A new method for hexosamine detection using 5-dimethylaminonaphthalene-sulfo-derivatives and separation by thin-layer chromatography

Several methods have been developed for the detection and quantitative estimation of glucosamine and galactosamine. With some of them it is possible to determine the total hexosamine content in natural products[1–5]. The existing methods of separate determination are not completely satisfactory and do not always yield reproducible results[6]. Paper or ion-exchange chromatography have been used for the separation either of free amino sugars[7,8] or their DNP*-derivatives[9,10]. Gas chromatography[11–13] has also been described, and quite recently chromatography of free amino sugars on a thin layer of powdered cellulose[14].

In view of the fact that natural products contain either only glucosamine or only galactosamine, their preliminary qualitative estimation would make the existing methods more precise. We have attempted here to develop a simple and substantially more sensitive method for determining glucosamine and galactosamine in a mixture by use of their DNS-derivatives. The R_F value of DNS-glucosamine is already known[15].

Materials and methods

α-D-Glucosamine hydrochloride (Koch-Light Laboratories, Ltd., England) and D-galactosamine hydrochloride (LOBA-Chemie, Austria), homogeneous in paper chromatography, were used. An apyrogenic preparation of glucose (SPOFA, Č.S.S.R.), DNS-chloride (Calbiochem, U.S.A.), and Kieselgel G für Dünnschichtchromatographie nach Stahl (Merck A.G., Germany) were also used.

The standard thin-layer chromatographic technique according to STAHL[16] with ascending development was used.

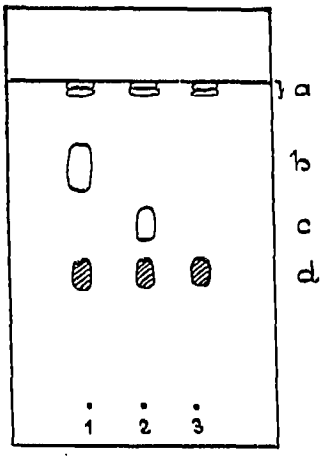
The DANSYLation was carried out according to GRAY AND HARTLEY[17] with several modifications: The hexosamine solution (GLA and GAA) was evaporated in a small tube, containing between 0.003 and 0.005 μmole of each substance. The residue was mixed with 20 μl 0.8% $NaHCO_3$ and 25 μl acetone solution of DANSYL-chloride (1 mg/ml). The tube was closed and left for 3 h at 4° or for 2 h at 15–20° in the dark. The reaction mixture was evaporated in a desiccator over NaOH and the DNS-derivatives were dissolved in acetone before placing on the thin-layer plate.

The chromatographic layer had the following composition: 20 ml 0.2 *M* $Na_2B_4O_7$, 45 ml water, 30 g Kieselgel G, with a thickness of 0.2 mm. The plates were dried in air for 10–12 h and then for 3 h at 130°. Before use they were heated for 10 min to 105°. The following solvent systems were found to be the most satisfactory: (I) ethyl acetate–pyridine–water (61:31:8, v/v), (II) petroleum ether–glacial acetic acid–*tert.*-butanol (75:15:15, v/v)[18], and (III) chloroform–methanol–glacial acetic acid (75:25:5, v/v)[19]. When a two-dimensional technique was used, the plates were dried after development in the first direction for 10 min at 105°. The DNS-derivatives were detected by examining the plates under transmitted U.V. light while still wet, when they appear as yellow fluorescent spots.

Results and discussion

One-dimensional chromatography in system (I) resolves DNS-GLA, DNS-GAA

* Abbreviations used: DNP- = dinitrophenyl-; DNS- (DANSYL-) = 5-dimethylaminonaphthalenesulfonyl-; GLA = glucosamine; GAA = galactosamine; GL = glucose.



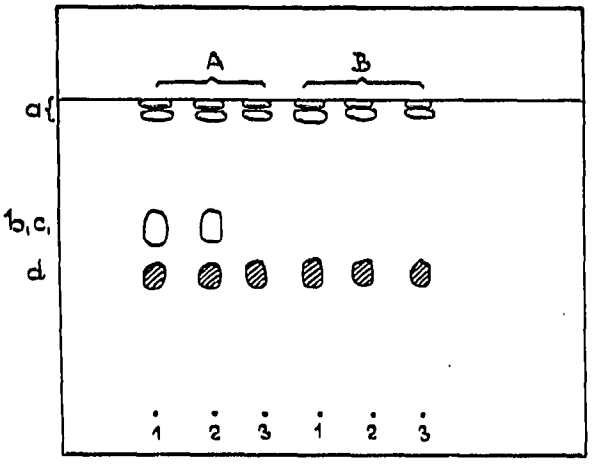


Fig. 1. Chromatogram of DNS-derivatives in system (I) using Kieselgel G with borate. DANSYLated samples: 1 = GLA; 2 = GAA; 3 = GL; a = DNS-NH_2 and artifacts; b = DNS-GLA; c = DNS-GAA; d = DNS-OH.

Fig. 2. Chromatogram of DNS-derivatives in system (III) using Kieselgel G with borate. A = sample DANSYLated by the standard procedure; B = samples incubated in the DANSYLation mixture at 105° for 1 h. Other symbols as in Fig. 1.

and DNS-GL within 40–50 min, the front travelling about 14–16 cm (Fig. 1). The sensitivity of the method is $3–5 \cdot 10^{-3}$ μmole hexosamines. DNS-hexosamines show some properties whereby they differ from the DNS-derivatives of amino acids and these can be made use of for their differentiation.

Thus, *e.g.* DNS-GLA and DNS-GAA in aqueous solution are highly temperature-sensitive. After incubation in the basic medium of the DANSYLation mixture at 37° for 1 h the spot intensity diminishes markedly. After 1 h at 105° the spots disappear completely (Fig. 2). Similarly, after hydrolysis in 6 *N* HCl at 105° the hexosamine

TABLE I

R_F VALUES OF DNS-DERIVATIVES OF GLA, GAA AND SOME AMINO ACIDS DURING THIN-LAYER CHROMATOGRAPHY USING KIESELGEL G WITH SODIUM BORATE

DNS-derivative	*Solvent system**	
	I	*II*
-GLA	0.65	0.00
-GAA	0.50	0.00
-Val, -Lys, -Tyr, -Phe, -Leu, -Ile	0.45–0.65	0.15–0.60
-Cys,-Pro,-Ser, -Thr,-Glu,-Gln, -Asp,-Asn,-Gly	0.40	0.10
-OH	0.40	0.00
-NH_2	0.92–0.96	0.09
-GL	1.00	0.90

* Composition given in the text.

spots disappear. On the other hand, the DNS-amino acids under identical conditions are quite stable.

The solvent system (III) was used for identifying artifacts and for following the stability of DNS-hexosamines. It was shown that DNS-GL as an O-DNS-derivative gives a weak spot in the front of system (I).

The separation of DNS-hexosamines from DNS-amino acids was attempted. During two-dimensional chromatography, when system (II) was used in the first direction, it separated DNS-GLA and DNS-GAA from most amino acids. In the second direction (system I) the hexosamines are distinguished from each other. The R_F values of DNS-hexosamines and DNS-amino acids in the two systems are shown in Table I where it can be seen that the R_F values of most DNS-amino acids differ from those of DNS-hexosamines. Experiments along these lines are being continued since the present separation is not considered as fully satisfactory.

The use of DNS-derivatives of GLA and GAA for the chromatographic separation described permits a very sensitive and simple detection, as well as a separation of both hexosamines within 3.5 h. Some properties of DNS-hexosamines were established which make it possible to distinguish them from DNS-amino acids.

The authors are indebted to Academician F. ŠORM for making this work possible.

Institute of Organic Chemistry and Biochemistry,
Czechoslovak Academy of Sciences, Prague (Czechoslovakia)

A. A. GALOYAN*
B. K. MESROB**
V. HOLEYŠOVSKÝ

1 L. A. ELSON AND W. T. MORGAN, *Biochem. J.*, 27 (1933) 1824.
2 W. T. MORGAN AND L. A. ELSON, *Biochem. J.*, 27 (1934) 988.
3 S. GARDELL, F. HEIJKENSKÖLD AND A. ROCHNORLUND, *Acta Chem. Scand.*, 4 (1950) 970.
4 N. BOAS, *J. Biol. Chem.*, 204 (1953) 553.
5 D. EXLEY, *Biochem. J.*, 67 (1957) 52.
6 S. GARDELL, *Biochem. Soc. Symp. (Cambridge, Engl.)*, 20 (1961) 39.
7 J. JONES, in R. L. WHISTLER AND M. L. WOLFROM (Editors), *Methods in Carbohydrate Chemistry*, Vol. I, Academic Press, New York, 1962, p. 21.
8 S. GARDELL, *Acta Chem. Scand.*, 7 (1953) 207.
9 P. KENT, G. LAWSON AND A. SENIOR, *Science*, 113 (1951) 354.
10 S. LESKOWITZ AND E. KABAT, *J. Am. Chem. Soc.*, 76 (1954) 4887.
11 M. PERRY, *Can. J. Biochem. Physiol.*, 42 (1964) 451.
12 J. RICHEY, H. RICHEY AND R. SCHRAER, *Anal. Biochem.*, 9 (1964) 272.
13 J. KÄRKKÄINEN, A. LEHTONEN AND T. NIKKARI, *J. Chromatog.*, 20 (1965) 457.
14 H. GÜNTHER AND A. SCHWEIGER, *J. Chromatog.*, 17 (1965) 602.
15 Z. DEYL AND J. ROSMUS, *J. Chromatog.*, 20 (1965) 514.
16 E. STAHL, *Dünnschichtchromatographie*, Springer-Verlag, Berlin, Göttingen, Heidelberg, 1962.
17 W. GRAY AND B. HARTLEY, *Biochem. J.*, 89 (1963) 59P.
18 B. MESROB AND V. HOLEYŠOVSKÝ, *J. Chromatog.*, 21 (1966) 135.
19 N. SEILER AND J. WIECHMAN, *Experientia*, 20 (1964) 559.

Received April 14th, 1966

* Present address: Institute of Biochemistry, Academy of Sciences of Armenian SSR, Erevan.
** Present address: Institute of Chemical Technology, Sofia.