determination of the optimum conditions for use of several of these compounds is now in progress. However, it is clear that most of these compounds will give adequate results with the amounts of iron porphyrins most conveniently used in paper and thin-layer chromatography.

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