A RE-EVALUATION OF PLASMA SIALIC ACID DETERMINATION USING THE PERIODATE RESORCINOL METHOD VERSUS THE ENZYMATIC METHOD

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Abstract. The concentration of plasma sialic acid was estimated using the modified chemical method and the more sensitive enzymatic method in 20 subjects with impaired glucose tolerance and 20 control subjects. The mean sialic acid concentration values of the control subjects and subjects with impaired glucose tolerance using the enzymatic method were 1.747 ± 0.047 and 2.583 ± 0.070 mmole/l and 1.753 ± 0.067 and 2.591 ± 1.02 mmole/l for the chemical method. The intra-assay coefficient of variation for the control subjects and for the subjects with impaired glucose tolerance were 1.963% and 1.583%, respectively, for the enzymatic assay and 2.728% and 2.431%, respectively, for the chemical assay. The inter-assay coefficient of variation for the control subjects and for the subjects with impaired glucose tolerance were 2.686% and 2.723% for the enzymatic assay, and 3.819% and 3.95% for the chemical assay. Since the values do not differ significantly, the chemical assay is a cost effective method that can be used in large epidemiological studies.

INTRODUCTION

The steps by which Type 2 diabetes mellitus (T2DM) causes atherosclerotic vascular disease are not clear. Emphasis is shifting from elucidation of risk factors, such as insulin resistance, to an understanding of the process occurring at the vasculature (Playford and Watts, 1999). An increase in the concentration of serum sialic acid has been shown to be a possible cardiovascular risk factor in patients with non insulin dependant diabetes (Crook *et al*, 1993). The earliest event associated with atherosclerosis is the accumulation of low density lipoprotein cholesterol (LDL) and fibrinogen /fibrin in the affected arterial wall (Smith and Staples, 1980). It is therefore important to understand the mechanisms, which govern the endothelial binding, uptake, and transport of these macromolecules across the vessel wall as a prerequisite to the prevention of atherogenesis. The role of the luminal endothelial plasma membrane may be particularly relevant because it is the first interface between the vessel wall and circulating blood components. The luminal surface of the endothelium is rich in sialoglycated proteins and thus provides an anionic barrier for the receptor mediated uptake of LDL. It has been shown that the removal of sialic acid, as well as glycosaminoglycans, increases the internalization of LDL by 20 fold (Gorog and Pearson, 1984). Thus, desialylation at the endothelium could be an early event in the atherosclerotic process of cardiovascular disease and in NIDDM.

Recent evidence suggests that inflammatory processes play a part in the cause of atherosclerotic cardiovascular disease (Ross, 1999) and that the increase in acute phase proteins rich in sialoglycated proteins in serum reflects the atherosclerotic process in the endothelium (Pickup *et al*, 1997). Thus, an increase in the acute phase proteins may partly explain the elevation of serum sialic acid in Type 2 DM. Since sialic acid is a marker for the early atherosclerotic process and is present in most acute phase proteins, there is a need for a specific assay, which is cost effective, and can be employed in large scale epidemiological studies. In our laboratory, we have modified the periodate resorcinol method (Jourdian *et al*, 1971) for the measurement of total sialic acid, and compared the values to those obtained with the enzymatic assay (Simpson *et al*, 1993).

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MATERIALS AND METHODS

Subjects

Twenty control subjects were studied along with 20 first-degree relatives with asymptomatic impaired glucose tolerance (IGT), who were age matched. The control subjects with a body mass index of < 30kg/m^2 were chosen randomly from the Klang Valley, Kuala Lumpur, through the distribution of questionnaires. Any subject with a family history of diabetes, hypertension, or coronary artery disease were excluded from the study. First degree relatives with an impaired glucose tolerence test (IGT) were chosen from diabetic families through the diabetic clinic, University of Malaya Medical Center, Kuala Lumpur. The study was approved by the Ethics Committee of the Medical Center. None of the subjects had received hypolipidemic drug therapy, nor had any renal, hepatic or thyroid disease affecting glucose or lipid metabolism.

Sialic acid determination

Fasting blood was collected in bottles containing disodium ethylene diamine tetraacetate dehydrate (EDTA), and the plasma was separated immediately by centrifugation at 3,000 rpm for 15 minutes at 4ºC and the sialic acid was measured using the enzymatic method or the periodate-resorcinol method. Sialic acid was determined by the enzymatic method as described by Simpson *et al* (1993). The glycoprotein was hydrolysed by neuraminidase, and the sialic acid was cleaved by the second enzyme N-acetyl neuraminic acid adolase, which produced pyruvate. The pyruvate was oxidized by the pyruvate oxidase in the presence of FAD and the hydrogen peroxide released was measured colorimetrically by peroxidase in the presence of 4-amino antipyrine and N-ethyl-N-2-hydroxyethyl-3-toluidine, when a red product was formed. The optical density was measured at 630 nm using a MRX ELISA reader. The enzymatic assay was done using the reagents supplied by the manufacturers (Boehringer Manheim). Reproducible results were obtained when the plasma was filtered with the Millipore filters supplied by the manufacturer (Qiagen). Total sialic acid was determined chemically as described by Jourdian *et al* (1971). The plasma samples were filtered and a sample containing not more than 0.2 μmol of N-acetyl neuraminic acid in a total of 0.5 ml was added to 0.1 ml of 0.04 M periodic solution. The solutions were thoroughly mixed and allowed to stand in an ice bath for 20 minutes. After the addition of the resorcinol reagent, the solutions were mixed and allowed to stand in an ice bath for 5 minutes. This mixture was heated at 100ºC, for 15 minutes and cooled under tap water. *Tert*–butyl alcohol was added to the mixture and mixed vigourously to give a single phase. The tubes were then transferred to a 37ºC water bath for 3 minutes to stabilize the color and cooled to room temperature. One hundred thirty microliters of the mixture was transferred to microtiter plates and the absorbance was determined at 630nm (λ max =626nm) using a MRX ELISA reader. The above assay was adapted so that the reaction could be carried out on microtiter plates. Standard curves for both the enzymatic and resorcinol-periodate method ranging from 0 to 1.0 μmole were plotted using N-acetyl neuraminic acid obtained from the manufacturer (Sigma). Pyruvate, in the range of 0.3-0.7 mg/100 ml, did not interfere with the chemical assay.

Statistical analysis

Data were expressed as mean ± standard deviation. The coefficient of variation was calculated using SPSS and Microsoft EXCEL packages.

RESULTS

From Table 1, the mean sialic acid concentration for the control subjects using enzymatic assay method was 1.747±0.047 mmole/l and for the chemical method it was 1.753±0.067 mmole/l. For the enzymatic assay, the intra- and inter-assay coefficients of variation were 1.96% and 2.69%, respectively. The corresponding values for the chemical assay were 2.73% and 3.82 %, respectively. The mean sialic acid concentration for the subjects with IGT using enzymatic assay method was 2.583±0.070 mmole/l and for the chemical method it was 2.591±1.02 mmole/l. For the enzymatic assay, the intra- and inter-assay coefficients of variation were 1.58% and 2.72%, respectively. The corresponding values for the chemical assay were 2.43% and 3.95 %, respectively.

Table 1
Comparison of enzymatic and chemical assays.

	Control subjects		Subjects with IGT	
Measure	Enzymatic	Chemical	Enzymatic	Chemical
Mean ± SD (mmole/l)	1.747 ± 0.047	1.753 ± 0.067	2.583 ± 0.070	2.591 ± 1.02
Intra-assay CV	1.96%	2.73%	1.58%	2.43 %
Inter-assay CV	2.69%	3.82%	2.72%	3.95%

IGT=Impaired glucose tolerance; CV=Coefficient of variation

Table 2
95% Confidence Intervals for mean concentrations.

Control subjects		Subjects with IGT	
Enzymatic	Chemical	Enzymatic	Chemical
1.726, 1.768	1.724, 1.782	2.552, 2.614	2.144, 3.038

From Table 2, the 95% confidence intervals for the mean sialic acid concentrations for control subjects using the enzymatic and chemical methods were 1.726, 1.768 and 1.724, 1.782, respectively. For subjects with IGT, the 95% confidence interval for the mean sialic acid concentration using the enzymatic and chemical methods were 2.552, 2.614 and 2.144, 3.038, respectively.

DISCUSSION

The inter- and intra-assay coefficients of variation for the chemical method is quite similar to that of enzymatic method. Thus, the accuracy of estimation is about the same for the two methods. For both control subjects and subjects with IGT, the 95% confidence intervals for mean sialic acid concentrations using chemical and enzymatic methods overlap each other. Furthermore, the point estimates for the chemical methods fall within the respective interval estimates for enzymatic methods. Thus, the chemical method is not significantly different from the enzymatic method in estimating the mean sialic acid concentration. Therefore, the chemical assay ia a cost-effective method and can be used in large epidemiological studies

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