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Separation on Dowex 50 Ion Exchange Resin of Glucosamine and Galactosamine and their Quantitative Determination

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Glucosamine (chitosamine) and galactosamine (chondrosamine) occur simultaneously as components of a number of natural polysaccharide mixtures. Morgan *et al.*[1] *e.g.* demonstrated that both of them are found in the blood group A substance isolated from the pseudomucin of ovarian cysts.

The amino sugars are usually identified as their hydrochlorides which crystallize from methanol on the addition of acetone or ether. Glucosamine can be identified fairly easily in this way, even when it is present in small amounts, but galactosamine hydrochloride is difficult to crystallize because of its solubility. Other methods have therefore been suggested, such as the isolation of the carbobenzoxy derivative [2] and isolation of various Schiff bases, such as those formed with 2.4-dihydroxybenzaldehyde [3] and with 2-hydroxynaphtaldehyde [4]. For the identification of small amounts of amino sugar Blix, Svennerholm and Werner [5] used X-ray spectrography.

Paper chromatography has been used by Partridge [6], Aminoff and Morgan [7], Gardell, Heijkenskjöld and Roch-Norlund [8], and also by Kent, Lawson and Senior [9]. A chromatographic method for the isolation and identification of small amounts of amino sugars in mixtures, based on separation of their N-dinitrophenyl derivatives has been worked out by Annison, James and Morgan [10].

Quantitative methods for the determination of amino sugars were elaborated by Elson and Morgan [11], Dische [12] and Tracy [13]. With none of these methods, however, is it possible to differentiate between glucosamine and galactosamine.

Flood, Hirst and Jones [14] used paper chromatography for quantitative determination of single monosaccharides occurring in mixtures. Morgan *et al.*[1] used the same principle for the determination of mixed amino sugars. After separation in a collidine water mixture the different components were eluted

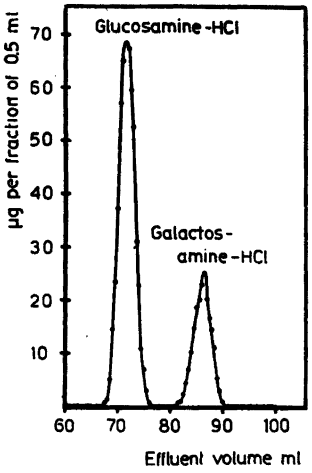

Fig. 1. Separation of 500 µg glucosamine-HCl and 225 µg galactosamine-HCl. Column 0.7 × 39.0 cm.

separately and the amount of amino sugar determined colorimetrically by the Elson and Morgan method. With this technique, not more than 30 per cent of the amino sugar was recovered, possibly due to destruction caused by the alkaline collidine.

The present author has endeavoured to avoid this source of error in the paper chromatographic method by separating the components in acid solvents. In none of the solvent systems tried (butanol, propanol and hydrochloric acid; propanol and hydrochloric acid; acetone, methanol and hydrochloric acid; methanol and hydrochloric acid, and others) was it possible to achieve a good separation. Separation in phenol is possible [6], but only if the chromatography is done with ammonia in the atmosphere, when the same source of error appears as with collidine.

Moore and Stein [15] and Partridge [16], in connection with their work on the separation of the amino acids, demonstrated that glucosamine migrates on columns of ion exchange resins.

In the present paper a chromatographic method is described for the separation of glucosamine and galactosamine hydrochloride on Dowex 50 ion exchange resin with 0.3 *N* hydrochloric acid as solvent. The liquid-chromatogram principle was applied and the effluent was collected in fractions of equal size, which were analyzed essentially by the Elson and Morgan method as modified by Blix [17]. By constructing concentration-effluent curves the resolving power of the column can be demonstrated directly. The effluent volumes corresponding to the peaks of the two amino sugars are so different when 0.3 *N* hydrochloric acid is used, that the separation of the components will be complete, thus making a quantitative determination of each of them possible. It is even pos-

sible to get sufficient amounts for crystallization. Fig. 1 shows a chromatogram made on a column 39.5 cm long and having a diameter of 0.7 cm.

PROCEDURE

Preparation of the column. Dowex 50, 250—400 mesh *, is washed with 4 *N* hydrochloric acid until the filtrate is colourless. The mass is then suspended in one volume of 4 *N* hydrochloric acid and is stored in this form. In preparing a column the mass is resuspended and an amount sufficient for a column of 35—40 cm length with a diameter of 0.6—0.7 cm is poured into a chromatographic tube (45 × 0.6—0.7 cm) fitted with a glass filter plate at the bottom and dilated at the top (2 × 5 cm). The tube is filled to a point about 0.5 cm from the upper edge. With the aid of a rubber stopper, the upper part of the tube is connected with a separatory funnel. A pressure of 10 cm of mercury is exerted on the funnel with the aid of compressed air. When the mass has formed a deposit of constant length the pressure is released and the funnel is filled with 0.3 *N* hydrochloric acid. A pressure of 7 cm of mercury is now applied and the column is washed with 0.3 *N* hydrochloric acid until the concentrations of the inflowing and outflowing acid are identical. This takes about 10 hours. The rubber stopper is now removed and the dilated upper part including the uppermost 1—4 cm of the tube are emptied of exchange resin and hydrochloric acid. The tube is once more connected to the funnel and a pressure of 10 cm applied until the upper surface of the column becomes dry without any air entering the column. The column is now ready for use. After use, the resin is removed, suspended in 4 *N* hydrochloric acid and washed with several volumes of 4 *N* hydrochloric acid.

Hydrolysis of the polysaccharide. The polysaccharide is hydrolysed with hydrochloric acid. After the hydrolysis the mixture is placed in a small beaker and evaporated to dryness in a desiccator over sodium hydroxide. The residue is then taken up in a suitable volume of 0.3 *N* hydrochloric acid, centrifuged to remove insoluble particles and chromatographed.

Application of the amino sugar mixture. 0.1—0.4 ml of amino sugar solution in 0.3 *N*, or stronger (up to 1 N), hydrochloric acid containing 60—600 μg of each component is added to the column and allowed to run into the column by gravity. When the amino sugar mixture is being added, care is taken to see that the upper surface of the column is not stirred up. The walls above the column are washed three times with 0.1 ml of 0.3 *N* hydrochloric acid without extra pressure, and the space in the tube, above the column, is filled with 0.3 *N* hydrochloric acid. By means of a tightly fitting rubber stopper and a piece of tubing the chromatographic tube is connected with a 500 ml flask containing the same acid and the position of the flask is regulated so that the rate of flow will be 1.5—2 ml an hour.

Collection of the effluent. The column is connected to a fraction collector ** and the effluent is collected in thin-walled test tubes, 15.4—16 × 120 mm, in 0.5 ml fractions. The volume of the effluent is measured from the moment when the amino sugar mixture is applied to the column.

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* From Microchemical Specialities, Berkeley 3. California.

** A modification of the apparatus described by Stein and Moore [18] was used. The apparatus was made by Svenska Mätapparater Fabriks Aktiebolag, Stockholm, Sweden, and is described in a paper by Carlander and Gardell [19].

Analysis. Reagents. Standard solutions of glucosamine and galactosamine hydrochloride in 0.3 *N* hydrochloric acid containing 10, 20, 50, 75 and 100 μg/0.5 ml. These solutions keep for about one month if stored in a refrigerator at + 4° C.

Acetylacetone solution. 1.5 ml of pure, colourless acetylacetone is diluted to 50 ml with 1.25 *N* sodium carbonate. The solution can only be kept at room temperature for three hours at the most. The stability is not appreciably increased in the cold.

The Ehrlich reagent. 1.6 g of p-dimethylaminobenzaldehyde, A. R., is dissolved in 30 ml of concentrated hydrochloric acid and 30 ml of 96 per cent alcohol is then added. The solution is pale yellow in colour and can be used for about two months if stored in the cold.

Technique. To each test tube containing a whole fraction in a volume of 0.5 ml, 1 ml of acetylacetone solution is added. The tubes are thoroughly shaken and placed in a rack equipped with a cooling system as described in a previous publication [20]. The openings of the tubes are covered with aluminium caps and the rack is heated in a water bath at 96° C for 60 minutes. A standard series with the above-mentioned five concentrations, 0.5 ml of each, and two blanks (0.5 ml of 0.3 *N* hydrochloric acid), all containing acetylacetone, are heated simultaneously. After heating, the tubes are placed in a cooling bath (tap water) for 5 minutes. The tubes are then removed from the bath, and 10 ml of 96 per cent alcohol followed by 1 ml of Ehrlich's reagent are added. The contents of the tubes are mixed carefully by blowing a stream of air through a capillary into the solution. After one hour, readings are made in a Beckman model B spectrophotometer or other similar apparatus with a 1 cm cell at 535 mμ. The standard series is read off first against the blank. As a correct blank for the amino sugar fractions is necessary for quantitative determinations, and as this blank does not necessarily need to be the same as that of the standard series, a few fractions of the effluent not containing amino sugar are included in each analysis. These fractions are read off against the blank of the standard series and the fraction giving a value most closely corresponding to the mean of these readings is used as blank in analyzing the amino sugar fractions. This value should not diverge from the blank of the standard series by more than ± 0.020 with a 1 cm cuvette.

Calculation. A standard curve is compiled for each amino sugar. In order to know which component corresponds to the respective amino sugar, it is necessary to know the effluent volume for both of the amino sugars. As this varies slightly for different batches of the Dowex resin it must be determined with the pure amino sugars. For one and the same batch of resin used in the same chromatographic tube, the effluent volume is constant and proportional to the length of the column. For columns of different diameters it is also proportional to the area of the cross section.

When the peaks have been identified each fraction belonging to the same peak is read off against the standard curve for the corresponding amino sugar. The total amount is obtained by adding the amounts of the different fractions belonging to the same peak. The separation can be illustrated by constructing concentration-effluent curves, the amount of amino sugar in each fraction being given on the ordinate and the total amount of ml of effluent on the abscissa.

Identification of the components. This can be done with the aid of the effluent volume for each peak. When there is any uncertainty as to the identity of the amino sugar, especially if there is only one peak, the presumed component is added to the hydrolysate in a new test and the mixture is chromatographed. The presence of only one peak in the concentration-effluent curve indicates with a high degree of certainty the identity of

the amino sugar. If large amounts of material are present the hydrolysate can be chromatographed on a column of larger dimensions and the components crystallized (see below).

RESULTS

Recovery. The recovery from a series of chromatograms in which varying amounts of the two components were chromatographed is demonstrated in Table 1.

Table 1. Recovery

Glucosamine hydrochloride				Galactosamine hydrochloride		
Running no.	μg	Recovered in the first component μg	Percentage recovery	μg	Recovered in the second component μg	Percentage recovery
1	200	201.8	100.9	400	380.0	95.0
2	300	286.0	95.3	—	—	—
3	200	225.0	112.5	400	427.0	106.8
4	400	415.0	103.8	400	426.0	106.5
5	400	397.9	99.5	400	397.7	99.4
6	250	279.0	111.6	500	499.4	99.9
7	600	547.5	91.3	60	64.8	108.0

Identification of the components by crystallization. In order to determine which amino sugar corresponded to the respective peaks a mixture of 50 mg of each component was chromatographed. A column having the dimensions 3.9 × 48 cm was used. This was prepared in the same way as those of smaller dimensions described above. The substances were dissolved in 2 ml of 0.3 N hydrochloric acid and the chromatography was performed in the usual manner with a rate of flow of about 5 ml an hour. The effluent was collected in 7.5 ml fractions. An amount of 0.5 ml was pipetted off from every third fraction and analyzed as described above. Fractions belonging to the same component were pooled and the hydrochloric acid was removed by repeated evaporation *in vacuo*. It was finally concentrated to a volume of 2 ml. The solution was mixed with 2 ml of methanol and was placed in a refrigerator at −10° C. Cold acetone was added dropwise to opalescence and the solution was set aside in the cold overnight and more acetone was then added. When crystallization was complete the crystals were collected and washed first with methanol : acetone : 1 *N* hydrochloric acid (2 : 4 : 1) and then with pure acetone. After

drying *in vacuo* the substance was weighed and dissolved in 1 ml of water. Optical rotation was determined in a 0.5 dm tube. The results are set out in Table 2.

Table 2. Identification of the components

Component I Glucosamine-HCl		Component II Galactosamine-HCl	
Found	Theoretical	Found	Theoretical
$(\alpha)_D^{20}$ final + 71.8°	+ 72.5°	+ 96.2°	+ 96.4° (Levene)

As may be seen from the table, the first component is glucosamine hydrochloride and the second galactosamine hydrochloride.

DISCUSSION

Size of the fractions. By collecting fractions of 0.5 ml pipetting is avoided. Larger fractions do not allow the resolving power of the column to be fully utilized.

Length and diameter of the column. As the method is intended to be a micromethod, the dimensions of the column was chosen to comply with this. However, the method can be scaled up for larger amounts.

Use of the column for several experiments. As the amino sugars are quantitatively recovered in the effluent the same column can be used again when dealing with pure substances. When hydrolysates are analyzed there is a possibility that other substances may be present which can disturb the quantitative analysis.

Amount of amino sugar. Amounts of 800 μg of each component have been separated on columns of the dimensions 0.6 × 40 cm but the yield tends to be less satisfactory as a number of fractions are too concentrated to permit accurate quantitative analysis by the colorimetric procedure. If amounts larger than 600 μg of each component are to be separated, larger columns should be used and the effluent must be collected in larger fractions and an aliquot pipetted off for analysis.

Relationship between the components. A ratio of 1 : 5 between the amounts of the components makes no difference to the analytical results as compared with the ratio 1 : 1. If the differences are larger, the determination of the smaller component will involve relatively large errors. It is then necessary to make two chromatograms with different amounts of the hydrolysate. By reducing the rate of flow, ratios slightly greater than 5 : 1 can be analyzed.

Rate of flow. An increase in this means that the concentration-effluent curve will be flatter and broader; a larger number of fractions will then have concentrations lower than desirable for analysis by the colorimetric method, and the errors will be larger. For a column of the dimensions mentioned, a rate of flow of 1.5—2 ml an hour has been found to be optimal for good separation. As a reduction in the rate of flow gives a slightly sharper separation it is sometimes of value to reduce the rate, especially if one of the components is present in small amounts, this making it possible to determine the smaller component with greater accuracy.

Hydrochloric acid concentration. If the hydrochloric acid concentration is increased the effluent volume for the peaks will be smaller while the separation will be less satisfactory. A decrease in the concentration of hydrochloric acid produces an increase in the effluent volume. The difference between the maxima for glucosamine and galactosamine will be greater but the curves will be flatter and broader, with the above-mentioned disadvantages as the result.

Technique of analysis. Blix [17] has demonstrated that an acetylation time of 60 minutes is necessary for concentrations of more than 0.09 mg hexosamine hydrochloride and is applicable up to 0.14 mg in 26 ml. The upper limit for the technique described in this paper seems to lie at 0.1 mg hexosamine hydrochloride in the final volume of 12.5 ml. The portions should not be neutralized before the addition of acetylacetone solution since the pH of the mixture before boiling is 9.7 and after boiling 9.4, an alkalinity which according to Sørensen [21] is optimal for the colour development. The volume of the fractions is not allowed to vary more than $\pm$ 5 per cent.

Influence of other sugars and amino acids. The common hexoses, methylpentoses and pentoses do not interfere with the separation and the analysis as their effluent volume is very small. Amounts of up to 600 μg of each of the common amino acids have no effect on the separation of the amino sugars.

Analysis of a polysaccharide fraction from the cornea. A polysaccharide fraction obtained from cornea after digestion with proteolytic enzymes and removal of the proteolytic split products by means of Lloyd's reagent, was analyzed.

Preliminary paper chromatographic experiments had shown that both amino sugars were present as found also by Woodin [22].

114.5 mg of the polysaccharide dissolved in 5 ml of 6 *N* hydrochloric acid were heated under reflux. After 2, 8 and 24 hours 1 ml samples were pipetted off, placed in a small beaker and cooled. They were then evaporated to dryness *in vacuo* over sodium hydroxide. The residue was taken up in 3 ml of 0.3 *N* hydrochloric acid and 0.4 ml samples were analyzed with the technique described.

The results are given in Table 3.

Table 3. Glucosamine and galactosamine in the corneal polysaccharide

Time of hydrolysis h	Percentage of amino sugar expressed as hydrochloride				
	Component				Total
	I	II	III (glucosamine)	IV (galactosamine)	
2	2.0	1.8	13.7	7.9	25.4
8	1.4	—	14.9	9.6	25.9
24	Trace	—	15.1	9.4	24.5

It is evident from the table that besides the components III and IV identified by means of their effluent volumes as glucosamine and galactosamine hydrochloride respectively there are other components giving the Elson-Morgan reaction. After 2 hours of hydrolysis there are two of them and after 8 hours one is still left. The identity of these components has not been further investigated. They, however, diminish in quantity with time of hydrolysis. They are probably products of partial hydrolysis. The value for the total amount of hexosamine corresponds fairly well to the amount of 25 per cent of glucosamine hydrochloride as found by direct determination after hydrolysis of the polysaccharide.

SUMMARY

A chromatographic technique is described for the separation of glucosamine hydrochloride and galactosamine hydrochloride when they occur together in hydrolysates.

The separation is carried out on a column of Dowex 50 cation exchange resin with 0.3 *N* hydrochloric acid as the developing solvent.

The effluent is collected in 0.5 ml fractions which are analyzed quantitatively by the Elson and Morgan method as modified by Blix. A concentration-effluent curve demonstrated the resolving power of the column.

Using this technique the individual amino sugars could be analyzed quantitatively in amounts between 60 and 600 μg. The technique can be modified for the separation of larger quantities. A polysaccharide from the cornea has been analyzed by the procedure developed.

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REFERENCES

1. Aminoff, D., Morgan, W. T. J., and Watkins, W. M. *Biochem. J.* **46** (1950) 426.
2. Chargaff, E., and Bovarnick, M. *J. Biol. Chem.* **118** (1937) 421.
3. Neuberger, A. *Biochem. J.* **32** (1938) 1435.
4. Jolles, Z. E., and Morgan, W. T. J. *Biochem. J.* **34** (1940) 1183.
5. Blix, G., Svennerholm, L., and Werner I. *Acta Chem. Scand.* **4** (1950) 717.
6. Partridge, S. M. *Biochem. J.* **42** (1948) 238.
7. Aminoff, D., and Morgan, W. T. J. *Nature* **162** (1948) 579.
8. Gardell, S., Heijkenskjöld, F., and Roch-Norlund, A. E. *Acta Chem. Scand.* **4** (1950) 970.
9. Kent, P. W., Lawson, G., and Senior, A., *Science* **113** (1951) 354.
10. Annison, E. F., James, A. T., and Morgan, W. T. J. *Biochem. J.* **48** (1951) 477.
11. Elson, L. A., and Morgan, W. T. J. *Biochem. J.* **27** (1933) 1824.
12. Dische, L., and Borenfreund, E., *J. Biol. Chem.* **184** (1950) 517.
13. Tracy, M. V. *Biochem. J. Proc. Biochem. Soc.* **49** (1951) xx.
14. Flood, A. E., Hirst, E. L., and Jones, J. K. N. *J. Chem. Soc. London* (1948) 1679.
15. Moore, S., and Stein, W. H. *J. Biol. Chem.* **192** (1951) 663.
16. Partridge, S. M. *Biochem. J.* **45** (1949) 459.
17. Blix, G. *Acta Chem. Scand.* **2** (1948) 467.
18. Stein, W. H., and Moore, S. *J. Biol. Chem.* **176** (1948) 337.
19. Carlander, A., and Gardell, S. *Arkiv Kemi* **4** (1952) 461.
20. Gardell, S. *Acta Chem. Scand.* **5** (1951) 1011.
21. Sørensen, M. *Compt. rend. trav. lab. Carlsberg. Sér. Chim.* **22** (1938) 487.
22. Woodin, A. M. *Biochem. J.* **51** (1952) 319.

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