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SEPARATION AND QUANTITATIVE DETERMINATION OF GALACTOSAMINE AND GLUCOSAMINE AT THE NANOGRAM LEVEL BY SULPHONYL CHLORIDE REACTION AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Two chromatographical systems for the separation and determination of glucosamine and galactosamine are described. In the more rapid and less sensitive of the two systems, the hexosamines are treated with toluene sulphonyl chloride and subsequently separated in a reversed-phase system. In this mode 0.4–50 μg of the hexosamines are chromatographed within 10 min. In the second and more sensitive system, the hexosamines are treated with Dns-Cl and subsequently chromatographed in a similar reversed-phase column. With this method, the separation of 0.02–2 μg is possible within 25 min. When internal standard was used, the procedure was lengthened by *ca.* 10 min. Both determinations can also be used when there are large differences in the relative amounts of the two hexosamines.

INTRODUCTION

The separation and characterization of hexosamines is of great importance in the study of glycosaminoglycans as well as in that of glycoproteins and glycolipids. D-Glucosamine and D-galactosamine and their N-acetylated derivatives are the most frequently encountered of these aminoglycans. A variety of methods, including ion-exchange chromatography[1,2], thin-layer chromatography (TLC)[3], paper chromatography[4] and gas–liquid chromatography[5], have been described for

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the study of these substances. These methods are either rather laborious or insensitive when only a few micrograms or less are available.

The development of high-performance liquid chromatographic (HPLC) techniques offers possibilities for scaled-down and rapid separation. Attempts to separate these amino sugars using different kinds of resin were, however, less successful owing to poor resolution in the columns as well as low sensitivity of the detectors used.

Sulphonyl chlorides such as 5-dimethylaminonaphthalene-1-sulphonyl chloride (Dns-Cl) have previously been used to detect primary amino groups in proteins[6] as well as in hexosamines[7]. Apart from being more easily detected, such derivatives seemed suitable for chromatographical separation in reversed-phase systems. The aims of this investigation were therefore to develop suitable procedures for the reaction with sulphonyl chlorides and to find optimal conditions for the subsequent chromatographic separation of the glucosamine and galactosamine derivatives. Two systems were tested, employing toluene sulphonyl chloride and Dns-Cl, respectively.

MATERIALS AND METHODS

Proteoglycan aggregates were prepared from puppy epiphyseal cartilage according to the method of Hascall and Sajdera[8]. Glycosaminoglycans were obtained from Sigma (St. Louis, MO, U.S.A.) as their "grade I" preparations. [^{3}H]Glucosamine hydrochloride was obtained from Amersham (Arlington Heights, IL, U.S.A.) and further purified chromatographically according to Antonopoulos[1]. Brij-35 (polyoxyethylene ether or lauryl alcohol) was obtained as a 30% solution from Sigma. The acetonitrile used was of a reagent grade (Baker, Phillipsburg, NJ, U.S.A.) and LiOH was of a puriss grade. All other chemicals used were of p.a. grade.

As the typical experiment for hexosamine determination, the following procedure was followed. The hexosamine-containing materials were hydrolyzed for 3 h with 8 *M* HCl at 95°C[9]. After lyophilization, protein-rich materials were purified using a modified Boas procedure[10]. Here the hydrolysates were dissolved in water and added to Dowex 50-X8 columns (8 × 3 mm I.D.). These columns were washed with 0.5 ml of water and the hexosamines were eluted with 0.2 ml of 2 *M* HCl. This fraction was lyophilized over NaOH pellets. The material was then dissolved in 25 μl of 0.1 *M* lithium acetate, which was prepared from a LiOH solution by adjusting the pH to 8.5 with acetic acid. Subsequently 75 μl of the sulphonyl chloride as a 1 mg/ml solution in acetone was added. Dns-Cl was used to detect amounts of hexosamines a few micrograms or less, while for larger amounts, toluene sulphonyl chloride was preferred. The tubes were subsequently sealed and left in the dark overnight. Prior to chromatography, particulate materials were removed by centrifugation at 10,000 *g* for 5 min.

For both kinds of derivative reversed-phase columns were used. The substances separated similarly in the different octadecylsilane resins tested. These were: Hypersil ODS (Shandon Southern Products, London, Great Britain) and LiChrosorb 5 RP-18 (Merck, Darmstadt, G.F.R.) packed in 200 × 4.6 mm I.D. columns; Waters C_{18} (Waters Assoc., Milford, MA, U.S.A.) packed in a 300 × 3.9 mm I.D. column (custom packed); and Supelcosil RC-18 (Supelco, Crans, Switzerland) in a custom-packed column (150 × 4.6 mm I.D.). The toluene sulphonyl derivatives were separated

in 150 × 4.6 mm I.D. Hypersil ODS columns. The material was mostly added in portions of 25 μl or less, using a loop injector.

The Dns derivatives were eluted with an 18% mixture of acetonitrile in water containing 0.1% Brij-35. With the Hypersil ODS resin and a flow-rate of 1.3 ml/min, a backpressure of 14 MPa (2000 p.s.i.) was obtained. Between each chromatogram the column was washed with ten column volumes of 99.7% acetonitrile, containing 0.1% Brij-35, followed by four column volumes of the 18% acetonitrile solution prior to the next injection. Using an Altex Model 420 programmer the washings could be performed automatically, enabling one chromatogram to be run every 35 min. The Dns derivatives could, however, be purified prior to the HPLC procedure, whereby a few daily washings sufficed. This purification was performed as follows. The reaction mixture was kept *in vacuo* for 2 h, reducing the volume by 75%, corresponding to the acetone volume. Water was added to restore the original volume, and the material was injected in a Sep-Pak C_{18} cartridge (Waters Assoc.). Most of the front material was washed away with 7 ml of 20%methanol, and virtually all hexosamine material was recovered with 3 ml of 60% methanol. Aliquots of this fraction could be taken directly for chromatography, or the material could be concentrated by desiccation, The recordings were made using a Schoeffel FS 970 fluorometer (Schoeffel Instruments, Kratos Inc.), with an excitation wavelength of 340 nm and a 470-nm cut-off filter for emitted light.

Toluene sulphonyl derivatives were eluted with 8% acetonitrile in water containing 0.1% Brij-35, and the eluent was pumped at a flow-rate of 2 ml/min (10 MPa). These columns were similarly washed after each chromatogram and one run could be performed each 12 min. The toluene sulphonyl derivatives could also be purified using a Sep-Pak C_{18} procedure, thereby reducing the need for column washes. Here front material was washed out with 1.5 ml of 10% methanol and the hexosamines were recovered with 3 ml of 30% methanol. The eluted peaks were recorded using an LDC spectromonitor III UV detector at 247 nm.

An internal standard was used when the absolute amounts of the respective hexosamines were to be determined. Tris(hydroxymethyl)aminomethane (Tris) seemed suitable for this purpose. It was added as the Dns or toluene sulphonyl derivative prior to the centrifugation. The washings were delayed 10 and 3 min, respectively, when this internal standard was present.

RESULTS AND DISCUSSION

The sulphonyl chlorides readily reacted with the hexosamines under the conditions described. Most material could be recovered within the two main peaks, indicating a recovery exceeding 95%, when radioactively labelled glucosamine was used. Contrary to earlier reports[7], this recovery was not achieved until after several hours, and the resulting products were stable for weeks. This discrepancy with the earlier results probably arises because better quantitation is obtained with HPLC than with TLC.

When the reaction was performed according to the original description, using a carbonate buffer instead of an acetate one, the fluorimetric recordings were spoiled by considerable tailing of the front. This tailing was probably caused by carbonate microprecipitates, and when acetate was used this tailing disappeared.

Even if this buffer did not have the same capacity, it was sufficient to keep the reaction mixture on the alkaline side of the pK_s values of the hexosamines and the rate of the reaction was thus not affected.

The Dns derivatives of the two hexosamines studied were eluted with their main peaks at 18 min for galactosamine and at 20 min for the glucosamine derivatives (Fig. 1). For both amino sugars a smaller peak was obtained shortly after 12 min. When this peak was collected and rechromatographed, the original pattern with two peaks was obtained. The same was also found on the rechromatography of pooled material from the main peaks. These findings, and the fact that longer retention times caused tailing from both of these peaks towards the other, show that the two peaks interchange in a short time. It can therefore be suggested that these peaks represent the α and β anomers of the hexosamine. According to the chromatograms, 10–20% of the amino sugar derivatives were recovered in the first (probably β) peak.

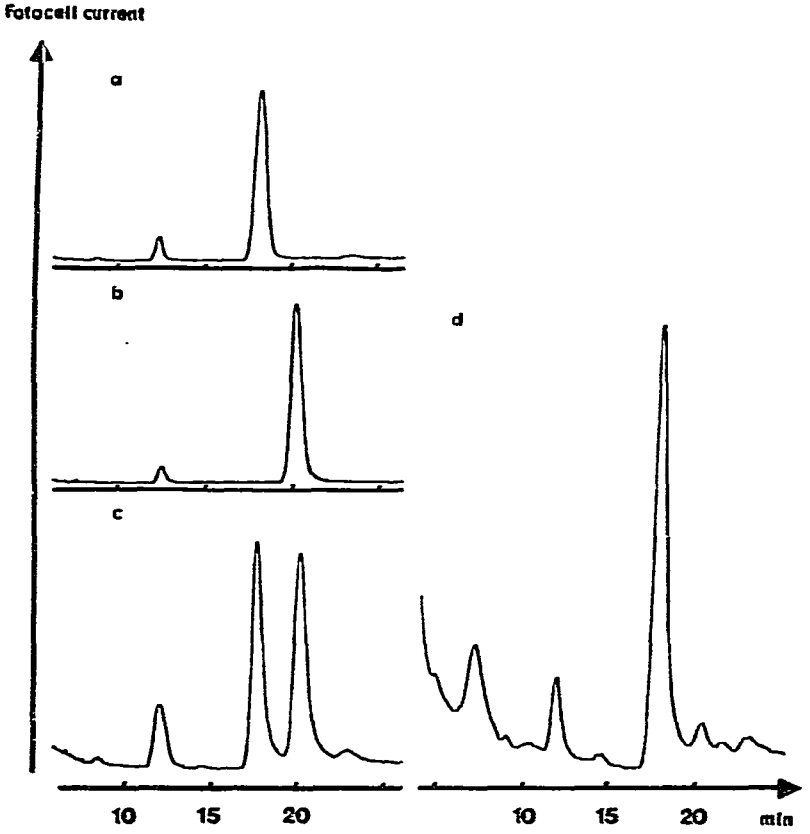

Fig. 1. Chromatography of glucosamine and galactosamine as their Dns derivatives on ODS-Hypersil. The main peaks are eluted at 18 min (galactosamine) and 20 min (glucosamine). (a) 130 ng of galactosamine; (b) 100 ng of glucosamine; (c) 160 ng of galactosamine plus 120 ng of glucosamine; (d) hydrolysate of epiphyseal cartilage proteoglycan aggregate corresponding to 250 ng of hexosamine and showing a galactosamine:glucosamine ratio of 25:1.

No further peak was obtained from glucosamine, whereas one additional peak was recorded after 23 min from the galactosamine preparations (Fig. 1). The material of this additional peak also seems to be transformed into the main-peak material during chromatography, necessitating a rapid chromatography to minimize the interference with glucosamine, which elutes in between. Since no corresponding peak was obtained from the glucosamine material, it seems to be dependent on the configuration of the hydroxyl group on the fourth carbon atom. In fact, evidence for an interaction between vicinal *cis*-hydroxyl groups and surrounding cations has been reported by Goulding[11]. The third and fourth hydroxyl groups of galactosamine have such a *cis*-relationship and this peak might therefore represent galactosamine with a cation associated with these hydroxyl groups. To minimize this effect, a lithium salt was chosen to buffer the sulphonyl chloride reaction (ammonium ions were incompatible with these reagents). Sodium, which is the cation present in Fig. 1c, caused a larger peak at 23 min, and if potassium was used this peak became still larger.

Under the conditions described, with lithium acetate buffering the reaction, the interval between these peaks at 18 and 23 min showed a baseline elevation of only 1% of the preceding galactosamine main peak height. Furthermore, this elevation was the same in the whole interval, and it therefore did not interfere with the estimation of the glucosamine peak area, also when high galactosamine:glucosamine ratios were studied. The Dns-Tris derivative used as internal standard eluted at 30 min.

When these Dns derivatives were chromatographed the main peaks separated with an α-value of 1.14, giving a baseline separation with the Hypersil, LiChrosorb or Waters resins. The use of a Supelcosil resin, with its high bottom-plate number, gave only slightly better performance. No significant reduction of the elution times could thereby be obtained. The toluene sulphonyl derivatives could be more readily separated owing to a higher α value (1.37) for the main peaks (Fig. 2). The detector

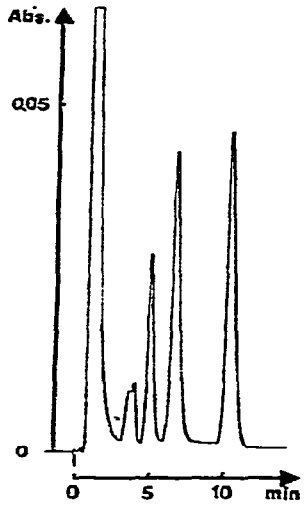

Fig. 2. Chromatography of equimolar amounts (3 μg each) of glucosamine and galactosamine as their toluene sulphonyl chloride derivatives. The main peaks are eluted at 5 min (galactosamine) and 7 min (glucosamine). The peak at 10 min is the Tris derivative used as internal standard.

sensitivity for these derivatives is lower than for the Dns ones. When larger amounts (a few micrograms or more) of hexosamines are available, however, this seems to be the method of choice, since the main peaks can be separated in less than 8 min owing to the high α value. When the Tris derivative was added this material was recovered in 10 min.

The detection limit, expressed as twice the baseline noise, was for Dns derivatives of the order of 0.5 ng of hexosamine added to the column. It was also shown that 20 ng could be determined within a 5% confidence interval, this latter figure including other errors such as pipetting errors. The corresponding values for toluene sulphonyl derivatives were 10 ng and 400 ng.

The standard curves were linear and they showed different recordings for equimolar amounts of glucosamine and galactosamine, the former being 30–35% higher for the two kinds of separation. These differences may partly be due to differences in molar absorption and to differences in the ratios of α- and β-anomers. Therefore, standards containing both glucosamine and galactosamine should preferably be used.

Dns-Amino acids were also tested, but none of them was found to interfere with the hexosamines tested. To avoid interference with amino acids in large amounts, however, the hydrolysates of protein-rich compounds should preferably be treated with the Boas procedure as described above. From the Boas columns the recovery was complete, and breakthrough did not occur with less than 1 mg of hexosamine added. Nevertheless the proteoglycan preparation showed several small and unidentified peaks (Fig. 1d), in spite of the Boas procedure. It seems as if such a hydrolysate contains other kinds of Dns-reactive material, which in smaller amounts may pass the preceding Dowex column together with the hexosamines. The obtained galactosamine:glucosamine ratios were, however, the same as those obtained by other methods[2], and we thus have no indication of interfering materials being eluted together with the main hexosamine peaks.

In the study of polymeric carbohydrates, methanolysis is sometimes a necessary alternative to hydrolysis. Attempts were therefore made to separate the 1-methylhexosamines obtained from the methanolysis of different glycosaminoglycans. This was done by eluting the Dns derivatives with 24% acetonitrile at a flow-rate of 1.3 ml/min. Different products were thereby nicely separated, but a large number of fluorescent derivatives were obtained. Thus 13–16 different retarded and separated peaks could be identified for each of the glycosaminoglycans tested, and the determination of galactosamine:glucosamine ratios thus became troublesome and inaccurate. It therefore seems that methanolysis is less suitable for such determinations than hydrolysis.

Washing of the column between each chromatographic run was necessary with the sulphonyl derivatives, where at least six different minute peaks could be distinguished during the washing procedure. In fact, if this washing was not performed, the chromatographic conditions altered considerably, indicating some kind of partition chromatography with contaminants as a stationary phase. In fact, the separations were even slightly better this way, but the conditions for good separations were not constant, giving variations in retention times. With the preparative Sep-Pak procedure described above, the separation did not change during twenty chromatographic runs. Thus after such a preparation a few daily washings suffice.

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