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QUANTITATIVE GAS CHROMATOGRAPHIC MEASUREMENT OF GLYCOSAMINOGLYCAN HEXOSAMINES IN URINE AND PLASMA

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SUMMARY

A method is described for the quantitative determination of urine and plasma glycosaminoglycans (GAGs) by gas chromatography of the acetylated amino sugars. GAGs were first recovered by precipitation from urine with alkyltrimethylammonium bromide and from plasma by mini-column chromatography after papain digestion. Urine samples (24) analysed for total hexosamines by gas chromatography and for uronic acid by colorimetry had a correlation coefficient of 0.85. The within-run coefficient of variation (C.V.) for nineteen samples from a pooled urine was 5.2% for total hexosamines and that for the ratio of galactosamine to total hexosamines was 3.7%. The corresponding C.V. values for twelve plasma samples from a common pool were 6.5 and 3.7%. The mean ratio of galactosamine to total hexosamine in ten pre-breakfast spot urines was 51.5%. The corresponding ratio in the plasma from twenty adolescent blood donors was 76.3% and the mean total hexosamine content of the GAGs was 47.36 μmol/l.

INTRODUCTION

Glycosaminoglycans (GAGs) are widely distributed in human tissues and fluids and are integral components of the ground substance of connective tissues, including bone [1]. In plasma, GAGs can be separated into two fractions, a low-charged species, under-sulphated chondroitin sulphate, and a highly charged fraction composed mainly of heparan and chondroitin sulphates. Plasma GAGs have a wide range of molecular masses, but a large proportion of the plasma heparan and chondroitin sulphates have molecular masses of less than 5000. It is this lower-molecular-mass, highly charged fraction of chondroitin and heparan sulphates which are the predominant GAGs found in normal urine [1,2]. While the origin of urine GAGs was thought by some to be equivocal, it now seems clear that they are mostly filtered from the plasma [2,3].

Recently we measured the urinary 24-h output of GAGs in a reference population, using the colorimetric assay for uronic acid [4,5]. In women we found a

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bimodal distribution for the ratio of GAGs to creatinine. Of women with a ratio of less than 2.1, 75% were premenopausal and 62% of those with a ratio greater than 2.1 were postmenopausal. The significance of this finding is not known, but we are currently investigating whether or not GAGs can be used as a biochemical marker of a senile and postmenopausal osteoporosis.

To gain information on the composition of the GAGs, an investigation was made into measuring quantitatively by gas chromatography (GC), GAG galactosamine and glucosamine in both urine and plasma. These hexosamines are, respectively, the predominant amino sugars of chondroitin and heparan sulphates. These studies are the subject of this report.

GC of urinary GAGs has been previously used to classify the various mucopolysaccharidoses [6]. The most common methods have been acid hydrolysis of the GAGs, reduction with sodium borohydride and then derivatization to the alditol acetates. While the chromatographic properties of these derivatives are excellent, these procedures have previously required the removal of borate after reduction, an extremely tedious operation. Recently Blakeney et al. [7,8] reported a simpler procedure avoiding this step, which was applicable to both neutral and amino sugars. This method has been used here.

EXPERIMENTAL

Chemicals

Alkyltrimethylammonium bromide, cysteine, papain (P4762), tris(HCl), phenyl N-acetyl-α-D-glucosaminide, glucosamine·HCl and galactosamine·HCl were all obtained from Sigma (St. Louis, MO, U.S.A.). Trichloroacetic acid, disodium EDTA, sodium chloride, sodium acetate, lithium chloride, anhydrous sodium sulphate, sodium borohydride, dichloromethane and dimethyl sulphoxide were purchased from BDH (Poole, U.K.) and were either Analar grade or general purpose reagent grade. Hydrochloric acid and ammonia came from J.T. Baker (Phillipsburg, NJ, U.S.A.). 1-Methylimidazole was purchased from Fluka (Buchs, Switzerland) and DEAE-Sephacel from Pharmacia (Uppsala, Sweden). EDTA-Vacutainer tubes came from Becton Dickinson (Rutherford, NJ, U.S.A.), Glycogel columns from Pierce (Rockford, IL, U.S.A.), and OV-225 and Gas-Chrom Q from Alltech Assoc. (Deerfield, IL, U.S.A.).

Isolation of GAGs

Urine was collected from healthy children and adults either as spot pre-breakfast samples or as 12-h collections without the addition of preservative. Samples were frozen until required. The GAGs were isolated from 5-ml aliquots of urine by precipitation with alkyltrimethylammonium bromide (46 g/l) [9], washed with ethanol and dissolved in 0.7 ml of water. Trichloroacetic acid (0.61 *M*, 0.7 ml) was added and after standing for 30 min the solution was centrifuged (600 *g*, 5 min). Aliquots of the supernatant were then taken for the determination of uronic acid (0.3 ml) [4] or for hexosamine analysis by GC (0.7–1.0 ml).

Plasma-protein-bound and free GAGs were isolated by digestion with papain followed by mini-column chromatography on DEAE-Sephacel using similar pro-

cedures to those of Staprans and Felts [2], Friman and Juvani [10] and Calatroni et al. [11]. Blood was collected into EDTA-Vacutainer tubes from adolescent blood donors. Plasma (2 ml) plus buffer (2 ml) (0.005 *M* cysteine, 0.05 *M* disodium EDTA, 0.15 *M* sodium chloride, 0.1 *M* sodium acetate, pH 6.0) and 2 mg of papain were mixed and digested for 18 h at 56°C. Trichloroacetic acid (0.61 *M*, 2 ml) was then added, the solution vortexed and chloroform (2 ml) added. After vigorously remixing, the solution was centrifuged (600 *g*, 5 min) and 5 ml of the supernatant were recovered. This was then washed three times with 5-ml aliquots of *n*-butanol to reduce the trichloroacetic acid content. Some concentration of the aqueous phase occurs in this process. To the remaining aqueous phase were added 1 ml of 0.15 *M* lithium chloride containing 0.02 *M* Tris, pH 8.6, and 8 ml of water. This solution was then applied to the columns. A further rinsing of the tube with 0.15 *M* lithium chloride–0.02 *M* Tris buffer, pH 8.6, ensured quantitative transfer.

Polystyrene Glycogel columns (6 cm×0.75 cm I.D.) with a fritted-glass base were packed to varying heights (usually 3.5 cm) with DEAE-Sephacel and washed with the 0.15 *M* lithium chloride buffer (15 ml). After applying the plasma extract the column was washed with two 5-ml aliquots of 0.15 *M* lithium chloride–0.02 *M* Tris buffer, pH 8.6. The GAGs were eluted with four 2-ml aliquots of 2.0 *M* lithium chloride containing 0.02 *M* Tris, pH 8.6. The collected eluates were evaporated to near dryness under a stream of air at 80°C. The residue was taken up in 12 ml of ethanol containing 10 m*M* sodium acetate and the GAGs were allowed to precipitate by standing at 4°C for 16 h. The tubes were centrifuged (600 *g*, 5 min) decanted and dried in a gentle stream of air. The precipitate was then taken up in 1 ml of distilled water.

Hydrolysis and derivatization of the GAGs

Concentrated hydrochloric acid was added to 1 ml of the aqueous solution containing either the plasma or urine GAGs, to give a final concentration of 3 *M* hydrochloric acid. The tubes were flushed with nitrogen, capped and autoclaved at 125°C for 45 min [12]. After cooling, 0.2 ml of 2 *M* lithium chloride was added and the tubes were evaporated to dryness at 56°C in a stream of air. The internal standard (20 μl) was added (phenyl N-acetyl-α-D-glucosaminide, 1.6 m*M* in water) followed by 2 *M* ammonia (50 μl) and sodium borohydride in dimethyl sulphoxide (0.5 ml, 1.5 g per 100 ml). The mixture was then heated at 37°C for 4 h. Glacial acetic acid (50 μl), 1-methylimidazole (100 μl) and acetic anhydride (1 ml) [7] were added. After mixing and standing for 15 min at room temperature, water (2 ml) was added, the solution mixed and after a further 5 min, dichloromethane (3 ml) added. This solution was vigorously mixed, the organic phase recovered and washed four times with water (2 ml). The extract was dried over solid anhydrous sodium sulphate, decanted and evaporated to dryness. The residue was taken up in ethyl acetate–heptane (50:50, v/v) just before GC and 3–5 μl were injected.

Gas chromatography

GC was carried out on a Philips 204 gas chromatograph equipped with a flame ionisation detector. The alditol acetates of the amino sugars were analysed on

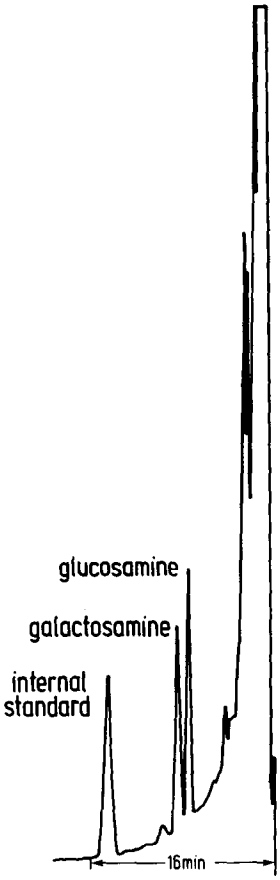

Fig. 1. GC separation of glycosaminoglycan hexosamines as acetates on a 1.5-m column containing 1.5% OV-225 on Gas-Chrom Q. Temperature 230°C.

glass columns (1.5 m×0.4 cm I.D.) packed with 1.5% OV-225 on Gas-Chrom Q 100–120 mesh at oven temperatures between 220 and 235°C depending on the column's condition. Injector and detector temperatures were set at 250°C and the nitrogen carrier gas flow-rate was 30 ml/min. Quantitation was by peak heights, comparing the hexosamine to internal standard ratio with a standard curve. Aliquots of standards, D(+)-glucosamine·HCl and D(+)-galactosamine·HCl in water (1.0 m*M*) were taken through the procedure to obtain the standard curve.

RESULTS AND DISCUSSION

Gas chromatography

A typical chromatogram of the separation of the hexosamines present in GAGs is shown in Fig. 1. A complete chromatogram can be run within 16 min. There is very little contamination apart from that buried in the solvent front for either plasma or urine specimens. Recently it was found that even this contamination can be greatly reduced by avoiding solvent contact with the screw-cap liners on

the extraction tubes. OV-225 was chosen as the column phase. Less polar phases such as OV-101 gave inadequate resolution while strongly polar phases such as SP-2340 (Supelco) require high temperatures to complete a chromatogram in a reasonable time. The standard curve is linear to over 30 μg of each standard added in the initial step and remains stable from day to day. The equation relating the ratio of peak heights (y, hexosamine/internal standard) to the concentration of hexosamine (x, μg per tube) is $y = 7.92\,x$. The two hexosamines give an equimolar response with the flame ionisation detector.

Hydrolysis of the GAGs

An investigation was made of the effects of hydrolysis in 3 *M* hydrochloric acid on the recovery of the standards. The mean recovery of glucosamine added in amounts from 5.8 to 29.0 μg to a urine pool or as pure standards taken through the complete procedure was 92.4%. The corresponding value for galactosamine was 82.1%. These results are in broad agreement with those reported elsewhere [13]. Since hexosamines might be expected to be less liable to degradation while bound into the GAG polymer, the values above may be taken as a worst case. No correction has been applied for recovery in the results that follow.

In early attempts to assay plasma GAGs it was observed that small amounts of lithium chloride present in the hydrolysis mixture, as a contaminant following column chromatography, gave an increased recovery of galactosamine. This effect was tested by adding increasing amounts of lithium chloride to a series of samples both before and after hydrolysis. Since the effect was observed in both situations, it was concluded that small amounts of lithium chloride inhibit the breakdown of galactosamine during the evaporation of the hydrolysis mixture. Loss of galactosamine at this step has also been reported by others [6]. As little as 0.01 g of lithium chloride per tube was effective. Increasing the amount of lithium chloride to 0.04 g gave no further improvement. In a typical assay on urine specimens the improvement in the ratio of galactosamine to total hexosamine was from 46.5 to 50.5%. Because of this effect lithium chloride was added to all samples after hydrolysis but before evaporation.

Isolation of GAGs from urine and plasma

Urine GAGs were precipitated for convenience with alkyltrimethylammonium bromide, although column methods are claimed by some to be superior [14]. Trichloroacetic acid was added when resolubilizing the GAGs to avoid taking up any glycoprotein, which is sometimes present [6] and which would effect the hexosamine ratio.

In the Niebes and Schifflers [15] assay for plasma GAGs, plasma is first digested with a pronase and the GAGs are precipitated with alkyltrimethylammonium bromide. The difficulty with this assay is the low colour density when quantitating the GAGs by the uronic acid method and suspected losses in the precipitation step. To overcome this the mini-column method was developed. Staprans and Felts [2] recently published interesting results on the determination of plasma GAGs, isolating them on macro-columns of DEAE-Sephacel using large volumes of plasma. In a clinical situation, however, the volume of plasma is limited to a

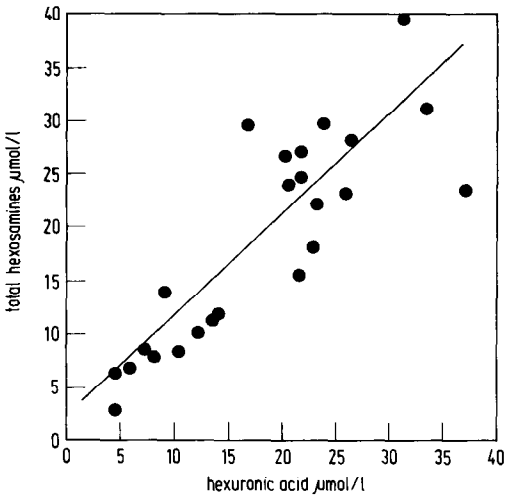

Fig. 2. Comparison of the quantitative determination of urine glycosaminoglycans by GC assay of the total hexosamines and by colorimetric assay of uronic acid ($r=0.85$).

few millilitres. The method reported here is adapted from their method. Staprans and Felts [2] hint that protein-bound GAGs may be isolated from plasma with trichloroacetic acid precipitation alone or by adding sodium dodecyl sulphate thus avoiding protein digestion. Preliminary experiments with either of these agents were unsuccessful for a variety of reasons. It was found necessary to digest with papain first. A trichloroacetic acid precipitation was then added before column chromatography as without it traces of protein are often recovered with the GAGs. The trichloroacetic acid is conveniently reduced to minimal levels with *n*-butanol extraction. The amount of DEAE-Sephacel required to recover quantitatively the GAGs extracted from 2 ml of plasma was assessed by packing Pierce mini-columns (6×0.75 cm) to varying heights with Sephacel. Packing to a height of 2 cm gave a recovery of 88% of that from a 3-cm column. Increasing the height to 4.5 cm gave no additional recovery. For routine experiments columns were packed to 3.5 cm. In a second set of experiments GAGs were extracted from 1, 2 and 3 ml of pooled plasma and the sample was applied to columns packed to 3.5 cm. Over this range the amount of GAG recovered was proportional to the volume of plasma extracted. For routine investigations 2 ml of plasma are extracted as this gives good peak heights at the GC step.

Quantitation of GAGs by gas chromatography

Fig. 2 shows the results of a parallel analysis of GAGs (GC versus colorimetric assay of uronic acid) isolated from 24 spot urines from a wide variety of healthy and sick children and adults. The mean uronic acid content of the samples was 17.81 µmol/l (S.D. 8.88) and the mean total hexosamine content 19.16 µmol/l (S.D. 9.15). The mean ratio of total hexosamine to total uronic acid was 1.09 (S.D. 0.085). There was a good correlation between the two assays ($r=0.85$), considering they measure different molecular entities. According to present

TABLE I

PRECISION OF THE GC ASSAY OF GAG HEXOSAMINES

Within-run C.V. values for the complete assay of pooled samples of plasma ($n=12$) and urine ($n=19$).

	Total hexosamines		Galactosamine/total hexosamines	
	Mean (μmol/l)	C.V. (%)	Mean (%)	C.V. (%)
Plasma	53.9	6.5	75.08	3.7
Urine	37.04	5.2	50.38	3.7

knowledge of GAG structure, GAGs (except keratan) are composed of repeating disaccharide units containing uronic acid and an hexosamine, so that the theoretical ratio of hexosamine to uronic acid is 1.0 [1], close to that found here. It is concluded that GAGs can be quantitatively determined by GC.

Precision and detection limit of the assay

Table I lists the within-run coefficients of variation (C.V.) for the complete assays, for both plasma and urine using pooled samples. A C.V. of less than 4% for the hexosamine ratio (galactosamine/galactosamine+glucosamine) is satisfactory by clinical chemistry standards. This ratio has been used to give some comparison with reports using electrophoretic methods. The higher C.V. in the quantitative determination of the total hexosamine content is less satisfactory but equal to or better than that obtained by other GC or colorimetric methods [6,16]. The detection limit for both glucosamine and galactosamine is equivalent to a urine concentration of 0.8 μmol/l.

Plasma and urine GAG concentrations

By the methods described here the total hexosamines in GAGs and the hexosamine ratio were measured in twenty plasma samples from adolescent blood donors. At 76.3% of total hexosamines, galactosamine was the predominant hexosamine in plasma GAGs (Table II). This is consistent with the results of Staprans and Felts [2], who by column and electrophoretic methods found the main

TABLE II

HEXOSAMINE CONTENT OF GLYCOSAMINOGLYCANS IN PLASMA FROM ADOLESCENT BLOOD DONORS ($n=20$)

	Glucosamine (μmol/l)	Galactosamine (μmol/l)	Total hexosamines (μmol/l)	Galactosamine/total hexosamines (%)
Mean	11.24	36.12	47.36	76.3
S.D.	1.68	4.5	6.1	1.25

GAG to be low-charged chondroitin sulphate in which galactosamine would be expected to be predominant. According to their data 92% of plasma GAGs are chondroitin sulphates. Calatroni et al. [11] also found chondroitin sulphate to be the main plasma GAG. The GAGs are heterogeneous in composition so that while galactosamine is the predominant hexosamine in chondroitin sulphate, it is not the exclusive hexosamine. This may account for the lower value reported here. A reference value for total plasma GAGs is not known with any certainty. The mean value reported here (47.36 μmol/l) on blood donors is comparable to that reported by Staprans and Felts on six people (49.34 μmol/l) [2]. Earlier reports using different methods cite lower values still, ca. 16–33.0 μmol/l [10,11].

GAGs were also isolated from spot urines collected before breakfast from ten adult males who were believed to be in good health. The mean GAG/creatinine ratio in these samples was 1.18 (μmol/mmol) (S.D. 0.29) and the mean galactosamine/total hexosamine ratio was 51.5% (S.D. 6.98).

To see whether the method might detect a diurnal variation in GAG composition, consecutive 12-h collections were made from two healthy boys aged 8 and 9 years, from 8 a.m. to 8 p.m. and 8 p.m. to 8 a.m., respectively. The galactosamine to total hexosamine ratio went from 75.8% for the night specimen to 63.6% for the day collection in one boy and in the other from 63.6 to 59.5%.

The composition of urinary GAGs has been most commonly elucidated by electrophoretic methods. Poulsen [1] found in adults chondroitin sulphate (47%) dermatan sulphate (13%) and chondriotinase ABC-resistant GAGs (mostly heparan) (40%); Staprans et al. [17] found chondroitin sulphate (74%) and heparan sulphate (25%), whereas Wessler [18] found chondroitin sulphate (60%) heparan sulphate (15%) and keratan sulphate (18%). Staprans and Felts [2] could find no keratan sulphate and others [19,20] found only traces of dermatan sulphate. These values are similar to the data reported here, though as for plasma the galactosamine percentage is lower than the reported percentages of chondroitin and dermatan sulphates in urine. It has also been reported that the proportion of chondroitinase ABC-resistant GAGs (mostly heparan sulphate) increases during the daytime, consistent with the observed decrease in galactosamine observed in the two boys [1].

Comparison of the plasma and urine data shows that the plasma is much richer in galactosamine-containing GAGs. This agrees with the results of Staprans and Felts [2], who found that low-sulphated chondroitin sulphate is the main GAG in plasma but does not appear in the urine of healthy adults. Thus the urine of healthy adults is relatively enriched in glucosamine-containing GAGs.

It is concluded that GAGs in urine and plasma may be satisfactorily analysed both qualitatively and quantitatively by GC methods.

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