Fluorometric determination of inulin using 5-quinolineboronic acid and inulinase

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Abstract

Inulin is a polysaccharide composed mainly of D-fructose units, and is the most reliable indicator of glomerular filtration rate. We have proposed an inulin detection method that involves the hydrolysis of inulin to D-fructose using inulinase and the selective binding of D-fructose from inulin using 5-quinolineboronic acid. In this method, the fluorescence of 5-quinolineboronic acid increases depending on inulin concentration. For inulin in plasma, the detection and quantitation limits were calculated to be 3.7 and 11 μg/mL, respectively.

Keywords;

Boronic acid; Fluorescent assay; Glomerular filtration rate; Inulin; Inulinase.

Inulin is a polysaccharide composed mainly of D-fructose units. In clinical use, inulin has been the most reliable indicator of glomerular filtration rate (GFR), which is an important parameter in the evaluation of the renal function of patients with kidney disease [1, 2]. Intravenously injected inulin is completely filtered at the glomerular capillary level. It is neither reabsorbed nor secreted by the tubules, and it is not bound by plasma proteins. Therefore, inulin has been used clinically as a highly accurate indicator of the GFR.

In the past, inulin has been quantitatively characterized using the anthrone-sulfuric method, which is commonly used for various other sugars [3, 4]. In this process, inulin is first hydrolyzed with sulfuric acid, which yields D-fructose. Then, the D-fructose is dehydrated with sulfuric acid to produce furfural. Finally, furfural reacts with anthrone, which results in the formation of optically detectable products. However, this method is unreliable because furfural derivatives are produced from other sugars, such as D-glucose, which is mainly present in biological fluids. In fact, a positive influence of 38.7% was observed for the anthrone method at a D-glucose concentration of 2 mg/ml, which is comparable to post-meal blood D-glucose levels [4].

To avoid the effect of endogenous D-glucose, alternative enzymatic approaches have been proposed [4-9]. In most cases, inulinase was used to prepare D-fructose from

inulin and further enzymatic reactions were needed to determine the presence or absence of D-fructose itself. However, selective D-fructose determination is not as simple to perform as it seems. For example, a method for quantitatively identifying D-fructose needs seven reagents, i.e., hexokinase, phsophoglucose isomerase, glucose-6-phosphate dehydrogenase, ATP, and $NADP^+$ [5, 6]. Another method needs five reagents, i.e., fructose-dehydrogenase, peroxidase, 1-methoxy-5-methylphenazinium, *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-*m*-toluidine, and 4-aminoantipyrine [4]. Sample preparation that entails using a lot of reagents is troublesome and the many reaction steps that are involved in such an experiment could cause experimental errors.

In this report, we propose a method for selectively detecting D-fructose formed from inulin; the proposed method involves the use of arylboronic acids. Many types of arylboronic acids have been investigated as recognition motifs that bind preferentially to sugars and other vicinal diols [10-12]. Fortunately, monoboronic acid derivatives have a higher affinity for D-fructose than for other sugars. When using monoboronic acid, we needed to only use a single reagent to quantitatively and selectively determine the presence of D-fructose. 5-quinolineboronic acid (5-QBA) was used to differentiate D-fructose from inulin [13]. 5-QBA is essentially non-fluorescent in neutral aqueous solution. However, upon addition of D-fructose, the fluorescence intensity of 5-QBA

increases dramatically in a concentration-dependent manner.

First, we evaluated the selectivity of 5-QBA in pH 7.2 solution because 5-QBA shows a good response in neutral pH [13]. Fig. 1 shows the change in fluorescence intensity of 5-QBA as a function of sugar concentration. Within the range 0–90 μg/mL, the fluorescence intensity was proportional to the concentration of D-fructose (Fig. 1). D-glucose did not affect the fluorescence intensity of 5-QBA. In addition, we confirmed that the coexistence of D-glucose at plasma level concentrations (2 mg/mL) had no effect on the reactivity of 5-QBA toward D-fructose. This D-fructose selectivity was due to the inherent selectivity of monoboronic acid [10-12]. The most abundant sugars after D-glucose are D-fructose and D-galactose, which are usually present in blood at a very low level (< 0.1 mM) [14]. Since D-galactose shows a low affinity for boronic acids like D-glucose, we consider the role of endogenous D-galactose as slight in this analytical method [10-12]. On the other hand, D-fructose shows a high affinity for boronic acids and it is difficult to differentiate the endogenous D-fructose and the D-fructose from inulin. This will be a subject of further study.

To determine the concentration of inulin, 2.5 μL of an endo-inulinase solution (EC 3.2.1.7, Sigma, cat no. I-2017) was added to 2.5 mL of inulin solution (50 mM acetate buffer, pH 4.5), and the solution was maintained at 50°C because this condition is

suitable for the inulinase reaction [4]. After the elapse of a certain period of time, 2.5 mL of 5-QBA solution (11 μg/mL in 200 mM phosphate buffer, pH 7.4) was added to the sample solution. The pH of the resulting solution was confirmed to be 7.2, which is appropriate for the 5-QBA–D-fructose interaction [13]. To fix the enzyme reaction time, we carried out the procedure with varying the enzyme reaction time. The fluorescence intensity increased up to a steady value with increasing the enzyme reaction time. The fluorescence intensity of 5-QBA reached a plateau within 20 min, so that the reaction time for hydrolysis was set to 30 min. Fig. 2 (a) shows the fluorescence intensity of 5-QBA against inulin concentration. Note that the abscissa shows the inulin concentration in the acetate buffer. The sugar was doubly diluted in the final solution. Depending on the concentration of inulin, fluorescence intensity increased. We confirmed that the response for inulin was similar to that for the same concentration of D-fructose, which means that the concentration of D-fructose derived from inulin was successfully determined. The inulin determination was carried out three times and the standard deviation (σ) was calculated. Based on the σ for the signal of blank, the detection and quantitation limits were calculated using equations (2) and (3), respectively.

Detection limit = 3.3 σ/S (2)

Quantitation limit = 10 σ/S (3)

The value of S is the slope of the calibration plot. The detection and quantitation limits were calculated to be 1.6 and 4.8 μg/mL, respectively. Assay accuracy was demonstrated by inulin recovery at different levels. Table 1 shows results in inulin contents ranging from 27 μg/mL to 135 μg/mL. Recoveries ranged from 100 to 104%, with an average value of 102%. These values were comparable to those obtained using an enzymatic inulin assay method that involved the use of D-fructose dehydrogenase [4].

In the next step, we tried to determine the level of inulin in bovine plasma. However, this proved difficult. When bovine plasma was treated using the above-mentioned procedure, the plasma itself showed a high level of fluorescence. In addition, the plasma interfered with the activity of 5-QBA toward D-fructose. We postulated that plasma proteins such as albumin interfered with the 5-QBA and found that deproteination by heating was effective for determining the level of inulin in the plasma. Plasma containing inulin was treated as follows. Plasma (2.5 mL) containing inulin was treated at 80°C for 4 min. To avoid complete solidification, 2.5 mL of 100 mM acetate buffer (pH 3.0) was added. Again, the mixture was treated at 80°C for 4 min. After that, the sample

was centrifuged at 10,000 rpm for 5 min to remove coagulated material. The pH of the supernatant was 4.8, which was appropriate for the inulinase reaction to occur. The inulinase solution (2.5 μL) was added to the supernatant (2.5 mL) and the sample was incubated at 50°C for 30 min. Then, the solution was mixed with phosphate buffer containing 5-QBA (55 μg/mL). Fig. 2(b) shows that the level of inulin in the plasma was successfully determined. Note that the abscissa shows the inulin concentration in the acetate buffer and that the sugar was diluted fourfold in the final solution. The inulin determination was carried out three times and the detection and quantitation limits were calculated using equations (2) and (3), respectively. The detection and quantitation limits were calculated to be 3.7 and 11 μg/mL, respectively. The value of the detection limit indicates that our proposed method using inulinase and 5-QBA would be used for GFR measurements because plasma inulin concentrations typically encountered in practice are around 100 and 150 μg/mL [15]. However, the intercept in Fig. 2 (b) is relatively high, which means some interferences exist in the plasma treated with heating. It is necessary to investigate the effect of interferences in plasma such as proteins, endogenous low-molecular compounds, medicinal product, and anticoagulants. For practical use, more detailed experiment using human plasma is necessary.

In summary, the concentration of inulin was successfully determined using inulinase

and 5-QBA. Deproteination by heating was effective for determining the inulin level in plasma. This is the first report of inulin determination using an arylboronic acid. The development of a wide variety of inulin-determination methods using arylboronic acids can be expected, because arylboronic acids employed for the development of sugar-sensitive systems based not only on fluorescence but also on visible absorption, electrochemistry, holography etc [10-12].

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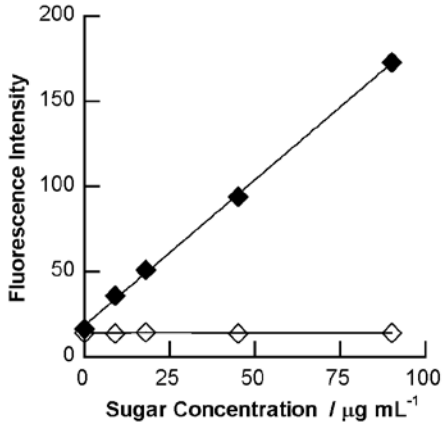

Fig. 1. Fluorescence intensity change of 5-QBA (5.5 μg/mL) solution as a function of sugar concentration at pH 7.2 (λ_{ex} = 315 nm, λ_{em} = 426 nm). Closed diamond, D-fructose; Open diamond, D-glucose.

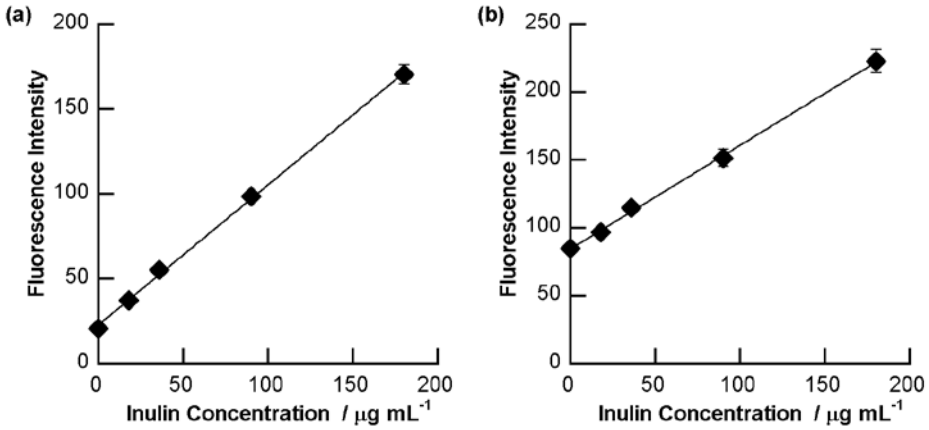

Fig. 2. Fluorescence intensity change in treated sample solutions as a function of inulin concentration (λ_{ex} = 315 nm, λ_{em} = 426 nm). (a) Inulin samples prepared with acetate buffer solution (n = 3). (b) Inulin samples prepared with bovine plasma (n = 3).

Table 1. Inulin recovery from buffered solutions.

added (μg/mL)	found[a] (μg/mL)	recovery (%)	CV (%)
27	28 ± 0.95	104	3.5
63	64 ± 3.36	101	5.3
135	134 ± 2.87	100	2.1

[a] Mean ± SD of three measurements.