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Estimation and separation of N-acetyl-beta-D-glucosaminidase isoenzymes in urine

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ESTIMATION AND SEPARATION OF N-ACETYL-B-D-GLUCOSAMINIDASE ISOENZYMES IN URINE

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ABSTRACT

N-acetyl-B-D-glucosaminidase is a lysosomal enzyme made up of two isoenzymes (A and B). It has been used extensively as a marker for kidney damage. However, its estimation in urine has not been standardized. We have established a method for the estimation and separation of NAG isoenzymes by ion-exchange chromatography. In 19 experiments done so far, this method has given reproducible results. The significance of this method is that with a single experiment, one can estimate total as well as individual isoenzyme activity. Furthermore, urine constituents do not appear to interfere in this assay (JPMA 42: 64,1992).

INTRODUCTION

The monitoring of N-acetyl-B-D-glucosaminidase (NAG; EC 3.2.1.30) in urine is one of the most promising, early non-invasive tests of renal injury[1-4]. During the last decade, great efforts have been made to develop rapid, reliable and cost-effective methods for assay of NAG in urine. All the methods currently used involve incubation of the enzyme with a phenolic N-acetyl-glucosaminide and can be divided into two groups. The first group utilizes endpoint methods[5-7] in which an alkaline buffer is added to stop the enzyme reaction and to develop the colour of the released chromogenic phenol molecule. In contrast, the second group includes several recently developed kinetic rate assays, or continuous methods[8-10], in which the substrate incorporates a phenolic aglycon with a low pICa value for the free phenol. When the aglycone is released at pH 4.8-6.25, a colour develops, obviating the need for addition of alkaline-stopping buffer. NAG is now being used in clinical nephrology to detect early changes of renal functions[1-4]. but unfortunately it has not been used in our country. In Pakistan, clinicians are mostly recommending the classical tests, i.e., creatinine clearance or plasma urea to assess the kidney functions[11,12]. We have standardized a method for the estimation of total NAG activity and its isoenzymes in urine.

PATIENTS AND METHODS

Normal urine was taken from the clinical laboratory Of the Aga Khan University Hospital (AKUR), Karachi. The urine of the kidney stone patients was taken directly from the patients in the kidney ward. Some urine samples were also taken from the patients just before and after lithotripsy treatment from the lithotripsy section of the surgery department. The urine samples were centrifuged for about 10 min at 5000 rpm and used as such, or frozen at -70°C. Some of the urine samples were also dialyzed against 10 mm sodium phosphate buffer, pH 7.0, for about 24 hrs. NAG-A from human uterus for standardizing the DEAE-column was partially purified according to the method described elsewhere[13].

DEAE-Sepharose column chromatography

One ml urine sample was applied on a minicolumn of DEAE-Sepharose (1.5x3 cm) pre-equilibrated with 10 mm sodium phosphate buffer, pH 7.0. The flow rate of the column was about 40 mI/hr. The column was washed with 10 ml equilibrating buffer. The NAG-A was then eluted with 0.3 m NaCl in

equilibrating buffer. 1 ml fraction was taken. The NAG activity was determined in alternate fractions.

NAG activity

The NAG activity in fractions after DEAE-Sepharose chromatography was measured according to the method described by Severint et al[14] by incubating (37°C, 15 min) 0.1 ml fraction with 0.9 ml of 20 mm citrate/phosphate buffer (pH 4.4) containing 0.1 mm methylumbelliferyl glucosaminide. To stop the reaction, we added 2 ml 0.5 M glycine buffer (pH 10.4). The fluorescence of 4-methylumbelliferone (4 MU) was measured with Jasco spectrofluorimeter at excitation and emission wave lengths of 368 and 448 nm, respectively and corrected for blank fluorescence. To calibrate the method, we used freshly prepared 4 MU standards (in 0.2 M sodium carbonate) of known concentration. One unit of NAG activity was defined as umole of 4 MU produced/mm under assay conditions. Total NAG activity was calculated by adding the activities of NAG-A and NAG-B.

RESULTS

The results of DEAE-Sepharose chromatography of one typical experiment is shown in Figure 1.

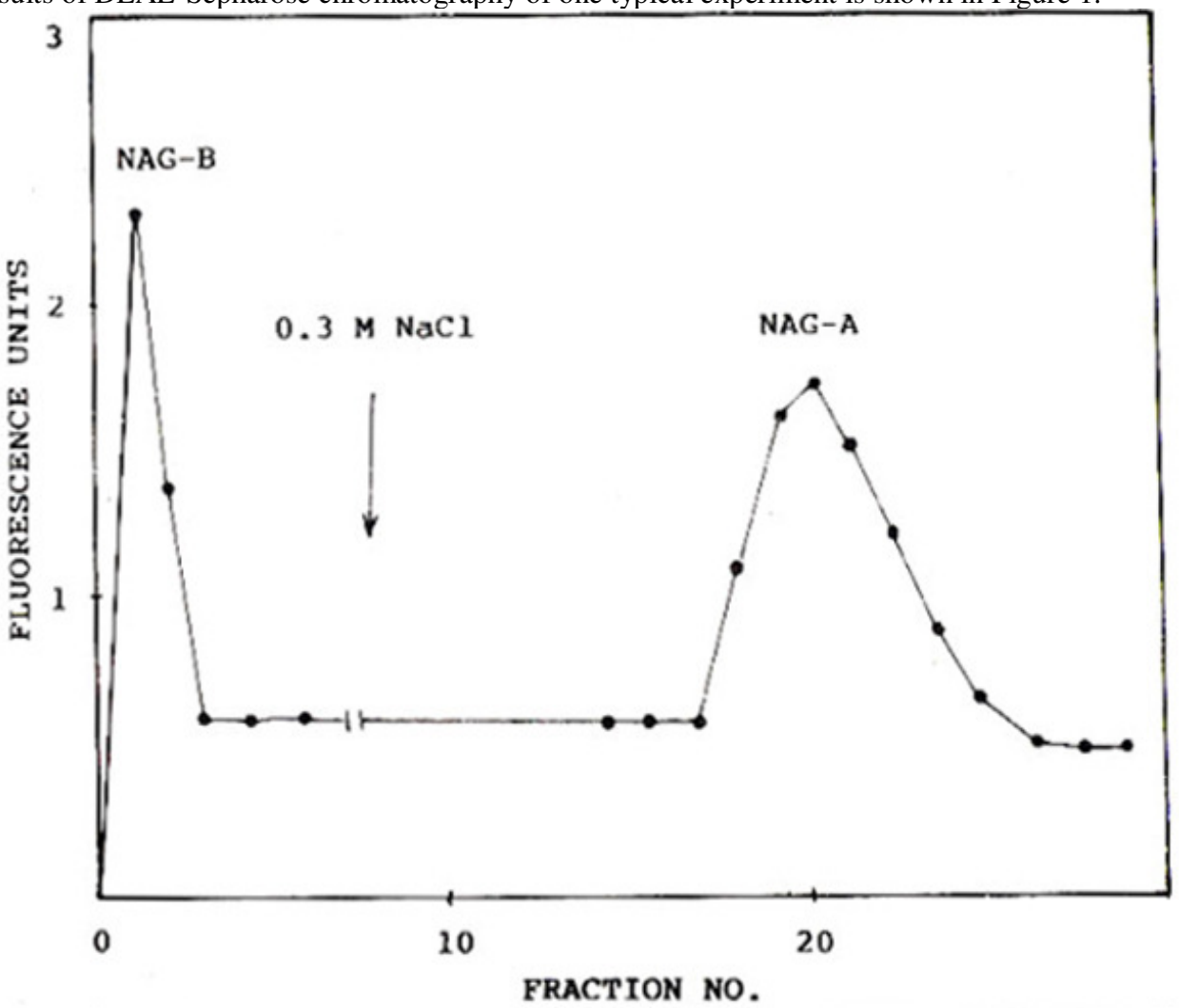

Figure 1. Typical chromatogram of NAG isoenzymes (NAG-A and NAG- B) of urine eluted from DEAE-sepharose. Experimental conditions are explained in "Patients and Methods".

This figure shows that NAG-B does not bind with the column while NAG-A is eluted by 0.3 M NaCl as a single peak. We have estimated the NAG activity in 19 samples of urine from normals and patients.

In all samples NAG-A was always eluted at almost same place. Only two samples (which were from kidney stone patients) showed NAG-B. In five normal urine samples, only NAG-A was present. Dialysis of urine has shown no effect on the elution profile of NAG-A. When we calibrated the column with known amount of NAG-A, we found that the activity in normal urine samples was 2.55± 1.29 U/L and in stone patients sample was 18.25 U/L (Table).

TABLE. NAG activity (U/L) in urine samples

No.	Normal	Kidney stone patients
1	4.43	45.90
2	1.78	17.18
3	1.73	44.93
4	3.73	20.36
5	1.08	3.51
6	-	14.80
7	-	15.50
8	-	18.95
9	-	6.75
10	-	31.75
11	-	9.13
12	-	11.66
13	-	4.27
14	-	10.96
Mean	2.55	18.25
S.D.	1.29^{5}	13.11^{14}

DISCUSSION

Increased urinary NAG level is a sensitive biochemical marker of tubular insult and the estimation of urinary NAG in foreign countries is being used in clinical nephrology to detect early changes of renal function[1-4]. However, in Pakistan it has not yet been applied for assessing renal functions. Different laboratories are using various methods for its estimation in urine[5-10]. Each method has its own merits and demerits. For example, p-nitrophenyl N-acetyl-B-D-glucosaminide is a reliable substrate for most NAG determinations; but because of the colour of the p-nitrophenolate ion is similar to that of urine,

pre- treatment of each urinary specimen by gel filtration has been recommended[5]. In Japan, Yagi et al[9] have synthesized 3, 4- dinitrophenyl N-acetyl-B-Dglucosaminide for direct (continuous) assay of NAG. However, this substrate is not yet available in the market. Moreover, it is sparingly soluble in water and the reagent mixture is slightly unstable after dissolution[10]. Our method for the estimation of NAG in urine has been found highly reproducible and reliable. There is no problem of interference of endogenous low-molecular mass activators or inhibitors present in human urine[15], because these have been removed in DEAE-exchange chromatography. The results (Table) clearly showed that kidneys have been damaged by stones because normal urine samples have very low NAG activity as compared to stone patients. The main problem is that it is a time consuming method and also it involves spectrofluorimeter which is a costly equipment and usually not available in many pathology laboratories. The increased total NAG activity has been associated with renal damage but this method alone cannot explain the mechanism of damage. A more convenient method is necessary, such as analysis of NAG isoenzymes in urine[14-16]. It has been shown that NAG-B increased in urine in membrane-proliferative glomerulonephritis, focal glomerulosclerosis but not in proliferative glomerulonephritis or membranous glomerulonephritis. Analysis of NAG isoenzymes has been performed using DEAE-cellulose column chromatography[14]. Very recently Shibasaki et al[16] have separated the NAG isoenzymes using automated fast protein liquid chromatography system. HPLC has also been used for estimation and separation of isoenzymes of NAG[17]. But these methods are very expensive. Using linear gradient of NaCl (0-0.3 M) for the elution of NAG from DEAE-column, NAG has been differentiated into B-, I- and A-forms[14,16]. We have also used the linear gradient of NaCl (0-0.5 M) for the elution of NAG from human uterus cytosol (Figure 2)

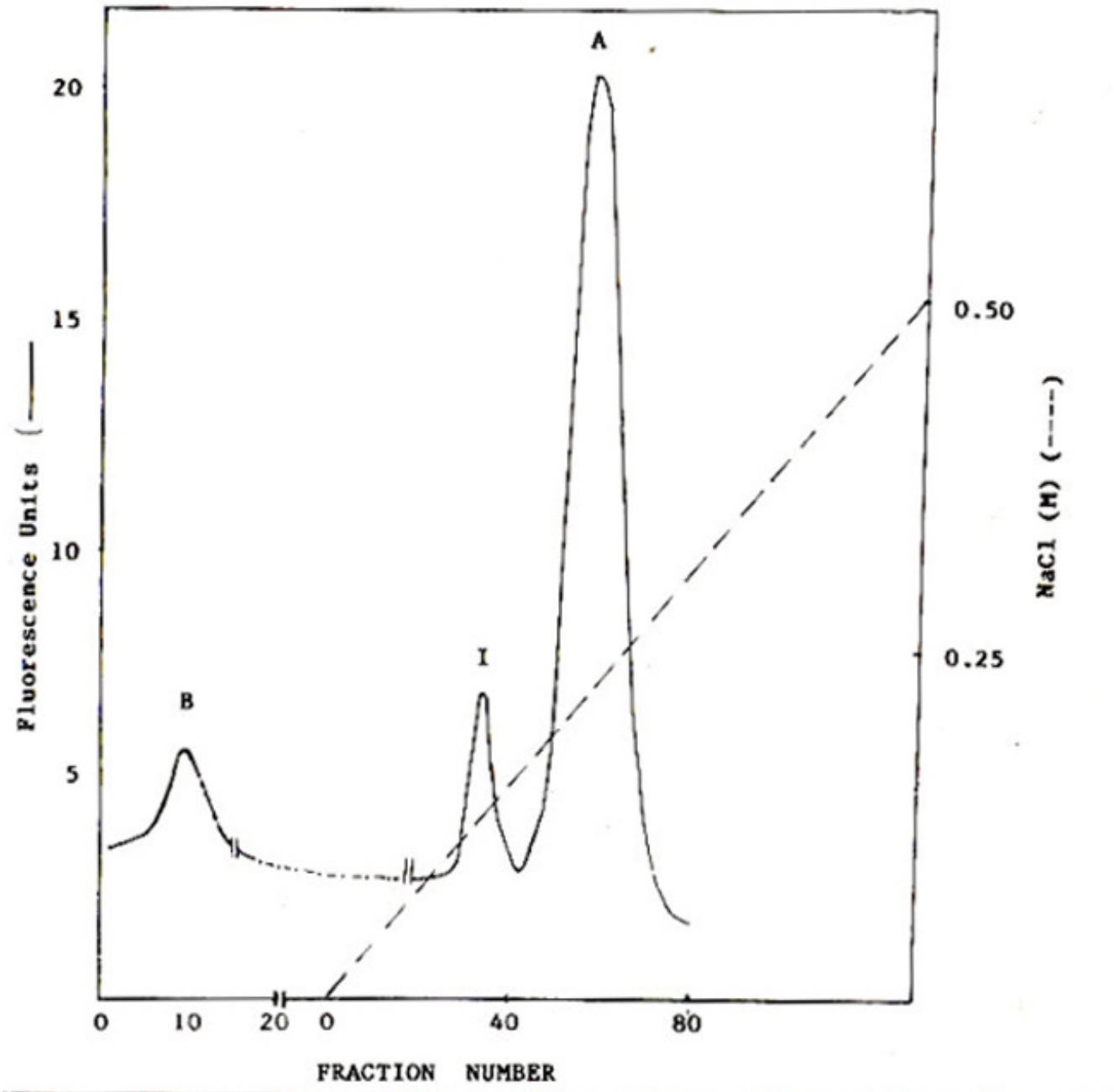

Figure 2. Typical chromatogram of NAG isoenzymes (A, I and B) of human uterus cytosol eluted from DEAE-sepharose. Experimental conditions are explained elsewhere[13].

and we have obtained almost identical results. In the present study our aim was not to differentiate the NAG isoenzymes. The aim was only to determine the activity of NAG in urine samples of various kidney diseases. After establishing the method for NAG activity and its isoenzymes in urine, we would like to apply this methodology for the assessment of renal disease and for monitoring the secondary involvement of the kidney in disorders such as diabetes, hypertension and rheumatoid arthritis. The method may also be applied in testing renal functions in the screening of new drugs.

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