Measurement of urine total sialic acid: comparison of an automated ultraviolet enzymatic method with a colorimetric assay

M.A. CROOK, S. KARGBO and P. LUMB
Clinical Chemistry Department, 5th Floor Tower, Guy's Hospital, London SE1 9RT, UK.

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ABSTRACT

An automated ultraviolet (UV) enzymatic assay for urine total sialic acid (SA), performed on a Cobas Fara analyser, is described and compared with the colorimetric Warren method, which is used widely to determine urine SA. Intra-assay coefficient of variation (CV) for urine total SA determination was 0.83% for the UV assay and 3.5% for the Warren method. Inter-assay CVs were 1.8% and 5.6%, respectively. Recovery of urine total SA ranged from 89% for the UV assay to 61% for the Warren method. Both were linear over a range of urine SA from 20 to 240 mg/L The UV assay was automated, took approximately 20 min to produce a result and avoided the need for solvent extraction; however, the reagents were expensive in comparison to those required for the Warren method. Urine samples with a creatinine concentration >14 mmol/L were diluted with distilled water to optimise SA recovery by the UV method. Urine SA:creatinine ratios for normals were 4.7 (±1.7) g/mol with the Warren method and 4.5 (±1.0) g/mol for the UV method. Similarly, in type-2 diabetic patients, urine SA:creatinine ratios were 7.6 (±2.3) g/mol ($P<0.001$) and 8.5 (±2.9) g/mol ($P<0.001$), respectively.

KEY WORDS: Enzyme tests.
Sialic acids.
Urine analysis.

Introduction

Sialic acid (SA) is a major component of glycoproteins and glycolipids, in which it commonly forms the terminal constituent of saccharide side chains.[1] The term 'sialic acid' is a generic one that describes a group of derivatives of neuraminic acid – a nine-carbon sugar. SA is highly negatively charged, giving rise to active or repulsive forces between different cell surfaces, and can influence membrane receptor and glycoprotein conformational states. Sialic acids often form part of antigenic determinants of glycolipids or glycoproteins.[1]

Urine total SA (TSA) is increased in diabetic patients, possibly due to alterations in neuraminidase and sialytransferase activities,[2] and also in individuals with chronic glomerulonephritis.[3] In addition, there are a number of inherited inborn errors of SA metabolism, such as sialidosis, galactosialidosis, Kanzaki disease and mucolipidosis II and III, in which an increase in urine total SA is found.[4-6]

The majority of urine SA is bound to glycopeptides and oligosaccharides; however, some 40% is unbound and exists in free form. This free fraction is filtered by glomeruli but not reabsorbed by the renal tubules. Urine SA also can be increased (predominantly as free SA) in other inborn errors of metabolism such as infantile sialic acid storage disease, sialuria and Salla disease.[7-9]

The purpose of this study is to establish a simple, automated and relatively quick assay to measure urine TSA using commercially available reagents on a Cobas Fara discrete analyser. This ultraviolet (UV) enzymatic method is based upon that reported for serum samples by Arakai *et al*.[10] In addition, we compare this with the Warren method,[11] as modified by Roboz,[12,13] which is a colorimetric method using thiobarbituric acid and solvent extraction used traditionally to assay urine SA. A rapid automated assay to determine urine SA would be useful clinically in facilitating the diagnosis of some of the inborn errors of SA metabolism mentioned above.

Correspondence to: Dr Martin Crook
Email: martin.crook@gstt.sthames.nhs.uk

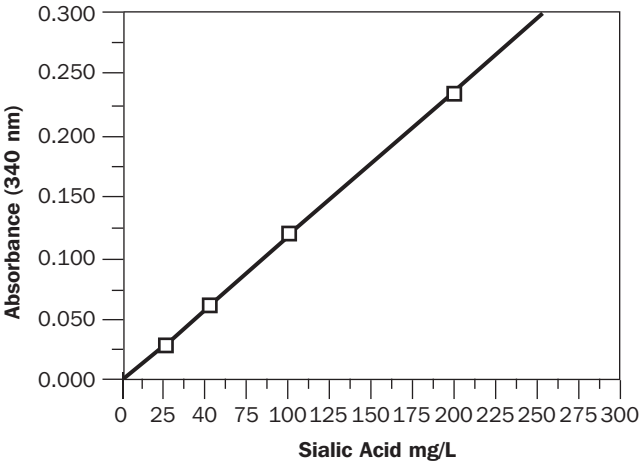
Materials and methods

The assays used for urine SA were performed using reagents (including all enzymes) supplied by the Sigma Chemical Company, Poole, Dorset, UK. The standards (primary) used for both assays were prepared in-house from *N*-acetylneuraminic acid (NANA, type VIII; Sigma) over a range from 20 to 500 mg/L. Calibration was checked with SA standard material supplied by Roche Diagnostics, Lewes, Sussex, UK.

Ultraviolet enzymatic assay

Briefly, the UV automated enzymatic assay of Arakai *et al*.[10] is a coupled enzyme assay, incorporating neuraminidase (*Clostridium perfringens*), *N*-acetylneuraminic acid aldolase and lactate dehydrogenase (LDH). Liberated NADH is converted to NAD^+, which can be measured kinetically by following the reaction absorbance at 340 nm. The method was set up for use on a Cobas Fara analyser (Roche, Welwyn, Herts, UK). In this study we prepared three reagent mixtures.

Fig. 1. Linearity for urine sialic acid achieved with the UV assay.



Reagent 1: 10 mmol/L Tris buffer (pH 7.5) in 2 mL distilled water containing 40 μL LDH at 1000 units/mL and 2mg NADH.

Reagent 2: 100 units neuraminidase in 10 mL Tris buffer (pH 7.5).

Reagent 3: NANA aldolase (25 units) dissolved in 2.5 mL Tris buffer (pH 7.5).

The settings for the Cobas Fara were as follows: 20 µL sample, 10 µL distilled water, 20 µL Reagent 1 and 20 µL Reagent 2. The mixture was incubated for 5 min, after which 16 µL Reagent 3 and 14 µL distilled water were added. The mixture was incubated for a further 20 min and the reaction was followed at 340 nm, with readings taken against a blank. Incubation times were based upon those used by Arakai *et al*.[10]

The sequence of reactions in this assay was as follows:

$$\text{sialic acid (bound)} \xrightarrow{\text{neuraminidase}} \text{sialic acid (free)}$$

$$\text{sialic acid (free)} \xrightarrow{\text{aldolase}} \text{pyruvate} + \textit{N}\text{-acetylmannosamine}$$

$$\text{pyruvate} + \text{NADH} \xrightarrow{\text{lactate dehydrogenase}} \text{lactate} + \text{NAD}^+$$

Warren method

Briefly, the Warren method consists of the oxidation of NANA with periodate, which is terminated by the addition of arsenite. This is followed by the addition of thiobarbituric acid, resulting in formation of a red colour that can be extracted in cyclohexanone and read at 549 nm.

The method used was as follows: 400 µL 0.063mol/L sulphuric acid was added to 100 µL urine and incubated at 80°C for 1 h. The tubes were cooled in iced water for 5 min and then 150 µL 0.2mol/L sodium periodate was added and the tubes allowed to stand for 20 minutes. The reaction was terminated by adding 1 mL 10% (w/v) sodium arsenite and each tube was vortex-mixed.

Subsequently, 3 mL 0.6% (w/v) thiobarbituric acid made up in 0.5 mol/L sodium sulphate was added and the mixture boiled for 5 min. After cooling in iced water, the pH of the reaction mixture was adjusted to 5.6-6.0 with 5 mol/L sodium hydroxide (300 µL). Cyclohexanone (4 mL) was added, the tubes were centrifuged at 900 x*g* for 5 min at room temperature and the upper organic layer discarded. The pH of the aqueous layer was readjusted to 1.75-1.95 with 5 mol/L hydrochloric acid (300 µL), a further 4 mL cyclohexanone was added and the tubes centrifuged again.

Absorbance of the upper organic layer was measured at 549 nm in a Phillips PU870 scanning spectrophotometer. Urine creatinine was measured by the Jaffe reaction in a Vitros 250 (Johnson and Johnson, Amersham, Bucks) using reagents supplied by the manufacturer. The intra-assay coefficient of variation (CV) for this assay was < 5%.

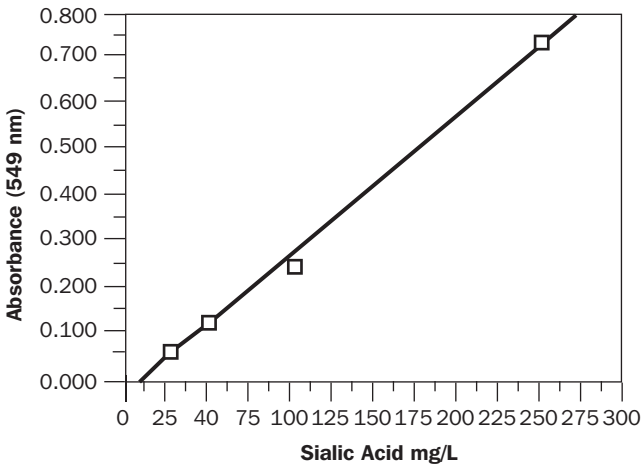
Samples

Thirty-six fresh urine samples were collected in the early morning from 18 healthy individuals and 18 type-2 diabetic patients. These two subject groups were chosen in order to study urine TSA across a wide range of concentrations. To our knowledge, none of the individuals included in the study had suffered recent urinary tract infection, malignant or inflammatory disease, or had an inborn error of SA metabolism.

In total, the two groups comprised 16 females and 20 males with a mean age (± SD) of 30 (± 10) years, range 22-55 years. Urine was collected into a plastic universal container without preservative and stored at 4°C for a couple of day before analysis. Prior to assay, the samples were mixed gently and returned to room temperature.

Intra-assay CVs were calculated on at least 10 determinations. Inter-assay CVs for urine TSA were also calculated on 10 different SA concentrations on three consecutive days. Linearity for urine TSA assay was studied up to a concentration of 240 mg/L using serial dilutions with distilled water.

Fig. 2. Linearity for urine sialic acid achieved the Warren method.



Recovery for the urine TSA assay was studied by 'spiking' urine samples with 50 mg/L NANA. Before-addition and after-addition urine SA was determined for each sample and the percentage recovery calculated.

Statistics

Results are presented as the mean (± SD) or, if the data were not normally distributed, the mean (range). CV was calculated as follows: SD of assay x 100%/mean of assay results. Comparison of the two urine SA assays was made using the method of Altman and Bland. This is recommended for assay comparison where CVs cannot be assumed to be similar.[14] Differences between data means were compared using the Student's *t*-test. Statistical significance was taken as $P<0.05$.

Results

Intra-assay CVs for urine TSA determination using the UV assay and the Warren method were 0.83% and 3.5%, respectively. Inter-assay CVs were 1.8% and 5.6%, respectively. Recoveries for the urine TSA assays ranged from 88% to 108% for the UV assay and 50% to 61% for the Warren method. However, it was noted that recoveries for the UV method decreased to between 65% and 88% when urine creatinine concentration was >14 mmol/L. Assay linearity for the UV assay is shown in Figure 1 and for the Warren method in Figure 2. An Altman-Bland plot comparing the two urine SA assay methods is shown in Figure 3. Urine SA:creatinine ratio for normals was 4.7 (± 1.7) g/mol with the Warren method and 4.5 (± 1.0) g/mol with the UV method. Similarly, in type-2 diabetic patients, urine SA:creatinine ratios were 7.6 (± 2.3) g/mol ($P<0.001$) and 8.5 (± 2.9) g/mol ($P<0.001$), respectively. There was no significant difference between the respective results for the Warren and UV methods.

Discussion

Data showing the performance characteristics of a urine TSA UV enzymatic assay for use on a Cobas Fara analyser are presented. The UV assay was found to be of good analytical precision with intra-assay CVs <1% and inter-assay CVs <5% across the range of urine SA measured. Similarly, the assay showed good linearity and percentage recoveries.

To our knowledge, this is the only reported study to have looked at a urine SA assay based on an enzymatic method using commercially available reagents in a Cobas analyser system. In our hands, the Warren method performed poorly compared to the UV method in the recovery experiments; however, the number of manual steps in the former may have contributed to this finding. The Altman-Bland plot shows a positive bias with the Warren method, which may be not too surprising given that the UV assay utilised an enzyme system supposedly specific for SA.

The majority of work on the UV assay method has been performed with serum or plasma samples and we are unaware of a comparative study with the Warren method – a urine TSA assay used commonly in the routine clinical laboratory. The UV assay gave better CV values and recoveries; however, it did give lower recoveries when urine creatinine concentration was >14 mmol/L. Thus, we would advocate caution when using concentrated urine samples and suggest that they be diluted with distilled water to reduce the creatinine concentration. In view of this, it may be useful to express SA as a creatinine ratio in urine samples.

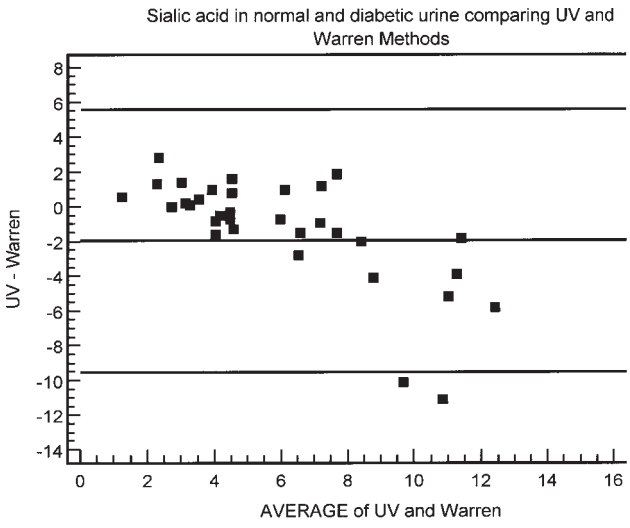
Both assays were linear over the urine SA concentration ranges found in our samples. Urine SA:creatinine ratio was significantly higher in the type-2 diabetic patients by both methods; however, there were no significant differences between the two methods. It is known that increased glomerular permeability occurs in diabetes mellitus and the results may reflect leakage of sialylated proteins into the urine.[2]

Many workers measure urinary SA by modifications of the Warren method,[12,13] which is a colorimetric assay that utilises thiobarbituric acid. However, there has been criticism of the method because of interference with 2-deoxyribose and therefore the pH correction procedure devised by Roboz *et al*.[13] should be applied. The SA UV enzymatic assay used in the present study is not prone to this interference and is thought to provide greater specificity for SA. This would be important because of possible interference from drugs or from other 'sugar' molecules.

It should be noted, however, that the two methods compared here may give different results not only because of differences in specificity but also because of methodological variations in the assay procedure and the liberation of bound SA. The Warren method uses acid hydrolysis and heating of the sample at 80°C, whereas the UV assay employs the enzyme neuraminidase to liberate bound SA. Interestingly, however, not all sialoglyco-conjugates are cleaved by neuraminidase. It may also be possible to measure urine free SA using the UV assay by measuring SA before and after treatment of urine with neuraminidase. Proteinuria may result in increased urine total SA because some urine proteins are sialylated.

Previously, we described a colorimetric assay for the determination of serum TSA using a Cobas Bio analyser[15] – a method that incorporated pyruvate oxidase and peroxidase with a colorimetric dye.[16,17] However, the manufacturer of the

Fig. 3. Altman-Bland plot comparing the UV assay and method.



manual kit for this method does not recommend its use with urine samples because of interfering substances, and was one of the reasons we used the urine UV SA method reported here. Furthermore, there are a number of high-performance liquid chromatography techniques to assay SA[18-20] but these are considered to be mainly for research purposes, being too tedious for routine clinical use.[12]

In conclusion, the UV assay reported here is quick to perform and is automated. Assay time is considerably shorter (approximately 20 min to allow the assay reaction to complete) than the Warren method, which requires heating at 80°C for one hour and subsequent organic solvent extraction. Furthermore, the sodium arsenite used in the Warren method is toxic and can be difficult to purchase. Conversely, the reagents used in the UV assay are expensive (approximately £1 per test). Therefore, we developed the assay using the Cobas Fara analyser, an automated machine which requires only small reagent volumes.

Finally, urine samples with a creatinine concentration >14 mmol/L should be diluted to ensure adequate SA recovery when using the UV assay, and the higher SA:creatinine ratio observed in our type-2 diabetic patients merits further study in a larger cohort. □

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