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Note

Fluorescamine as a tool for amino sugar analysis

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In the course of our investigations on chitin metabolism in insects, a need arose for a rapid and sensitive assay for glucosamine in solution and on paper chromatograms. Although there are numerous assays for amino sugars and for carbohydrates in general, they were found to be either laborious or were limited in their applicability. For example, a simple and sensitive fluorometric method utilizing *o*-phthaldehyde (OPA)[1] was investigated and found suitable only for solution analysis.

Because we desired a simple, rapid, single method for the analysis and detection of amino sugars, the possibility of using fluorescamine (4-phenylspiro[furan-2(3H),1′-(3′H)-isobenzofuran]-3,3′-dione)[2] was investigated. Fluorescamine has been successfully used in the analysis of amino acids[3,4], peptides[5,6], and proteins[7,8]. Since this reagent reacts extremely well with free amino groups, it should be applicable to amino sugars. The advantages of this reagent are (1) high sensitivity due to high fluorescence yield, (2) rapid reaction and completeness which allow its use as a spray, (3) ready hydrolysis in aqueous solutions to form a non-fluorescent product, and (4) extreme ease of application.

In this communication we report the application of fluorescamine to the quantitative and qualitative analysis of amino sugars and to the determination of chitin concentration.

EXPERIMENTAL

The hydrochloride salts of D-glucosamine (GlcN) and D-mannosamine (ManN) were purchased from Sigma (St. Louis, MO, U.S.A.), and D-galactosamine (GalN) from Calbiochem (San Diego, CA, U.S.A.). [6-^{3}H]GlcN (21.0 Ci/mmol) was a product of New England Nuclear (Boston, MA, U.S.A.). Fluorescamine was obtained from Pierce (Rockford, IL, U.S.A.). Regenerated chitin was prepared as described by Molano *et al.*[9]. Hydrolysis of chitin was performed as previously described[10].

Quantitative analysis of amino sugars

Reactions were carried out in disposable 12 × 75 mm borosilicate culture

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tubes without prior washing. Aqueous samples (0.1 ml) containing 0.02–100 nmol of amino sugars were mixed with 1 ml of Na_2HPO_4 (50 m*M*). While mixing on a vortex mixer, 0.05 ml of fluorescamine solution (3.0 mg/ml in acetonitrile, good for at least two weeks at room temperature) was added. Fluorescence was determined with a ratio spectrofluorometer (Aminco, Silver Springs, MD, U.S.A.) in 1-cm light path cuvettes with excitation at 395 and 485 nm emission. The light-slit arrangement was 1.0-mm primary slit, 2.0-mm secondary slit, and 3.0-mm slit at the photomultiplier tube. GlcN concentration was determined in two chitin hydrolysis samples by this method. Determinations were also carried out with *p*-(dimethylamino)benzaldehyde[11] for comparison.

Paper chromatography

Aqueous solutions containing various amounts of amino sugars were applied (1 μl) to Whatman No. 1 chromatography paper, and descending chromatography was performed at room temperature for 16 h with 1-butanol–pyridine–water (6:4:3)[9]. After air drying, chromatograms were sprayed with fluorescamine in acetonitrile (0.2 mg/ml) and the fluorescent spots detected by long-wave UV light. When the effects of the fluorescent reaction products on radioactivity measurements were analyzed, *ca.* 100,000 dpm of [^{3}H]GlcN were included in GlcN samples. The fluorescent spots were located and cut into 1-cm segments. These cut segments were placed in 3 ml of Bray's solution (New England Nuclear) in Wheaton polypropylene Omni vials (Fisher Scientific, Pittsburgh, PA, U.S.A.) and the radioactivity measured with a liquid scintillation spectrometer. Controls were co-chromatographed with test samples but were not sprayed.

Ascending chromatography was performed similarly for 25 min on glass-fibre sheets (ITLCSG, Gelman, Ann Arbor, MI, U.S.A.) impregnated with 1% NaH_2PO_4 using chloroform–methanol–ammonium hydroxide–water (130:63:7:4)[12].

RESULTS AND DISCUSSION

The quantum yields for the ManN-, GalN- and GlcN-fluorescamine derivatives appear to be the same because there is little or no difference in the fluorescence intensities at identical concentrations (Fig. 1). Also, Fig. 1 shows that fluorescence is linearly dependent on the amount of amino sugar over a range of 0.05–100 nmol. Although the OPA fluorescence method[1] is more sensitive (*ca.* eight-fold) we found the fluorescamine assay to be sufficiently sensitive and much more versatile. Moreover, laborious labware cleaning and the use of ultra-pure chemicals were not necessary with fluorescamine as they are with OPA[1]. An additional advantage is that the fluorescamine reaction is almost instantaneous and the product is stable for hours. However, since fluorescamine reacts readily with water[3], it is essential to mix the reactants vigorously.

An attempt was made to increase the fluorescence intensity of the sugar derivatives by adding 2-mercaptoethanol, as was successfully used with the OPA method[1]; however, the fluorescence was severely quenched at 0.1%. Other concentrations were tried but proved unsuccessful in increasing fluorescence. The possibility of increasing the fluorescence yield by decreased solvent polarity was also examined. Ethanol, methanol, and acetonitrile were all found to have no effect.

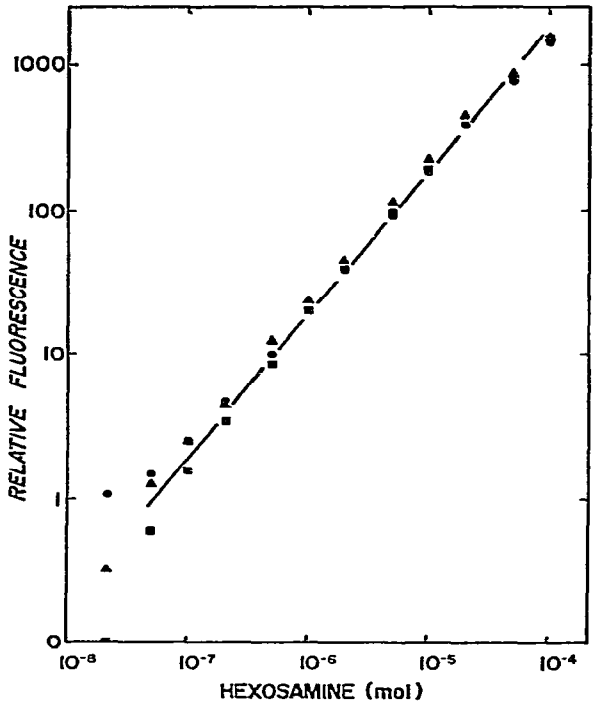

Fig. 1. Standard curve showing fluorescence intensity in relation to the amount of amino sugar in the reaction mixture. A logarithmic scale is used to accommodate the wide range of fluorescence and concentration. The amino sugars are GalN (●), GlcN (■) and ManN (▲). Each point is the mean of triplicate determinations. The standard deviations are not included because they do not exceed the size of each point.

Advantage was taken of the extremely rapid reaction between fluorescamine and primary amines by using this reagent as a spray for the detection of amino sugars on paper chromatograms. We could easily detect spots containing 1 nmol of the sample in an area of 1.5 cm^2. This is *ca.* ten times more sensitive than alkaline $AgNO_3$ treatment[13]. The OPA method could not be used to detect samples on chromatograms. This method can also be used qualitatively if amino sugar standards are co-chromatographed with the test samples.

In addition, the method can be used with internal standards on chromatograms containing radioisotopes. After visualization, the fluorescent spots can be removed for scintillation measurement. The presence of fluorophore or unreacted fluorescamine does not interfere with the measurement of radioactivity. This is demonstrated in Table I as the cpm values for sprayed samples were the same as for non-sprayed samples. This application can also be used with carbohydrate samples not containing amino sugars by utilization of R_F values relative to an internal GlcN standard.

When ascending chromatography was performed on glass-fiber sheets and sprayed with fluorescamine we were able to visually detect 0.5 nmol of GlcN in a spot of 0.8 cm^2. This is *ca.* a 50-fold increase in sensitivity over the charring method detected by densitometry[12]. In our experience the visual detection limit for charred samples was 23 nmol. As with paper chromatography, spraying on glass-fibre sheets with fluorescamine did not affect the measurement of radioactivity (Table I).

TABLE I

EFFECTS OF FLUORESCAMINE ON RADIOACTIVE MEASUREMENTS

A 1-μl volume of GlcN (5 nmol containing 100,000 dpm [^{3}H]GlcN) was chromatographed. After spraying, the radioactivity of the fluorescent section was measured in 1-cm segments in 3 ml of Bray's solution in a liquid scintillation spectrometer. The corresponding sections on chromatograms without spraying were treated in the same manner.

Chromatogram	*Fluorescamine spray*		*t-test*
	Yes [cpm ± S.D. (n = 3)]	*No [cpm ± S.D. (n = 3)]*	
Whatman No. 1 paper	12,975.7 ± 424.5	13,289.1 ± 161.1	$p > 0.05$
ITLCSG glass-fiber sheets	31,618.2 ± 349.7	32,440.9 ± 550.3	$p > 0.05$

Chitin concentration has been traditionally determined in terms of N-acetyl-D-glucosamine equivalents by the Morgan–Elson reaction[11] after exhaustive digestion of the polymer by chitinase and chitobiase[9]. Alternatively, it can be acid-hydrolyzed and the resultant GlcN quantified by the Elson–Morgan procedure[14] or by the Morgan–Elson procedure after acetylation[11]. All these methods are multi-step procedures and cumbersome. However, with fluorescamine the determination of GlcN in the acid hydrolysate is not only simple but 100-fold more sensitive than the Morgan–Elson reaction (50 pmol *vs.* 5 nmol detection limit). A comparison of the two procedures showed that a suspended chitin preparation had 680 ± 12.7 (S.D.) nmol GlcN eq/100 μl by the present method while the Morgan–Elson procedure[11] yielded 645 ± 22.6 (S.D.) nmol GlcN eq/100 μl. The close correlation between results obtained with the two methods shows the validity of the fluorescamine quantitation of GlcN. Furthermore, the improved reproducibility of the fluorescamine method makes it the more desirable.

The current reports extends the use of fluorescamine to the analysis of amino sugars. This method is simple, rapid, and sensitive and should facilitate future analyses of amino sugars.

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