Journal of Chromatography, 490 (1989) 293–299
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4697

ANALYSIS OF ASPARTYLGLUCOSAMINE AT THE PICOMOLE LEVEL BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(First received October 3rd, 1988, revised manuscript received January 23rd, 1989)

SUMMARY

A sensitive method for quantitative analysis of aspartylglucosamine as its dabsyl chloride derivative by high-performance liquid chromatography is described. Precolumn-derivatized aspartylglucosamine and internal standard (carboxymethylcysteine) are separated on a reversed-phase column with a mobile phase consisting of phosphate buffer and acetonitrile and monitored by UV–VIS detection at 436 nm. Aspartylglucosamine acts in the assay like a polar amino acid, and it can be separated from interfering substances in urine with a retention time of ca. 13 min. Its detection limit is ca. 0.3 μM in water and 0.5–1.0 μM in urine and other biological samples, which permits its quantitation in normal urine, for example. The within-day coefficient of variation is less than 4.7% and the day-to-day coefficient of variation is less than 8.3%. The present method is applicable to the direct analysis of aspartylglucosamine in body fluids and tissues without any prepurification and, in combination with automated liquid chromatography, allows rapid assay of a large number of samples in the detection of aspartylglycosaminuria. The sensitivity of the assay also allows direct quantitation of aspartylglucosamine in normal urine and leukocytes of aspartylglycosaminuria patients, and may thus be used in metabolic studies of the compound.

INTRODUCTION

Aspartylglucosamine (2-acetamido-1-L-β-aspartamido-1,2-dideoxy-β-D-glucose; GlcNAc-Asn) is the linkage region between so-called N-glycosidic carbohydrate units and the protein chains in glycoproteins [1]. The compound is normally catabolized by a lysosomal enzyme, aspartylglycosylaminase, N^4-(β-N-acetylglucosaminyl)-L-asparaginase (EC 3.5.1.26) to N-acetylglucosamine, aspartic acid and ammonia [2]. In an inherited autosomal recessive disease

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0378-4347/89/$03.50

called aspartylglycosaminuria (AGU) (McKusic 20 840) [3], the catabolism of GlcNAc-Asn is inhibited because of deficient activity of aspartylglycosylaminase, and GlcNAc-Asn among other glycoasparagines accumulates in body fluids and tissues [4]. In detection of the disease, the elevated concentration of GlcNAc-Asn in urine can be demonstrated qualitatively by thin-layer chromatography (TLC) [5] and quantitatively by gas chromatography–mass spectrometry (GC–MS) [6] or high-performance liquid chromatography (HPLC) [7]. The HPLC assay of underivatized aspartylglucosamine [7] requires prepurification of urine and is not sensitive enough for direct detection of GlcNAc-Asn in normal urine and tissues, and its internal standardization has not been possible. Fluorometric assay of dansylated glycoasparagines by HPLC has also been described [8].

We describe here a sensitive HPLC assay for aspartylglucosamine in unpurified samples with UV–VIS detection. The method is based on precolumn derivatization of aspartylglucosamine with dabsyl chloride (dimethylaminoazobenzene-4′-sulphonyl chloride; Dabs-Cl) [9–12] and separation on a reversed-phase column. The method was applied to quantitative analysis of aspartylglucosamine in body fluids and tissues of normal individuals and AGU patients.

EXPERIMENTAL

Reagents

GlcNAc-Asn and S-carboxymethyl-L-cysteine (CmCys) were from Sigma (St. Louis, MO, U.S.A.). HPLC-grade acetonitrile was from Merck (Darmstadt, F.R.G.). Dabs-Cl was from Fluka (Buchs, Switzerland), and it was used after recrystallization [10]. The bicinchoninic acid (BCA) protein assay kit was from Pierce (Rockford, IL, U.S.A.) and 6% dextran (Macrodex) was from Leiras (Turku, Finland). Potassium sodium phosphate buffer (28 m*M*, pH 7.2) was prepared in HPLC-grade water. All the other reagents were of analytical grade and they were used without further purification.

Samples

The urine samples of eleven AGU patients (aged 8–36 years), eight obligatory heterozygotes (aged 28–60 years) and six healthy control individuals (aged 20–35 years) were analysed. Random urine samples were collected without dietary restrictions and stored frozen until used. The leukocytes were isolated from EDTA blood samples of six AGU patients (aged 8–36 years), eight obligatory heterozygotes (aged 28–60 years) and six healthy control individuals (aged 20–35 years). The cells were allowed to sediment in the presence of 6% dextran. The upper layer, containing the leukocyte mixture, was removed and centrifuged, and contaminating erythrocytes were hemolysed with water. The cells were suspended in 1 ml of 50 m*M* potassium phosphate buffer (pH 7.2), which contained 0.1% of Triton X-100 and stored frozen. The protein concen-

tration was determined using the BCA protein kit with bovine serum albumin as a standard.

Derivatization

The Dabs-Cl derivatives were prepared according to the method of Lin and Wang [11].

For urine samples, 100 μl of normal or AGU urine was dispensed to a glass tube. A 1-nmol amount of CmCys, the internal standard, in 10 μl of water was added to the tubes with normal urine. For urine from AGU patients the amount of CmCys was increased to 75 nmol per 10 μl water. The liquid phase was evaporated under nitrogen, and the residue was dissolved in 200 μl of 0.1 *M* sodium carbonate buffer (pH 9.1). A 400-μl volume of 10 m*M* Dabs-Cl in acetone was added, and the mixture was heated at 70°C for 15 min. The liquid mixture was evaporated under nitrogen, and the precipitate was dissolved in 400 μl of ethanol–50 m*M* phosphate buffer, pH 6.0 (7:3, v/v).

For leukocyte samples, proteins were removed from the leukocyte homogenate by incubating the tubes in boiling water for 5 min. The tubes were centrifuged, and 10 nmol of CmCys were added in 10 μl of water to 100 μl of the supernatant. The samples were derivatized in the same way as the urine samples.

High-performance liquid chromatography

A Series 4 liquid chromatograph equipped with a variable-wavelength UV–VIS detector, an ISS-100 autosampler and an LCI-100 computing integrator, all from Perkin-Elmer (Norwalk, CT, U.S.A.), were used. The column was a Spherisorb S3 ODS2 (150 mm×4.6 mm I.D.) and the mobile phase was 28 m*M* potassium sodium phosphate buffer (pH 7.0)–acetonitrile. The flow-rate was 0.8 ml/min. The following programme was used: a linear gradient of 20–26% acetonitrile in 15 min, a linear gradient of 26–95% acetonitrile in 2 min, 95% acetonitrile for 2 min and down to 20% acetonitrile in 2 min. Aliquots of 5–10 μl were injected onto the column. The detection wavelength was 436 nm and the attenuation was 0.5 a.u.f.s.

Testing of the method

The linearity of the detector response was established by analysing GlcNAc-Asn added to normal urine in concentrations of 0 to 300 μM. In the concentration range 0–10 μM GlcNAc-Asn, 1 nmol of CmCys (10 μM) was used, and in concentrations over 10 μM GlcNAc-Asn, 75 nmol of CmCys (750 μM) were added. The peak areas of GlcNAc-Asn and the internal standard (CmCys) on the chromatograms were measured, their ratio was calculated, and the concentration of GlcNAc-Asn determined with the aid of a standard curve. For sensitivity testing, a 1 μM aqueous solution of GlcNAc-Asn was further diluted with water. The reproducibility was tested by using an AGU patient's urine

containing 550 μM GlcNAc-Asn. The results were correlated with those obtained by direct analysis of underivatized GlcNAc-Asn in urine [7].

RESULTS

Chromatograms of normal urine, AGU urine and leukocyte homogenate of an AGU patient are shown in Fig. 1. The retention times for CmCys (peak 1) and GlcNAc-Asn (peak 2) were 10.3 and 13.7 min, respectively. A good separation of the compounds was achieved during the first 15 min by programming the eluents, and the whole assay required only 20 min.

The detector response for GlcNAc-Asn from 0.3 to 300 μM was linear ($y=0.008x+0.070$, $r=0.998$, $n=6$). The detection limit of the assay at a signal-to-noise ratio of over 2 was 0.3 μM GlcNAc-Asn in water. In urine and other biological samples it was 0.5–1.0 μM because of interfering substances in chromatograms. The analytical recovery of GlcNAc-Asn in urine was 93% (range 82–102%) of that in water. The within-day coefficient of variation (C.V.) was less than 4.7% ($n=10$) and the day-to-day C.V. with the same sample was less than 8.3% ($n=10$).

The applicability of the method was tested on urine and leukocyte samples from AGU patients, heterozygotes and control individuals. The urinary excretion of GlcNAc-Asn in the patients was, according to the present method, 230–2380 μM (mean 916 μM, $n=11$), or after correlation to creatinine excretion, 113–253 μmol/mmol of creatinine (mean 169 μmol/mmol of creatinine) (Ta-

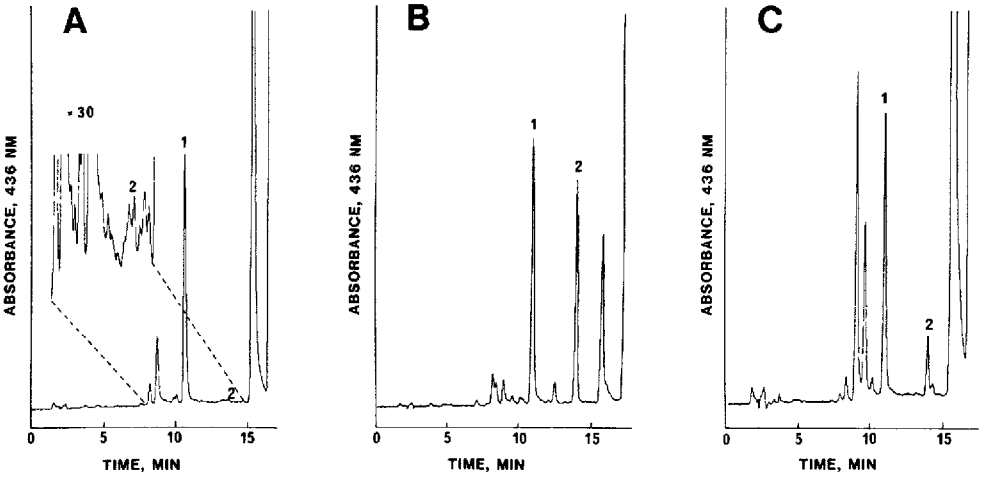

Fig. 1. Chromatograms of normal urine (A), AGU urine (B) and AGU leukocytes (C). Peaks: 1 = CmCys, retention time 10.0 min; 2 = GlcNAc-Asn, retention time 13 0 min. The concentration of CmCys in A and B was 750 μM and in C 100 μM. The concentration of GlcNAc-Asn in normal urine (A) was 2 μM, in AGU urine (B) 550 μM and in AGU leukocytes (C) 22 μM.

ble I). The corresponding values obtained by the reference method [7] were 280–3240 μM or 130–360 μmol/mmol of creatinine (mean 1344 μM or 212 μmol/mmol of creatinine) (Table I). Regression analysis of the results obtained by present method and the reference method is shown in Fig. 2. The data indicate a statistically highly significant positive linear correlation between these two methods ($r=0.87, p<0.001, n=11$).

The urinary excretion of GlcNAc-Asn in heterozygotes and control individuals was at the same, very low level (0.2–1.0 μmol/mmol of creatinine), and

TABLE I

ASPARTYLGLUCOSAMINE CONCENTRATIONS IN URINE AND LEUKOCYTE HOMOGENATES OF AGU PATIENTS, HETEROZYGOUS CARRIERS OF THE DISEASE AND CONTROL INDIVIDUALS

Sample	n	GlcNAc-Asn in urine		GlcNAc-Asn in leukocytes (μmol/g of protein)
		μmol/mmol of creatinine	μM	
AGU				
Mean ± S.D.	11	169.5 ± 42.6	902 ± 617	18.3 ± 7.3[a]
Range		113–253	230–2378	11.0–29.3
Heterozygotes				
Mean ± S.D.	8	0.5 ± 0.3	2.8 ± 0.9	< 0.5
Range		0.2–1.0	1.8–4.2	–
Controls				
Mean ± S.D.	6	0.4 ± 0.3	2.7 ± 1.1	< 0.5
Range		0.2–1.0	0.8–3 6	–

[a] $n=6$.

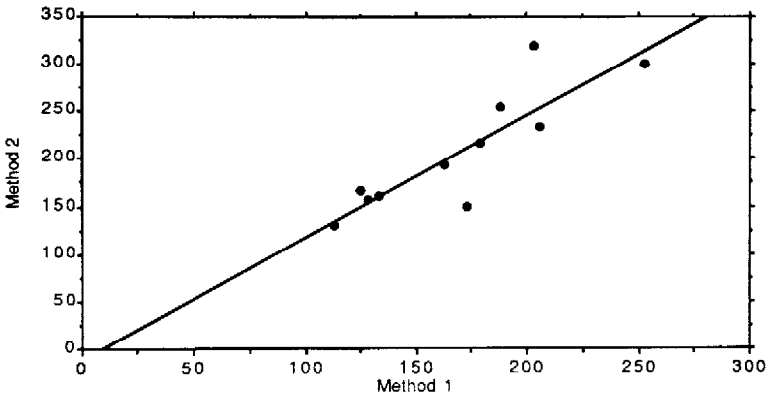

Fig. 2. Concentration of GlcNAc-Asn in urine of eleven AGU patients The values obtained by present method (x-axis) and by the earlier HPLC method [7] (y-axis) from the same urine samples are presented as μmol GlcNAc-Asn/mmol of creatinine. The positive linear correlation is statistically highly significant ($r=0.87; p<0.001$).

the values overlapped (Table I). The GlcNAc-Asn concentration in leukocytes of six AGU patients was 11–29 μmol/g of protein (mean 18 μmol/g). GlcNAc-Asn in leukocytes of heterozygotes and controls could not be detected (Table I).

DISCUSSION

The assay for GlcNAc-Asn described in this report has some significant advantages over the methods described earlier. Compared with the permethylation in the GC assay [6], the derivatization with Dabs-Cl is rapid and simple and the analysis time is short (20 min). Compared with the HPLC assay of underivatized aspartylglucosamine [7] or its dansyl derivative [8], the present method is 30 or 50 times more sensitive, respectively. Internal standardization could be used, and CmCys was chosen since it is not found in normal urine or tissues and because it is eluted close to GlcNAc-Asn. The Dabs-Cl derivative of GlcNAc-Asn was found to be stable. This is an advantage over the rapidly fading process of dansyl derivatives that may hamper the quantitative determination of these derivatives [13].

Two aspects of the derivatization require special consideration. The molar ratio of the Dabs-Cl reagent and amino acids has to be 5:1 or more, and the pH during the derivatization has to be 9.0 [11]. Since the presence of ammonia in the samples may exceed the capacity of the Dabs-Cl reagent and lead to underderivatization of the substances in the mixture [11], the liquid phase of the sample must be evaporated before the derivatization step. These points were considered in our derivatization procedure, which proved to give highly reproducible and reliable results. None of the several hundred samples analysed so far has been rejected because of interfering compounds in chromatograms or suspicion of under-derivatization.

The excretion of GlcNAc-Asn in urine from eleven AGU patients correlates well with the results obtained by the HPLC assay of underivatized GlcNAc-Asn on an amino column [7]. In the earlier studies, the excretion of GlcNAc-Asn in normal individuals gave values of 0.07–1.6 μmol/mmol of creatinine [14,15], which is in the same range as the present method (0.2–1.0 μmol/mmol). In the excretion between heterozygotes and controls, no differences were found as shown previously [15]. The GlcNAc-Asn concentration detected in leukocytes of six AGU patients was of the same order (11–29 μmol/g of protein) reported in lymphocytes (23 μmol/g of protein) by GC–MS [16]. The concentration of GlcNAc-Asn in the leukocytes of carriers of AGU and normal controls was below 0.5 μmol/g of protein, indicating effective catabolism of the compounds in cells. The combined evidence indicates that this assay procedure may be applicable to the quantitative assay of other carbohydrate–amino acid conjugates and glycopeptides.

ACKNOWLEDGEMENTS

The authors are grateful to Dr. Eila Airaksinen, M.D., for collaboration in collecting the samples from AGU patients and carriers. This work was financially supported by grants from the Academy of Finland, the Association of Finnish Chemical Societies and the North-Savo Fund of The Finnish Cultural Foundation.

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