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Sequential injection-capillary immunoassay system for determination of sialoglycoconjugates

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ABSTRACT

An automatic immunoassay system for an assay of sialoglycoconjugates was developed based on the sequential injection technique. A cost effective plain glass capillary tube was used as a solid surface for immobilization of biomolecules via a simple physical adsorption which is adequate to tolerate the force of solution flowing through the capillary during the multi-steps immunoassay process. Immunoassay could be performed with many improvements—rapidity per sample as compared to the conventional micro-plate format (40 min vs. 5–8 h); lower cost and simpler as compared to fused silica capillary with covalent immobilization; and without problem of back pressure as compared to flow injection-bead based immunoassay. Performance of the sequential injection-capillary immunoassay was demonstrated by assay of sialoglycoconjugates level in human serum to differentiate cancer patients from healthy people.

1. Introduction

Sialic acid in human body is presented in the form of N-acetylneuramic acid, a nine carbon sugar, as a component of glycoproteins, glycolipids and polysaccharides found in the outer cell membrane [1,2]. Here the term "sialoglycoconjugates" is used rather than "sialic acid" because most of the sialic acid binds to glycoprotein and glycolipid and free sialic acid is normally only found in very small concentration. It has been reported that cancer cells are composed of sialic acid rich glycoproteins and it might contribute to the ability of cancer cells to adhere to various organs and decrease the ability of host defense mechanisms to destroy cancer cells. It was found that cancer patients, especially ones with tumor growth status, have an elevated level of sialoglycoconjugates in their serum. Thus, sialoglycoconjugates are thought to be a potential biomarker for various cancers, though other conditions such as heart disease, inflammation and pulmonary disease have also been reported to have some relationship with this biomarker [3,4].

A variety of techniques including colorimetry [5,6], fluorimetry [7], enzymetry [8], HPLC [9], CE-MS [10] and immunochemical methods [11,12] have been used for quantitative analysis of sialic acid and sialoglycoconjugates. Among all these techniques, immunoassay is superior to other techniques in terms of high specificity and requirement of small amount of sample and reagents. In conventional 96-well micro-plate immunoassay, operation still involves many steps including incubation and washing which are time consuming and subjected to human errors.

Sequential injection analysis (SIA) is the second generation of flow injection analysis (FIA) technique where chemical reaction takes place in small tubing using a bi-directional pump to control the order of reagent aspiration. A computer program is used to manage the various operational steps with high precision in timing and small volume manipulation. SIA has been applied for various bioassays, especially with a special unit integrated to a multi-selection valve called a "lab-on-valve" (LOV) [13,14]. Solid phase for immobilization of antibody or ligands has evolved from micro-well into forms that are more suitable to operate with the flow system. Beads are the most popular new immobilization surfaces due to their mobility in solution and their retainability to facilitate the separation process of bound and unbound components. Beads may be packed into the channel of the LOV which acts as a bioassay reactor. However, using beads in a flow system may cause a back pressure problem which would alter flow rate and precision of the analysis. An open tubular surface such as fused silica capillary has been reported as successfully used in immunoassay [15,16]. While the use of high cost fused silica material is essential for immobilization processes that require high heat activation, a plain glass capillary may be adequate for some biomolecules with high physical affinity to glass surface.

In this study, a low cost plain glass capillary tube was introduced as an alternative solid surface for the immobilization of the specific receptor used in the competitive immunoassay of

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sialoglycoconjugates. A capillary tube is well suited for use in the flow system because it acts as part of the tubing where the solution flows through. This helps to eliminate the back pressure problem and reduce cost of analysis. Bovine submaxillary mucin (BSM) which has sialic acid as the main acidic carbohydrate constituent was chosen for immobilization onto the inner wall of a glass capillary tube and also for the competition standard to construct a calibration curve. Sialic acid in BSM and sialoglycoconjugates in serum sample competed to bind with a limited amount of lectin conjugated to biotin (b-lectin). Anti-biotin conjugated with enzyme horseradish peroxidase (anti-b-HRP) and substrate TMB were used for detection of bound b-lectin which in turn inversely relates to the amount of sialoglycoconjugates in the sample. This SIA system which was developed for better flow using a capillary tube as reactor, instead of a bead reactor, also provides automatic operation of the immunoassay steps. The system was applied to determine the amount of sialoglycoconjugates in healthy and cancer subjects.

2. Experimental

2.1. Materials and apparatus

Normal glass capillaries used as a solid phase for immobilization of mucin were commercial plain haematocrit tubes with the dimension of 75 (±0.5) mm in length and 1.15 (±0.05) mm i.d. (Vitrex). Stock solution of mucin from bovine submaxillary glands, Type I-S (BSM) was prepared at the concentration of 0.0100 g ml^{-1} in 0.02 M carbonate buffer pH 9.6. Working solution used for coating the capillary was diluted to 100 μg ml^{-1}, except for the study on suitable blocking reagent to reduce non-specific binding, 10 μg ml^{-1} BSM solution was used. BSM was immobilized onto the inner wall of glass capillary by physical adsorption, followed by incubation of a suitable blocking reagent. These two steps were done off-line in order to prepare many capillaries in one batch. Various blocking solutions (0.1% Tween20, 1% gelatin, 5% non-fat milk, 5% skim milk, and 1% BSA) prepared in 0.01 M phosphate buffer, pH 7.4 (PB) were tested. Biotinylated *Maackia amurensis* Lectin II (MAL II, Vector Laboratories) was diluted to 1:50 ratio with PBS (0.01 M PB with 0.15 M NaCl, pH 7.5). Mouse anti-biotin conjugated with horseradish peroxidase (Zymed) was diluted to 1:1000 ratio with PB. Ready to use substrate, 3,3′,5,5′-tetramethylbenzidine (TMB, KLP) was employed. Standard calibration curve was constructed from a series of BSM standard solutions prepared in 6% BSA (Sigma) to compensate for the protein matrices of real serum samples. Carrier solution in the flow system was citric phosphate buffer pH 5.0, prepared by mixing 0.05 M citric acid with 0.10 M di-sodium hydrogen phosphate dehydrate.

Serum samples were prepared by centrifugation of fasting bloods, collected in the Vacutainer tube without EDTA (BD), to separate out the blood cells. They were kept at −20 °C and were used without further dilution. Serum samples of patients with cancer were obtained from the Thailand Excellence Center for Tissue Engineering, Department of Biochemistry, Faculty of Science, Chiang Mai University Hospital. Serum samples of healthy people were from volunteers.

The SI system was set up using a syringe pump (XL 3000, CAVRO Scientific Instrument) with 1 ml barrel. A 10 ports selection valve (C25-3180 EMH, Valco Instrument) was used to accommodate all the reagents, air, detector and waste connections. All tubings were PTFE tubings. Spectronic 21 (Spectronic Instrument) with a flow through cell of 8 μl (Pye Unicam) was set at 650 nm. SIA control software (FIA Lab3000, FIA Instrument) is used for controlling the flow rate, volume of reagents and incubation time.

3. Results and discussion

3.1. Manifold and operation steps

The manifold design is shown in Fig. 1. To perform the competitive immunoassay illustrated step by step as in Fig. 2, the operational steps controlled by a computer software program are summarized in Table 1. Volume, flow rate and time for each step were obtained from optimization as described in more detail in the section “optimization”.

3.2. Blocking solution

High non-specific binding of either b-lectin and/or anti-b-HRP onto the uncoated-capillary (capillary without immobilized BSM) was observed. This could cause high background signal and error in the use of a coated capillary if there was empty space where BSM was not bound. To reduce this problem, various blocking reagents were tried. The detergent based blockers such as Tween20 are believed to prevent hydrophobic adsorption of the other proteins that may cause non-specific binding to the solid surface. The small protein based blockers such as BSA, milk protein, and gelatin are believed to fill in the empty solid surface not occupied by the immobilized receptor [17].

The results compared between uncoated capillary (blank) and BSM-coated capillary (analytical signal) were used to identify the best blocking reagent. Fig. 3(a) shows the differences between the assay systems that gave blank and analytical signals. Fig. 3(b) clearly indicates that 0.1% Tween20 was the most suitable blocking solution because the analytical signal is much higher than blank which means that Tween20 did not interfere with the assay. This also implied that the non-specific binding in this assay system may be due mainly to hydrophobic binding of non-specific components to the glass surface which could be reduced by detergent based blocker.

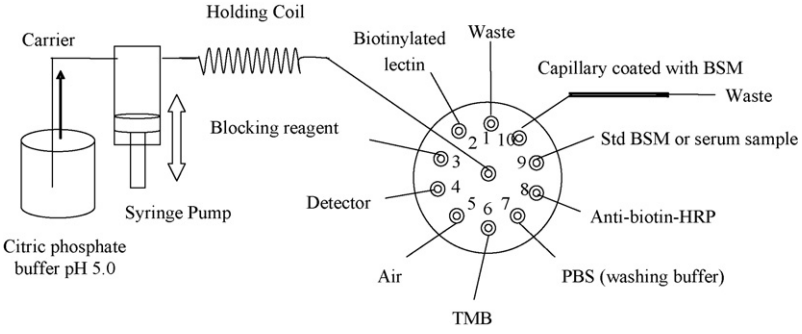

Fig. 1. Sequential injection-capillary immunoassay (SI-CI) system.

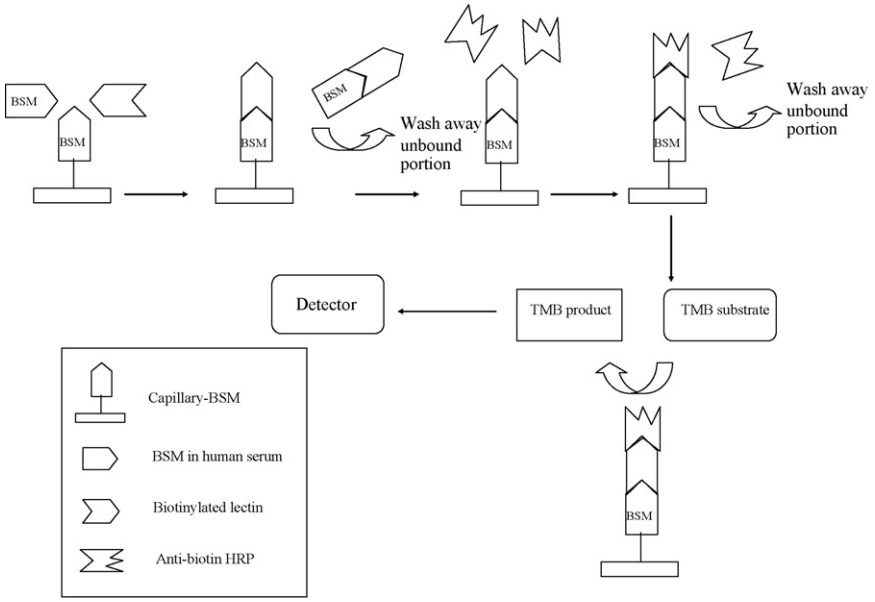

Fig. 2. Diagram illustrating the competitive immunoassay process of BSM.

Table 1
Summarization of operational steps.

Step no.	Operation	Volume (μl)	Flowrate (μl s^{-1})
1	Washing capillary with PBS	1500	50
2	Aspiration of air to the holding coil	20	10
3	Aspiration of 0.1%Tween in PBS solution to the holding coil	80	10
4	Aspiration of air to the holding coil	20	10
5	Sending 0.1%Tween in PBS solution to capillary and incubate for 3 min	80	10
6	Removing unbound species by washing capillary with PBS	1000	50
7	Aspiration of air to the holding coil	20	10
8	Aspiration of standard BSM solution or serum sample to the holding coil	10	10
9	Aspiration of biotinylated lectin to the holding coil	60	10
10	Aspiration of standard BSM solution or serum sample to the holding coil	10	10
11	Aspiration of air to the holding coil	20	10
12	Mixing standard BSM solution and biotinylated lectin by moving solution back and forth three times	–	–
13	Sending mixture to BSM coated capillary and incubate for 10 min	80	10
14	Removing unbound species by washing capillary with PBS	2000	50
15	Aspiration of air to the holding coil	20	10
16	Aspiration of anti-biotin HRP to the holding coil	80	10
17	Aspiration of air to the holding coil	20	10
18	Sending anti-biotin HRP to the capillary and incubate for 10 min	80	10
19	Removing unbound species by washing capillary with PBS	2000	50
20	Washing with citric phosphate buffer	1000	50
21	Aspiration of air to the holding coil	20	10
22	Aspiration of substrate TMB to the holding coil	80	10
23	Aspiration of air to the holding coil	20	10
24	Sending substrate TMB to capillary and incubate with anti-biotin HRP for 2 min	80	10
25[a]	Discarding air		30
26[a]	Sending the solution product to the detector	50	10

[a] To completely prevent an air bubble from entering the detection flow cell, the air bubble was discarded before sending the product zone into the flow cell. The discarded volume (30 μl) needs to be higher than its aspiration volume (20 μl). Therefore, the volume (50 μl) of the product zone left to be sent to the detector was lower than the aspirated solution volume (80 μl).

Note that the signal profiles were peak down due to the electronic data acquisition system used in this work that gave the signal in voltage which relates to transmittance.

3.3. Optimization

3.3.1. Concentrations of BSM, b-lectin, and enzyme conjugates

The suitable concentration of BSM for coating the capillary was obtained from the previous independent studies of coating BSM on the surface of the conventional micro-well plate. The surface area of a micro-well is approximately the same as that of the glass capillary used in this study (2.7 cm^2) [18]. Therefore, the same optimum concentration of BSM at 100 μg ml^{-1} was used in the immobilization step to ensure an excess amount of BSM. Amounts of other reagents and the incubation time for each step were optimized as follows.

The amount of biotinylated lectin (b-lectin) is an important parameter. An inadequate amount would be used up when binding with a small amount of sialoglycoconjugates in serum and therefore, would not be able to differentiate various higher sialoglycoconjugates concentrations. In contrast, having too much excess b-lectin would lead to unnecessary expense and increase in analysis cost. It was found that the signals were increased with the increased amount of b-lectin. However, in this study, the highest concentration used was limited at 1:50 dilution ratio to save reagent.

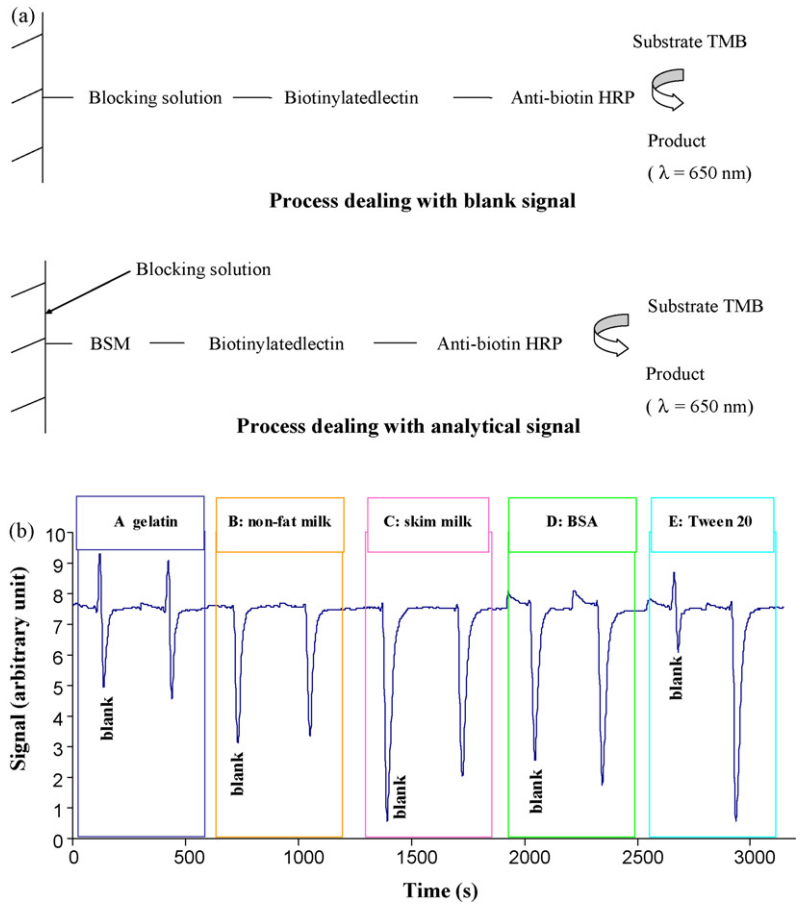

Fig. 3. Study on suitable blocking reagent to reduce nonspecific binding in the BSM coated glass capillaries using the SI-CI system. (a) Diagram illustrating the process of obtaining blank signal and analytical signal. (b) Comparing blank and analytical signal profiles when using (A) 1% gelatin in PBS, (B) 5% non-fat milk in PBS, (C) 5% skim milk in PBS, (D) 1% BSA in PBS and (E) 0.1% Tween20 in PBS, as blocking reagents. The first peak and the second peak of each signal set are blank and analytical signals, respectively. Each capillary was coated off-line with 10 μg ml^{-1} BSM at 8 °C for 24 h, followed by addition of 1:200 dilution b-lectin and 1:2000 anti-b-HRP and TMB. Product was detected with the SI system.

The optimum amount of anti-biotin conjugated with HRP (anti-b-HRP) was determined by varying the dilution ratio in the range of 1:5000, 1:2000 and 1:1000. Anti-b-HRP should be present in sufficient amount to bind to all bound b-lectin. However, too much excess anti-b-HRP would require extensive washing and unnecessary waste of time and reagents. The signals were found to increase with the increase of the amount of anti-b-HRP and leveled off at the concentration between 1:2000 and 1:1000 dilution ratios. Although dilution of 1:2000 seemed to be adequate, dilution ratio of 1:1000 was chosen for further experiments to compensate for the loss of enzyme activity which might occur due to unfavorable storage conditions. (i.e. storage temperature not maintained).

3.3.2. *Incubation time*

There were four different steps in which incubation time should be optimized; immobilization of BSM onto the glass capillary, incubation of the mixture of b-lectin and BSM in serum/standard to the immobilized BSM, incubation of anti-b-HRP to the bound b-lectin, and incubation of the bound anti-b-HRP with substrate TMB. The longer incubation time yielded better sensitivity but reduced sample throughput. Incubation times of each step were chosen to compromise sensitivity and analysis time. Immobilization of BSM onto the glass capillary was done off-line for the convenience in preparation of many capillaries at once and to reduce overall analysis time in the flow system.

Efficiency of immobilization of BSM onto the glass capillary depended on both time and temperature. Three sets of conditions were studied; in the refrigerator (8 °C) for 1, 5 and 24 h; at room temperature (25 °C) for 1, 5 and 8 h; and in the incubator (37 °C) for 1, 3 and 5 h. The results are shown in Fig. 4. Immobilization was best at 8 °C for 5–24 h or, alternatively, at room temperature for 1 h. The higher temperature of 37 °C was not suitable, possibly owing to

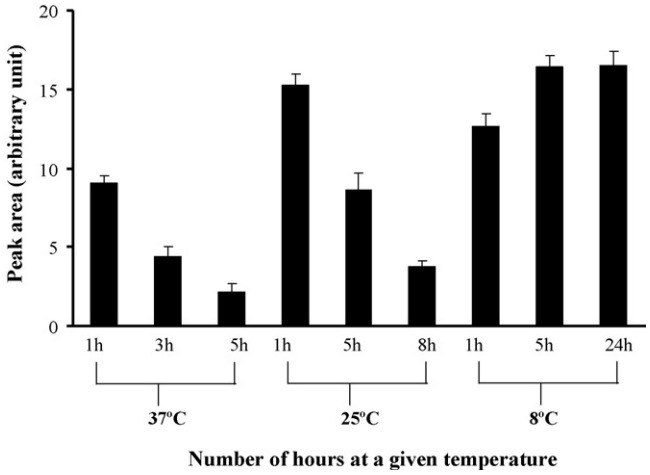

Fig. 4. Effect of temperature on efficiency of immobilization of BSM onto glass capillary. Each capillary was coated with 100 μg ml^{-1} BSM at various times and temperatures, followed by addition of 1:100 dilution b-lectin in 6% BSA and 1:1000 dilution anti-b-HRP and TMB.

degradation or conformational change of BSM. Here immobilization of BSM at 8 °C between 5 and 24 h was chosen for the convenience of preparation of many capillaries. However, if online immobilization is to be done one at a time, the performance of immobilization of BSM at 25 °C for less than 1 h should be explored.

The b-lectin was introduced into the sequential injection-capillary immunoassay (SI-CI) system and mixed with standard BSM prior to being sent to stop in the capillary. Immobilized BSM and BSM in serum/standard competed for a limited amount of b-lectin. The degree of binding depends on incubation time. Longer incubation time allows for higher interaction and better analytical sensitivity. Incubation times of 1, 5, 10, 20 and 40 min were studied. Signals increased from 1 to 10 min, after that the signals remained unchanged. This indicated that the interaction between b-lectin and BSM was completed in 10 min and therefore this incubation time of 10 min was chosen for further experiments.

Similarly, the longer incubation time between bound b-lectin and anti-b-HRP led to higher sensitivity. The increasing of sensitivity was observed from 1 to 10 min. After that (10–40 min), the signal was stable. This implied that the binding between b-lectin and anti-b-HRP reached equilibrium at 10 min, so this was selected for further experiments.

The longer incubation time between anti-b-HRP and excess amount of TMB yielded more enzyme-substrate product which resulted in higher analytical signal. However, to avoid auto-oxidation of TMB and to shorten up the analysis time, incubation time of 2 min was chosen for this step as it gave sufficient sensitivity.

It should be noted that the fused silica capillary with small diameter (e.g. 0.53 mm [16]) has advantages in high ratio of the surface area to the volume and flexible length of choice. The plain glass capillary used in this work is relatively larger in diameter (~1.15 mm) with length limited in commercial availability. Therefore, this glass capillary may provide lower sensitivity and require longer reaction time as compared to fused silica capillary. However, the much cheaper plain glass haematocrit capillary makes its usability worth the investigation. In addition, the bigger diameter of the glass capillary would actually facilitate the flow of solution and reduce back pressure in the system.

3.4. Performances

3.4.1. Precision

Within-run precision or repeatability was determined by analyzing a normal serum sample spiked with a 50 μl of 100 ng ml^{-1} BSM standard. Analysis was done repeatedly 10 times within a day. This study was carried out using 2 different samples. The relative standard deviations (RSDs) were practically the same in both samples (2.8% and 2.3%).

Between-run precision or reproducibility was determined by considering the calibration curve of standard BSM equivalent constructed in 5 different days (1, 4, 10, 20 and 33 days apart). The investigated concentrations were in the range of 1–1000 ng ml^{-1}. The percentages of relative standard deviation (%RSDs) were 0.7 for 1 ng ml^{-1}, 0.7 for 100 ng ml^{-1}, 1.1 for 250 ng ml^{-1}, 2.2 for 500 ng ml^{-1} and 2.8 for 1000 ng ml^{-1}.

The precision of system decreased as the concentrations of BSM increased. This may be due to the fact that in this competitive immunoassay, when the concentration of analyte increased, less b-lectin was bound to BSM on the capillary wall, leading to lower signal (peak area). The precision of measurement was therefore decreased. However, all were in an acceptable range.

3.4.2. Stability of the coated capillary

The aim of this study was to estimate the stability or shelf life of BSM coated glass capillary tubes kept in different conditions. This was done by incubating BSM solution with glass capillaries 24 h and dividing them into four groups; the first group was kept in phosphate buffer (PB) pH 7.4, the second group was kept in PB containing 0.05% Tween20 pH 7.4, the third group was kept in carbonate buffer pH 9.6, and the last group was kept dry. All capillaries were sealed with parafilm on both ends. The capillaries were refrigerated at 8 °C for future use. The stability of capillary was investigated in 1, 3, 5, 7 and 15 days as compared to freshly prepared coated capillary. The peak areas obtained from capillaries kept at various conditions were calculated against the peak areas obtained from freshly prepared capillaries which were set at 100%.

Table 2
Efficiency of BSM coated capillaries kept in various conditions.

Day	Efficiency relative to freshly prepared capillaries			
	PB pH 7.4	PB with 0.05% Tween20	Carbonate buffer pH 9.6	Dry
1	100	99	84	99
3	97	94	76	82
5	92	89	65	70
7	82	79	62	59
15	72	70	41	33

Percentages of signal were compared to those obtained from freshly prepared capillaries. Results from freshly prepared capillaries are set as 100%.

Table 2 shows that the signals obtained from the capillaries kept in PB pH 7.4 for 1 and 3 days were not significantly changed as compared to the freshly prepared capillaries. Capillaries kept in PB longer than 3 days tended to be degraded. This might be because the immobilization of BSM with glass capillary was not through covalent bonding. Passive adsorption may be too weak of a force to hold the BSM on the capillary surface for a long time. It also

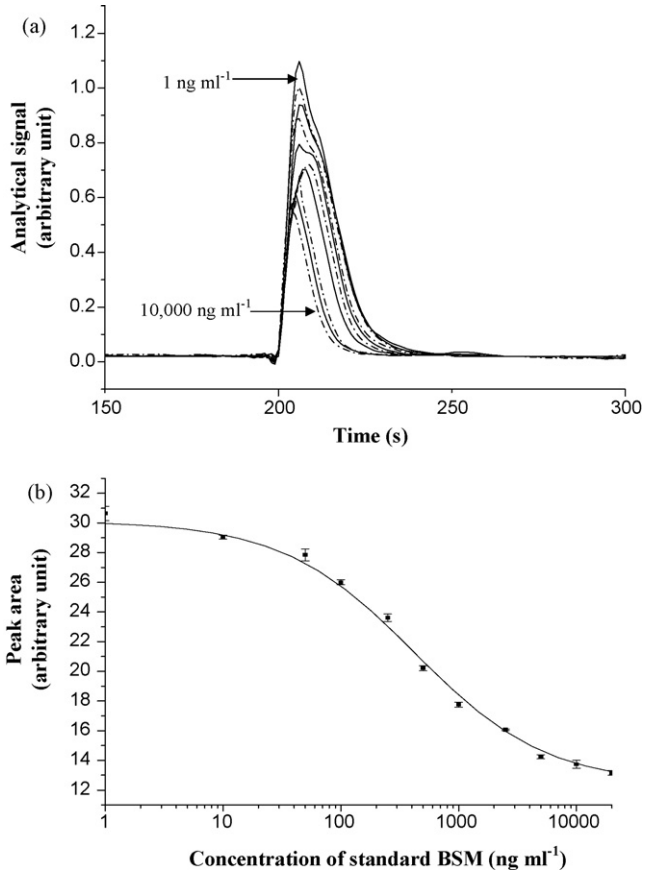

Fig. 5. (a) Analytical signal profiles of standard BSM of various concentrations: 1, 10, 50, 100, 250, 500, 1000, 2500, 5000 and 10,000 ng ml^{-1}, obtained from the SI-CI system (peaks were rotated for easier viewing) and (b) the corresponding calibration curve for determination of sialoglycoconjugates (BSM equivalent) using SI-CI system.

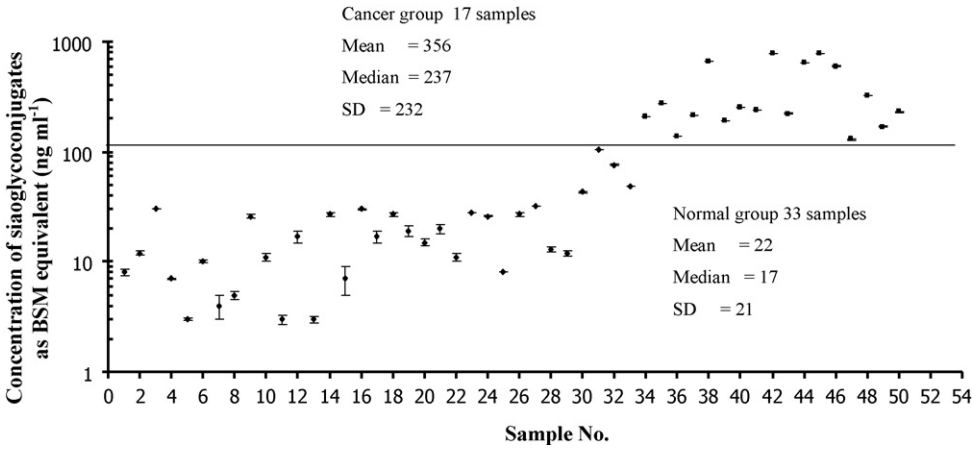

Fig. 6. Comparison of the concentrations of sialoglycoconjugates as BSM equivalent obtained from 33 healthy and 17 cancer diseases samples. All normal subjects have sialoglycoconjugates lower than 110 ng ml^{-1} BSM equivalent determined using the proposed SI-CI system. Note that *Y*-axis is in log scale.

showed that Tween20 was not necessary for keeping capillaries. The capillaries that were kept dry showed the decrease in signal after 1 day. This might be because the BSM was degraded and lost the specific binding abilities when the capillaries were kept dry. The BSM coated onto glass capillary could not be kept in carbonate buffer pH 9.6 because the conformation of BSM might be altered and could no longer bind to the b-lectin.

Since the binding of BSM on the glass capillary is through physical adsorption, the ability to recycle of BSM coated capillary is not expected to be effective. However, reusing of the plain capillary after removing all reagents may be possible and this will be investigated further.

3.4.3. Calibration curve

Calibration curve of BSM standard (sialoglycoconjugates equivalent) was investigated in the concentration range of 1–10,000 ng ml^{-1} to cover the amount of sialoglycoconjugates normally found in a normal person and various types of cancer patients. The profiles of analytical signals and the corresponding calibration curve are as shown in Fig. 5(a) and (b), respectively. Here, the signals were turned up side down as compared to those shown in Fig. 3 for easier viewing and relating the concentration of product to the absorbance.

Calibration curve is the sigmoidal (logistic) fit obtained from the computer software (Origin version 7.0). The calibration curve is expressed in sigmoid equation as follows:

$$Y = [(A_1 - A_2)/(1 + (X/X_0^p))] + A_2 \quad (R^2 = 0.9967),$$

where Y is analytical signal as peak area, X is concentration of BSM equivalent in ng ml^{-1}, A_1 is initial Y value (30.12), A_2 is final Y value (12.37), X_0 is X value at Y equal to half of the limit A_1 and A_2 (424.48), and P is power (0.77).

From this calibration curve, the working range was found to be 50–5000 ng ml^{-1}. The lowest detectable concentration of BSM equivalent was 10 ng ml^{-1}, determined as the lowest distinguishable analytical signal ± 2S.D. with 95% confidence from blank signal while the highest detectable concentration of BSM equivalent was 5000 ng ml^{-1}, determined similarly on another end of the calibration curve. This range very well covers the sialoglycoconjugates level found in normal human and at various elevated levels in patients with cancer diseases.

3.4.4. Real samples

The SI-CI system was developed for determination of sialoglycoconjugates in serum samples. Normal human serum samples (no. 1–33) were from 12 male (age 23–41 years old), 14 female (age 22–74 years old) and 7 subjects with unknown gender and age. Cancer serum samples (no. 34-50) were from 11 male (age 32–78 years old) and 6 female (age 41–82 years old) subjects. The concentrations read from the calibration graph are not true concentrations of sialoglycoconjugates because the calibration graph was constructed from BSM standard. Therefore, the sialoglycoconjugates concentration is reported as BSM equivalent. The concentrations of BSM equivalent obtained from healthy people and cancer patients using the proposed SI-CI system are shown in Fig. 6. The over all trends showed that the concentrations of sialoglycoconjugates as BSM equivalent in normal samples are much lower than that found in cancer subjects (average 22 vs. 356 ng ml^{-1}). All normal subjects have sialoglycoconjugates lower than 110 ng ml^{-1} BSM equivalent. However, with the limited number of samples, it is not possible to pinpoint the cut-off value. The use of a higher number of samples and chemometrics may help in better categorization of the two groups with the suitable cut-off value. Nevertheless, the distinctive difference between the average levels of sialoglycoconjugates found in the two groups demonstrated the performance of the SI-CI system for automatic quantification of sialoglycoconjugates in real clinical matrices.

4. Conclusion

The SI-CI system for determination of sialoglycoconjugates in human serum samples was developed. The ordinary commercially available glass capillary which is cost-effective can be used as a solid surface to immobilize BSM. The opened tubular glass capillary can eliminate the backpressure which is a drawback of packed bead column. In addition, the glass capillary is much cheaper than other solid surfaces such as fused silica capillary and bead. BSM could be adsorbed directly onto the glass surface without the need of modification, but careful selection of blocking reagents is necessary. The small size of the capillary can reduce the diffusion time for lectin to reach the immobilized BSM on the surface, resulting in a shorter assay time per sample as compared to the conventional microplate assay. Therefore, the glass capillary is suitable to be coupled to the sequential injection system to make the immunoassay process more automated. The results obtained from the SI-CI system can differentiate cancer disease serums from normal healthy serums based on the amount of sialoglycoconjugates, though the cut-off value should be further investigated with a larger number of samples and the aid of chemometrics. The system may be used as an alternative analysis system for sialic acid and the authors hope that the results from this work would benefit the researchers that are evaluating the use of this biomarker.

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