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DANSYL HYDRAZINE AS A FLUORIMETRIC REAGENT FOR THIN-LAYER CHROMATOGRAPHIC ANALYSIS OF REDUCING SUGARS

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SUMMARY

Reducing sugars, particularly aldoses, react readily with dansyl hydrazine. The fluorescent hydrazones produced can be separated by thin-layer chromatography and determined quantitatively by spectrofluorimetry after elution from the chromatograms.

INTRODUCTION

Dansyl hydrazine [(1-naphthalenesulfonyl-5(dimethylamino)-hydrazide] has been suggested as a fluorimetric reagent for the analysis of ketosteroids[1,2]. It has been applied as a staining reagent for periodate oxidized glycoproteins separated by acrylamide gel electrophoresis[3,4]. In the present communication we report the use of dansyl hydrazine for the fluorimetric analysis of reducing sugars employing thin-layer chromatographic (TLC) procedures. The method described here for sugars is similar in principle to the many, commonly employed procedures available for the analysis of amino acids and other amines which use dansyl chloride as the fluorimetric reagent[5–20].

MATERIALS AND METHODS

Materials

D-[^{14}C(U)]Glucose was obtained from New England Nuclear (Boston, Mass., U.S.A.) and diluted to give a stock solution of 10 m*M* glucose with a specific activity of 0.2 μCi/μmole. Dansyl hydrazine was purchased from Pierce (Rockford, Ill., U.S.A.). Other chemicals used were obtained from Fisher (King of Prussia, Pa., U.S.A.) and Sigma (St. Louis, Mo., U.S.A.). TLC was carried out on Eastman-Kodak No. 13179 silica gel sheets and on Anasil G plates (Analabs, North Haven, Conn., U.S.A.). Fluorescence was measured in a spectro-fluorimeter[21] equipped with a 300-W xenon lamp and using cuvettes with a 1.0-cm light path. Radioactivity was measured with 87% efficiency for counting ^{14}C using the Aquasol liquid scintillation cocktail (New England Nuclear).

Reagents

Reagents used were: 4% (w/v) trichloroacetic acid, 1% dansyl hydrazine in ethanol, 1% (v/v) acetic acid in ethanol, 10 m*M* D-glucose (or other reducing sugar) stock solution.

Procedure

A 100-μl sample containing 0.04–2 μmoles reducing sugar is mixed with 100 μl trichloroacetic and 200 μl dansyl hydrazine solutions. The mixture is heated for 10 min at 80°, then cooled to room temperature. The same procedure can also be performed using smaller samples (2–10 μl) of test solution, keeping the same proportions of reagents as described above. A control tube containing no sugar is used as a reference system to aid in the detection of dansyl hydrazine and its degradation products.

Samples of the reaction mixture (2–10 μl, containing 2–100 nmoles sugar) are applied to starting points on silica gel plates and then developed by standard TLC procedures using solvents as described in Table I. After development and drying at room temperature, the plates are illuminated with a long-wave ultraviolet lamp. Sugar hydrazones appear as intensely fluorescent bright yellow spots. Dansyl hydrazine itself appears as a yellow fluorescent spot which is usually well separated from the

TABLE I

MOBILITY OF SOME SUGAR DANSYL HYDRAZONES ON TLC PLATES

Numbers indicate R_F values obtained on Anasil-G plates. Mobilities on Eastman-Kodak silica gel sheets differed only slightly from those reported here. Solvent systems: I, Benzene–pyridine–acetic acid (16:4:1); II, toluene–triethylamine–acetic acid (13:5:2); III, chloroform–ethylacetate–1% boric acid in methanol (3:5:2); IV, chloroform–*tert.*-butanol–acetic acid (5:4:1); V, benzene–chloroform–1% sulfamic acid in dimethylformamide (5:3:2).

Compound	R_F				
	I	*II*	*III*	*IV*	*V*
Maltose	0.01	0.11	0.15	0.08	0.20
Melibiose	0.01	0.09	0.10	0.06	0.12
D-Glucose	0.17	0.21	0.42	0.36	0.42
D-Galactose	0.13	0.19	0.27	0.29	0.38
D-Mannose	0.15	0.20	0.36	0.35	0.40
2-Deoxy-D-glucose	0.23	0.26	0.53	0.49	0.61
D-Fructose	0.23	0.23	0.46	0.50	0.54
L-Sorbose	0.25	0.25	0.50	0.60	0.47
D-Xylose	0.35	0.27	0.62	0.54	0.59
L-Arabinose	0.30	0.25	0.52	0.47	0.54
D-Ribose	0.41	0.32	0.59	0.52	0.66
L-Rhamnose	0.40	0.35	0.63	0.64	0.62
D-Fucose	0.42	0.36	0.58	0.56	0.65
2-Amino-2-deoxy-D-glucose	0	0.28	0.45	0.20	0
2-N-Acetylamido-2-deoxy-D-glucose	0.07	0.16	0.41	0.30	0.24
N-Acetylneuraminic acid	0	0.14	0	0.10	0.19
D-Glucuronic acid	0	0.14	0	0.04	0.23
DL-Glyceraldehyde	0.52	0.44	0.67	0.77	0.68
Dansyl hydrazine	0.86	0.56	0.94	0.96	0.98
Dansyl-OH	0.10	0.37, 0.51	0.26, 0.22	0.35, 0.18	0.25

hydrazones. A small amount of degradation products of dansyl hydrazine, mostly dansyl sulfonic acid (dansyl-OH), appear as green-blue fluorescent spots[5], easily distinguishable from the dansylhydrazones.

Elution and quantitative analysis

The yellow fluorescent spots are scraped off the plate (or cut out when Eastman-Kodak sheets are used) into a standard conical centrifuge tube. The hydrazones are then eluted by 10 min shaking with 2 ml of 1% acetic acid in ethanol. Particles are removed by centrifugation in a desk-top clinical centrifuge. The supernatant is measured in the spectrofluorimeter using the wavelength of 360 nm for exitation and 510 nm for emissions. If a radioactive sugar is used, a sample (0.1–1.0 ml) of this supernatant is counted by scintillation spectrometry.

RESULTS AND DISCUSSION

Optimal conditions for the formation of glucose dansyl hydrazone were worked out so as to formulate the analytical procedure described above. The optimum pH range for the reaction was found to be between 2 and 3. The rate of hydrazone formation at less acidic solutions was appreciably slower. Acetic acid (0.1 *M*) could substitute trichloroacetic acid in the reaction mixture. Hydrazone formation was found to be complete within less than 10 min when the system was heated up to 80°. The sugar dansyl hydrazones were relatively stable in the acidic solution and could be chromatographically and fluorimetrically analyzed with very little loss of fluorescence intensity also after 48 h if kept in the dark. The lowest limit of visual detection of the fluorescent spot on the TLC plate was found to be in the range of 1–2 nmoles glucose dansyl hydrazone. This is similar to the level of sensitivity described for the detection of dansyl amino acids on TLC plates[5,6,8]. The lowest level for the quantitative fluorimetric determination of the glucose dansyl hydrazone in solution after elution of the spot from the chromatographic plate was between 2 and 4 nmoles. This level of detection can most probably be lowered significantly through the use of a more sensitive instrumentation suitable for the fluorimetric analysis of smaller volumes than those used in the present study.

The results presented in Fig. 1 indicate that the procedure can be conveniently employed for the quantitative analysis of glucose. The use of [^{14}C]glucose in this experiment provided an internal control which indicated that about 90% of the glucose present was converted to its hydrazone under the assay condition described.

Many other reducing sugars yielded dansyl hydrazones with discrete mobilities which could be detected by TLC (Table I). The rate and yield of hydrazones formed with free aldoses was similar to that observed for D-glucose. However, 2-amino-2-deoxy sugars as well as ketoses (*e.g.*, D-fructose and L-sorbose) reacted very poorly with dansyl hydrazine and yielded low levels of hydrazones under the conditions described. Prolonged heating seemed to increase somewhat the yield of these hydrazones. Also, it was noted that if hydrazone formation was conducted at pH 4–6, ketoses and 2-amino-2-deoxy aldoses did not interact significantly with dansyl hydrazine even if heated to 100°, whereas glucose and other aldoses reacted readily under these conditions. A complete and comparative kinetic analysis of the interaction of ketoses, aminosugars and aldoses with dansyl hydroazine has not yet been performed.

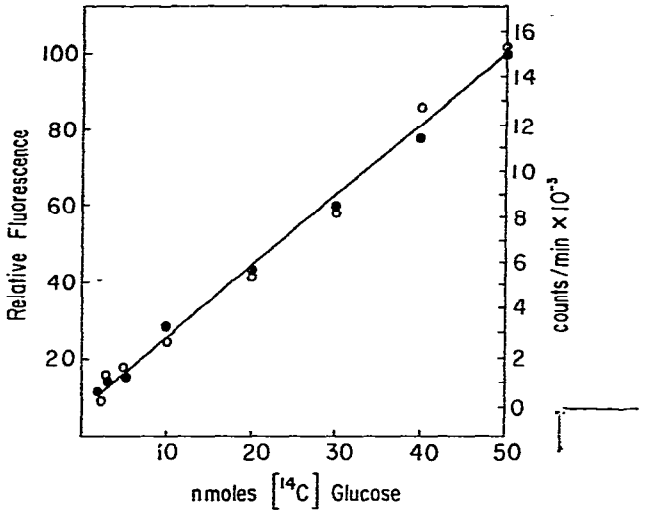

Fig. 1. Relationship between fluorescence intensity and glucose dansyl hydrazone concentration. Samples (10 μl) of reaction mixtures containing the indicated quantities of [^{14}C]glucose ($3 \cdot 10^5$ counts/min·μmole) were analyzed by TLC and subsequent elution as described in the text.

The arsenal of chromatographic, spectrophotometric and enzymatic procedures available to the analyst for the assay of reducing sugars is very large, but only a very limited number of reliable fluorimetric methods are available for this purpose. The method described here does not intend to substitute for any of the popularly employed procedures for the micro determination of reducing sugars in solution. However, it should be considered to be a useful addition which may occasionally provide a simple and helpful method for the detection of submicromolar quantities of sugars, primarily aldoses, in a mixture. Such occasions often exist during the analysis of acid hydrolysates of complex carbohydrates isolated in very small quantities from various biological preparations.

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