A PERIODATE-RESORCINOL MICROASSAY FOR THE QUANTITATION OF TOTAL SIALIC ACID IN HUMAN SERUM

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Abstract A periodate-resorcinol microassay for the quantitation of total sialic acid (TSA) has been developed. A 96-well microtiter plate format was used as a single reagent mixing well. The principle of this newly-developed assay is the periodate-resorcinol procedure. Conditions for the quantitation of sialic acid in serum, such as the concentration of periodate, resorcinol, incubation time and human serum volume, were optimized. It was found that the optimal concentration of periodic acid and resorcinol reagent were 1.3 mM and 0.6%, respectively. An incubation time of 60 minutes for the reaction of periodic acid and resorcinol with samples was found to give the highest absorbance. These new procedures were used for the quantitation of sialic acid in human serum. It was found that only 5 µL samples were needed to give a varied coefficient of the intra- and inter-assay of 0.79% and 4.68%, respectively. Furthermore, this method demonstrated the recovery percentage of 94.25% by the addition of a known amount of pure sialic acid. This has been used to quantitate TSA in normal healthy and cancer serum and a significant difference between these two groups \(p<0.0001\) was found. These results suggest that the periodate-resorcinol microassay is sensitive, reliable, economical, requires less sample volume and is suitable for the quantitation of total sialic acid in biological fluid and tissue extraction samples.


Sialic acid, a class of important ketoses that contain nine carbon atoms, are acetylated derivatives of neuraminic acid (2-keto-5-amino-3,5-dideoxy-D-nonulosonic acid).1 The unique structural features of this molecule, which includes a negative charge owing to a carboxyl group, enable it to play an important role in cellular functions, such as cell-to-cell repulsion, recognition, transportation of positively charged compounds and tumor cell metastasis.2 Sialic acid is a common terminal sugar unit of the oligosaccharide of glycoproteins and glycolipids, which are cell surface constituents. These sialic acids apparently enter the circulation by either shedding or cell lysis and are of considerable interest because of their potential diagnostic value.2 Elevated levels of serum sialic acids have been reported in patients with lymphoma, malignant melanoma and lung cancer as well as cancers of the prostate, colon, brain, stomach, bladder and gastrointestinal system.3-6 A widely used technique for quantitation of free sialic acid is the periodate-thiobabituric acid method. The periodate-resorcinol assay is used for total sialic acid (TSA).1 Bhavanandan and Sheykhnazari1 reported a convenient micro-assay for simultaneously measuring sialic acid in a large number of samples and
specifically for monitoring fractions during fractionation of biological samples. They have adapted the popular periodate-resorcinol method to a 96-well microtiter plate format.

This present paper reports a modification and optimization of the periodate-resorcinol method, which is more suitable for biological fluids such as human serum samples. It is based on the periodate-resorcinol reaction for TSA, and the modified assay has been used to quantitate TSA in the serum of normal healthy humans and that from cancer patients. Furthermore, it has been compared with the Ehrlich assay, previously described.

**Materials and methods**

All chemicals were of analytical grade unless otherwise stated. N-acetylneuraminic acid (Cat. No. A-9646) was from Sigma Chemical Co., MO, USA. Periodic acid and resorcinol were from Fluka Chemika, Germany. Sodium acetate was from Merck, Germany.

**Samples collection**

Serum healthy individuals samples were collected from (n=100) who presented for a medical check-up at the Department of Clinical Chemistry, Faculty of Associated Medical Sciences, Chiang Mai University Thailand. The serum samples were obtained from 50 patients with various types of cancer, as diagnosed by medical doctors in Maharaj Nakorn Chiang Mai Hospital. All samples were measured for carcinoembryonic antigen (CEA) in the tumor marker laboratory at the Department of Biochemistry, Faculty of Medicine, Chiang Mai University. Venous blood was collected (5 mL) in a glass tube and left at room temperature for 30-60 minutes before being centrifuged. The serum was then collected, divided into aliquots, and stored at -20°C until used.

**Measurement of TSA by the periodate-resorcinol microassay.**

TSA determination was modified from the method. It was performed as follows: 40 µL of pure standard sialic acid sample (2-10 µg/well) were added to the wells of a 96-well microtiter plate (polystyrene, Nunc, Cat. No. 269620). Then, 50 µL of 1.3 mM periodic acid (prepared from stock 0.32 M) was added to each well and mixed by shaking the plate for 5 min on a microplate shaker at room temperature. The plate was placed (floated) in an ice box for 60 minutes, then 100 µL of 0.6 g/dL of resorcinol reagent (prepared from stock 6 g/dL) was added and mixed by shaking, as described above. The plate was covered with a glass plate and heated 80°C for 60 minutes in a water bath it was then removed, placed on the shaker and mixed for about 2 minutes, while the contents cooled down to room temperature. Then, 100 µL of 95% tert-butyl alcohol was added to each well and the contents were mixed once again, as described above. The absorbance at 620 nm was measured immediately by a microtiter plate reader.

**The measurement of TSA by the standard Ehrlich’s method.**

Samples of 200 µL were mixed with 400 µL of 0.2 N H₂SO₄ and incubated in a waterbath at 80°C for 1 hour. Then, 1 mL of 10% trichloroacetic acid (TCA) was added and mixed, after which the solution was centrifuged at 3,000 rpm for 5 minutes. The supernatant (500 µL) was collected for analysis. It was diluted with 2 mL of distilled water and a further 500 µL of Ehrlich reagents were added. The solution was boiled in the waterbath for 30 minutes. The reaction was stopped by cooling the sample tubes in an icebath. Absorbance of the color was measured by a spectrophotometer at 565 nm. The pure sialic acid (20-100 µg/tube) was used as the standard and 0.2 N H₂SO₄ - 10% TCA was used as a blank.
Optimization of serum sample volume for the TSA periodate-resorcinol microassay.

Serum samples were added in the range of 5,10, 20, 25, 30, 35 µL to the microtiter well and the volume was adjusted to 40 µL by distilled water before being subjected to the periodate-resorcinol microassay for TSA, as described above.

The optimized volume of the sample was used for quantitation of TSA in individual samples in triplicate, and the same sample was quantitated in parallel by the standard Ehrlich method,\(^7\) using both normal and pathological serum samples.

Evaluation of precision and accuracy of the newly-developed method.

The intra- and inter-assay, using the periodate-resorcinol microassay procedure for TSA, was studied in pooled human serum samples. The intra-assay was performed on 20 identical aliquots of pooled serum within the same plate. The inter-assay was performed on separate plates under the same conditions. The recovery percentage of pooled TSA was determined by adding a known amount of sialic acid before analysis by the periodate-resorcinol microassay. The amount found was compared with theoretical values.

Statistical analysis

The results of TSA from the assay in normal healthy controls and cancer patients was statistically tested for a significant difference using the students unpaired t-test.

Results

The principle of this method is based on a periodate-resorcinol reaction with sialic acid in the sample. The amount of periodic acid must be titrated for the optimal color development without sample precipitation. As shown in Figure 1, it was found that the dilution of periodic acid at 1:250, which is equal to a concentration of 1.3 mM, was selected for TSA periodate-resorcinol microassay, due to its sensitivity and stronger absorbance.

The incubation period of the periodic acid with the sample or standard condition is an important factor for this periodate-resorcinol microassay. It was found that color production increased with an incubation time of up to 60 minutes and decreased when it was incubated longer, as shown in Figure 2. The incubation period of periodic acid with samples at 30–60 minutes was selected for the standard condition in the periodate-resorcinol microassay for TSA.

The optimal concentration of resorcinol was also studied. When using various concentrations of resorcinol in the standard curve, it was found that the dilution of 1:10 from the stock solution gave a good linear standard curve and quite strong absorbance signal, as shown in Figure 3. This experiment shows that the color production decreased according to the concentration of resocinol.

The maximum absorption at the end of the reaction was obtained after heating the resorcinol with reaction mixture for 60 min at 80°C, as shown in Figure 4. This incubation period was selected for further use in the quantitation of TSA in human serum samples.

When the optimal conditions of the new periodate-resorcinol microassay for TSA was used to evaluate the precision and accuracy of the assay, it was found that the intra- and inter-assays (N=20) were 0.79% (CV) and 4.68% (CV), respectively. The percentage of recovery, which was studied by the addition of a known amount of pure sialic acid was found at 94.25% (ranged between 76.23–105.23%; data not shown).

The result of the comparison between the newly-developed periodate-resorcinol microassay for TSA and the previous standard Ehrlich method is shown in Figure 5. The two methods were found to correlate with each other at the correlation coefficient of 0.70 (p<0.0001).
Fig. 1. Evaluation of the effect of various concentrations on periodic acid. Periodic acid was diluted from stock solution (0.32 M) with acetate buffer pH 4.5 at a dilution of 1:50, 1:100, 1:150, 1:250, 1:300, 1:350 and then used in the periodate-resorcinol microassay for TSA human pooled serum samples.

Fig. 2. Evaluation of the effect on the incubation time of periodic acid in the reagent mixture. The periodic acid had a range of 10, 20, 30, 60, 90 and 120 minutes. The periodic acid was used at a concentration of 1.3 mM and then incubated with the sample for the period previously described.
Fig. 3. Evaluation of the optimal concentration of resorcinol reagent. The reagent was diluted from stock solution at 1:5, 1:10, 1:15, 1:20, 1:25, 1:30. The illustrated standard curve was obtained by using various dilutions of the resorcinol reagent and the experiment was completed.

Fig. 4. Evaluation of the effect of time on the incubation resorcinol reagent in the periodate-resorcinol microassay for TSA. The resorcinol was fixed at one concentration and the variation of incubation with reaction mixture was studied at 30, 60, 90 and 120 minutes.
Fig. 5. Correlation curve between a developed periodate-resorcinol microassay and the Ehrlich method for TSA. The same sample was divided into two separated aliquots and subjected to both methods for TSA.

Fig. 6. Bar-graph demonstration of the TSA quantity of both normal healthy and cancer human serum samples by periodate-resorcinol microassay (p<0.0001).
The quantitation of TAS in the serum of normal healthy humans and that from cancer patients.

The newly-developed periodate-resorcinol microassay was used to determine the quantity of TAS in both normal and pathological human serum samples. It was found that the amount of TAS in normal human serum samples (n=100) was 49.40±4.6 mg%, which was significant difference ($p<0.0001$) from the pathological (cancers) ones (n=50) that had 74.93±12.8 mg%, as shown in Figure 6.

Discussion

Sialic acid levels in serum and urine have been investigated and reported as diagnostic markers for patients with inflammatory disorders, cancer, sialuria, and many other diseases. A variety of methods are available for the detection and estimation of free and glycosidically-bound sialic acids. These can be broadly classified as colorimetric, fluorometric and enzymatic methods as well as the highly sensitive high performance liquid chromatographic (HPLC) method. The most widely used procedures are the colorimetric method including the Ehrlich method\(^7\) the periodate-thiobabituric acid method\(^9\) and the periodate-resorcinol assay.\(^10\)

The procedure described by Warren\(^8\) is typical of the periodic and thiobabituric acid procedure, which measures only free sialic acid that is released after an initial hydrolysis step. The procedure is reported to have an assay coefficient of variation of approximately 3%.\(^11\) A significant rise in total sialic acid was found in patients with cancer of the stomach, breast, colorectal region and gall bladder. The periodate-resorcinol microassay presented in this report has several advantages over the original one. These include the use of smaller samples (5 µL), and the larger the number of samples that can be simultaneously analyzed, with the fast addition of reagents with multichannel or repeating pipetter, the greater the speed in measuring absorbance by the microtiter plate spectrophotometer. This will enable the direct transfer of data to a computer\(^1\) or even an adaptation to an automated system in the future. This experiment reported the optimal conditions of a micromethod for the amount of sialic acid in a range of between 2–10 µg/well. The color production, when using a 1:50 dilution (equal to 6.4 mM) of periodic acid, gave a lower absorbance than other dilutions because of interference by the brown color caused from excess periodic acid. The absorbance decreased when used at 1:300 dilution (1.06 mM), which may be because the periodic acid concentration was not enough to oxidize N-acetylneuraminic acid (Figure 1). Although, the optimal incubation time of periodic acid and resorcinol reagent was 60 minutes, which gave maximum absorbance, a shorter incubation time of 30 minutes could be used that would still give a linear standard curve and high absorbance signal (Figure 2 and 3).

The volume of the human serum sample used in this study was only 5 µL, which was enough to give an absorption level that could be read by a microtiter plate reader. A larger volume of human serum could cause a precipitation of the protein from hydrochloric acid in the resorcinol reagent, which could interfere by forming turbidity in the sample well.

The newly-developed periodate-resorcinol microassay has shown compatibility with the Ehrlich method ($r=0.70, p<0.0001$), which has been widely used. When employing this developed method, it was found that the mean concentration of TAS was significantly higher in cancer patients than normal controls $p<0.0001$ (Figure 6). The method described herein demonstrates an intra- and inter-assay coefficient variation of 0.79% and 4.68%, respectively, which would be acceptable in a clinical chemistry analysis. Furthermore, the recovery percentage of this method is 94.25%, which has been proven as
an accurate assay. Attempts to find new markers have led to sialic acid assays in cancer patients. The measurement of TSA using this newly-developed method could be a useful diagnostic determinant in a variety of neoplastic conditions. It is suggested that this periodate-resorcinol microassay is simpler, faster, easier and more suitable than other methods from previous reports.

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References